



Terminal Respiratory Oxidases: A Targetable Vulnerability of Mycobacterial Bioenergetics?

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Recently, ATP synthase inhibitor Bedaquiline was approved for the treatment of multi-drug resistant tuberculosis emphasizing the importance of oxidative phosphorylation for the survival of mycobacteria. ATP synthesis is primarily dependent on the generation of proton motive force through the electron transport chain in mycobacteria. The mycobacterial electron transport chain utilizes two terminal oxidases for the reduction of oxygen, namely the *bc₁-aa₃* supercomplex and the cytochrome *bd* oxidase. The *bc₁-aa₃* supercomplex is an energy-efficient terminal oxidase that pumps out four vectoral protons, besides consuming four scalar protons during the transfer of electrons from menaquinone to molecular oxygen. In the past few years, several inhibitors of *bc₁-aa₃* supercomplex have been developed, out of which, Q203 belonging to the class of imidazopyridine, has moved to clinical trials. Recently, the crystal structure of the mycobacterial cytochrome *bc₁-aa₃* supercomplex was solved, providing details of the route of transfer of electrons from menaquinone to molecular oxygen. Besides providing insights into the molecular functioning, crystal structure is aiding in the targeted drug development. On the other hand, the second respiratory terminal oxidase of the mycobacterial respiratory chain, cytochrome *bd* oxidase, does not pump out the vectoral protons and is energetically less efficient. However, it can detoxify the reactive oxygen species and facilitate mycobacterial survival during a multitude of stresses. Quinolone derivatives (CK-2-63) and quinone derivative (Aurachin D) inhibit cytochrome *bd* oxidase. Notably, ablation of both the two terminal oxidases simultaneously through genetic methods or pharmacological inhibition leads to the rapid death of the mycobacterial cells. Thus, terminal oxidases have emerged as important drug targets. In this review, we have described the current understanding of the functioning of these two oxidases, their physiological relevance to mycobacteria, and their inhibitors. Besides these, we also describe the alternative terminal complexes that are used by mycobacteria to maintain energized membrane during hypoxia and anaerobic conditions.

Keywords: *Mycobacterium*, oxidative phosphorylation, electron transport chain, *bc1-aa3* supercomplex, cytochrome *bd* oxidase, Q203, respiratory inhibitors, Aurachin D

INTRODUCTION

Mycobacterium tuberculosis (Mtb) causes tuberculosis (TB) and remains one of the leading causes of human deaths worldwide from a single infectious agent (W.H. Organisation, 2019). Management of TB relies on the WHO recommended chemotherapeutic regimen known as directly observed therapy short-course (DOTS) (W.H. Organisation, 2010). DOTS utilizes the administration of four antibiotics for 6 months. Such a lengthy treatment is associated with compliance issues, and is considered as one of the reasons for the emergence of drug resistance. The number of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB cases are steadily rising over the years (Seung et al., 2015). Given that the antimycobacterials used in DOTS were discovered several decades back, there is an urgent need for the development of newer drugs with distinct mechanisms of action. Fortunately, recently Bedaquiline (BDQ) (W.H. Organization, 2013), Pretomanid (Keam, 2019), and Delamanid (Ryan and Lo, 2014) were approved for the treatment of MDR-TB. Of these, Pretomanid and Delamanid belong to the class of nitroimidazole. Pretomanid targets cell wall biosynthesis as well as the respiratory electron transport chain (ETC) of Mtb and thus kills both replicating and non-replicating mycobacterial cells (Manjunatha et al., 2009). Delamanid primarily inhibits mycolic acid biosynthesis in Mtb (Thakare et al., 2015). BDQ belongs to the diarylquinoline class of drugs and inhibits ATP synthesis of Mtb (Andries et al., 2005). BDQ is capable of killing both actively replicating and non-replicating persistent mycobacterial cells (Rao et al., 2008). Mtb utilizes respiratory flexibility to survive under varying environmental conditions (Trivedi et al., 2012). Due to the presence of parallel and alternative components, ETC was considered a poor drug target (Iqbal et al., 2018). However, Pretomanid and BDQ both target Mtb bioenergetics and thus have established it as a validated target. Several reviews have earlier discussed the possibility of targeting oxidative phosphorylation to develop potential therapeutic antimycobacterials (Cook et al., 2014; Bald et al., 2017; Cook et al., 2017; Iqbal et al., 2018). Two reviews were recently published emphasizing the importance of respiratory terminal oxidases in mycobacterial physiology and their potential as drug targets (Lee et al., 2020; Mascolo and Bald, 2020). In line with these reviews, here we will discuss recent studies on the contribution of respiratory terminal oxidases to mycobacterial physiology, the recent development of inhibitors

targeting them, and how these could be synergistically targeted for the development of a novel regimen for the treatment of TB. Besides, we will also describe the alternative electron acceptors utilized by mycobacteria for re-oxidizing the electron carrier menaquinone for maintaining an energized membrane.

Mycobacterial Electron Transport Chain

ETC is utilized by microorganisms for extracting reducing power from the reduced cofactors generated during catabolic processes. It utilizes membrane-anchored dehydrogenases that accept electrons from NADH/FADH₂ and other reduced substrates and then transfer these electrons between a series of membrane-bound multi-protein complexes, finally transferring it to the enzymes catalyzing the reduction of oxygen to water, known as terminal oxidases (Magalon and Alberge, 2016). In this process of electron transfer, protons are pumped into the periplasm, generating a proton gradient that manifests a proton motive force (PMF) (Kashket, 1985; Cook et al., 2009). This force is utilized for ATP synthesis through ATP synthase (Walker, 2013). Mycobacterial cells utilize a large number of dehydrogenases for feeding electron into the ETC (Cook et al., 2014). However, NADH/menaquinone and succinate dehydrogenase (SDH) act as primary electron feeders in mycobacterial cells (Cook et al., 2014; Iqbal et al., 2018). Mtb contains a proton-pumping type I NADH dehydrogenase encoded by *nuoABCDEFGHIJKLMN* operon and two non-proton pumping type II NADH dehydrogenases encoded by *ndh* (Rv1854c) and *ndhA* (Rv0392c) (Cook et al., 2014; Iqbal et al., 2018). Most of the mycobacterial species contain two isoforms of SDH; SDH1 and SDH2 (Pecsi et al., 2014). In Mtb, the deletion of *sdh1* disturbs the rate of respiration and leads to the inability to survive the long-term stationary phase. During aerobic growth, SDH1 functions as SDH, while SDH2 is dispensable for this catalysis (Hartman et al., 2014). SDH couples tricarboxylic acid cycle (TCA) with the ETC and feeds electron to the menaquinone pool. The pool of reduced menaquinones is then oxidized by two distinct terminal oxidases, namely menaquinol-cytochrome *c* oxidase (also known as cytochrome *bc₁-aa₃* complex) and cytochrome *bd*-type menaquinol oxidase (*bd* oxidase) (Cook et al., 2014; Iqbal et al., 2018). These terminal oxidases transfer electrons from menaquinol to oxygen, and during this process, reduce oxygen to water. PMF generated through this cascade of electron transfer is utilized for the synthesis of ATP via ATP synthase (Figure 1). Here it must be noted that, unlike *Escherichia coli* and many other bacterial species that can survive using substrate-level phosphorylation on fermentable carbon source, Mtb cannot grow using substrate-level phosphorylation and is dependent upon ATP synthase for ATP synthesis and growth on fermentable and non-fermentable carbon sources (Tran and Cook, 2005).

Terminal Respiratory Oxidases

As mentioned above, mycobacterial cells use two terminal oxidases, namely, cytochrome *bc₁-aa₃* supercomplex and cytochrome *bd* oxidase (Cook et al., 2014; Iqbal et al., 2018). Both of these contribute to the generation of PMF through the release of protons from menaquinol into periplasmic space. However, cytochrome *bc₁-aa₃* complex is energetically more efficient as it pumps additional protons into the periplasm. In

Abbreviations: *Mycobacterium tuberculosis*; TB, Tuberculosis; WHO, The world health organization; DOTS, Directly observed therapy short-course; MDR, Multidrug-resistant; XDR, Extensively drug-resistant; ETC, Electron transport chain; ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; NADH, Reduced nicotinamide adenine dinucleotide; FADH, Reduced flavin adenine dinucleotide; SDH, Succinate dehydrogenase; NTMs, Non-tuberculous mycobacteria; SAR, Structure-activity relationship; PRSAF1, Prokaryotic respiratory supercomplex association factor 1; SOD, Superoxide dismutase; ROS, Reactive oxygen species; BCG, Bacillus Calmette Guerin; IP, Imidazo[1,2-a]pyridine; IPA, Imidazopyridine amides; MIC, Minimum inhibitory concentration; PK, Pharmacokinetic; ADME, Absorption, distribution, metabolism and excretion; TCA, Tricarboxylic acid cycle; GSK, GlaxoSmithKline; QOAs, 2-(quinolin-4-yl-oxo)acetamides; PAB, Phenoxy-alkyl benzimidazole; LPZ, Lansoprazole; LPZS, Lansoprazole sulfide; ROS, Reactive oxygen species; RNA, Ribonucleic acid.

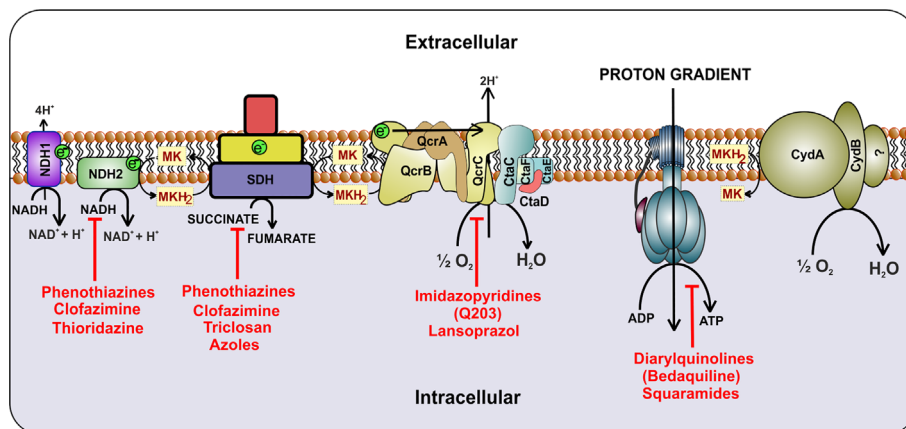


FIGURE 1 | Schematic representation of the electron transport chain of mycobacteria. NADH dehydrogenase (Complex I) oxidizes NADH and transfers the electrons to cytochrome *bc*₁-*aa*₃ oxidoreductase (complex III-IV) by reducing menaquinone. Alternatively, succinate dehydrogenase (complex II) uses succinate as substrate and transfers electrons to complex III via menaquinone. Electrons are then transferred to cytochrome *bc*₁-*aa*₃ and cytochrome *bd*-type menaquinol oxidase (*bd* oxidase), which finally passes the electrons to the terminal electron acceptor, oxygen. During this process, a proton gradient is generated, which helps in the synthesis of ATP by ATP synthase. Inhibitors of the respiratory complexes are shown in red color.

