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Drug Discovery & Development: State-of-the-Art and Future Directions" on the topic of "Targets":

OXPHOS as a target space for tuberculosis: success, caution, and future directions

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Chapter summary

The emergence and spread of drug-resistant pathogens, and our inability to develop new antimicrobials to combat resistance, has inspired scientists to seek out new targets for drug development. The *Mycobacterium tuberculosis* complex is a group of obligately aerobic bacteria that have specialized for inhabiting a wide range of intracellular and extracellular environments. Two fundamental features in this adaptation are the flexible utilization of energy sources and continued metabolism in the absence of growth. *M. tuberculosis* is an obligately aerobic heterotroph that depends on oxidative phosphorylation (OXPHOS) for growth and survival. However, several studies are redefining the metabolic breadth of the genus. Alternative electron donors and acceptors may provide the maintenance energy for the pathogen to maintain viability in hypoxic, nonreplicating states relevant to latent infection. This hidden metabolic flexibility may ultimately decrease the efficacy of drugs targeted against primary dehydrogenases and terminal oxidases. However, it may also open up opportunities to develop novel antimycobacterials targeting persister cells. In this review, we discuss the progress in understanding the role of energetic targets in mycobacterial physiology and pathogenesis, and the opportunities for drug discovery.

Keywords

bacterial energetics; respiration; drug targets; physiology

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OVERVIEW OF RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN *MYCOBACTERIUM TUBERCULOSIS*

The genus *Mycobacterium* comprises a group of obligately aerobic bacteria that have adapted to inhabit a wide range of intracellular and extracellular environments. Fundamental to this adaptation is the ability to respire and generate energy from variable sources, and sustain metabolism in the absence of growth. The pioneering work of Brodie and colleagues on *Mycobacterium phlei* established much of the primary information on the electron transport chain and oxidative phosphorylation system in mycobacteria (reviewed in (1)). Mycobacteria can only generate sufficient energy for growth by coupling the oxidation of electron donors derived from organic carbon catabolism (e.g. NADH, succinate, malate) to the reduction of O_2 as a terminal electron acceptor. Mycobacterial genome sequencing revealed that branched pathways exist in mycobacterial species for electron transfer from many low potential reductants, via quinol, to oxygen (Figure 1).

During aerobic growth, electrons are transferred to oxygen via two terminal respiratory oxidases: an *aa*$_3$-type cytochrome *c* oxidase (encoded by *ctaBCDE*) belonging to the heme-copper respiratory oxidase family and cytochrome *bd*-type menaquinol oxidase (*cydABCD*) (Figure 1). Despite the acknowledged importance of oxygen in the physiology and pathobiology of *M. tuberculosis*, the molecular mechanisms governing the regulation of terminal oxidase expression remains largely unknown. In the absence of oxygen, mycobacterial growth is inhibited, even if alternative electron acceptors are present (e.g. nitrate, fumarate). Despite growth being inhibited, mycobacteria are able to metabolize exogenous and endogenous energy sources under low oxygen for maintenance functions. The electron acceptors and mechanisms to recycle reducing equivalents under these conditions are poorly understood. ATP synthesis is obligatorily coupled to the electron transport chain and the F_1F_0-ATP synthase, irrespective of the oxygen concentration or the protonmotive force (*pmf*), but the reasons for this remain unexplained. The aim of this chapter is to discuss the progress in understanding the role of energetic targets in mycobacterial physiology and pathogenesis, and the opportunities for drug discovery.

TARGETING THE PROTONMOTIVE FORCE IN *MYCOBACTERIUM TUBERCULOSIS*

All bacteria require a protonmotive force (*pmf*) to grow and remain viable under replicating and non-replicating conditions. During respiration, energy is conserved by the generation of a *pmf* across a proton-impermeable membrane. The *pmf* (electrochemical potential) consists of two gradients: an electrical potential ($\Delta\psi$), due to the charge separation across the membrane ($positive_{outside}/negative_{inside}$), and a chemical transmembrane gradient of protons (ΔpH, $acidic_{outside}/alkaline_{inside}$) (Figure 2). At neutral pH, the *pmf* is predominantly in the form of a $\Delta\psi$, but as the external pH drops, the ΔpH increases and the $\Delta\psi$ decreases to maintain a constant *pmf*. Dissipation of the *pmf* leads to a rapid loss of cell viability and cell death.

A variety of mechanisms are used to generate the *pmf* in mycobacteria (Figure 2). Mycobacteria generally grow at neutral pH and under these conditions generate a *pmf* of approximately −180 mV (2). Under hypoxia, *M. tuberculosis* generates a total *pmf* of −113 mV, (−73 mV of Δψ and −41 mV of ZΔpH) (3). In obligately aerobic bacteria like *M. tuberculosis*, the generation of a *pmf* is mediated primarily by the proton-pumping components of the electron transport chain (Figure 2, mechanism 3). As oxygen becomes limiting for growth, many bacterial pathogens switch to alternative electron acceptors (e.g. nitrate, fumarate) and proton release is coupled to a terminal reductase (e.g. nitrate reductase) via a *pmf* redox loop mechanism (4) (Figure 2, mechanism 2). *M. tuberculosis* harbors both nitrate reductase and fumarate reductase, but little direct experimental data has accumulated to suggest that they contribute to *pmf* generation under hypoxia. The membrane-bound F_1F_0-ATP synthase can usually operate as a reversible ATP-driven proton pump to generate the *pmf* (5). However, in *M. tuberculosis* the enzyme shows extreme latency in the hydrolysis reaction (6). End-product (e.g. lactate) efflux can generate a *pmf* (7) (Figure 2, mechanism 1) and it has been proposed that fumarate may be used as a mechanism to generate succinate as an excreted end product for maintenance of the membrane potential, under hypoxia, in *M. tuberculosis* (8).

There are a number of compounds that target the *pmf* in bacteria (Figure 3A), including agents that inhibit the major proton pumps (e.g. rotenone) and those that facilitate proton transport through the cytoplasmic membrane (protonophores, e.g. CCCP). The majority of protonophores are non-specific and functional in both prokaryotic and eukaryotic cell membranes. Individual components of the *pmf* can be collapsed using specific inhibitors. For example, the membrane potential can be collapsed by compounds that catalyze electrogenic cation transport across the cell membrane (e.g. valinomycin) (Figure 3A). Valinomycin is a dodecaepsipeptide that forms a macrocyclic molecule allowing for rapid K^+ movement down its electrochemical gradient. The chemical transmembrane gradient of protons (ΔpH) can be collapsed by nigericin through its K^+/H^+ antiporter (electroneutral) activity (Figure 3A). Growth of mycobacteria is sensitive to compounds that dissipate the membrane potential (e.g. protonophores and valinomycin) and these compounds are bactericidal towards growing and non-growing (aerobic or hypoxic) cells, further highlighting the importance of the membrane potential in mycobacterial viability (2, 3). Rao *et al.* (3) have reported that thioridazine, a compound purported to target NDH-2, results in dissipation of the Δψ and significant cell death. They suggest that NADH is an important electron donor for the generation of the Δψ, under hypoxic conditions. Inhibitors of succinate dehydrogenase (SDH) (e.g. 3-propionate) are also able to dissipate the Δψ under hypoxia, suggesting SDH is an important generator of the Δψ under hypoxia (9).

As the external pH of the growth medium changes and becomes mildly acidic, mycobacteria are able to generate a considerable transmembrane pH gradient (ZΔpH) and maintain a constant *pmf* (2). While proton translocation *via* the respiratory chain generates the *pmf*, during respiration with oxygen as the terminal electron acceptor, it is not clear how the *pmf* is established in the absence of oxygen under microaerobic growth conditions. Anaerobic bacteria are able to generate a significant *pmf* (−100 mV) using their membrane-bound F_1F_0-ATP synthase in the ATP hydrolysis direction (5). The ATPase activity (proton-pumping) of the enzyme is fuelled by ATP produced by substrate level phosphorylation.

This mechanism does not appear to operate in mycobacterial cells where the F_1F_0-ATP synthase has been reported to have latent ATPase activity when measured in inverted membrane vesicles (6, 10). Whether the enzyme is also latent in actively growing cells is not known and therefore the potential does exist for this enzyme to function as a primary proton pump in the absence of oxygen and a functional respiratory chain to generate the *pmf*. The mechanisms controlling this extreme latency in the ATP hydrolysis direction is an area that could unlock new avenues for drug development and requires further investigation at a molecular and structural level.

The clinically-approved antimycobacterial bedaquiline (Sirturo™, TMC207) is a potent nanomolar inhibitor of the mycobacterial F_1F_0-ATP synthase that binds to the enzyme's oligomeric c ring to inhibit ATP synthesis (11–16). We have recently reported that bedaquiline activates respiration and is a potent uncoupler of respiration-driven ATP synthesis in mycobacteria (17). However, unlike classical uncouplers/protonophores, bedaquiline does not translocate protons *per se* but perturbs respiration by binding to F_0 (oligomeric c ring) of the ATP synthase, likely disrupting the subunit a-c subunit interface in F_0, thereby uncoupling proton flow from ATP synthesis by the F_1F_0-ATP synthase (Figure 3B). This uncoupling is electroneutral, consistent with no observed change in the membrane potential. Feng et al. (18) further demonstrate the potential of targeting the *pmf* of *M. tuberculosis* and report that a number of tuberculosis drugs (e.g. clofazimine, BDQ, SQ109) are active uncouplers of the *pmf* in addition to binding to enzyme targets, highlighting the multi-targeting nature of these molecules.