Gram-negative bacteria and the mitochondria of the eucaryotic cells, complex III (cytochrome *bc*₁) is linked to the complex IV (cytochrome *aa*₃) by a soluble cytochrome *c*. However, in *Mycobacterium*, complex III (cytochrome *bc*₁) is fused with the complex IV (cytochrome *aa*₃) to make a super complex (Megehee et al., 2006; Kim et al., 2015). This arrangement precludes the requirement of a soluble cytochrome *c*. A similar respiratory supercomplex is found in several genera under the phylum *Actinobacteria* (Kao et al., 2016), including *Corynebacterium* (Niebisch and Bott, 2003) and *Rhodococcus* (Sone et al., 2003). Although cytochrome *bd* oxidase is less energy efficient than the cytochrome *bc*₁-*aa*₃ complex, it is more versatile, has a higher affinity for oxygen (D’Mello et al., 1996), is induced under hypoxic stress (Parish et al., 2003), and also could help with the detoxification of hydrogen peroxide (Lindqvist et al., 2000) and antibacterials (Mascolo and Bald, 2020). Besides these main terminal oxidases, mycobacterial cells are equipped with alternative oxidases/hydrogenases that function during hypoxia or absence of oxygen. These oxidases/hydrogenases help mycobacteria in the sustenance of bioenergetics but are unable to support mycobacterial growth. Thus, these are believed to play an essential role in persistence and survival during stress conditions. In the following sections, we will try to make a case for the terminal oxidases as an important and synergistic drug target aimed at the development of novel antimycobacterials.

CYTOCHROME *BC*₁-*AA*₃ COMPLEX AND ITS INHIBITORS

In the following section, we will describe the evolving understanding of the role of the cytochrome *bc*₁-*aa*₃ complex in mycobacterial physiology. We will also summarize the current knowledge of the inhibitors of the mycobacterial, the cytochrome *bc*₁-*aa*₃ complex.

*bc*₁-*aa*₃ Oxidoreductase Complex

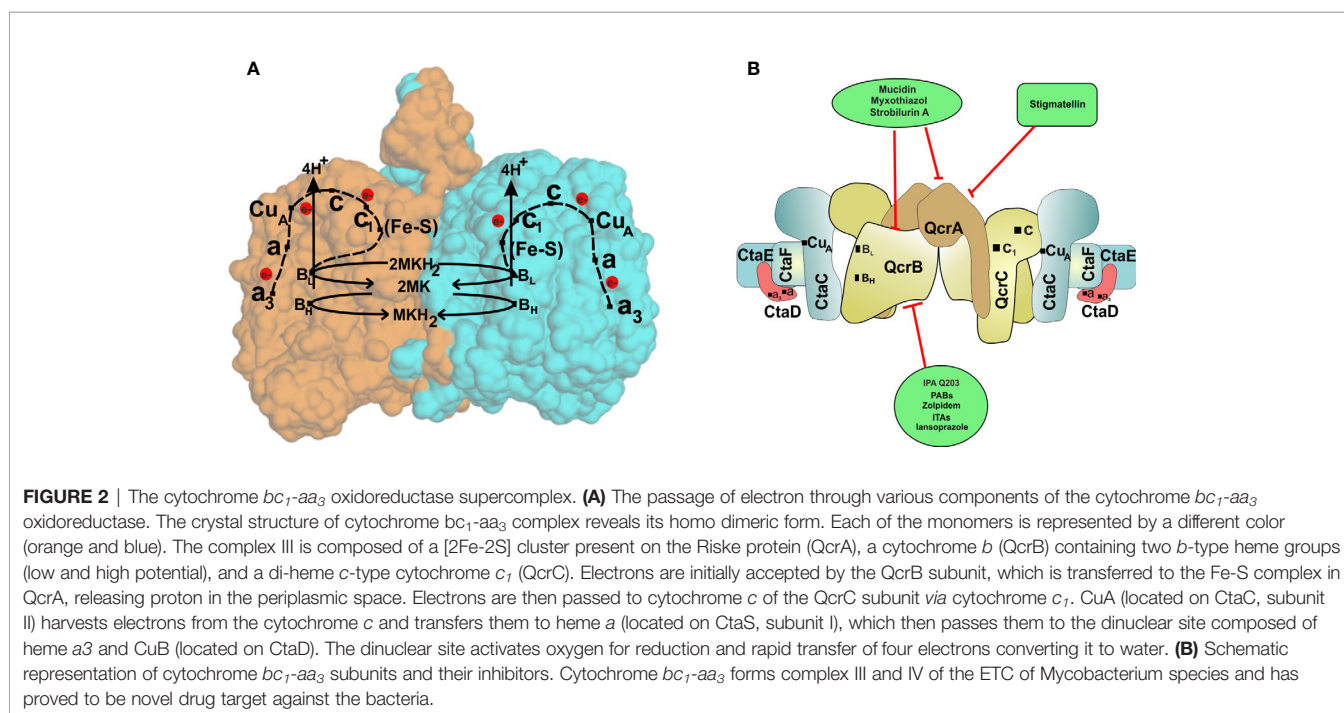
In bacterial and mitochondrial respiratory chains, cytochrome *bc*₁ (or complex III) extracts electrons from ubiquinone/menaquinone and then transfers them to membrane-anchored cytochrome *c*. Cytochrome *bc*₁ pumps protons in the periplasmic space during electron transfer to cytochrome *c* (Berry et al., 2000). Cytochrome *c* then passes electrons to cytochrome *c* oxidase or cytochrome *aa*₃ complex (also known as complex IV) that uses these electrons for reducing oxygen and couples the electron flow with proton translocation across the membranes (Capaldi, 1990). In mycobacteria, the complex III consists of a 2Fe/2S iron-sulfur cluster present on the Rieske protein (QcrA), a cytochrome *b* (QcrB) containing two *b*-type heme groups (low and high potential), and a di-heme *c*-type cytochrome *c*₁ (QcrC) (Niebisch and Bott, 2001; Cook et al., 2017). The *bc*₁ complex is a membrane-bound “hub” involved in the ETC of phylogenetically diverse species (Mulikidjanian, 2007). This complex is involved in the oxidation of menaquinol/ubiquinol. The Q-cycle mechanism has been proposed for explaining the functioning of the *bc*₁ complex (Mitchell, 1976). According to the Q-cycle hypothesis, two electrons from ubiquinol/menaquinol are transferred to two different chains at the Q_o-site of the complex. The first electron from quinol is transferred to the [2Fe-2S] cluster of the Rieske protein, that further transfers it to *c* type heme, and then later, the electron is passed on to complex IV. The second electron from quinol is passed to cytochrome *b*, harboring a low potential heme (*b*_L) and a high potential heme (*b*_H). These electrons are delivered to a second quinone on the Q_i-site, and the quinone is reduced to quinol (Crofts et al., 2003). Complex IV (cytochrome *aa*₃) contains four redox active sites, namely CuA, CuB, heme *a*, and heme *a*₃ (Scott, 1995). CuA (located on CtaC, subunit II) harvests electron from the cytochrome *c* and passes them to heme *a* (located on CtaS, subunit I), which then passes them to the dinuclear site composed of heme *a*₃ and CuB (located on

CtaD). The dinuclear site activates oxygen for reduction and rapidly passes four electrons converting it in water. During this process, besides consuming four scalar protons for protonation of oxygen, four vectoral protons are pumped out (Scott, 1995). The CtaS and CtaC are the primary electron acceptors from the bc₁ complex, while the a₃-CuB unit is the oxygen-reducing element (Cook et al., 2014).

In recent years, the concept of respirasomes has emerged (Krause et al., 2004). This concept suggests that different respiratory complexes associate with each other to make a supramolecular complex called “respirasome.” The organization and composition of such supercomplexes are believed to be dynamic and change with cellular energy requirements (Krause et al., 2004). In mitochondria, mostly complex I, complex III, and complex IV associate with each other in such supercomplexes (Krause et al., 2004; Lenaz and Genova, 2010; Dudkina et al., 2011). In line with these studies, a respiratory supercomplex consisting of complex III and complex IV has been reported for mycobacteria (Megehee et al., 2006; Kim et al., 2015). The mycobacterial cytochrome bc₁-aa₃ supercomplex contains tightly associated menaquinol reductase and an aa₃ oxidase (Megehee et al., 2006; Kim et al., 2015; Gong et al., 2018). Importantly, cytochrome *c* is fused to the bc₁ complex in *Mycobacterium* (Megehee et al., 2006). Another important difference of mycobacterial bc₁-aa₃ super complex from mitochondrial and eukaryotic III-IV supercomplex is that the former is tightly associated while the latter is only loosely associated. The atomic structure of cytochrome bc₁-aa₃ complex of *M. smegmatis* was recently elucidated using cryo-electron microscopy (Gong et al., 2018; Wiseman et al., 2018). These studies revealed detailed features of supercomplex, which are specific to mycobacteria. These studies suggested that around 20

subunits associate to form the respiratory supercomplex in mycobacteria. The core of the supercomplex is composed of a bc₁ dimer that is sandwiched between aa₃ complexes on each side (Gong et al., 2018; Wiseman et al., 2018). Interestingly, QcrA of mycobacteria possesses a roof-like structure towards the periplasm that facilitates the dimerization of the bc₁ complex. This feature is unique to mycobacterial QcrA. The cryo-EM structure also revealed the role of previously unknown subunits, namely, Cta I, Cta J, LpqE, and prokaryotic respiratory supercomplex association factor 1 (PRSAF1) (Gong et al., 2018; Wiseman et al., 2018). LpqE and PRSAF1 are present at the interface of the bc₁ complex and aa₃ complex. The structural analysis also revealed an association of superoxide dismutase (SOD) with the bc₁-aa₃ complex supercomplex (Figure 2A). These studies suggested that SOD may play a role in the detoxification of ROS formed by the bc₁ complex.

Importantly, since the bc₁-aa₃ complex supercomplex is critical for the optimal growth of mycobacterial cells, the expression of its subunits is tightly controlled. Under conditions favoring aerobic respiration, the bc₁-aa₃ supercomplex is the primary respiratory route in Mtb, yielding more ATP, and thus is essential for the mycobacterial growth (Matsoso et al., 2005; Cook et al., 2014). In response to the optimal oxygen levels, the expression of the two oxidases is regulated by mycobacteria to maximize the utilization of the terminal electron acceptor. It is assumed that the oxygen affinity of two-terminal oxidases is different (Cook et al., 2014), but whether the same is true is not analyzed. Notably, the presence of cytochrome bc₁-aa₃ supercomplex in *M. smegmatis* bd mutant enables it to grow at a similar rate to the wild type, suggesting that bc₁-aa₃ supercomplex alone can fulfill the energy needs of mycobacteria under normoxic conditions (Kana et al., 2001; Megehee and Lundrigan, 2007; Lu et al., 2015). These findings are



in agreement with the observation that mutants of *M. smegmatis* and Mtb lacking QcrCAB are attenuated for growth (Kana et al., 2001; Matsoso et al., 2005; Beites et al., 2019). However, during the early phase of mice infection, the expression of the *bc₁-aa₃* complex is downregulated and stabilizes by around three weeks (Shi et al., 2005a). These observations are supported by the delayed growth of Mtb *qcrCAB* mutant in mice (Beites et al., 2019). Although a mutant of Mtb lacking a functional *bc₁-aa₃* complex can be generated, suggesting that this terminal oxidase is not essential in Mtb (Small et al., 2013; Beites et al., 2019), but several inhibitors of this complex can inhibit the Mtb growth in cultures, in macrophages, and during the infection emphasizing on its significance for mycobacterial growth.

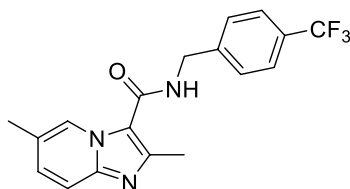
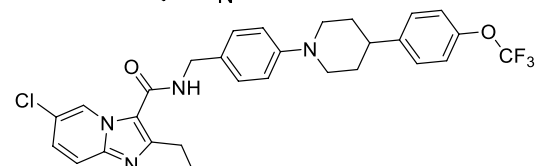
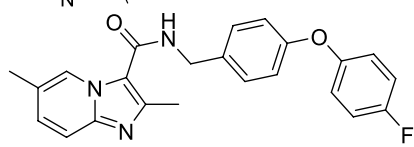
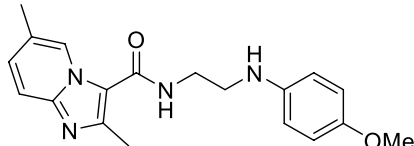
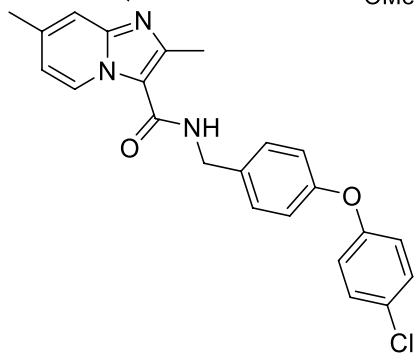
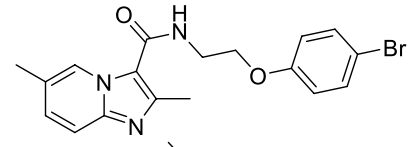
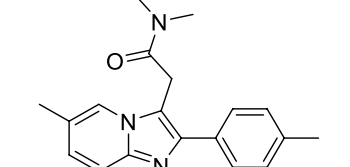
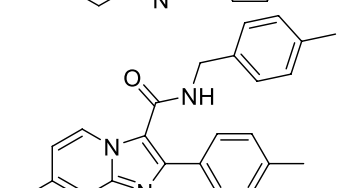
***bc₁-aa₃* Oxidoreductase Inhibitors**

The role of the *bc₁-aa₃* supercomplex in optimal growth and its distinct structure and function from the mammalian respiratory system make it a relevant druggable target. Various well-characterized inhibitors of the mitochondrial, as well as mycobacterial *bc₁-aa₃* complex, are known. Crystallographic studies and deducing their mechanism of action has led to the development of various other inhibitors for therapeutic purposes. Usually, these inhibitors target the catalytic domains of complex utilizing the structure analogous to quinone or quinol (Abrahams et al., 2012). A variety of chemical compounds and antibiotics are known to inhibit the *bc₁-aa₃* complex for a long time. Myxothiazol from *Myxococcus fulvus*, a well-known antibiotic targeting the mitochondrial cytochrome *b*, was first characterized in 1984. It was shown to interact with both cytochrome *b* as well as the iron-sulfur protein of the complex resulting in the displacement of a quinone from the high-affinity binding site of the iron-sulfur protein (von Jagow et al., 1984). Antifungal antibiotics, like mucidin (from basidiomycetes *Oudemansiella mucida*) and strobilurin A (from *Strobilurins tenacellus*), also inhibit the complex by binding at the same site as that of myxothiazol (Von Jagow et al., 1986). However, another antibiotic, antimycin binds to a different location, inhibiting the oxidation of the cytochrome *b* subunit (Kucera et al., 1988). Stigmatellin, an antibiotic synthesized by *Stigmatella aurentica*, contains a 5,7-dimethoxy-8-hydroxychromone aromatic headgroup with a hydrophobic alkenyl chain in position 2. It directly binds to the cytochrome *b* Q₀ site, associating with QcrA (von Jagow and Link, 1986).

To target a pathogen, it is important to identify compounds that are specific to the bacterial respiration pathway. This is achieved mainly by using high throughput screening (HTS), using which a plethora of cytochrome *bc₁-aa₃* inhibitors have been discovered until now (Table 1). One such screening identified the imidazo[1,2-*a*]pyridine (IP) series of compounds. IPs were discovered as potent inhibitors of Mtb and *M. bovis* BCG. Four IP inhibitors were specifically targeting QcrB, forcing the bacterium to use energetically less efficient cytochrome *bd* oxidase. Among these compounds, 2,6-Dimethyl-*N*-(4-(trifluoromethyl)benzyl)imidazo[1,2-*a*]pyridine-3-carboxamide with a minimum inhibitory concentration (MIC) of 0.03 μM was found to be the most potent molecule (Abrahams et al., 2012). In

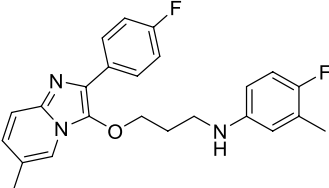
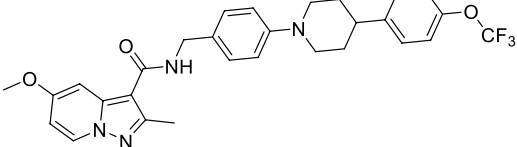
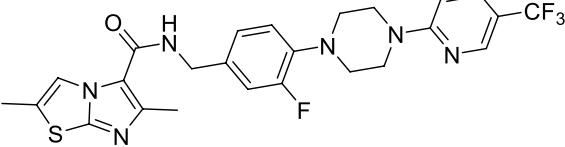
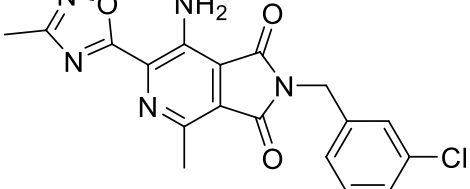
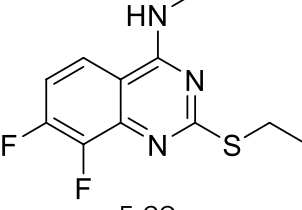
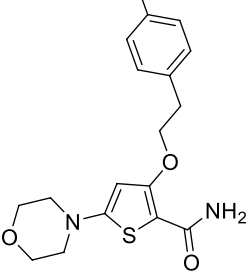
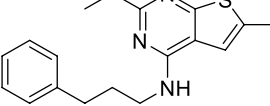
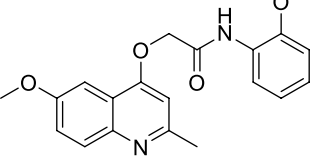
2013, Pethe et al. reported a more active and less toxic class of imidazopyridine amides (IPA), targeting the Mtb cytochrome *bc₁*. The compound, Q203 (Telacebac), was found to be active against Mtb H37Rv at an MIC₅₀ of 2.7 nM *in vitro* and MIC₅₀ of 0.28 nM inside the macrophages (Pethe et al., 2013). However, it must be noted that Q203 is bacteriostatic only, as mycobacteria can utilize cytochrome *bd* oxidase for survival in presence of Q203 (Kalia et al., 2017a). In agreement with these observations, Q203 is bactericidal for Mtb strains lacking cytochrome *bd* oxidase (Kalia et al., 2017a). This compound was further optimized against MDR and XDR clinical isolates in the nanomolar range (Kang et al., 2014). This leading drug candidate has recently progressed to the clinical development phase II under U.S. FDA investigational new drug application. A screen for the spontaneous resistant mutants identified the cytochrome *bc₁* complex (*qcrB*) as the target of Q203. A mutation of Thr313 to either alanine or isoleucine was specifically involved in the resistance to Q203 (Pethe et al., 2013). In a cytochrome *bd* oxidase knockout mutant, Q203 completely inhibited the respiration (Lamprecht et al., 2016). Also, the chemical inhibition of cytochrome *bd* oxidase by aurachin D has been shown to turn the bacteriostatic activity of Q203 into bactericidal activity in Mtb and *M. smegmatis* (Lu et al., 2018). It is already evident that efflux pumps play a significant role in drug resistance in Mtb (da Silva et al., 2011). Verapamil (efflux pump inhibitor), in combination with Q203, increases the potency of the drug, showing the importance of efflux pumps mediated resistance and combination therapy with inhibitors to combat the problem (Jang et al., 2017). Putative 3D structures of the wild-type and T313A mutant along with the docking analysis with Q203 have been published, which can give insights for the resistance mechanism and will be beneficial for making better compounds within the IPA series (Ko and Choi, 2016). Apart from that, Q203 has also been evaluated against non-tuberculous mycobacteria (NTM) *M. abscessus* (Mabs), which is also quickly emerging as a health concern worldwide. It was shown that Q203 is ineffective against Mabs due to a genetic polymorphism in the target gene (QcrB). However, the complementation of Mabs Δ*qcrCAB* with chimeric Mabs *qcrCAB* (amino acids changed to Mtb *qcrCAB*) led to the susceptibility of Mabs towards this drug, suggesting the involvement of respiratory pathways in the intrinsic resistance mechanism towards drugs in Mabs (Abrahams et al., 2012). Recently, AWE402, another inhibitor belonging to the IPA class, was synthesized, which is structurally related to Q203 and shows good activity with MIC of 0.005 μM towards Mtb (Moraski et al., 2013; Ward et al., 2017). Later, a report of identification of an imidazo[1,2-*a*]pyridine-3-carboxamide bearing a variety of different linkers revealed 2,6-Dimethyl-*N*-[2-(phenylamino)ethyl]imidazo[1,2-*a*]pyridine-3-carboxamide as a potent anti-TB compound with MIC of 0.041 μM - 2.64 μM against both drug-sensitive as well as drug-resistant strains of Mtb (Lv et al., 2017). A compound belonging to this class, *N*-(4-(4-Chlorophenoxy)benzyl)-2,7-dimethylimidazo[1,2-*a*]pyridine-3-carboxamide (ND-09759) was also identified as a potent anti-TB molecule with an MIC of ≤0.006 μM or 0.0024 μg/ml. Its *in vivo*

TABLE 1 | Summary of cytochrome *bc₁-aa₃* supercomplex inhibitors, their chemical structures and MIC values. Number in the parentheses denotes the references.