M. tuberculosis encounters acidic microenvironments in the host and must maintain their intracellular pH homeostasis to survive. Compounds that dissipate the transmembrane pH gradient lead to a rapid loss in cell viability at acidic pH and the lethal intracellular pH for mycobacterial species in the pH range 5.5–6 (2). To address pH homeostasis as a drug target, Nathan and colleagues developed a whole cell screen to identify compounds that dissipate the ΔpH gradient in *M. tuberculosis* (19). This study identified a number of candidate molecules, including PZA, that disrupted intracellular pH homeostasis and caused cell killing (loss of viability) highlighting this as a potential pathway for drug development (19) (Figure 3B).

TARGETING PRIMARY DEHYDROGENASES IN *MYCOBACTERIUM TUBERCULOSIS*: THE UNTAPPED SOURCE OF METABOLIC DRUG DISCOVERY

M. tuberculosis encodes several primary dehydrogenases that serve as direct reductants for electron transport (Figure 1). Many of these have attractive properties for drug development, such as essentiality or lack of presence in the human genome. Unfortunately, the unifying feature of these enzymes is a paucity of information regarding their physiological and biochemical roles. Increasing our understanding of these processes will likely reveal mechanisms to perturb the viability within, and reactivation from, dormant mycobacteria.

NADH:Menaquinone Oxidoreductases

Mycobacterium tuberculosis possesses two classes of NADH:menaquinone oxidoreductase to couple the oxidation of NADH from central metabolism to energize the electron transport chain (20) (Figure 1). Like mitochondria, *M. tuberculosis* harbours a proton-pumping type I NADH dehydrogenase complex (NDH-1, complex I) that transfers electrons to menaquinone, conserving energy by translocating protons across the membrane to generate a *pmf* (Figure 1 and Figure 2). In *M. tuberculosis*, the *nuo* operon is neither essential for growth nor persistence in an *in vitro* Wayne model (3). Moreover, the *nuo* operon has been lost from the genome of the intracellular parasite *Mycobacterium leprae* except for a single remaining *nuoN* pseudogene (21). These data suggest NDH-1 does not represent a compelling target for drug development. However, *M. tuberculosis* mutants lacking the NDH-1 subunit *nuoG* had reduced virulence in mice (22). There is evidence that *nuoG* and potentially other subunits of NDH-1 are anti-apoptosis factors and are indeed potential candidates for vaccine development.

The second class of NADH:menaquinone oxidoreductase is the non-proton translocating type II NADH dehydrogenase (NDH-2) that does not conserve energy (Figure 1). NDH-2 is a small monotopic membrane protein (50–60 kDa) that catalyses electron transfer from NADH via FAD (non-covalently bound redox prosthetic group) to quinone. *M. tuberculosis* harbors two copies of NDH-2 (*ndh* Rv1854c and *ndhA* Rv0392c) (20), which are well conserved among slow-growing mycobacterial species. In *M. tuberculosis*, Ndh (1392 bp) and NdhA (1413 bp) share 65% identity and the FAD- and NADH-binding motifs are highly conserved. The Ndh and NdhA proteins of *M. tuberculosis* have been shown to be functional NADH dehydrogenases that transfer electrons to the quinone pool via a two-site ping-pong reaction mechanism (23, 24). Several studies have suggested that *ndh* is essential for growth of *M. tuberculosis* (20, 25, 26), but the reasons for this essentiality remain unknown.

Unlike NDH-1, NDH-2 has not been reported in mammalian mitochondria, leading to the proposal that NDH-2 may represent a potential drug target for tuberculosis. Several antimycobacterial compounds have been reported to target NDH-2 (3, 20, 23, 27–30) (Figure 4). For example, drugs of the phenothiazine family (e.g. thioridazine, trifluoperazine, chlorpromazine) have potent activity in vitro against drug-susceptible and drug-resistant *M. tuberculosis* strains (31, 32) and show activity in a mouse model of pulmonary tuberculosis (20). However, the levels of phenothiazines required for antitubercular activity (>0.5 mg/L of plasma) appear to be clinically unachievable in patients (33); the development of phenothiazines as antitubercular drugs is currently limited by the wide range of potentially serious off-target effects displayed during use, including cognitive effects in the central nervous system at concentrations lower than their antimycobacterial activity (34). In contrast to the phenothiazines, quinolinyl pyrimidines inhibit NDH-2 from *M. tuberculosis* in the nanomolar range and show no toxicity to the eukaryotic organism *S. cerevisiae*, and no membrane disruption activity in a red blood cell haemolysis assay (30). The high potency and preliminary lack of toxicity against higher order species are promising. Scopafungin, gramicidin S, and polymyxin B have been identified as inhibitors of *M. smegmatis* NDH-2 and further work with *M. tuberculosis* is needed to determine their efficacy against the pathogen (35, 36).

Mode of action studies are required to understand how NDH-2 inhibitors work at a molecular and structural level and why inhibition of NDH-2 activity leads to cell death. Yano et al. (37) have shown that clofazimine (CFZ), a long-standing clinical drug for leprosy, is a redox-active (phenazine derivative) prodrug activated by NDH-2. The authors propose a model in which the drug inhibits the growth of mycobacteria by a redox cycling pathway involving the enzymatic reduction of CFZ by NDH-2 following by non-enzymatic reoxidation of CFZ by O_2, leading to the production of toxic reactive oxygen species (ROS) (37). Hartkoorn et al. (38) report that CFZ-resistant mutants of *M. tuberculosis* map to the transcriptional regulator *Rv0678* leading to the upregulation of the multisubstrate efflux pump, MmpL5. The authors show that CFZ-resistant mutants are cross-resistant to bedaquiline suggesting a common mechanism of resistance (38). In slow- and fast-growing mycobacterial species, reduction in NDH-2 activity has been linked to isoniazid and ethambutol resistance (39). Taken together these studies suggest that the development of NDH-2 inhibitors will need to determine what effect these compounds have on current drug therapy regimens, particularly in regard to the development of cross resistance.

Structure-aided drug design against NDH-2 is now possible with the first high-resolution bacterial structures published (40, 41). The bacterial NDH-2 structure reveals a homodimeric organization and localization to the cytoplasmic membrane by the membrane-anchoring domain highlighted with two amphipathic C-terminal helices (40, 41). Unique binding sites for quinone and NADH sites were also proposed, allowing concomitant oxidation of NADH from the cytoplasm with reduction of quinone from the membrane with the ability of both substrates to access the FAD cofactor sequentially (40). This implies that NDH-2 harbours two potential drug target sites and both warrant investigation. Most common structure-based drug design approach relies on the protein-ligand complex structure model (42). The ligand-complex structures have been solved for both yeast (43) and bacterial NDH2 (Y. Nakatani, unpublished data) allowing for the rational design of small inhibitor molecules targeting the NADH-binding site, and with further quinone-ligand structures, the quinone-binding site.

Succinate:Quinone Oxidoreductase: A Chink in the Carbon-Metabolic Armor

Succinate dehydrogenase (SDH), or complex II (Figure 1), enzymes are well known for their role in the citric acid cycle. They couple the oxidation of succinate to the reduction of quinone via both FAD and heme co-factors (44), thereby playing important roles in both carbon metabolism and *pmf* generation. *M. tuberculosis* encodes two SDH enzymes (*sdh1*, *Rv0249c-Rv0247c*; *sdh2*, *Rv3316-Rv3319*), as well as a separate fumarate reductase with possible bidirectional behaviour. It is unprecedented in the current literature for an organism to encode three functionally redundant enzymes for this reaction, which complicates both physiological analysis and drug design. Furthermore, while a vast amount of literature exist regarding the SDHs of *E. coli* and mitochondria, the SDHs encoded by mycobacteria have distinct phylogeny, prosthetic groups and predicted biochemistry (9, 45). Despite these challenges, using SDHs as drug-targets remains promising, as succinate is a major focal point in both the central carbon metabolism and respiratory chain of *M. tuberculosis*. The bacilli must find a way to drive the endergonic oxidation of succinate (E_m = +113 mV versus E_m = −74 for menaquinone); a reaction required for generating a membrane potential under

hypoxia (9) and maintaining the balance of menaquinone:menaquinol (46). Under hypoxia, succinate can he electronically secreted or stored until a suitable electron acceptor is accessed (47). The versatility of succinate therefore suggests that disrupting its oxidation may result in clinically advantageous outcomes: inhibitors may have primary lethality, force a premature exit from non-replicating persistence or compromise bacilli reactivation, depending on which aspect of succinate metabolism is affected by modulating SDH activity.

Drug development targeting SDH enzymes will need to consider how to achieve selectivity for the multiple *M. tuberculosis* enzymes without off-target effects on human counterparts. Fortunately, several key differences between these homologues exist within the hydrophobic, menaquinone-binding portion of the complex. Enzymes are classified as Type A-E according to their heme content and number of transmembrane subunits (45, 48). Sdh2 is a Type A enzyme (2 subunits, 2 hemes) (49), while Sdh1 was proposed to be similar to the *Bacillus*-like Type B enzymes (1 subunits, 2 hemes) (9). The mammalian SDH and *M. tuberculosis* FRD are of different types (Type C and D respectively), and so are different in terms of electron-transfer and menaquinone-reduction. It follows that compounds targeting the hydrophobic subunits of SDH are ideally suited for achieving selective inhibition.

There have been no reported screens for inhibitors of mycobacterial SDH activity. A commonly used inhibitor of SDH is 3-nitropropionate, targeting the A subunit (50, 51), which has been reported to inhibit mycobacterial SDHs (9, 47). However, inhibitors developed against the dicarboxylate-binding site are likely to also inhibit mitochondrial SDH due to high A subunit similarity and hence this is likely to be a poor direction for lead candidate identification. Despite this, routine inhibitors such as the A subunit-targeting carboxin and quinone-mimic HQNO have been found to display selectivity between organisms (52) and so this enzyme may yet serve as a lesson about development of drugs against targets also present in mitochondrial genomes.