Chemical Class	Name of the Compound	Chemical Structure	MIC
Imidazopyridine amides (IPAs)	2,6-Dimethyl- <i>N</i> -(4-(trifluoromethyl)benzyl)imidazo[1,2- <i>a</i>]pyridine-3-carboxamide (Megehee and Lundrigan, 2007)		0.03 μM
	Telacebac (Q203) (Abrahams et al., 2012)		2.7 nM ^a
	AWE402 (Kang et al., 2014; Lamprecht et al., 2016)		0.005 μM
	2,6-Dimethyl- <i>N</i> -[2-(phenylamino)ethyl]imidazo[1,2- <i>a</i>]pyridine-3-carboxamide (Lu et al., 2018)		0.041 μM
	<i>N</i> -(4-(4-Chlorophenoxy)benzyl)-2,7-dimethylimidazo[1,2- <i>a</i>]pyridine-3-carboxamide (ND-09759) (da Silva et al., 2011)		0.006 μM
	<i>N</i> -(2-(4-Bromophenoxy)ethyl)-2,6-dimethylimidazo[1,2- <i>a</i>]pyridine-3-carboxamide (IMB-1402) (Ko and Choi, 2016)		0.027 μg/ml
	Zolpidem (Ward et al., 2017)		10-50 μM
	7-Methyl- <i>N</i> -(4-methylbenzyl)-2-(<i>p</i> -tolyl)imidazo[1,2- <i>a</i>]pyridine-3-carboxamide (Ward et al., 2017)		0.004 μM

(Continued)

TABLE 1 | Continued

Chemical Class	Name of the Compound	Chemical Structure	MIC
Imidazopyridine ethers (IPEs)	4-Fluoro- <i>N</i> -(3-((2-(4-fluorophenyl)-6-methylimidazo[1,2- <i>a</i>]pyridin-3-yl)oxy)propyl)-3-methylaniline (Moraski et al., 2013)		0.03 μM
Pyrazolopyridine carboxamide (PPA)	TB47 (Lv et al., 2017)		0.016 μg/ml
Imidazo[2,1-<i>b</i>]thiazole-5-carboxamide	ND-11543 (Moraski et al., 2016a)		0.004 μM
Pyrrolo[3,4-<i>c</i>]pyridine-1,3(2<i>H</i>)-dione	7-Amino-2-(3-chlorobenzyl)-4-methyl-6-(3-methyl-1,2,4-oxadiazol-5-yl)-1 <i>H</i> -pyrrolo[3,4- <i>c</i>]pyridine-1,3(2 <i>H</i>)-dione (Wu et al., 2016)		0.065 μM
Quinazolinamine	2-(Ethylthio)-7,8-difluoro- <i>N</i> -methylquinazolin-4-amine (Compound 11726148) (Moraski et al., 2015)		0.05 μg/ml
Morpholinothiophene	5-Morpholino-3-phenethoxythiophene-2-carboxamide (Tantry et al., 2017)		0.024 μM
Thieno-pyrimidines	CWHM-1023 (Lu et al., 2019)		83 ± 5.4 nM ^a
2-(Quinolin-4-yl)oxy Acetamide	GSK 358607A (Moraski et al., 2020)		1.11 μM

(Continued)

TABLE 1 | Continued

Chemical Class	Name of the Compound	Chemical Structure	MIC
	2-((6-Methoxy-2-methylquinolin-4-yl)oxy)-N-(naphthalen-2-yl)acetamide (INCT-TB422) (Cleghorn et al., 2018)		0.05 μM
	2-((6-Methoxy-2-methylquinolin-4-yl)oxy)-N-(4-pentylphenyl)acetamide (Cleghorn et al., 2018)		0.005 μM
Arylvinyloxy piperazine amide	AX-35 (Ballell et al., 2013)		0.3 μM
Benzimidazole	N-(3-(2-Ethyl-6-methyl-1H-benzo[d]imidazol-1-yl)propyl)aniline (IDR-0578347) (Pitta et al., 2016)		0.056 μM^{b}
	Lansoprazole (Pissinate et al., 2016; Borsoi et al., 2020)		1.13 μM
Quinolinone	MTC420 (Foo et al., 2018)		0.14 μM^{c}
	SCR0911 (Ananthan et al., 2009)		272 μM^{a}

^aMIC₅₀ value. ^bMIC₉₉ value. ^cMIC₅₀ (MDR Mtb).

bactericidal activity in a mice infection model, and good pharmacokinetic (PK) properties established it as a potent anti-TB drug (Cheng et al., 2014). Imidazo[1,2-a]pyridine-3-carboxamide has also been shown to be active against *M. avium* strains *in vitro* and *in vivo* in the mouse infection model, showing the importance of these compounds as drug molecule against NTM infections as well (Moraski et al., 2016a). Using a scaffold hopping strategy, a series of novel IPA derivatives bearing an *N*-(2-phenoxy)ethyl moiety were designed, synthesized, and checked for *in vitro* inhibitory activity against both drug-

sensitive H37Rv and drug-resistant clinical isolates. Compound IMB-1402 (*N*-(2-(4-Bromophenoxy)ethyl)-2,6-dimethylimidazo[1,2-a]pyridine-3-carboxamide) displayed acceptable safety and PK properties with an MIC of 0.025 $\mu\text{g/ml}$ against H37Rv (Wu et al., 2016). Zolpidem (*N,N*-dimethyl-2-(6-methyl-2-*p*-tolyl-imidazo[1,2-*a*]pyridin-3-yl)acetamide; Ambien) is one of the best known and approved drug for the treatment of insomnia. Its striking structural similarity with imidazo[1,2-*a*]pyridine-3-carboxamides led to the discovery of Zolpidem's anti-TB activity (Moraski et al., 2015). The rational redesign of the structural

moieties found in Zolpidem resulted in the series of potent anti-TB compounds and 7-methyl-*N*-(4-methylbenzyl)-2-(*p*-tolyl)imidazo[1,2-*a*]pyridine-3-carboxamide was found to be the potent one with MIC of 0.004 μ M demonstrating the inherent *in vitro* potency, selectivity, and low toxicity of imidazo[1,2-*a*]pyridines (Moraski et al., 2015).

Another robust screening of 900,000 compounds and their SAR analysis revealed imidazo[1,2-*a*]pyridine ethers as inhibitors of ATP synthesis and targeting cytochrome *bc₁* oxidase of Mtb. This screening resulted in the discovery of 4-Fluoro-*N*-(3-((2-(4-fluorophenyl)-6-methylimidazo[1,2-*a*]pyridin-3-yl)oxy)propyl)-3-methylaniline as anti-TB agent having an MIC of 0.03 μ M against Mtb (Tantry et al., 2017).

Similarly, due to the structural similarities with Q203, TB47, a pyrazolo[1,5-*a*]pyridine-3-carboxamide, was identified as a potent anti-TB drug molecule, showing MIC between 0.016 and 0.500 μ g/ml against various clinical strains of sensitive, MDR as well as XDR strains of Mtb. It was shown to efficiently inhibit oxygen consumption in the cytochrome *bd* mutant strain of *M. smegmatis*. The target of this compound was found to be *cd2*-loop of *qcrB* (H190 in *M. smegmatis*). Metabolomics profiling suggested the accumulation of TCA cycle intermediates linked to reducing-equivalents upon TB47 treatment, suggesting the toxicity by the compound to be mediated by metabolic redox stress. Like other QcrB inhibitors, it shows a bacteriostatic effect. However, upon the deletion of cytochrome *bd* oxidase, it becomes bactericidal. Also, in mice model of Mtb infection, it shows promising synergy with pyrazinamide and rifampicin, proving it to be an effective lead compound for the development of novel anti-TB chemotherapy (Lu et al., 2019).

Later, another class of compound targeting QcrB, the imidazo[2,1-*b*]thiazole-5-carboxamides were identified as a promising new scaffold, showing potent anti-TB activity against Mtb *in vitro*, inside macrophages, and MDR-TB with very low cytotoxic activity (Moraski et al., 2016b). These compounds show MIC₉₀ ranging from 0.0625 μ M - 2.5 μ M against Mtb, along with potency towards mono-drug resistance strains in a concentration ranging from 0.0017 μ M - 7 μ M. A recent study demonstrated its good *in vitro* ADME properties including, protein binding, CaCo-2, human microsomal stability, and CYP450 inhibition. They also demonstrated the good efficacy of a tool compound, ND-11543, in the murine TB infection model (Moraski et al., 2020).

Another HTS of the library of small polar molecules led to the identification of compounds showing activity against Mtb. Still, the presence of ester linkage imposed a question over their metabolic instability. So the compounds were optimized to get good metabolic stability in mouse PK studies yielding more stable Pyrrolo[3,4-*c*]pyridine-1,3(2*H*)-dione and showing MIC₉₀ in the micromolar range against Mtb. Among those, 7-Amino-2-(3-chlorobenzyl)-4-methyl-6-(3-methyl-1,2,4-oxadiazol-5-yl)-1*H*-pyrrolo[3,4-*c*]pyridine-1,3(2*H*)-dione had an MIC of 0.065 μ M (van der Westhuyzen et al., 2015). These compounds are hyperactive against cytochrome *bd* oxidase mutants, and a point mutation (Ala317Thr) in *qcrB* results in resistance towards these

compounds, strongly indicating the target to be the QcrB subunit (van der Westhuyzen et al., 2015).

Further, new potent quinazoline derivatives were synthesized and investigated for anti-TB activity. This study yielded a series of 2-ethylthio-4-methylaminoquinazoline derivatives against Mtb. They tested 76 derivatives, out of which four (11626141, 11626142, 11626252, and 11726148) had good activity (MIC below 0.09 μ g/ml). These compounds had very low cytotoxicity in human hepatocytes. Compounds 11626252 (2-(ethylthio)-8-fluoro-*N*-methylquinazolin-4-amine) and 11726148 (2-(ethylthio)-7,8-difluoro-*N*-methylquinazolin-4-amine) were also active in mice model of TB infection, showing 0.51 log₁₀ CFU/organ upon treatment for 10 days. Surprisingly, the analysis of mutants showing resistance towards these compounds identified QcrA and QcrB as the target of these compounds. This was the first report which identified QcrA as an important druggable target. Compound 11626252 is bacteriostatic, but in a strain of Mtb lacking cytochrome *bd* oxidase (H37Rv Δ *cydAB*), it becomes bactericidal (Lupien et al., 2020). This proves that combination therapy of drug molecules targeting both the branches of Mtb aerobic respiration could be a new therapeutic regimen.

An aerobic whole-cell phenotypic screening of Eli Lilly corporate library against Mtb resulted in the identification of a cluster of a novel morpholino-thiophenes series. This screen resulted in the identification of tool compounds like 5-morpholino-3-phenethoxythiophene-2-carboxamide. These compounds had MIC₉₀ in the micromolar range with no cytotoxicity and were active in an acute murine model of infection. Again, the target of these compounds was QcrB, proving it to be a crucial druggable target (Cleghorn et al., 2018).

In another study, a class of QcrB inhibitors was discovered, known as 4-amino-thieno[2,3-*d*]pyrimidines, that are chemically distinct from previously identified inhibitors, adding to the growing chemical space that can be exploited for the generation of new compounds with anti-TB activity. The most potent compound from their SAR analysis yielded CWHM-1023 having MIC₅₀ of 83 \pm 5.4 nM against Mtb. In combination with Q203, CWHM-1023 efficiently decreased the ATP levels in *M. smegmatis* and Mtb. Also, upon the deletion of *cydA*, bacteria become more sensitive towards this compound, proving its efficacy as an anti-TB drug molecule (Harrison et al., 2019).

In 2013, GlaxoSmithKline (GSK) made the results of an anti-mycobacterial phenotypic screening publically available. A total of 177 hits were reported, belonging to different structural classes (Ballell et al., 2013). Out of them, five 2-(quinolin-4-*yl*oxy)acetamides (QOAs) exhibited potent anti-mycobacterial properties (MIC₉₀ of 0.3 μ M - 3.3 μ M). Out of them, GSK 358607A was further investigated for its anti-TB activity using a preliminary SAR, leading to the identification of four analogs that were more potent than the original inhibitor. This class was also shown to target QcrB (Phummarin et al., 2016). The problem with these compounds was their moderate metabolic stability, due to the amide group lability (Pitta et al., 2016; Giacobbo et al., 2017). SAR studies of QOAs resulted in the discovery 2-((6-Methoxy-2-methylquinolin-4-*yl*)oxy)-*N*-

(naphthalen-2-yl)acetamide (INCT-TB422), with an MIC of 0.05 μM and 2-((6-Methoxy-2-methylquinolin-4-yl)oxy)-N-(4-pentylphenyl)acetamide with an MIC of 0.005 μM against Mtb H37Rv (Pissinate et al., 2016). It was shown to be active against intracellular and drug-resistant Mtb strains as well (Giacobbo et al., 2017). Recently, another group conducted molecular simplification of these compounds yielding more stable and active even towards MDR-TB. The compounds thus generated were active in a macrophage model of TB infection (Borsoi et al., 2020).

Another study was focused on arylvinylpiperazine amides, which were also identified in the screen conducted by GSK. One of them was GW861072X (AX-35), which showed MIC₉₀ of 0.3 μM against Mtb and *M. bovis* BCG. Lead optimization of this compound led to the identification of analogs with potent activity against Mtb *in vitro* and inside macrophages with mild cytotoxicity. These compounds were also active in an acute mouse model of TB infection, proving to be a good candidate against Mtb (Foo et al., 2018).

Further, HTS of a chemical diversity library containing 100,997 compounds was screened to identify a specific class of compounds showing anti-TB activity. It led to the discovery of the phenoxy-alkyl benzimidazole (PAB) class of compounds. They were showing a nanomolar range of MIC₉₀ and very low cytotoxicity (Ananthan et al., 2009; Chandrasekera et al., 2015). Later, isolation of mutants resistant to PAB compounds showed mutations either in *rv1339*, a gene of unknown function, or *qcrB* (Chandrasekera et al., 2017). SAR studies of PABS yielded improved compounds with alkylbenzimidazole moiety. Several compounds were even active against intracellular bacteria, out of which the compound *N*-(3-(2-ethyl-6-methyl-1*H*-benzo[d]imidazol-1-yl)propyl)aniline (IDR-0578347) was most potent with MIC₉₉ of 0.056 \pm 0.020 μM (Chandrasekera et al., 2017). Already known gastric proton pump inhibitor, lansoprazole (LPZ, Prevacid), which is extensively used to treat acid-related stomach disorders, was found to possess intracellular activity against Mtb. Target identification studies revealed its target to be cytochrome *bc*₁ complex by intracellular sulfoxide reduction to lansoprazole sulfide. A single nucleotide polymorphism of leucine-176 to proline in the cytochrome *b* results in the resistance towards this drug. LPZ rapidly converted to lansoprazole sulfide (LPZS), a potent anti-mycobacterial agent, cannot bind to human H⁺K⁺-ATPase and thus can be used as a drug molecule (Rybniker et al., 2015). But *in vivo*, LPZ does not yield a sufficient amount of LPZS to be active against Mtb, while a single intravenous dose of pure LPZS does results in higher tissue concentration, adequate to kill Mtb (Mdanda et al., 2017). LPZS is highly specific and possesses potent activity against drug-resistant isolates of Mtb (Rybniker et al., 2015). Through modeling of mutant mycobacterial protein on the published QcrB protein, it was revealed that both L176P (mutation leading to resistance towards LPZS) and T313A (resistance towards IPA) were localized to the same site, i.e., ubiquinol oxidation (P site). But, L176P mutants remained susceptible to various IPA, and T313A mutants were susceptible to LPZS, revealing a different binding mechanism for them (Rybniker et al., 2015).

A compound of quinolinone class, MTC420, synthesized by Hong and coworkers, has been reported as the cytochrome *bc*₁ inhibitor with an MIC value of 0.14 μM (Hong et al., 2017). Another repurposing strategy led to the identification of an anti-malarial compound as an anti-TB agent, inhibiting cytochrome *bc*₁ oxidase, SCR0911. It showed an MIC₅₀ of 272 \pm 41 μM against *M. smegmatis* and 107 \pm 5.8 μM against *M. bovis* BCG. Also, SCR0911 resulted in rapid intracellular ATP depletion in mycobacteria, indicating the interruption of electron flow. Docking studies revealed the Q_i site of mycobacterial cytochrome *bc*₁ to be the potential target of this drug. This opens the door for the SAR analysis of this compound for improved and specific drug molecules against Mtb (Chong et al., 2020).

Despite the high vulnerability of this respiratory branch, it is surprising to note that the screening of compounds targeting the supercomplex has majorly identified the compounds inhibiting the narrow region of QcrB subunit (Arora et al., 2014; Rybniker et al., 2015) (**Figure 2B**). A recent optimal homology model of Mtb QcrB using *M. smegmatis* QcrB as a template was built. This could provide a more in-depth insight into the interaction of Mtb QcrB with its inhibitors (Pan et al., 2019). However, exciting drug-development has been done in recent times, the absence of *bc*₁-*aa*₃ supercomplex results in the upregulation of cytochrome *bd* oxidase to meet the energetics demand of Mycobacteria (Matsoso et al., 2005; Arora et al., 2014), which demands a much better understanding of the mycobacterial ETC to target the mycobacterial cytochrome *bc*₁-*aa*₃ oxidoreductase supercomplex. Although the same is not valid in the case of the clinical isolate, which regulates cytochrome *bd* expression more tightly (Rybniker et al., 2015).

CYTOCHROME BD OXIDASE AND ITS INHIBITORS

In the following section, we will describe the current understanding of the role of cytochrome *bd* oxidase in mycobacterial physiology. We will also describe currently known inhibitors of the mycobacterial cytochrome *bd* oxidase.

Cytochrome *bd* Oxidase

Cytochrome *bd* oxidase is confined to the prokaryotic world and thus is considered a plausible drug target. Unlike cytochrome *c* oxidase, it does not pump protons to the periplasm and therefore is not energy efficient (Borisov et al., 2011). However, it still helps in the generation of PMF through the vectorial charge transfer of protons (Borisov et al., 2011). It has been purified and characterized from *E. coli* (Miller and Gennis, 1983), *Azotobacter vinelandii* (Junemann and Wrigglesworth, 1995), *Corynebacterium glutamicum* (Kusumoto et al., 2000), and *Geobacillus thermodenitrificans* (previously *Bacillus Stearothermophilus*) (Sakamoto et al., 1996). The long-awaited crystal structure of cytochrome *bd* oxidases was recently solved for enzymes from *Geobacillus thermodenitrificans* and *E. coli* (Safarian et al., 2016; Safarian et al., 2019; Theßeling et al., 2019).

Mycobacterial cytochrome *bd* oxidase is not extensively studied and is assumed to share similar functions to the closest structural bacterial homologs. Analysis of the Mtb genome suggests that Mtb cytochrome *bd* oxidase comprises of two main canonical subunits, namely CydA and CydB. In the Mtb genome, genes encoding for CydA, CydB, CydC, and CydD are present in an operon. The function of CydC and CydD is not clear, but they are assumed to be cytochrome ABC type cysteine exporter, which may facilitate the proper assembly of the cytochrome *bd* oxidase (Cruz-Ramos et al., 2004). In *M. smegmatis*, the *cydDC* and *cydAB* are located on separate operons parted by 100 base pairs (Aung et al., 2014). Sequence comparison of CydA and CydB across several mycobacteria reveal higher similarity in the sequence of *cydA* compared to *cydB*. This observation is in concordance with the norm of asymmetrical evolution of cytochrome *bd* subunits (Hao and Golding, 2006). It has been demonstrated that sensitivity to respiratory inhibitors such as hydrogen cyanide and pyocyanin significantly varies by microevolution of *cydB* (Voggu et al., 2006); however, in mycobacteria, it has not been explored yet. The sequences of *cydA* and *cydB* in Mtb is 100% identical to *M. bovis* BCG, making BCG a useful model organism to study cytochrome *bd* oxidase. Subunit CydA is involved in the oxidation of the menaquinol. It contains a q loop that binds to menaquinol and three heme groups that act as redox centers for transferring the electron flux across the terminal oxidase. These three heme are low spin cytochrome *b*₅₅₈, high spin-*b*₅₉₅, and chlorin type heme d. Recently, the homology model of cytochrome *bd* oxidase has been created (Sviriaeva et al., 2020) by utilizing the crystal structures of the enzymes from *G. thermodenitrificans* and *E. coli* (Safarian et al., 2016; Safarian et al., 2019). Comparing the mycobacterial and bacterial amino acid sequence shows that the menaquinol binding Q loop region in mycobacterial cytochrome *bd* oxidase harbors various residues that are unique and different from bacterial orthologs (Sviriaeva et al., 2020). Given the uniqueness of the additional amino acid stretches in CydAB and the role of these in the function of cytochrome *bd* oxidase, these could be utilized in discovering mycobacteria specific inhibitors. In mycobacterial cytochrome *bd* oxidase, the presence of methionine residues around the catalytic site may be the reason for reactive oxygen species (ROS) scavenging and guarding the enzyme (Harikishore et al., 2020).

A variable regulation of cytochrome *bd* expression is seen across different mycobacterial species as the *cydABDC* genes can form a polycistronic operon in various bacteria (Kabus et al., 2007). *E. coli* cytochrome *bd* possesses a higher affinity for oxygen (D'Mello et al., 1996) and is induced under hypoxic conditions (Poole and Cook, 2000). Although the oxygen affinity of the mycobacterial CydAB has not been determined yet, it is known to be induced in response to hypoxia (Kana et al., 2001; Berney and Cook, 2010). Expression of *cydAB* is regulated by ubiquinone/ubiquinol sensor ArcA/ArcB in *E. coli* (Malpica et al., 2004), while in mycobacteria, it is regulated by the oxygen sensing two-component system SenX3/RegX3 (Roberts et al., 2011; Singh and Kumar, 2015) (Figure 3). Additionally, cyclic AMP receptor protein regulates the expression of *cydAB* in *M. smegmatis* under

hypoxic conditions (Aung et al., 2014). However, whether the same is true for the Mtb homolog of cyclic AMP receptor protein needs to be further explored. In line with these observations, the *cydAB* operon is induced in response to nitric oxide (Shi et al., 2005a) that inhibits mycobacterial respiration. Notably, the *cydAB* operon is also induced during chronic infection in mice lungs (Shi et al., 2005a), suggesting a role in mycobacterial survival in the mice lungs. Furthermore, Mtb *cydAB* mutants are attenuated for survival in mice during chronic infection (Shi et al., 2005a). These observations are in agreement with earlier observations wherein CydAB was shown to play an important role in virulence and survival of various bacterial pathogens inside the host (Juty et al., 1997; Zhang-Barber et al., 1997; Endley et al., 2001; Turner et al., 2003; Baughn and Malamy, 2004; Shi et al., 2005a; Yamamoto et al., 2005; Larsen et al., 2006). Although the precise role of CydAB in protection from the host system remains undefined, these observations suggest a role beyond maintaining cellular bioenergetics. Emerging evidence indicates that CydAB could play a role in imparting resistance towards oxidative and nitrosative stress (Junemann and Wrigglesworth, 1995; Hori et al., 1996; Winstedt et al., 1998; Lindqvist et al., 2000; Borisov et al., 2011), as well as to the low iron concentrations (Cook et al., 1998). These effects could be explained due to the capability of CydAB oxidase to detoxify ROS. *E. coli* CydAB is known to possess quinol peroxidase activity that leads to detoxification of hydrogen peroxide (Al-Attar et al., 2016). It is plausible that Mtb CydAB oxidase could also protect the bacilli from oxidative stress, but it remains to be analyzed. In agreement with this hypothesis, the cytochrome *bd* oxidase expression rises three-fold in Mtb strain lacking a functional cytochrome *bc₁-aa₃* supercomplex, leading to increased resistance against H₂O₂ (Small et al., 2013). In classical strains of *M. ulcerans* and *M. leprae*, a functional cytochrome *bd* oxidase is absent, whereas, in Mtb, the enzyme may considerably protect the bacteria from multiple environmental stresses, including low oxygen tensions and nitric oxide or peroxides during infection in the human host (Cole et al., 2001; Berney et al., 2014; Scherr et al., 2018). In *E. coli*, cytochrome *bd* oxidase is overexpressed in biofilms. Loss of this enzyme results in the impairment of biofilm formation (Beebout et al., 2019). The role of cytochrome *bd* oxidase in mycobacterial biofilm formation still remains to be established.