Alternative Dehydrogenases: OXPHOS Intrinsically Linked to Growth Reactivation

While mycobacteria are primarily heterotrophs, there is strong evidence that they can support chemolithotrophic on certain gases. *M. tuberculosis* is capable of supporting carboxydotrophic growth by utilizing carbon monoxide dehydrogenases (53) (CODH structural subunits, *Rv0375c-Rv0373c*) (Figure 1). These enzymes oxidize CO to CO_2 concomitant with the reduction of various types of acceptors, ferredoxins or cytochromes for example (54, 55). The mycobacterial enzymes have additionally been proposed to oxidize NO (56). Aerobic CODHs are three subunit enzymes that can be distinguished from their anaerobic counterparts by their molybdenum active sites (57, 58). They are typically induced under autotrophic conditions (59), although catabolite repression has been implicated in mycobacterial CODH regulation (60). Genetic essentiality has not been rigorously confirmed, although several transposon mutagenesis studies have putatively identified that the large subunit of CODH is essential (26, 61). The physiological electron acceptor of CODH has not been identified in mycobacteria and doing so would likely reveal a more robust site for drug design, as opposed to targeting the gas-binding catalytic site.

While CO is abundant during host infection (62) and is likely an important energy source for the bacillus, the possibility of deleterious effects during CODH inhibition must be

considered. CO and NO are inducers of the mycobacterial DosR response (63–65), both being apparent substrates of the mycobacterial CODH (56). As suggested previously (66), the metabolism of CO by CODH could serve as a reactivation signal by depleting the inducer. The potential metabolism of NO by CODH only further serves this hypothesis. It is therefore possible that inhibiting CODH will force a greater proportion of bacilli into a non-replicating persistent state, by allowing accumulation of DosR inducers. Instead, CODH is a very promising target for activators, as opposed to inhibitors, as it could force these drug-resistant persisters to reactivate growth. This would allow the repurposing of classical anti-tubercular compounds in effective short-term regimens. Currently only molecules with broad-spectrum inhibitory activity, such as cyanide and derivative isonitriles, are reported (67) and no activators have been reported at the time of writing.

Another potential respiratory electron donor in mycobacteria is $F_{420}H_2$. A low-potential two-electron carrier, F_{420} plays a unique and central role in the redox metabolism of mycobacteria, but is absent from human cells and gut microbiota (68). F_{420} is reduced during central carbon catabolism of *M. tuberculosis* by an F_{420}-dependent glucose 6-phosphate dehydrogenase (69). Multiple $F_{420}H_2$-dependent reductases in turn couple the reoxidation of $F_{420}H_2$ to the reduction of diverse endogenous and exogenous heterocyclic organic compounds (68, 70, 71), among them quinones. It has been demonstrated that three such reductases of the split β-barrel family in *M. tuberculosis*, Rv3547, Rv1558, and Rv3178, can reduce quinone compounds through hydride transfer (72). It has been proposed that this activity maintains the quinone pool in a reduced state during oxidative stress (72); while rapid activity has been observed with nonphysiological quinones (72), it has yet to be confirmed if $F_{420}H_2$ oxidation can reduce menaquinone and generate *pmf* in whole cells. Irrespectively, mutants unable to synthesize or reduce F_{420} are hypersusceptible to oxidative stress, antibiotic treatment, and hypoxia (72–74); hence inhibitors of the F_{420} system (including F_{420} analogs) might selectively kill persistent mycobacteria and would act synergistically with first-line antimycobacterials. However, there may be even more promise in exploiting the mycobacterial FDORs to activate prodrugs. The 5-nitroimidazoles delamanid and pretomanid are reductively activated by Rv3547, resulting in production of reactive nitrogen species and *des*-nitro products that are proposed to kill *M. tuberculosis* through a combination of respiratory poisoning and inhibition of mycolic acid synthesis (71, 75–77). Delamanid is the second new drug in 40 years (following bedaquiline) to be clinically-approved for TB treatment (78). Acquired resistance to delamanid in XDR-TB has been reported to be mediated by mutations in *fbiA* (F_{420} biosynthesis protein) and *fgd1* (F_{420}-dependent glucose-6-phosphate dehydrogenase) (79).

The enzymes discussed above demonstrate the potential array of treatment options that could be achieved with sufficient ingenuity. Valuable outcomes, such as direct lethality and modulation of growth state, can be hypothesized and the potential to overcome undesirable target properties are readily apparent. NDH-2 has been extensively studied and large high throughout screens for inhibitors performed with some success (20, 30, 80). Other primary dehydrogenases have received little attention. *M. tuberculosis* encodes far more primary dehydrogenases that are not covered here due to paucity of understanding in the context of mycobacterial oxidative phosphorylation. Most notably, there is need to understand the role of the tricarboxylic acid cycle-linked malate:quinone oxidoreductase (*Rv2852c*) in the redox

and ion homeostasis of *M. tuberculosis* (81, 82). Two quinone-linked glycerol-3-phosphate dehydrogenases (Rv3302c & Rv2249c) need to dissected from a further two NAD(P)H linked counterparts (Rv0564c & Rv2982c). In addition, the physiological conditions that would promote the activity of two *L*-Lactate dehydrogenases (Rv0694 & Rv1872) need to be assessed and it is not yet determined if quinone reduction by proline dehydrogenase occurs purely as a consequence of its previously demonstrated methylglyoxal-detoxification activity (83).

MENAQUINONE BIOSYNTHESIS: PROMISING DRUG TARGETS

Lipoquinones serve as the membrane-bound electron shuttles between primary dehydrogenases and terminal reductases in respiratory chains. Whereas ubiquinone (UQ) serves as the predominant quinone in mitochondria and many Gram-negative bacteria, menaquinones (MK) are the predominant lipoquinones of mycobacteria and many other Gram-positive bacteria (84). It has recently been reported that polyketide derived quinones (PkQ) are alternate lipoquinones that are expressed and function as electron carriers in mycobacterial biofilms (85). The biosynthesis of MK requires two separate pathways (Figure 5). 1,4-Dihydroxy-2-naphthoate is synthesized from chorismate. The naphthoate ring is then prenylated with a polyisoprenyldiphosphate, derived from isopentenyl diphosphate and dimethylallyl diphosphate, to form demethylmenaquinone and, subsequently, the C2 position of the ring structure is methylated. In mycobacteria the β-isoprene unit of the prenyl group is reduced to form MK-9 (II−H_2) after the formation of MK (86). MK synthesis has been relatively extensively studied in *E. coli* (due in part to the availability of the *men* mutants, which can easily be generated in this organism, as it can utilize ubiquinone as an electron carrier in aerobic conditions). In *E. coli* the synthesis of MK is accomplished by nine enzymes (MenA−MenI, and UbiE, see Figure 5). These enzymes are encoded by 2 clusters of genes. The *men* cluster consisting of the *menB*, *C, D, E, F, H* and a separate cluster containing *menA* and *ubiE*. It was originally thought that MenB catalyzed the conversion of 2-succinylbenzoyl-CoA to 1,4-dihydroxy-2-naphthoate; however recent evidence indicates that MenB forms 1,4-dihydroxy-2-naphthoyl-CoA, which MenI then hydrolyzes to 1,4-dihydroxy-2-naphthoate (87). MK synthesis in Gram-positives in general has largely been under-represented in the literature; however, the general pathway in *M. tuberculosis* appears to be similar to that of *E. coli*. In *M. tuberculosis* the *menA-E* genes appear to be found in a single cluster along with the two genes annotated as possible methyl transferases involved in lipoquinone synthesis (*Rv0558* and *Rv0560c*). One or both of these genes presumably encode the protein(s) (MenG, which has analogous function to UbiE in *E. coli*) that methylate demethylmenaquinone. The gene encoding the protein with the most similarity to MenF in *E. coli* is *Rv3215* annotated as *entC* (isochorismate synthase) and the gene encoding the protein most similar to MenI is *Rv1847*. In addition, *M. tuberculosis* harbours *Rv0561c*, which is clustered with *menA-E and* encodes MenJ the enzyme that reduces the β-isoprene of MK (86). Interestingly, *M. tuberculosis* does not have a gene that is easily identifiable as encoding a protein with similar function to MenH. Rv0045c, Rv1938 and Rv2715 are all potential candidates, although none encode a protein with a high degree of similarity to MenH from *E. coli*. The isoprenoid tail of the menaquinone must be generated by a isoprenyl diphosphate synthase as described above and

together with 1,4-dihydroxy-2-napthoic acid are the substrates for MenA (Rv0534c). However, the specific synthase generating this isoprenyl diphosphate has yet to be identified.

Surprisingly, not all of the enzymes involved in the mycobacterial MK biosynthetic pathway appear to be viable drug targets, or even essential. Initial studies (25) predicted that the mycobacterial *menC*, *menD*, *menE*, and *menF* genes were essential for bacterial survival. Subsequently, these predictions were supported by high-resolution phenotypic profiling experiments (26), which added *men*F, and *men*A to the list of predicted essential genes. MenA [demethylmenaquinone synthase, Rv0534c (88, 89)], MenB [1,4-dihydroxy-2-naphthoyl-CoA synthase, Rv0548c (20–22)], and MenE [*o*-succinylbenzoyl-CoA synthase, Rv0542c (90–92)] from mycobacteria are under study as potential drug targets having been genetically or pharmacologically demonstrated to be essential. This is exemplified by the development of Aurachin RE analogues, which inhibit MenA and result in growth inhibition of drug-resistant *M. tuberculosis* (89). It seems probable that MenC, D and F are potential drug targets in addition to MenA, B and E. MenG has yet to be definitively identified in mycobacteria. As noted above two genes are annotated as possible methyl transferases involved in lipoquinone synthesis (*Rv0558* and *Rv0560c*). Both of these genes reside in the Men cluster in *M. tuberculosis*; however, only *Rv0558* is predicted to be essential (25, 26, 93). Thus, further study is indicated.