Cytochrome *bd* Oxidase Inhibitors

Cytochrome *bd* oxidase is specifically present in bacteria and thus could be used as a target for anti-TB drug development (Figure 4). Furthermore, mycobacterial CydAB oxidase contains an extended menaquinone binding loop, and several additional stretches of amino acids essential for its function, making it amenable to Mtb specific drug targeting (Figure 5). But the non-essentiality of cytochrome *bd* oxidase represents a challenge to identify its inhibitors. Not many compounds are known to inhibit cytochrome *bd* oxidase (Table 2). The Aurachin class of compounds is a quinone analog, which has been reported to inhibit a variety of cytochrome oxidases (Meunier et al., 1995; Debnath et al., 2012; Li et al., 2013). Aurachin D is well known among this class of compounds known to inhibit *E. coli*

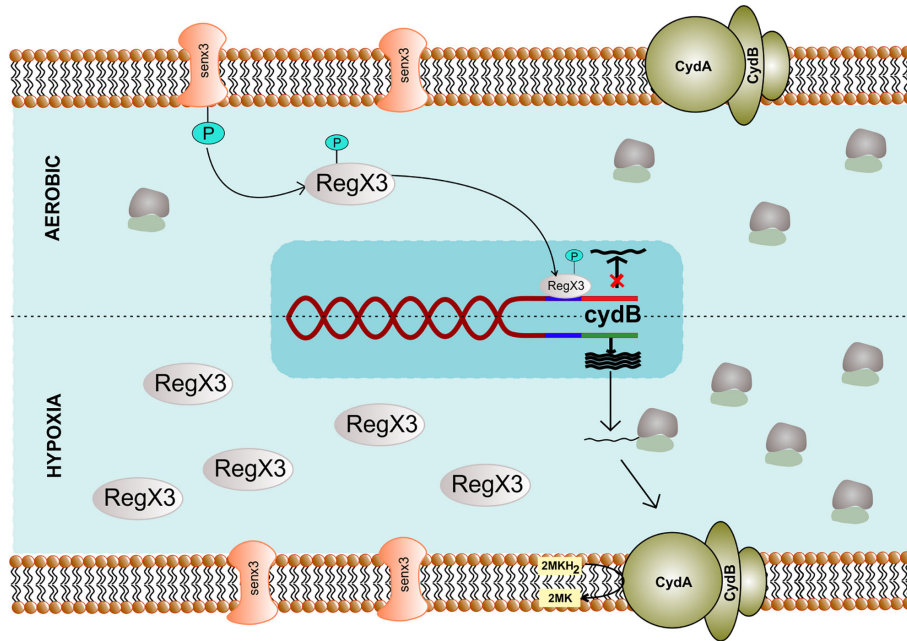


FIGURE 3 | Genetic regulation of cytochrome *bd* complex. The expression of *cydB* component of terminal oxidase cytochrome *bd* depends upon SenX₃ – RegX₃ two-component system specific to *Mycobacterium*. During the aerobic condition, phosphorylated RegX₃ binds to the promoter region of *cydB* and inhibits transcription. However, during hypoxia, RegX₃ gets dephosphorylated and does not bind to the promoter of *cydB*, thus facilitating its expression.

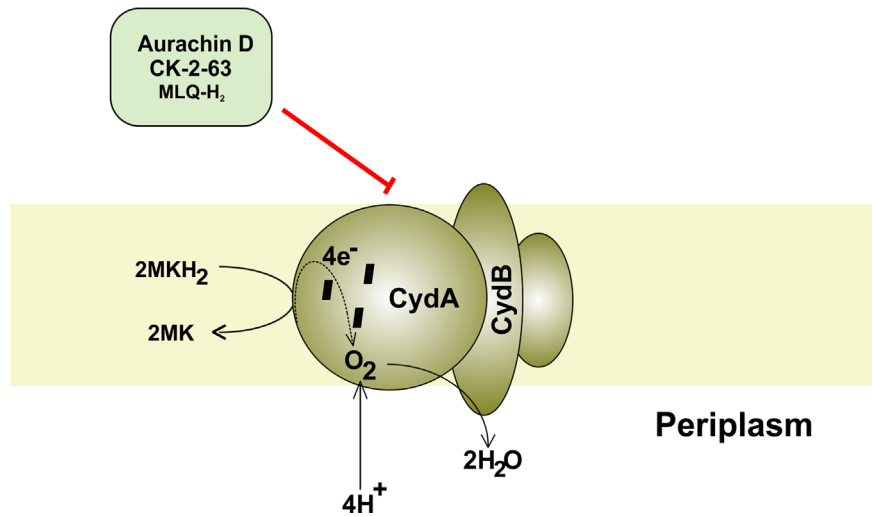


FIGURE 4 | Mycobacterial cytochrome *bd* oxidase. The components and inhibitors of terminal oxidase cytochrome *bd* and the passage of electron through its various subcomponents. The electron is finally accepted by oxygen, which accepts proton from the periplasm to form water.

cytochrome *bd* oxidase (Meunier et al., 1995). It inhibits oxygen consumption in *M. smegmatis* in a dose-dependent manner. Optimized, derivatives of Aurachin D, with better ability to penetrate the mycobacterial cell wall, can become a new class of antitubercular drugs (Lu et al., 2015). Gramicidin S represents another class of compounds capable of inhibiting *bd* oxidase

(Mogi et al., 2008). It is a cationic cyclic decapeptide and possesses potent antibiotic activity. Earlier models suggest that it perturbs the bacterial membrane to exert its antibiotic activity, but recent literature shows that it could also target *bd* oxidase (Mogi et al., 2008). Besides, Gramicidin S, another antimicrobial peptide Microcin J25 was shown to inhibit *E. coli* *bd* oxidase

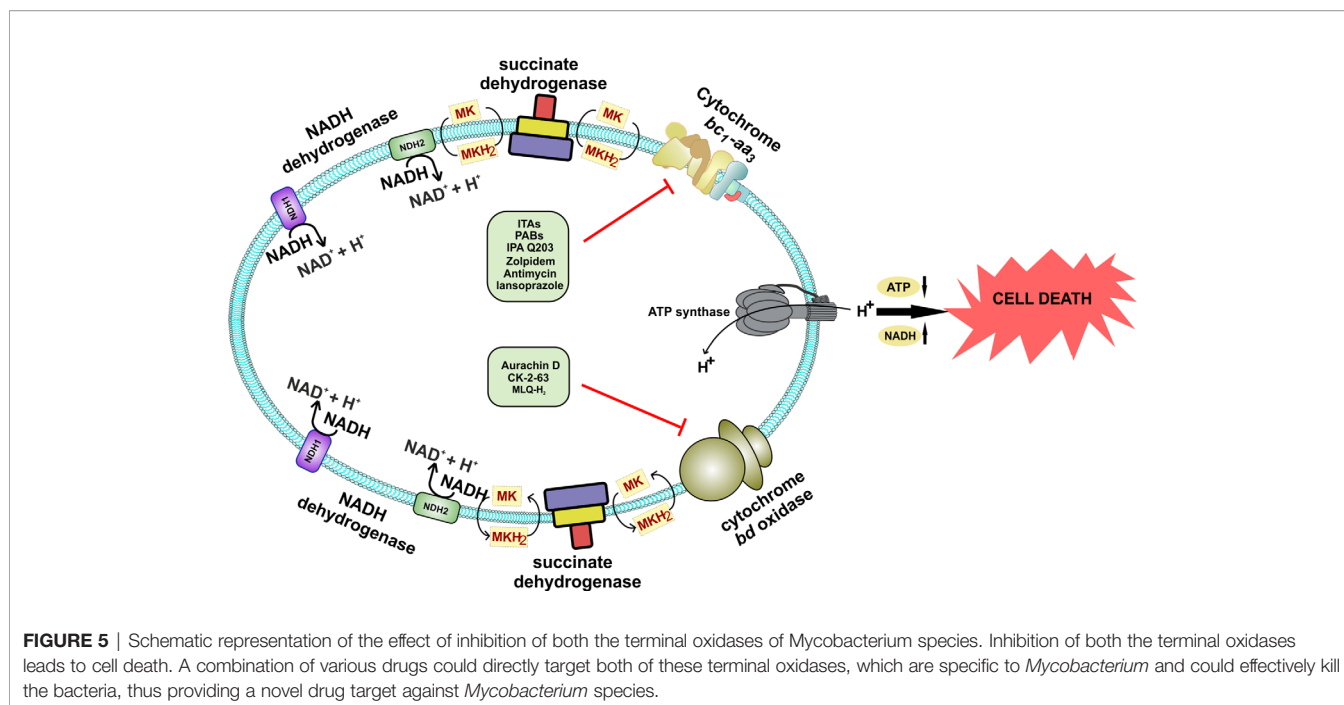


TABLE 2 | Summary of cytochrome *bd* oxidase inhibitors, their chemical structures and MIC values. Number in the parentheses denotes the references.

Compound name	Chemical structure	MIC value
Aurachin D (Lenaz and Genova, 2010)		>85 μM
MLQ-H₂ (Berney et al., 2014)		34 μM
CK-2-63 (Kang et al., 2014)		0.003 μM

(Galvan et al., 2019). However, it remains to be seen if these antimicrobial peptides could inhibit the respiration of mycobacterial cells. Another study identified the prenylphenols class of compounds from the Kitasato Institute for Life Sciences Chemical Library to inhibit *bd* oxidases. Interestingly, prenylphenols LL-Z1272 beta and epsilon were specifically

active against the *bd* oxidase (Mogi et al., 2009). Importantly, the utilization of the computational biology approach led to the identification of MQL-H2, a novel inhibitor of *bd* oxidase (Harikishore et al., 2020). This inhibitor is postulated to bind the menaquinol binding site of CydA and inhibits ATP synthesis in inverted membrane vesicles of *M. smegmatis* (Harikishore

et al., 2020). In an interesting development, a quinolone derivative CK-2-63, and its analogs were identified as potent inhibitors of mycobacterial *bd* oxidase inhibitors (Ward et al., 2017). CK-2-63 alone partially inhibits the growth of *Mtb* *in vitro* cultures. Interestingly, this inhibitor, in combination with cytochrome *bcc* inhibitors and/or ATP synthase inhibitor Q203 was capable of killing mycobacterial cells (Ward et al., 2017).