MenI has only recently been identified as the enzyme that hydrolyzes 1,4-dihydroxy-2-naphthoyl-CoA to 1,4-dihydroxy-2-naphthoate in *E. coli* (87). An orthologous gene has not been positively identified in mycobacteria, although *Rv1847* appears to be the highest probability match. This gene is predicted to be non-essential for mycobacterial survival (25, 26, 93). This is perhaps unremarkable as deletion of MenI in *E. coli* does not eliminate MK synthesis, only reducing the levels of MK in the bacteria by 67% (87), suggesting other non-specific thioesterase activities can compensate. Similarly *Rv0045c*, *Rv1938* and *Rv2715* encode potential, but low probability, candidates for 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase (MenH) in *M. tuberculosis*, none of which are predicted to be essential. In this case it should be noted that 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate can undergo spontaneous elimination to form 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (94). Thus, mycobacterial MenI and MenH do not appear to be likely drug targets. MenJ is unique among the *men* genes in that it is non-essential for growth in culture (25, 26, 86), but is essential for bacterial survival in mouse macrophages (86, 95). Thus, MK with partially saturated isoprenyl moieties appear to be a novel virulence factor and MenJ is a contextually essential enzyme and a potential drug target (86). Sulfated MK synthesis does not appear to present an important drug target. The function of this unique lipid is, as yet, unknown; however, it has been reported that the synthesis of sulfated MK, reduces the virulence of the organism in mouse infection models (96).

TARGETING OXYGEN REDUCTION IN MYCOBACTERIA

All mycobacteria sequenced to date harbour genes for a cytochrome *c* pathway consisting of a cytochrome bc_1 (related to the mitochondrial complex III) and an aa_3-type cytochrome *c* oxidase (complex IV) (Figure 1). The cytochrome bc_1 transfers electrons from menaquinol

to the cytochrome *c* oxidase, a process linked to proton translocation across the membrane. Since the cytochrome *c* oxidase is also capable of pumping protons, this pathway is the most energetically favourable respiratory branch in mycobacteria. In contrast to mitochondria, actinobacteria do not possess genes for soluble cytochrome *c* or any other *c*-type cytochrome (97, 98). Instead, the complex III and IV form a supercomplex that facilitates the direct transfer of electrons from menaquinol to oxygen (98–100). The bc_1 complex (encoded by the *qcrCAB* operon) is composed of the cytochrome *b* (*qcrB*) that contains two *b*-types heme groups, a 2Fe-2S iron-sulfur cluster located on the Riske protein QcrA, and a di-heme c-type cytochrome c_1 (*qcrC*), as initially described in *Corynebacterium glutamicum* (101).

The aa_3-type cytochrome *c* oxidase is encoded by the *ctaB*, *ctaC*, *ctaD* and *ctaE* genes. The genes *ctaD* and *ctaC* are in close proximity with the *qcrCAB* operon, whereas *ctaB* and *ctaE* are located elsewhere in the genome. The cytochrome *c* oxidase contains three redox centers: CuA (located on CtaC, subunit II) and the haem *a* (located on CtaD, subunit I) are the primary electron acceptors from the bc_1 complex, whereas the a_3-CuB unit (located on CtaD) is the oxygen-reducing element. The cytochrome *c* oxidase is annotated as essential (25), whereas attempts to delete *qcrCAB* in *M. tuberculosis* were unsuccessful (102), suggesting that the cytochrome *c* pathway is required for the survival of slow-growing mycobacteria. *qcrCAB* could be deleted in *Mycobacterium smegmatis*, but led to a profound growth impairment *in vitro* (102). Recently, the thioredoxin CcsX was shown to be required for haem insertion into membrane-bound heam-contaning proteins (103). Deletion of *ccsX* in *M. tuberculosis* had a marked growth defect due to a deficient haem insertion in membrane-proteins, including QcrC. Nevertheless, the mutant strain could still multiply (103), suggesting that the perturbation, or inactivation, of the cytochrome *c* branch may be viable under certain circumstances. Interestingly, the cytochrome *bd* oxidase was upregulated in the *ccsX* mutant strain, suggesting that the cytochrome *bd* oxidase can act as a robust alternate terminal oxidase when the integrity of the cytochrome *c* branch is compromised (103).

Several inhibitors of the cytochrome bc_1 are known. The archetype is stigmatellin, a natural antibiotic that inhibits most cytochrome bc_1 by impeding the interaction of the quinol with the QcrB subunit. The recent discovery of small-molecules targeting QcrB has triggered interest on the respiratory cytochrome *c* pathway (104–07). A number of groups have identified a series of imidazopyridine amide (IPA) compounds that interfere with energy metabolism (104–06, 108). The most advanced derivatives of the IPA series are active in the low nanomolar range *in vitro* (106, 108, 109). The series is surprisingly highly selective to mycobacteria since it does not inhibit the growth of any other bacteria or microorganism classes that were tested (104, 106). Whole-genome sequencing of spontaneous-resistant mutants to the IPA drugs revealed that a single amino acid substitution at the position 313 in QcrB confers high resistance to Q203 (104, 106). Subsequently, additional mutations were identified in a strain deficient for the expression of the cytochrome *bd* oxidase (105). Since all the mutations conferring resistance are in close proximity to the Qp menaquinone-binding site, it is likely that the IPA compounds inhibit respiration by interfering with the binding of menaquinol at the Qp site of QcrB. Even though target engagement remains to be demonstrated, the observation that the IPA series trigger a rapid ATP depletion (105, 106) suggests that the cytochrome bc_1 is the direct target. The drug candidate Q203 recently

progressed to clinical development phase I under a US-FDA investigational new drug application, marking the first step to validate the vulnerability of the cytochrome *c* pathway in human tuberculosis.

Several other compound series targeting QcrB have been reported (105, 107), arguing for a high vulnerability of this respiratory branch under conditions used for compound screening. Of particular interest, the approved drug lansoprazole is a prodrug targeting QcrB in mycobacteria (107). Despite the high vulnerability of the cytochrome bc_1, it is puzzling to note that all the inhibitors discovered to date bind to a narrow region of the QcrB subunit that is predicted to interact with menaquinol. A better understanding of the biology of the cytochrome *c* branch may allow for the identification of lead molecules that bind to alternate positions. Although exciting drug development advances have been made, much work remains to be done to understand the biology of the cytochrome bc_1, and under which conditions the cytochrome *c* branch is essential for survival. As such, it was observed that the high expression of the cytochrome *bd*-type oxidase in laboratory strains of *M. tuberculosis* can alleviate partly the potency of cytochrome bc_1 inhibitors (105). Although this was not the case for clinical isolates that seem to regulate more tightly cytochrome *bd* expression (105), it is imperative to clarify the synthetic genetic interaction between the two terminal oxidases to exploit the full potential of QcrB inhibitors for the treatment of tuberculosis. Furthermore, the conditions under which alternate terminal acceptors can compensate for the inhibition of the bc_1 complex must also be delineated in order to develop a rational drug combination targeting oxidative phosphorylation. It is interesting to note that ongoing reductive evolution in *Mycobacterium leprae* and *Mycobacterium ulcerans* resulted in the deletion of the cytochrome *bd* oxidase, nitrate reductase and fumarate reductase (21, 110), leaving the cytochrome *c* oxidase branch as the only functional terminal electron acceptor. Therefore, drugs targeting the cytochrome bc_1 hold great promise for the treatment of leprosy and Buruli ulcer infections.

Cytochrome *bd*-type Oxidase and CydDC

M. tuberculosis and other mycobacterial species harbour genes for the cytochrome *bd*-type menaquinol oxidase (*cydAB*) (111) (Figure 1). Cytochrome *bd* oxidase (CbdO) could be viewed as one of the most scientifically neglected and least understood respiratory enzymes in the electron transport chain of *M. tuberculosis*. The rather modest interest in this *M. tuberculosis* enzyme might stem from its dispensability for optimal growth and survival in mouse models (25, 61). In the absence of structural or biochemical data for CbdO of *M. tuberculosis* it is assumed that this enzyme functions similarly to its well characterized *E. coli* homolog as a high-affinity terminal oxidase that accepts electrons from menaquinol to reduce oxygen under hypoxic conditions (112–14). This enzyme activity contributes to the maintenance of a *pmf* and facilitates the scavenging of oxygen necessary to colonize oxygen poor niches or to protect oxygen labile enzymes (112, 114– 116). From an energetic point of view, cytochrome *bd* oxidase is less efficient than cytochrome *c* oxidases because it does not pump protons, but instead generates a *pmf* by transmembrane charge separation at an H^+/e ratio of 1 (113). This would appear useful for cells under conditions where the role of CbdO is not primarily to create a *pmf*, but serve as an electron sink, for example during reductive, oxidative, or nitrosative stress defenses (117) or during disulfide-bond formation (114).

Downstream of the *cydAB* gene locus of *M. tuberculosis* another operon, *cydDC*, encodes a putative ABC-transporter. In *E. coli* this transporter was shown to transport glutathione and cysteine to the periplasm where these molecules contribute to redox homeostasis and disulfide bond formation (114). Similar to CbdO, CydDC of *M. tuberculosis* is largely uncharacterized and its physiological role is unclear. Mouse infection studies with transposon mutant libraries indicate that mutants with insertions in CydDC are at a disadvantage compared to the wild-type cells (118). However mouse challenge with single *cydC* mutants show no growth attenuation and only a subtle decrease in bacillary loads in lungs during latent infection (119), although a subsequent study was unable to reproduce this phenotype (120). It is important to note that mice do not form hypoxic granulomas, which could explain why neither *cydAB* nor *cydDC* mutants are attenuated in this animal model.

Cytochrome *bd* oxidase and the CydDC transporter appear to protect mycobacteria from chemotherapeutic challenge. For example, disruption of *cydC* in *M. tuberculosis* caused increased bacterial clearance in mouse model infections treated with isoniazid when compared to wild-type infections (120). An even more striking observation has been the role of CbdO, specifically, in protection of *M. tuberculosis* from respiratory chain inhibitors. Berney *et al.* demonstrated that *cydA* deletion greatly enhances the early bactericidal activity, killing in the first seven days, in *M. tuberculosis* treated with the F_1F_0-ATP synthase inhibitor bedaquiline (121). In another study, *cydA* inactivation in *M. tuberculosis* H37*Rv* enhanced the minimum inhibitory concentration (MIC) of cytochrome *c* oxidoreductase inhibitors by more than four orders of magnitude (105). Consistent with these data, deletion of *cydA* in *M. smegmatis* greatly decreased the MIC of bedaquiline (17) and led to complete sterilization after clofazimine treatment for 72 hours, while effects on the wild-type and cytochrome bc_1 mutant were only bacteriostatic (122). However, the mechanism of action of this phenotypic resistance and the more pronounced role in protection from respiratory chain inhibitors is unclear.

CdbO and CydDC enzymes also appear important in adaptation to adverse conditions and persistence. Kana *et. al.* showed that inactivation of *cydA* in *M. smegmatis* inhibits cell growth under hypoxic conditions (111). It is intriguing to note that inactivation of the cytochrome c maturation pathway in *M. tuberculosis* led to an upregulation of CbdO concomitant with increased resistance to hydrogen peroxide (103). Accordingly *cydA* deletion in *M. smegmatis* (122) and *M. tuberculosis* (Berney, M. unpublished results) increases susceptibility to peroxide. Taken together, with the observed protection from antibiotics one can assume that cytochrome *bd* oxidase and potentially CydDC are important in pathogenicity and adaptation to adverse conditions. Interestingly, *cydAB* and *cydDC* are upregulated as part of hypoxia induced dormancy (123) and may facilitate *M. tuberculosis* transition to non-replicating persistence (124). Bacilli in this state are tolerant to antibiotics (125, 126), and it is possible that the phenotypic resistance to drugs is due in part to a CbdO-facilitated transition to persistence.

Neither cytochrome *bd* oxidase nor the CydDC transporter appears to be essential for the survival or normal growth of *M. tuberculosis* under standard conditions. However, their unique roles discussed above and the lack of mammalian homologues warrants their

investigation as potential drug targets. When *M. tuberculosis* is treated with bedaquiline, the dormancy regulon and ATP-generating pathways, including *cydAB and cydDC* genes, are activated and induce metabolic remodeling that delays bactericidal activity (127). As noted, inactivation of CbdO inhibits this phenotypic resistance and promotes *de novo* early bactericidal activity of BDQ (121). It is possible that similar mechanisms of resistance are employed in response to a broad range of chemotherapeutics (128). Furthermore, CbdO is likely the predominate terminal oxidase under low oxygen tensions (111, 129, 130) such as those found in hypoxic granulomas (131) and may facilitate the survival of latent or persistent bacilli (132, 133). Therefore, it is conceivable that inhibitors of CbdO or CydDC would enhance the eBA of existing chemotherapeutics and target non-replicating bacilli populations, which could effectively reduce the currently lengthy therapy timelines (134).

The only cytochrome *bd* oxidase-selective inhibitors currently identified are aurachin D and its analogues (135). Aurachin D is a quinolone-type drug that prevents menaquinol reduction (135) by competitive inhibition at the quinol-binding domain of CbdO (136). Its selective inhibition of CbdO has been validated in *M. smegmatis* by measuring oxygen consumption of membrane vesicles (122). Although its efficacy as a chemotherapeutic and toxicity to mammals has not been studied. To our knowledge, there have not been any inhibitors of the CydDC transporter that have been identified. This general lack of development is likely because inhibitors of these enzymes are not a promising source of stand-alone drugs for treatment of *M. tuberculosis* under aerobic conditions. However, the attributes of CbdO and CydDC discussed here suggest that they may be valuable drug targets to enhance efficacy and reduce treatment timelines of current chemotherapy regimens.

Alternative Reductases: Critical for Redox Homeostasis During Hypoxia

There is strong evidence that *M. tuberculosis* uses the alternative electron sinks nitrate, nitrite, and fumarate to maintain redox balance during hypoxia. *M. tuberculosis* exploits host defences in order to generate the respiratory electron acceptor nitrate. The organism converts the nitric oxide (NO) produced by host iNOS in the human macrophage to nitrate by secreting the nitric oxide dioxygenase HbN (137, 138). It in turn imports the nitrate produce with a specific transporter (NarK2), reduces nitrate to nitrite with a membrane-bound respiratory nitrate reductase (NarGHJI), and detoxifies the nitrite to ammonium with a cytosolic NADH-dependent nitrite reductase (NirBD) (139, 140). While nitrate reductase and nitrite reductase are constitutively expressed, the nitrate transporter is under tight transcriptional control by the NO- and hypoxia-induced DosS/DosT-DosR system (119, 141, 142), and hence the rate of nitrate reduction increases in hypoxic cells concurrent with reduction of the respiratory chain (140). While the physiological role of this pathway is incompletely understood, it appears to enhance the flexibility of mycobacteria in response to reductive stress: nitrate supplementation enhances the survival of *M. tuberculosis* cultures following sudden anaerobiosis or phenothiazine treatment (143, 144). The nitrite produced can be exported from the cell or alternatively reduced to ammonium; the nitrite reductase that mediates this process is essential for survival of *M. tuberculosis* both in the Wayne model and human macrophages, likely due to its combined roles in nitrite detoxification and nitrogen assimilation (139, 145, 146). Given the multifaceted roles of nitrate and nitrite reduction in this pathogen, there may be potential in developing small-molecule inhibitors of

nitrate reductase and nitrite reductase. Host-directed therapies aimed at reducing NO production also show promise (147), though could also be counterproductive given NO also has innate cytotoxic effects: administration of the iNOS2 inhibitor N^6-(1-iminoethyl)-L-lysine in fact accelerated progress of *M. tuberculosis* infection in a murine model (148).

There is also a weight of evidence that *M. tuberculosis* depends on fumarate reduction to adapt to hypoxia. Two groups have independently demonstrated that *M. tuberculosis* operates a reverse tricarboxylic acid cycle during hypoxia resulting in fumarate production and succinate excretion (8, 47). Given succinate is a multifunctional molecule, this remodelling may serve several purposes: i) respiratory electron transport to fumarate generates *pmf* by a redox-loop mechanism, ii) fermentative succinate excretion to the extracellular milieu dissipates excess reductant, and iii) succinate may be used for anaplerosis or respiration according to cellular needs (8, 47). In contrast to its saprophytic relatives, *M. tuberculosis* has acquired a canonical fumarate reductase that likely mediates the majority of these activities. There is evidence that the two aforementioned annotated succinate dehydrogenases in its genome (Sdh1, Sdh2) can operate in reversed direction to compensate for loss of this enzyme (8, 9, 46, 47). Consistent with such roles, both the canonical fumarate reductase and Sdh2 are strongly upregulated and highly active under hypoxia (8, 9, 46, 149). Given this functional redundancy, genes encoding these enzymes can be individually but not collectively deleted (8, 9, 47) and hence it may be difficult to develop effective inhibitors against the fumarate reduction pathway. However, an exciting precedent has been set by the discovery of nanomolar affinity natural products (e.g. nafured, verticipyrone) that inhibit eukaryotic parasites by targeting mitochondrial fumarate reductases (150). The finding that succinate may be excreted as a fermentative end-product in mycobacteria is also worthy of special attention (8). Other recent work has suggested that, while mycobacteria strictly requires respiration for growth, they may resort to fermentation if all respiratory electron acceptors are exhausted (151). It is important to gain a further understanding of the electron sinks that *M. tuberculosis* use to maintain redox balance in order to evaluate current and discover new drug targets in mycobacterial energetics.

ATP SYNTHESIS BY THE F_1F_o ATP SYNTHASE: A CLINICALLY-VALIDATED TARGET

In *M. tuberculosis* and other mycobacterial species, ATP is synthesized via substrate level phosphorylation and oxidative phosphorylation using the membrane-bound F_1F_0ATP synthases (encoded by the *atpBEFHAGDC* operon, Rv1304-1311). The F_1F_0ATP synthase catalyzes ATP synthesis by utilizing the electrochemical gradient of protons to generate ATP from ADP and inorganic phosphate (P_i) and operates under conditions of a high *pmf* and low intracellular ATP. The enzyme is also capable of working as an ATPase under conditions of high intracellular ATP and an overall low *pmf* (152). As an ATPase, the enzyme hydrolyzes ATP, while pumping protons from the cytoplasm to the outside of the cell. The ATP synthase of mycobacteria has been studied in detail at a biochemical level in *M. phlei* and shown to exhibit latent ATPase activity (10). ATPase activity could be activated by trypsin treatment and magnesium ions, but the mechanism of activation was not elucidated. Recent experiments with inverted membrane vesicles of *M. bovis* BCG and *M. smegmatis*

demonstrate latent ATPase activity that could be activated by methanol and the *pmf*, suggesting regulation by the epsilon subunit and ADP inhibition (6). The reason for the extreme latency in ATP hydrolysis of the mycobacterial ATP synthase is unknown, but may represent an adaptation to function at low *pmf* and under hypoxia. Hypoxic non-replicating cells of *M. tuberculosis* generate a *pmf* in the order of -100 mV and the ATP synthase inhibitor TMC207 is bactericidal towards these cells demonstrating that the ATP synthase still continues to function at relatively low *pmf* (3).