ALTERNATIVE RESPIRATORY COMPLEXES

Respiratory complexes *bc₁-aa₃* supercomplex and cytochrome *bd* oxidase utilize oxygen as a terminal electron acceptor. However, during severe hypoxia and anoxia, oxygen is not readily available to act as an electron acceptor. Under such conditions, *E. coli* cells utilize alternative electron acceptors such as nitrate and fumarate for oxidizing the reduced pool of the electron carrier menaquinone. Importantly, under oxygen limiting conditions and upon inhibition of respiration, NADH levels are tremendously reduced along with lower levels of ATP (Watanabe et al., 2011; Bhat et al., 2016). Reoxidation of NADH is critical for mycobacterial cells to deter reductive stress and survive during such conditions (Mavi et al., 2020). Mycobacterium alters its ETC and central metabolic pathways to endure low levels of oxygen. In this section, we will describe the role of nitrate reductase (NR) and fumarate reductase (FRD) as a component of an alternative respiratory chain utilized by mycobacteria for survival during hypoxia and anoxia. It must be noted that these modulations only help mycobacteria to survive but do not facilitate mycobacterial growth.

Nitrate Reductase

NRs are families of enzymes that catalyze the reduction of nitrate (NO_3^-) into nitrite (NO_2^-). The presence of this family of enzymes in bacteria was revealed way back in 1925 through the demonstration of nitrate aided anoxic bacterial growth (Quastel et al., 1925; Stewart, 1988). These enzymes are utilized for a multitude of functions ranging from respiration to assimilation and dissimilation of nitrate. A common defining characteristic of these enzymes is the utilization of the molybdenum cofactor for the reduction of nitrate (Morozkina and Zvyagilskaya, 2007). The catalytic molybdenum site is coordinated through bis-molybdopterin guanine dinucleotide (Diaes et al., 1999). Three types of NRs are found in bacteria, i.e., cytoplasmic assimilatory, membrane-bound respiratory, and periplasmic dissimilatory (Morozkina and Zvyagilskaya, 2007). Both the periplasmic dissimilatory and membrane-bound respiratory forms are known to assist growth during anaerobic conditions and are present in *E. coli* (Stewart et al., 2002; Morozkina and Zvyagilskaya, 2007). For this review, we will primarily focus on the membrane-anchored respiratory NR as it is the only functional NR in mycobacteria (Figure 6). Respiratory NR is composed of three subunits (Stewart, 1988; Morozkina and Zvyagilskaya, 2007). Subunit γ is encoded by gene *narI* and it anchors the enzymatic complex to the membrane and captures electrons from reduced menaquinone using heme cofactors. Electrons are then passed onto the subunit β encoded by *narH*. NarH contains [Fe-S] clusters that facilitate the transfer of electrons to subunit α encoded by *narG*. Subunit α utilizes [Fe-S] and molybdenum cofactor to reduce nitrate into nitrite in the cytoplasm (Stewart, 1988; Morozkina and Zvyagilskaya, 2007). The expression of *narGHIJ* operon encoding the enzyme complex is tightly

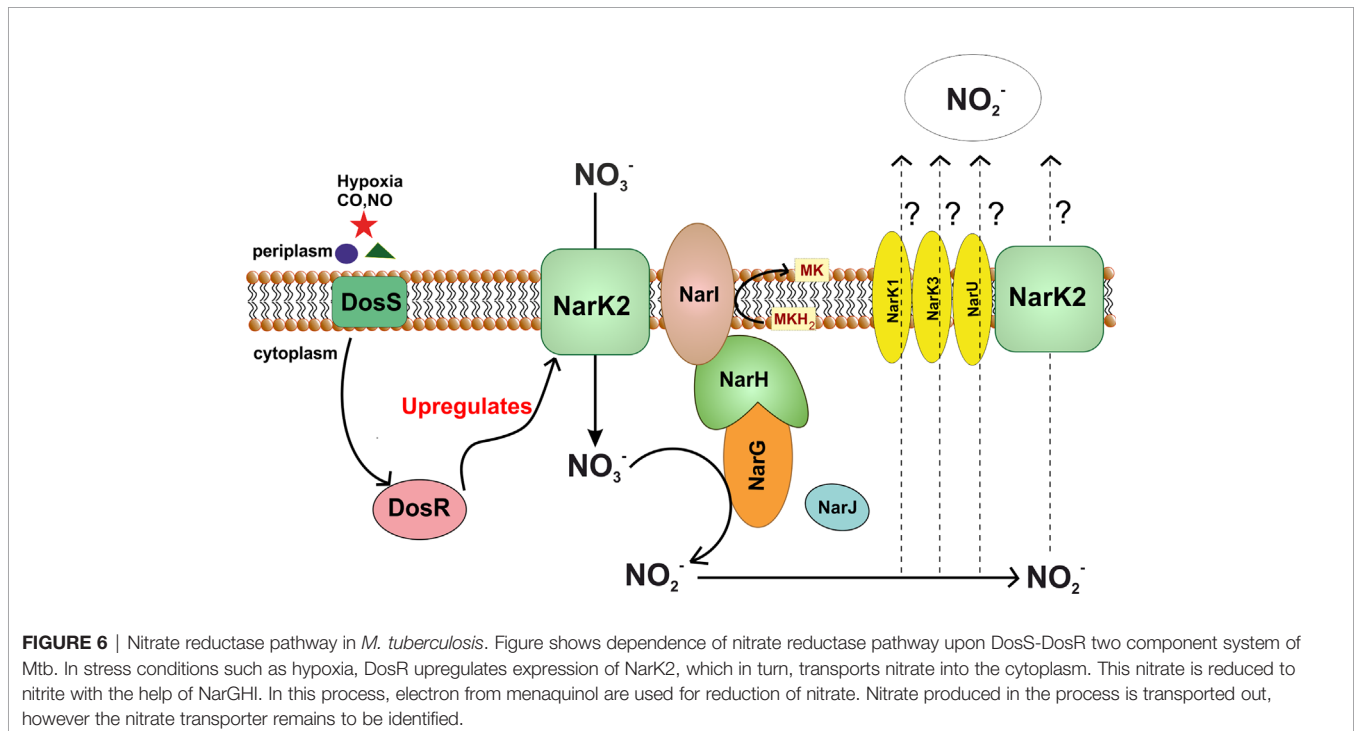


FIGURE 6 | Nitrate reductase pathway in *M. tuberculosis*. Figure shows dependence of nitrate reductase pathway upon DosS-DosR two component system of *Mtb*. In stress conditions such as hypoxia, DosR upregulates expression of NarK2, which in turn, transports nitrate into the cytoplasm. This nitrate is reduced to nitrite with the help of NarGHI. In this process, electron from menaquinone are used for reduction of nitrate. Nitrate produced in the process is transported out, however the nitrate transporter remains to be identified.

regulated by transcription factors like FNR, NarL, and NarP during anaerobic conditions or in the presence of nitrate (Stewart, 1994).

Mtb possesses the capability to reduce nitrate into nitrite while other related mycobacterial species lack this ability. Significantly, this ability is historically utilized for the discrimination of Mtb from *M. bovis* (Virtanen, 1960; Escoto and de Kantor, 1978). Mtb has two genetic clusters bearing homology to genes encoding dissimilatory NRs, namely *narGHJI* operon and the *narX* gene of the *narK2X* operon (Sohaskey and Wayne, 2003). The *narGHJI* operon encodes for the classical respiratory NR. Overexpression of this operon manifests NR activity in *M. smegmatis* (Weber et al., 2000), and can complement the *nar* mutant of *E. coli* for NR activity (Sohaskey and Wayne, 2003). On the other hand, NarX is a fused NR that seems to lack functional NR activity since the deletion of this gene does not affect the NR activity of Mtb (Sohaskey and Wayne, 2003). In comparison, the disruption of NarG leads to the attenuation of NR activity in Mtb. Interestingly, the aerobic cultures of Mtb possess basal levels of NR activity, which is enhanced during adaptation to hypoxia (Wayne and Hayes, 1998). However, unlike the *E. coli narGHJI* operon, that is induced upon hypoxia and in the presence of nitrate through transcriptional regulators FNR and NarL, the expression of *narGHJI* operon of Mtb is not induced in response to hypoxia or nitrate (Sohaskey and Wayne, 2003). On the contrary, the increase in NR activity of Mtb in response hypoxia is associated with increased expression of nitrate transporter NarK2X (Sohaskey and Wayne, 2003). Mtb possesses three isomers of nitrate transporter NarK2X, namely NarK1, NarK3, and NarU. Out of these, NarK2X is part of the *dos/dormancy* regulon regulated by the DosRST two-component system that responds to hypoxia, carbon monoxide and nitric oxide (Park et al., 2003; Voskuil et al., 2003; Kumar et al., 2007; Kumar et al., 2008; Trivedi et al., 2012). In line with this, the expression of *narK2* is induced during infection in mice (Shi et al., 2005b), suggesting that NR could aid in the survival of Mtb during hypoxia. However, the *narG* mutant is not attenuated for survival in hypoxia in the presence or absence of nitrate in the media (Sohaskey and Wayne, 2003). Furthermore, the expression of the *narK2* and *NarX* are downregulated upon biofilm formation (Trivedi et al., 2016). These observations raise doubts on the role of the *narGHJI* operon (and NRs) in mycobacterial survival and respiration under anaerobic conditions. However, NR protects the hypoxic Mtb cells from the stress of reactive nitrogen species and acidic pH (Tan et al., 2010).

Mtb is known for its capability to shift down its metabolic state and acquire the state of non-replicating persistence in response to a gradual decrease in the oxygen levels (Kumar et al., 2011; Bhat et al., 2012). However, Mtb does not survive sudden anaerobiosis; the presence of nitrate in culture medium aids in the survival of Mtb during sudden anaerobiosis. Furthermore, NarG plays an essential role in this survival against the sudden removal of oxygen (Sohaskey, 2008). In agreement with these observations, mycobacterial survival in the presence of respiratory inhibitor Thioridazine is enhanced by nitrate and a functional NR (Sohaskey, 2008). Inhibition of

respiration leads to the accumulation of NADH (Bhat et al., 2016). Accumulated NADH ultimately leads to oxidative stress (Mavi et al., 2020), and thus it is prudent for mycobacterial cells to modulate its respiration and metabolism to re-oxidize NADH.