The F_1F_0ATP synthase in *M. tuberculosis* and *M. smegmatis* has been shown to be essential for optimal growth (25, 153). In other bacteria, the F_1F_0-ATP synthase is dispensable for growth on fermentable carbon sources (154, 155), where increased glycolytic flux can compensate for the loss of oxidative phosphorylation. This strategy does not appear to be exploited by *M. smegmatis*: the F_1F_0-ATP synthase is essential for growth even on fermentable substrates, suggesting that ATP production from substrate level phosphorylation alone, despite increased glycolytic flux, may be insufficient to sustain growth of these bacteria (153). This may be due to an extraordinarily high value for the amount of ATP required to synthesize a mycobacterial cell, a possibility that requires further investigation (156). Alternatively, in conjunction with a high ATP demand for growth, the ATP synthase may be an obligatory requirement for the oxidation of NADH by providing a sink for translocated protons during NADH oxidation coupled to oxygen reduction (153). Such strict coupling would imply that mycobacteria do not support uncoupled respiration; either they lack a conduit for proton re-entry in the absence of the F_1F_0-ATP synthase or they are unable to adjust the proton permeability of the cytoplasmic membrane to allow a futile cycle of protons to operate. In this context, the cytoplasmic membrane of *M. smegmatis* has been shown to be extremely impermeable to protons (157). In *M. tuberculosis*, the *atp* operon is downregulated during growth in macrophages (158), the mouse lung and in cells exposed to NO or hypoxia (119). The *atp* operon of *M. bovis* BCG and *M. smegmatis* is downregulated in response to slow growth rate (159, 160). When slow growing cells of *M. smegmatis* (70 h doubling time) with low levels of *atp* operon expression are exposed to hypoxia (0.6% oxygen saturation), the *atp* operon is upregulated 3-fold, suggesting an important role for this enzyme during adaptation to hypoxia (159).

Several new anti-tubercular compounds have been reported that target oxidative phosphorylation in mycobacteria (11, 20, 88). The most promising compounds clinically, the diarylquinolines, have been shown to target the F_1F_0-ATP synthase and inhibit ATP synthesis (11–13). The FDA approved the use of a diarylquinoline (i.e. first-in-class compound bedaquiline, BDQ) for treatment of MDR-TB in 2012, which was the first drug licensed in forty years for TB disease. BDQ was developed in an attempt to improve outcomes in MDR-TB patients due to the sub-optimal effectiveness and toxicity of currently available drugs and regimens. BDQ has fast-acting bactericidal *in vivo* activity in different animal models and in TB patients against several mycobacterial species, both susceptible and resistant to all first-line and many second-line drugs, (161, 162). However, resistance to BDQ has already been reported (127) and Phase 2 clinical trials showed a higher mortality rate in subjects assigned to the bedaquiline cohort compared to the placebo group (163). Additionally, BDQ accumulates in tissues and has a prolonged half-life, taking 8 weeks to reach peak exposure and displaying a terminal half-life of 4 to 5 months.

Genome sequencing of both *M. tuberculosis* and *M. smegmatis* mutants that are resistant to diarylquinolines (i.e. TMC207) revealed that the target of these compounds is the oligomeric *c* ring (encoded by *atpE*) of the enzyme (11, 14, 15). The high resolution X-ray structure of the oligomeric *c* ring of *Mycobacterium phlei* has been solved complexed with BDQ. The structure reveals that BDQ interacts with the oligomeric c ring via numerous interactions (hydrophobic, hydrophilic, and electrostatic) completely covering the c-ring's proton-binding sites thus explaining the high-affinity (nM) binding of BDQ to the *M. phlei* c-ring and the measured low MIC values of the BDQ towards *M. tuberculosis* (11). The binding of BDQ to the c-ring prevents the rotor ring from acting as a proton shuttle and stalls ATP synthase operation (16). The structures explain how diarylquinolines specifically inhibit the mycobacterial ATP synthase and thus will enable structure-based drug design for next-generation ATP synthase inhibitors against *M. tuberculosis* (16, 164, 165).

When mycobacterial cells (growing or non-growing) are treated with BDQ, time-dependent (not dose-dependent) killing is observed (11, 127). The mechanism of killing is not clear, but does not involve the dissipation of the membrane potential, which is lethal to all living cells. A dose-dependent decrease in intracellular ATP has been observed when *M. tuberculosis* cells are treated with TMC207 (12, 13), but these data do not explain cell death because mycobacterial cells can be depleted of ATP and yet remain viable (166). We have shown that BDQ kills non-replicating mycobacterial cells by a unique mechanism that involves uncoupling of the electron transport chain (through ATP synthase), leading to a futile cycling of protons that causes cell death (17). A striking observation during this work was the activation of respiration by BDQ suggesting a protonophoric-like activity. As discussed above, this was due to binding the *c* subunit of the F_0 subunit.

A NEED TO UNDERSTAND ENERGETIC PLASTICITY AND ANTIMICROBIAL RESISTANCE/SUSCEPTIBILITY

The energetic targets discussed in this chapter play essential roles in mycobacterial metabolism and respiration under different host conditions. There is a need for continued fundamental research to clarify the molecular interactions and compensatory expression between various energetic targets in order to develop a rational drug combination targeting oxidative phosphorylation. The discovery of bedaquiline demonstrates that energetic targets provide a pathway to discover fast acting drugs that eradicate replicating and non-replicating cells. The mode of bedaquiline action further highlights the multi-targeting nature of these molecules. The promise of respiration and oxidative phosphorylation as a new target space is highlighted by the discovery that bacterial respiration is essential for the killing of *Escherichia coli* by ampicillin, gentamicin and norfloxacin (167). In *E. coli*, cytochrome *bo* and cytochrome *bd* mutants are resistant to the killing effects of ampicillin, gentamicin and norfloxacin (167). In contrast to *E. coli*, cytochrome *bd* mutants of *M. tuberculosis* (121) and *M. smegmatis* (17, 122) become hypersusceptible to bedaquiline, clofazimine, and hydrogen peroxide (17, 121, 122). These data suggest the inhibitors of cytochrome *bd* would indeed be synergistic with bedaquiline and clofazimine making this a priority target for inhibitor discovery.

When *M. tuberculosis* is grown in aerobic batch culture the rate of oxygen consumption is precisely regulated as a function of percent air saturation (46). Under these conditions, cells are able to direct electron flow to both terminal respiratory oxidases (cytochrome *bd* and aa_3-type cytochrome *c* oxidase) allowing the cell to rapidly adjust to changes in the *pmf* and direct electrons to the appropriate oxidase (proton pumping or non-proton pumping) in response to physiological demand (17, 122). The mechanisms that control the rate of oxygen consumption by *M. tuberculosis* are not known. Succinate dehydrogenase and NADH dehydrogenase mutants of *M. tuberculosis* are perturbed in oxygen management leading to higher rates of oxygen consumption during normal growth and a survival defect in stationary phase (46). These data suggest that the identification of molecules that activate respiration in *M. tuberculosis* may be effective in killing non-replicating cells and synergizing with current TB drugs. The scope that exists for modulating TB metabolism sets the scene for several exciting innovations and discoveries, a promising contrast to other targets that may soon reach the point of saturation.

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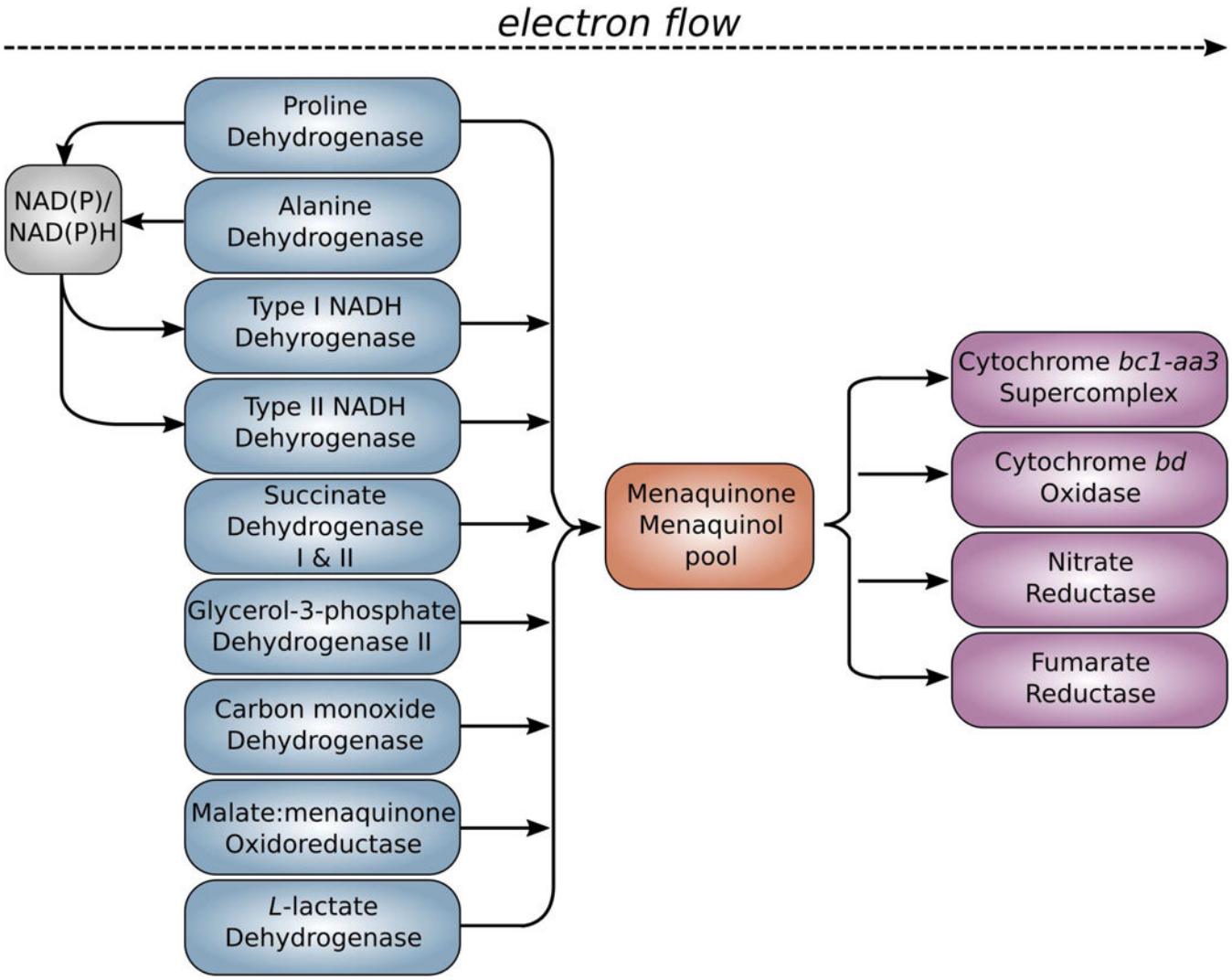