An interesting property of NR of Mtb is that it is not oxygen sensitive, unlike other bacterial NRs (Pichinoty and D'Ornano, 1961). Mtb utilizes this property of NR and leads to NarG dependent accumulation of nitrite in Mtb infected human macrophages (Cunningham-Bussell et al., 2013). Nitrite affects the metabolism and growth of Mtb (Cunningham-Bussell et al., 2013). It was suggested that NR aid in the maintenance of redox homeostasis in Mtb (Sohaskey, 2008). Importantly, transcripts of NarG were detected in the pericavity and the distant lung regions in clinical lung samples (Rachman et al., 2006). Furthermore, the evidence for the importance of nitrate respiration in TB pathogenesis comes from the vaccine strain *M. bovis* BCG. Although BCG exhibit the low activity of NR, however, this activity is important for survival and tissue-specific persistence of BCG in immunocompetent and immunocompromised mice (Fritz et al., 2002). Despite the importance of NR in TB pathogenesis, it has not been used as a target for drug development. Although, a few compounds like Pentachlorophenol are known to inhibit the NR. These are used primarily as a pesticide and a disinfectant. However, the establishment of NR as a valid drug target and development of Mtb specific inhibitors need further research.

Fumarate Reductase and Bidirectional Succinate Dehydrogenases

Fumarate reductases (FRD) are the enzymes that catalyze the reduction of fumarate into succinate (Van Hellemond and Tielens, 1994). These enzymes are critical for organisms utilizing fumarate as the terminal electron acceptor. These enzymes are very similar to succinate dehydrogenases that catalyze the reverse reaction and convert succinate into fumarate while donating electrons to ubiquinone/menaquinone. Mtb genome encodes for a fumarate dehydrogenase (FRD, encoded by *frdA-rv1556* operon) and two succinate dehydrogenases (SDH1, encoded by *rv0247c-rv0249c* and SDH2, encoded by *rv3316-rv3319*) (Cook et al., 2014; Iqbal et al., 2018). FRD can be found as a soluble enzyme, but the one that participates in the respiration is often membrane-anchored (Van Hellemond and Tielens, 1994). It is a polypeptide complex consisting of three to four subunits named; A, B, C, and D. Catalytic core is constituted by FRD-A and FRD-B, whereas other domain/s act as membrane anchor and harvest electrons from menaquinone/ubiquinone (Van Hellemond and Tielens, 1994). Importantly, the FRD of Mtb is induced upon exposure to hypoxia (Watanabe et al., 2011). Furthermore, hypoxic Mtb cultures accumulate succinate in media (Watanabe et al., 2011; Eoh and Rhee, 2013). Notably, during hypoxia, Mtb could utilize a reductive half TCA cycle to oxidize NADH (Eoh and Rhee, 2013). These observations suggest that FRD will be critical for the survival of Mtb during hypoxia and in animal models. However, the *frdA* deletion mutant is not attenuated for survival during hypoxia or inside animals (Watanabe et al., 2011). Furthermore, cultures of *frdA* deletion mutant in hypoxia lead to the accumulation of equivalent levels of succinate in the media, as is observed with wild type control. These observations point towards

redundancy in the FRD and SDH function in Mtb. In line with these, *sdh2* of *M. smegmatis* is upregulated during hypoxia while *sdh1* is induced during energy limiting conditions (Berney and Cook, 2010). These observations again suggest a functional redundancy (Figure 7). However, analysis of Mtb SDH1 and SDH 2 suggested a subtle difference in the two. It was observed that the deletion of SDH1 results in an alteration in the rate of respiration associated with the inability to recover from the stationary phase (Hartman et al., 2014). Nonetheless, such a functional redundancy is a major roadblock in targeting FRD/SDH of Mtb and suggests that inhibitors capable of inhibiting all three enzymes despite genetics and structural differences may target FRD/SDH of Mtb (Hards et al., 2020).

SYNERGY BETWEEN INHIBITORS OF TERMINAL RESPIRATORY OXIDASES

Emerging literature suggests that Mtb shows a high level of flexibility in utilizing both of its terminal respiratory oxidases for survival and/or growth (Arora et al., 2014; Kalia et al., 2017b; Moosa et al., 2017; Scherr et al., 2018; Beites et al., 2019). Knock

out strains of Mtb lacking *bc₁-aa₃* oxidoreductase (Beites et al., 2019) or cytochrome *bd* oxidase (Moosa et al., 2017) have been created, suggesting these oxidases could suffice each other's function and thus are redundant. These observations raise questions over the suitability of the terminal oxidases as a drug target. Notably, mutants lacking a functional cytochrome *bd* oxidase are hyper susceptible to the inhibitors of *bc₁-aa₃* oxidoreductase (Arora et al., 2014; Kalia et al., 2017a; Moosa et al., 2017), and mutants lacking functional *bc₁-aa₃* oxidoreductase are highly susceptible to inhibitors of *bd* oxidase (Lu et al., 2015). These studies suggest that simultaneous inhibition of both terminal respiratory oxidases is bactericidal *in vitro* and *in vivo* (Kalia et al., 2017a; Beites et al., 2019). Importantly, such an approach is successful at killing replicating as well as non-replicating persistent bacteria. We believe that the synergistic inhibition of both the terminal oxidases represents an ideal drug development strategy (Figure 5). It was recently demonstrated that *bc₁-aa₃* oxidoreductase inhibitor Q203 and ATP synthase inhibitor leads to increased respiration in Mtb cells (Lamprecht et al., 2016). Importantly, this study also elucidated that a combination of BDQ, Q203, and clofazimine is extremely potent at killing mycobacterial cells in culture medium (Lamprecht et al., 2016).

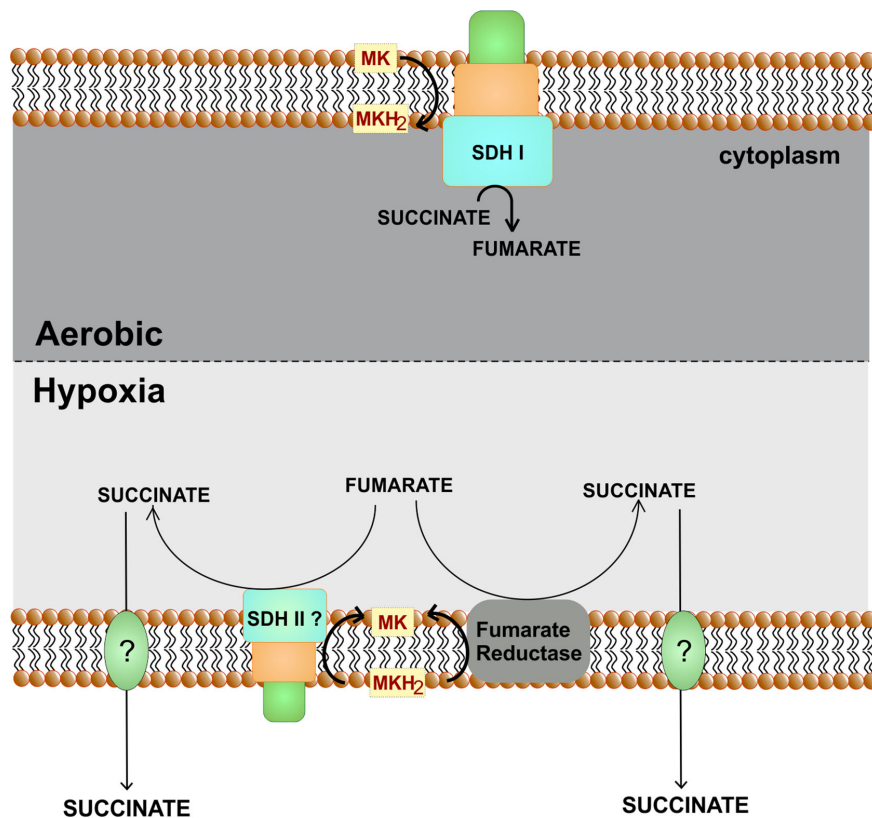


FIGURE 7 | Role of fumarate reductase/succinate dehydrogenase as an alternate respiratory pathway. Under hypoxic conditions, fumarate can act as an alternate electron acceptor. For this either fumarate reductase is utilized or succinate dehydrogenase perform the reverse reaction. Succinate is secreted out of the cells.

Testing animal infection with such a regimen and its comparison vis a vis isoniazid/rifampicin/pyrazinamide/ethambutol could further establish the validity of bioenergetics as one of the best drug targets available. It is important to note that BDQ lack bactericidal activity during the initial 3-4 days although its exposure leads to depletion of ATP within 3 h (Koul et al., 2014). It could be hypothesized that Mtb may survive during first few days in the presence of BDQ through limited substrate-level phosphorylation. Glycolysis is a major catabolic pathway that leads to production of ATP in Mtb cells. In the light of these, we propose that the inclusion of a glycolysis inhibitor such as 2-deoxyglucose (Jin et al., 2016) may synergize with the combination of BDQ, Q203, and clofazimine and thus could be very effective at killing mycobacterial cells *in vitro* and *in vivo*. Such a hypothesis needs to be tested to increase the efficacy of respiratory inhibitors in the mycobacterial killing. Furthermore, transposon mutagenesis based screens could be utilized for discovering pathways utilized by Mtb for generation of ATP in the presence of BDQ. Development of inhibitors of these pathways could be prioritized. Once such inhibitors are identified, they could be used in combination with BDQ and clofazimine.

CONCLUDING REMARKS

Mycobacterial ETC is highly flexible and efficiently synchronizes with the plasticity of the central metabolic system to facilitate the survival of Mtb in varied microenvironments ranging from diverse types of granulomas to sputum droplets. During the last few years, inhibitors of ETC have raced ahead in the clinical trials, and few are even approved for the treatment of MDR-TB and XDR-TB. Amongst the components of ETC, terminal oxidases stand out as a druggable target. Several classes of chemical compounds capable of inhibiting cytochrome *bc₁-aa₃* oxidase are known, including Imidazopyridine amides, Arylvinyloxy piperazine amide, Morpholinothiophene, Pyrrolopyridinedione, Quinazolinamines, Quinolinones, Benzimidazoles, Pyrazolopyridine amides, Imidazothiazole amide, Imidazopyridine ethers, 2-(Quinolin-4-ylxy)acetamides. However, only a few classes of chemical compounds capable of inhibiting the cytochrome *bd* oxidase are known, including quinone analogs of the Aurachin family, a

quinolone derivative CK-2-63, and a novel cytochrome *bd* oxidase specific inhibitor, MQL-H2, of imidazole class. Evidence emerging from hyper-susceptibility of one terminal oxidase mutant to the inhibitors of other terminal oxidase opens up the door for developing a new regimen where a combination of inhibitors targeting both the terminal oxidases can be used for the treatment of TB. In this direction, identifying novel cytochrome *bd* oxidase inhibitors should be a priority. Combination therapy could help in the shortening of the treatment duration. A regimen targeting several different pathways such as oxidative phosphorylation, cell wall biosynthesis, and RNA/protein biogenesis could emerge as a universal regimen capable of treating drug-susceptible, MDR-TB, and XDR-TB patients. TB community, along with the pharma companies, have embarked upon this mission, which has led to the strengthening of the TB drug pipeline. We are optimistic that this will lead to the evolution of new regimens for TB treatment in the future.

AUTHOR CONTRIBUTIONS

SB and AK conceptualized the structure of the manuscript. SB, NB, MK, GP, VC, and AK wrote the manuscript. NB and SB also helped with the illustrations of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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