Figure 1.

Generalized schematic overview of relevant electron transfer components of *Mycobacterium tuberculosis*. Complexes indicated in blue oxidize various substrates to reduce quinones. The resulting (mena)quinol molecules (orange) can be oxidized to result in reduction of various terminal electron acceptors, mediated by the complexes indicated in purple.

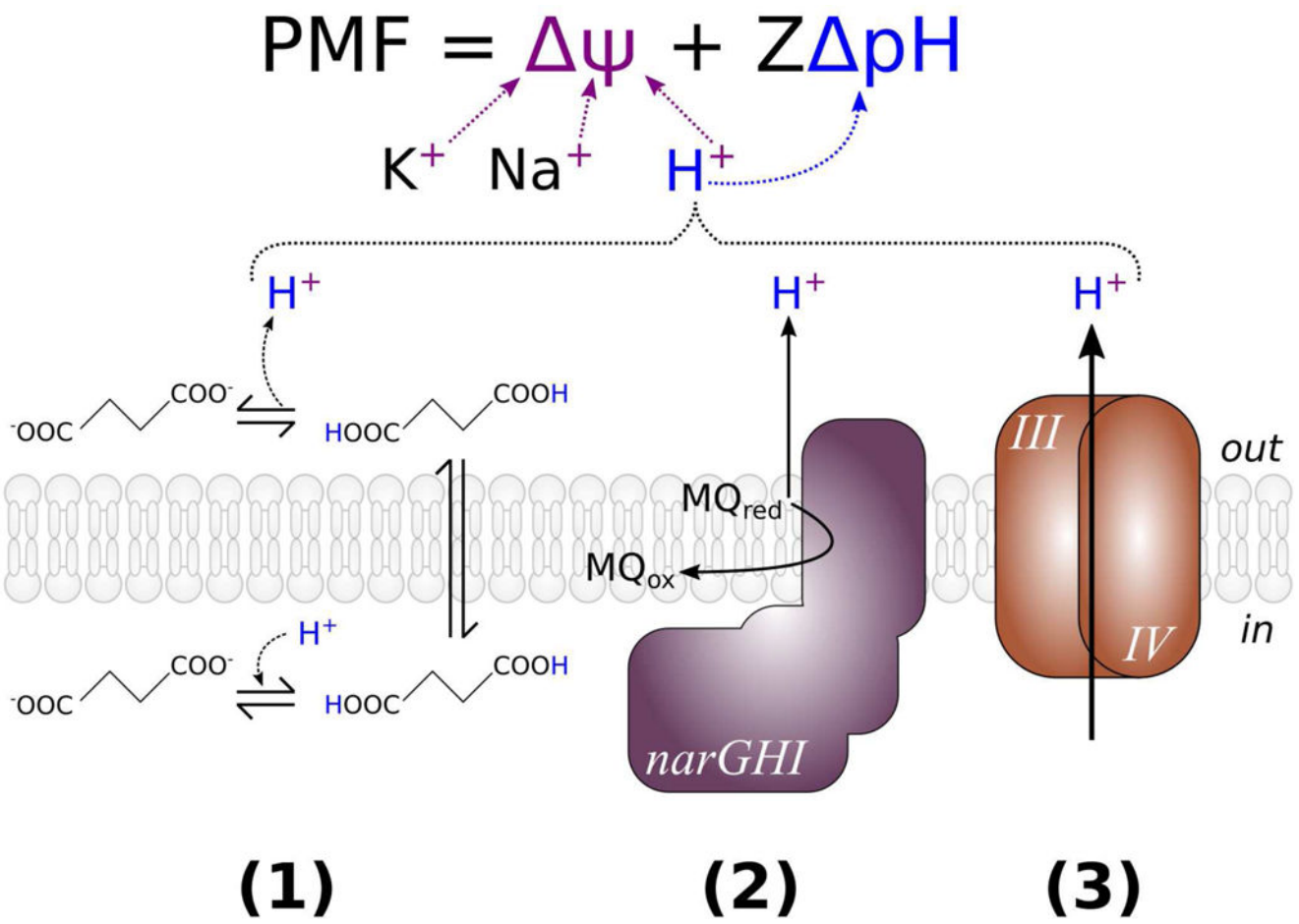

Figure 2. Mechanisms (1–3) by which a protonmotive [(membrane potential (Δψ) + transmembrane pH gradient (ZΔpH)] force can be generated in mycobacteria. (1) Co-transport of protons driven by solute (succinate) symport into the periplasm. (2) Redox-loop separation of charge; (mena)quinol oxidation results in proton release into the periplasm by virtue of (mena)quinol site proximity to the periplasm, while electrons are transferred to reduce a terminal electron acceptor (e.g. nitrate, fumarate) in the cytoplasm that results in neutralization of charge. (3) Proton translocation mediated by primary proton-pumping complexes (bc_1–aa_3 supercomplex).

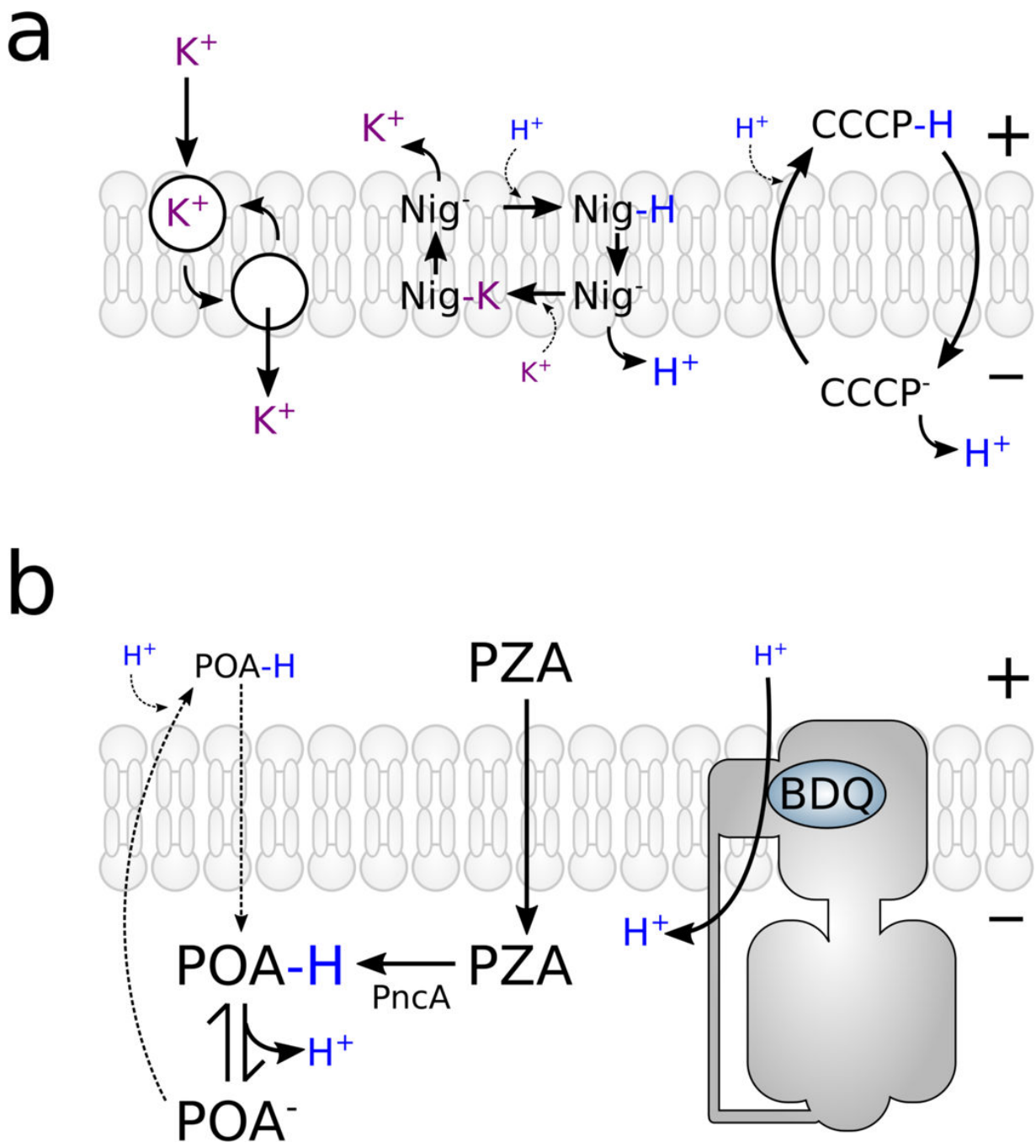

Figure 3. Traditional inhibitors of proton motive force generation

(A). Valinomycin is an ionophore, selective for potassium ions, which equilibrates the potassium gradient— dissipating the $\Delta\psi$ (electrogenic). Nigericin is a hydrophobic weak carboxylic acid, which can traverse the membrane as its either protonated acid or neutral salt. It dissipates chemical gradients (i.e. ΔpH) but maintains the charge (one positive charge exchanged for one positive charge—electroneutral). (3) Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) is an electrogenic protonophore. $CCCP^{-}$ is driven to the periplasm by the $\Delta\psi$, while CCCPH is driven to the cytoplasm by the ΔpH. It can equilibrate both $\Delta\psi$ and

ΔpH. (B) Model for uncoupling by either pyrazinamide (PZA) or bedaquiline (BDQ). *Left panel*. PZA diffuses into the cell and is converted to pyrazinoic acid (POA) by PncA (pyrazinamidase). Anionic POA could effectively inhibit growth through anion accumulation in the neutral pH of the cytoplasm and/or efflux from the cells to become protonated in the acidic extracellular environment (POA−H). POA−H would then diffuse back into the cell driven by the ΔpH gradient and dissociate in the cytoplasm (neutral pH) leading to intracellular acidification and cell death. *Right panel*. In a typical mycobacterial cell, the majority of ATP synthesis is respiratory, driven by the *pmf*. The binding of BDQ 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.

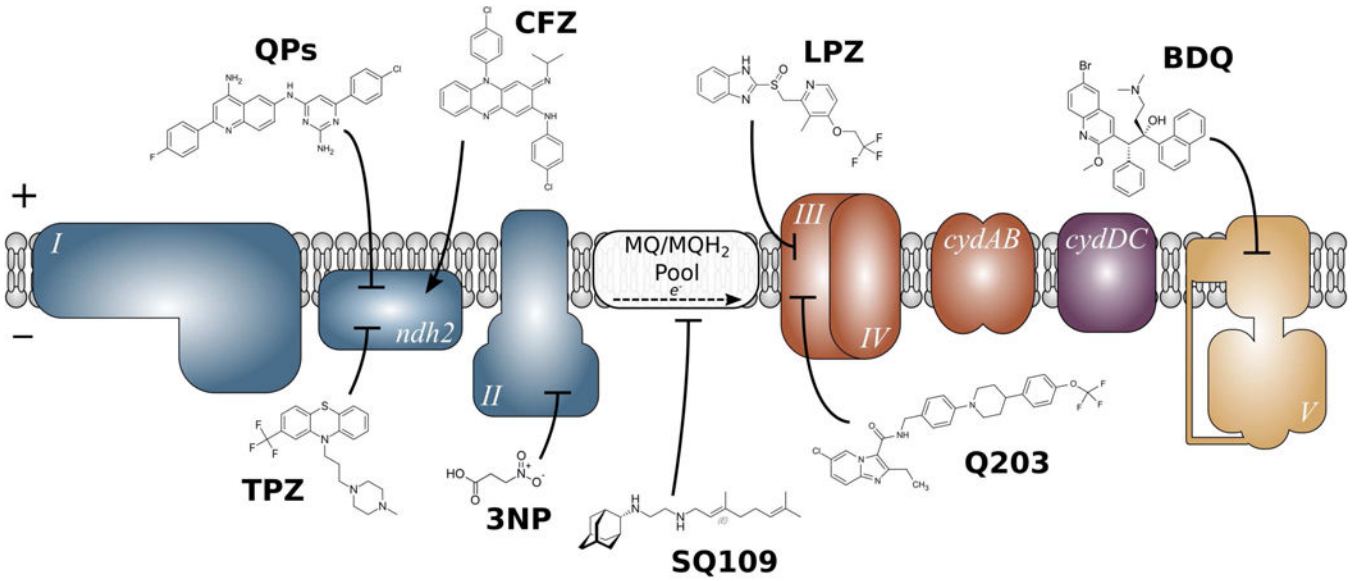

Figure 4. Inhibitors of the electron transport chain and F_1F_0-ATP synthase of *M. tuberculosis*

Selected inhibitors of these complexes are indicated with flathead arrows and do not reflect the binding site of the inhibitors. QPs = quinolinyl pyrimidines, TPZ = trifluoperazine, CFZ = clofazimine, 3-NP = 3-nitropropionate, SQ109 = *N*-Adamantan-2-yl-*N*'-((*E*)-3,7-dimethyl-octa-2,6-dienyl)-ethane-1,2-diamine, LPZ = Lansoprazole, Q203 = imidazopyridine amide, BDQ = bedaquiline.

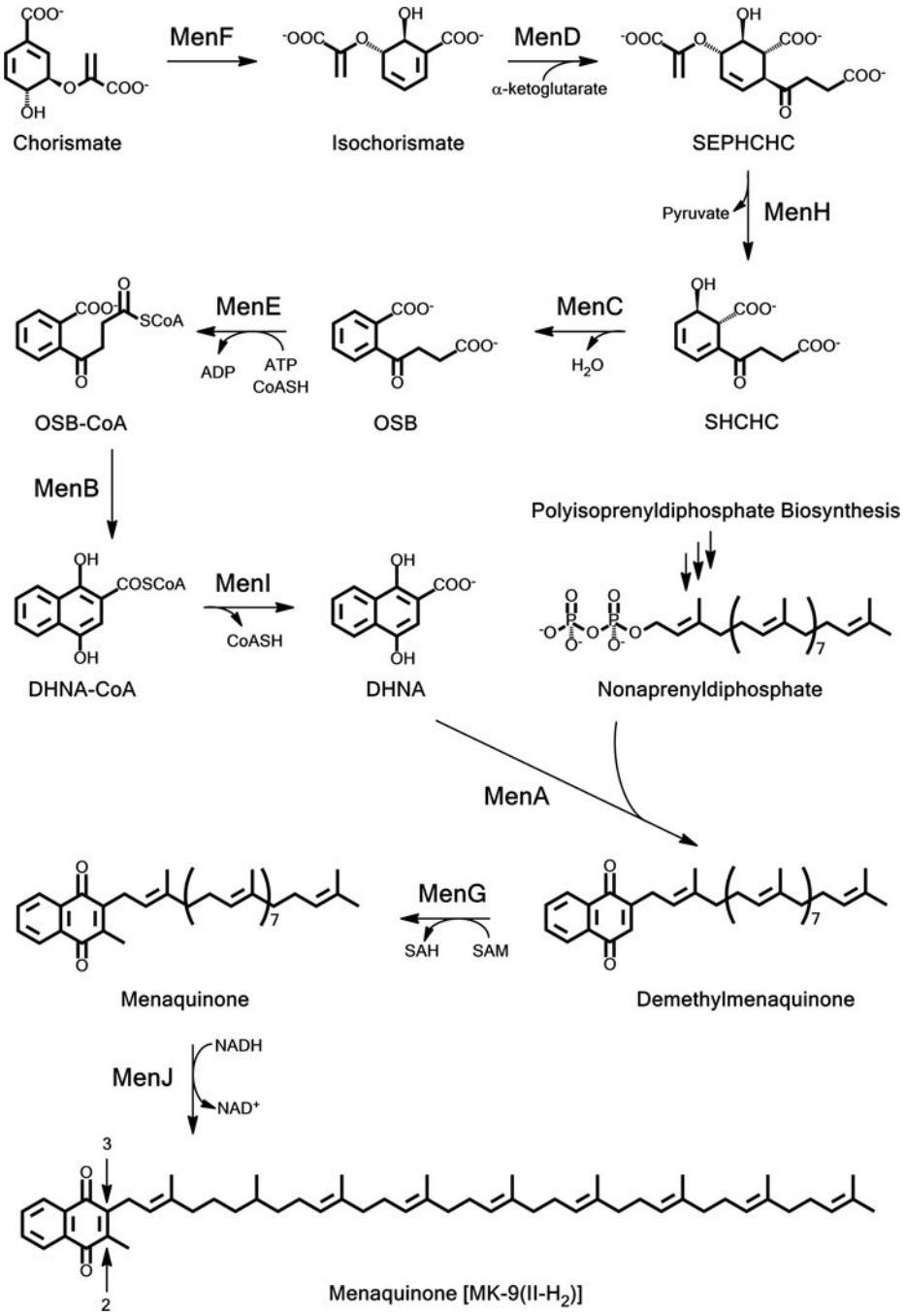

Figure 5. Proposed menaquinone biosynthesis pathway in mycobacteria based on the known pathway in *Escherichia coli*

In this scheme the product of MenA is depicted as the quinone rather than the quinol. This is consistent with the majority of the MK literature (168), which indicates that the oxidation from quinol to quinone is spontaneous, but differs from UQ synthesis. The arrows indicate C2 and C3 of MK-9(II−H_2). Abbreviations used: DHNA, 1,4-dihydroxy-2-naphthoate; DHNA-CoA, 1,4-dihydroxy-2-naphthoyl-CoA; OSB, *o*-succinylbenzoate; OSB-CoA, *o*-succinylbenzoyl-CoA ; SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-

cyclohexadiene-1-carboxylate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate.