Targeting the cytochrome oxidases for drug development in mycobacteria

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Abstract

Mycobacterium tuberculosis strictly depends on oxygen to multiply, and the terminal oxidases are a vital part of the oxidative phosphorylation pathway. The bacterium possesses two aerobic respiratory branches: a cytochrome *bcc-aa₃* and a bacteria-specific cytochrome *bd* oxidase. The identification of small-molecule inhibitors of the cytochrome *bcc-aa₃* under numerous experimental conditions reflects the essentiality of the pathway for the optimum growth of *M. tuberculosis*. Recent findings on the biology of the cytochrome *bcc-aa₃* as well as the report of the first high-resolution structure of a mycobacterial cytochrome *bcc-aa₃* complex will help in the characterization and further development of potent inhibitors. Although the aerobic cytochrome *bd* respiratory branch is not strictly essential for growth, the discovery of a strong synthetic lethal interaction with the cytochrome *bcc-aa₃* placed the cytochrome *bd* oxidase under the spotlight as an attractive drug target for its synergistic role in potentiating the efficacy of cytochrome *bcc-aa₃* inhibitors and other drugs targeting oxidative phosphorylation. In this review, we are discussing current knowledge about the two mycobacterial aerobic respiratory branches, their potential as drug targets, as well as potential drawbacks.

Keywords: telacebec, Q203, drug discovery, oxidative phosphorylation, terminal oxidase, bioenergetics

1. Introduction

Oxygen reductases, or oxidases, comprise of structurally distinct enzymes that are widely found in all kingdoms of life. Besides the well characterised family of heme-copper oxidases present in both prokaryotes and eukaryotes, other oxidases include cytochrome *bd* oxidases (cyt-*bd*) and cyanide-resistant alternative oxidases of mitochondria and chloroplasts found in plants and some protozoa (Borisov et al., 2011). Within the heme-copper oxidase family, the most widely known member is the complex IV of mitochondrial electron transport chain (ETC). Oxygen reductases play an important role in the oxidative phosphorylation (OXPHOS) pathway by conducting the final electron transfer from the electron transport chain (ETC) to molecular oxygen. Their enzymatic function prevents the system from an over-reduced state and recycles cofactors required for the function of the central carbon metabolism upstream.

In mycobacteria, oxygen is a strict requirement for optimum growth. Even in their persister state – which can occur when the pathogen encounters stress (Cunningham and Spreadbury, 1998; Wayne and Sohaskey, 2001), oxygen limitation, or a microenvironment with poor availability of nutrients – the maintenance of its proton motive force and ATP homeostasis is vital for their survival (Cook et al., 2017; Koul et al., 2008; Rao et al., 2008). Their reliance on the oxidative phosphorylation pathway thus highlights the importance of the terminal oxidases for the bacteria's growth and survival. Mycobacteria possess two types of oxidases: a heme-copper *aa₃*-type oxidase coupled with *bcc*-quinol reductase as a proton-pumping cytochrome *bcc-aa₃* supercomplex (cyt-*bcc-aa₃*) (Cole et al., 1998; Gong et al., 2018; Megehee et al.,

2006; Wiseman et al., 2018) and a *bd*-type oxidase (Cole et al., 1998; Kana et al., 2001). Research findings thus far support the attractiveness of the terminal oxidases for drug development: the cytochrome *bcc-aa₃* is a validated drug target (Chandrasekera et al., 2017; Moraski et al., 2016; Pethe et al., 2013), and the cytochrome *bd* oxidase influences the potency of *cyt-bcc-aa₃* inhibitors (Kalia et al., 2017; Moosa et al., 2017). However, our understanding of their function and their involvement in other metabolic pathways in the cells remain incomplete. Furthermore, the impact of these inhibitors on the action of existing drugs may prove to be essential to develop rational drug combinations. Here, we discuss the current understanding of the mycobacterial oxidases as well as the benefits and potential problems that might occur upon chemical inhibition of the respiratory terminal oxidases.

2. Cytochrome *bcc-aa₃*

2.1 Cytochrome *bcc-aa₃* complex

The mycobacterial cytochrome *bcc-aa₃* supercomplex consists of a *bcc* menaquinol reductase and an *aa₃* oxidase that are tightly associated, alleviating the need for a soluble cytochrome *c* component (Megehee et al., 2006). These two complexes are homologous to and structurally very similar to the mitochondrial complexes III and IV respectively (Gong et al., 2018; Wiseman et al., 2018).

The atomic structure of *cyt-bcc-aa₃* in *M. smegmatis* revealed features specific to mycobacteria (Gong et al., 2018; Wiseman et al., 2018). In the mitochondria, complexes III and IV form respiratory supercomplexes whose high-resolution structures are well established (Letts et al., 2016). While it was known that the mycobacterial *bcc* and *aa₃* complexes do interact and only function when associated (Megehee et al., 2006), it was the resolution of the atomic structure of a mycobacterial *cyt-bcc:aa₃* supercomplex, which unveiled its intricate structure and electron transfer pathway (Gong et al., 2018; Wiseman et al., 2018). The resolved supercomplex contains additional subunits to those encoded by *qcrCAB* and *ctaCDEF* (Cole et al., 1998; Megehee et al., 2006; Sakamoto et al., 2001), including new structural subunits of the *aa₃* oxidase, association factors that stabilize the supercomplex and, more surprisingly, a superoxide dismutase (SOD) (Gong et al., 2018; Wiseman et al., 2018) (Figure 1). The significance of the association of SOD within the supercomplex is not yet understood but may indicate a localisation at a site of maximal reactive oxygen species generation (Gong et al., 2018). The reported route of electron transport within the complex is also unique to mycobacteria: instead of using a soluble cytochrome *c* as a shuttle, these bacteria utilize a QcrC domain of *bcc* to shuffle electrons to the *aa₃* complex (Gong et al., 2018; Wiseman et al., 2018) by changing its conformation from closed to open (Wiseman et al., 2018). These novel observations emphasize gaps in our fundamental understanding about mycobacterial respiratory oxidases. In addition, they also showcase how basic research can contribute to the field of drug development by identifying vulnerabilities and thus new possibilities for the design of novel inhibitors.

2.2 Biological significance of *cyt-bcc-aa₃* in mycobacteria

The *bcc-aa₃*-oxidoreductase supercomplex is required for optimum mycobacterial growth: deletion of the *bcc-aa₃*-encoding genes in *M. tuberculosis* and *M. smegmatis* results in drastic elongation of generation time and changes in colony morphology (Beites et al., 2019; Matsoso et al., 2005). As an adaptation to the absence of the *cyt-bcc-aa₃*, the expression of the *cyt-bd* oxidase is upregulated in *M. smegmatis* in order for the bacteria to meet its energetics demand (Matsoso et al., 2005). In *M. tuberculosis*, the genetic knockout of cytochrome *bcc-aa₃* resulted in slowed growth *in vitro* as well as partial attenuation *in vivo* (Beites et al., 2019). Further, the

slow-grower's dependency on the complex for optimum growth is evident from the impact of chemical inhibition of the *cyt-bcc-aa₃*. Small molecule drugs acting on the *cyt-bcc-aa₃* effectively halts *M. tuberculosis* growth *in vitro* as well as in macrophage and fibroblast infection models (Foo et al., 2018; Pethe et al., 2013; Rybniker et al., 2015). The essentiality of *cyt-bcc-aa₃* *in vivo* however may depend heavily on the environment the pathogen resides in. The manifestation of granulomatous lesions is one of the key features in human tuberculosis disease, which exposes the pathogen to different microenvironmental conditions such as varying levels of oxygen (Barry et al., 2009), which could affect the expression of the *cyt-bcc-aa₃*. In *M. tuberculosis*, the expression of *cyt-bcc-aa₃* decreases in mouse and macrophage infection models, possibly in response to nitric oxide produced by macrophages (Schnappinger et al., 2003; Shi et al., 2005). Moreover, the expression of the homologous complex in *M. smegmatis* was downregulated when bacteria enter a steady-state growth condition (Berney and Cook, 2010). On the other hand, mycobacterial *cyt-bcc-aa₃* expression is either unaffected (Shi et al., 2005), or upregulated (Berney and Cook, 2010) under hypoxic conditions. The availability of carbon sources was also recently found to directly affect the essentiality of *cyt-bcc-aa₃* by altering the expression of *cyt-bd* (Kalia et al., 2019). While all these studies are informative of the effect individual factors have on the expression of *cyt-bcc-aa₃*, pathogens often encounter a combination of these conditions during infection (Beste et al., 2007; Schnappinger et al., 2003; Via et al., 2008). Thus how those factors affect the expression and function of *cyt-bcc-aa₃* in combination needs to be studied further.

Figure 1. Atomic structure of *cyt-bcc-aa₃* of *M. smegmatis* (PDB ID: 6ADQ) (Gong et al., 2018). The subunits of the dimeric complex are color-coded from red (complex *bcc*, in the middle) to green (complex *aa₃*, on the periphery). The SOD dimer is shown in cyan.

2.3 *Cyt-bcc-aa₃* as a drug target

The anti-mycobacterial potency of the imidazopyridine amide (IPA) series was first reported in 2011 (Moraski et al., 2011). The subsequent discovery of the IPA derivative Q203 (Telacebec) demonstrated that the QcrB subunit of the cytochrome *bcc* complex is the target of this chemical series in *M. tuberculosis* (Pethe et al., 2013). Q203 is the most advanced

member of the IPA series currently in phase 2 clinical trial. Over the following years, new classes of QcrB inhibitors were discovered: pyrrolo[3,4-c]pyridine-1,3(2H)-diones (van der Westhuyzen et al., 2015), 2-(quinolin-4-yloxy)acetamides (Phummarin et al., 2016), imidazo[2,1-b]thiazole-5-carboxamides (Moraski et al., 2016), phenoxyalkylbenzimidazoles (Chandrasekera et al., 2017), arylvinylpiperazine amides (Foo et al., 2018), morpholino thiophenes (Cleghorn et al., 2018), and 4-amino-thieno[2,3-d]pyrimidine (Harrison et al., 2019) (Table 1). In the effort to repurpose drugs for anti-TB therapy, gastric proton pump inhibitor lansoprazole and the sedative zolpidem were shown to inhibit the *M. tuberculosis* cytochrome *bcc-aa₃* (Moraski et al., 2015; Rybniker et al., 2015) (Table 1). Interestingly all the QcrB inhibitors published to date appear to share a similar binding pocket with Q203 near the menaquinol binding (Q_p) site in the structure of *bcc* reductase (Gong et al., 2018; Wiseman et al., 2018). The drug-target binding mode of imidazopyridine amides to cytochrome *b* was also demonstrated using in-cell NMR (Bouvier et al., 2019). The seven most commonly found single nucleotide polymorphisms in *qcrB* which conferred bacteria's resistance to Q203 were shown to drastically reduce the potency of other inhibitors (Cleghorn et al., 2018; Moraski et al., 2016; Phummarin et al., 2016; van der Westhuyzen et al., 2015). In the 2-(quinolin-4-yloxy)acetamide, phenoxyalkylbenzimidazole, and 4-amino-thieno-[2,3-d]pyrimidine series, their binding target was confirmed by the sequencing of resistant mutants harbouring SNPs in *qcrB* (Chandrasekera et al., 2017; Harrison et al., 2019; Phummarin et al., 2016). Using a computational model of *M. tuberculosis* *qcrB*, the SNPs conferring resistance to AX-35 (arylvinylpiperazine amide series) was found to cluster with resistance-conferring mutations of Q203 and lansoprazole (Foo et al., 2018), suggesting that the series share a similar binding site. While lansoprazole retained some potency against strains showing common polymorphisms such as T313A, the substitution L176P was sufficient to annihilate its inhibitory potency. Upon closer examination of the residue's localisation in the 3D model, it became apparent that Lansoprazole occupies the same binding pocket as Q203 near the Q_p site, albeit most likely relies on interaction with different residues in the pocket to attain its potency (Rybniker et al., 2015).

The sensitivity of *cyt-bcc-aa₃* to chemical inhibition by multiple distinct chemical series has left an impression of unspecificity and attracted the label of a "promiscuous target" (Cole, 2016; Laurent Roberto et al., 2016). However, the frequency of discovery of *cyt-bcc-aa₃* inhibitors may simply reiterate the vulnerability of the target to chemical inhibition (Lee and Pethe, 2018). Regardless, the fact that all known inhibitors of *cyt-bcc-aa₃* share the same binding site and most likely also the mechanism of action with Q203 is concerning. The drug pipeline has a notoriously high attrition rate due to the stringent requirements of drug safety and efficacy, which can be difficult to predict in the earlier stages of development. Thus having multiple series possessing the same binding target in development can improve the odds of bringing *cyt-bcc-aa₃* inhibitors to clinical use. Nonetheless, it also reveals that current work focuses only on a specific part of the supercomplex. With that in mind, the recently resolved three-dimensional structure of the mycobacterial *cyt-bcc-aa₃* supercomplex, as well as the proposed electron transfer pathway might prove to be useful in spearheading the development and discovery of "new generation" *cyt-bcc-aa₃* inhibitors targeting novel binding sites distinct from Q203. In light of the discovery, rational design may be relevant in aiding the search for targeted small molecules that selectively act to disrupt the function of the *cyt-bcc-aa₃* via a novel binding site.

Sequences and structural features of heme-copper oxidases are highly similar across the organisms (Sharma and Wikstrom, 2014), possibly due to the essentiality of the complex for growth in aerobic organisms. Given the strict conservation of this complex, cross-species activity within the *Mycobacterium* genus could occur. Thus far, the vulnerability of cytochrome *bcc-aa₃* to chemical inhibition has proved to be translatable only in *M. ulcerans*, the causative

agent of Buruli ulcer (Converse et al., 2019; Liu et al., 2019; Scherr et al., 2018). These findings demonstrated that inhibitors of the target resulted in rapid clearance of infection. Notably, the efficacy of *cyt-bcc-aa₃* inhibitors in the classical *M. ulcerans* lineages far exceeds that observed in *M. tuberculosis*. While Q203 is strictly bacteriostatic in *M. tuberculosis*, it is rapidly bactericidal *in vitro* and *in vivo* against *M. ulcerans* (Scherr et al., 2018). This exceptional potency is attributed to the absence of a functional *cyt-bd* in *M. ulcerans*. Similarly, *M. leprae*, the mycobacterium species responsible for leprosy, may also be highly susceptible to chemical inhibition of *cyt-bcc-aa₃* since this species also lacks *cyt-bd*-encoding genes (Cole et al., 2001).

The revelation on the hypersusceptibility of *M. ulcerans* to *cyt-bcc-aa₃* inhibition is important for our understanding of how respiration in mycobacteria operates. The classical mycobacterial respiratory chain is a robust pathway, and in case of a disruption in one complex, mycobacteria are capable of switching to an alternate pathway to survive. For most mycobacteria, the presence of the *cyt-bd* oxidase can rescue them from the detriments of a collapse in *cyt-bcc-aa₃* activity, as will be discussed in the next section.

Table 1. Structures of *qcrB* inhibitors of different series.

Chemical series	Compound name	Chemical Structure
Imidazopyridine amide	<i>N</i> -benzyl-2,7-dimethylimidazo[1,2- <i>a</i>]pyridine-3-carboxamide (Moraski et al., 2011)	
Imidazopyridine amide	Q203 (Pethe et al., 2013)	
pyrrolo[3,4- <i>c</i>]pyridine-1,3(2 <i>H</i>)-dione	Ethyl 7-amino-2-benzyl-1,3-dioxo-2,3-dihydro-1 <i>H</i> -pyrrolo[3,4- <i>c</i>]pyridine-6-carboxylate (van der Westhuyzen et al., 2015)	
2-(quinolin-4-yloxy)acetamide	GSK 358607A (Phummarin et al., 2016)	

imidazo[2,1-b]thiazole-5-carboxamide	ND-11543 (Moraski et al., 2016)
Phenoxyalkylbenzimidazole	2-ethyl-1-(3-phenoxypropyl)-1H-benzo[d]imidazole (Chandrasekera et al., 2017)
arylvinylpiperazine amide	AX-35 (Foo et al., 2018)
morpholino thiophene	5-morpholino-3-phenethoxythiophene-2-carboxamide (Cleghorn et al., 2018)
4-Amino-Thieno[2,3-d]pyrimidine	CWHM-1023 (Harrison et al., 2019)
	Lansoprazole (Rybniker et al., 2015)
	Zolpidem (Moraski et al., 2015)

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189 3. Cytochrome *bd*-type oxidase

190 3.1 Cytochrome *bd* complex

191 The cytochrome *bd* oxidases, present only in prokaryotic organisms, is evolutionarily distinct
192 from the most studied heme-copper oxidases (Borisov et al., 2011). Studies conducted in

several bacteria suggest that *cyt-bd* is not only involved in basic bioenergetic maintenance, but also enhances resistance to oxidative and nitrosative stress (Borisov et al., 2011; Hori et al., 1996; Junemann and Wrigglesworth, 1995; Lindqvist et al., 2000; Wall et al., 1992). Since the mycobacterial *cyt-bd* is not extensively studied, it is commonly assumed that it shares similar functions with its homologs in other species.

The mycobacterial *cyt-bd* oxidase comprises of two subunits encoded by *cydA* and *cydB*, while the neighbouring *cydD* and *cydC* encode a putative cytochrome ABC-transporter whose function is not yet studied. Even though the *cydABDC* genes form a cluster with a short gap of 100 base pairs between *cydB* and *cydD*, *cydAB* and *cydDC* form two separate operons in *M. smegmatis* (Aung et al., 2014). However, since the *cydABDC* genes can also form a polycistronic operon in other bacteria (Kabus et al., 2007; Winstedt et al., 1998), the regulation of *cyt-bd* expression might vary across different mycobacteria species. Moreover, one can observe that sequence identity of CydA is in general higher than for CydB across mycobacteria (Table 2 and 3), consistent with the finding that the evolution rates of *cydA* and *cydB* are asymmetrical (Hao and Golding, 2006; Voggu et al., 2006). It appears that the CydA subunit evolves slightly slower while a larger variance in the CydB subunit is observed in several species (Hao and Golding, 2006). Notably, the sequences of *M. tuberculosis* and *M. bovis* BCG are 100% identical, making BCG a good model organism to study *cyt-bd* (Table 2 and 3). The differences in protein sequences of *cyt-bd* across mycobacteria might result in variation in its function as previously reported in staphylococci (Voggu et al., 2006). The study demonstrated that microevolution of CydB led to variable sensitivity to respiratory inhibitors such as hydrogen cyanide and pyocyanin (Voggu et al., 2006). However, this is something that is yet to be explored in mycobacteria.

Table 2. CydA protein identity matrix identity across mycobacteria, *E. coli* and *G. thermodenitrificans*. Alignment was performed using Clustal Omega program.

	Geotn	Ecoli	Mabs	Msmeg	Mav	Mmar	Mbovis	Mtb
Geotn	100	-	-	-	-	-	-	-
Ecoli	29	100	-	-	-	-	-	-
Mabs	29	37	100	-	-	-	-	-
Msmeg	29	36	74	100	-	-	-	-
Mav	28	36	74	77	100	-	-	-
Mmar	30	37	72	77	80	100	-	-
Mbovis	28	35	73	79	80	84	100	-
Mtb	28	35	73	79	80	84	100	100

Geotn – *G. thermodenitrificans*; Ecoli – *E. coli*; Mabs – *M. abscessus*; Msmeg – *M. smegmatis*; Mav – *M. avium* (strain 104); Mmar – *M. marinum*; Mtb – *M. tuberculosis* H37Rv; Mbovis – *M. bovis* BCG.

Table 3. CydB protein identity matrix identity across mycobacteria, *E. coli* and *G. thermodenitrificans*. Alignment was performed using Clustal Omega program.

	Geotn	Ecoli	Mabs	Msmeg	Mav	Mmar	Mbovis	Mtb
Geotn	100	-	-	-	-	-	-	-
Ecoli	19	100	-	-	-	-	-	-
Mabs	20	37	100	-	-	-	-	-
Msmeg	20	36	63	100	-	-	-	-
Mav	20	34	63	69	100	-	-	-
Mmar	20	34	63	70	83	100	-	-
Mbovis	20	34	63	69	83	85	100	-
Mtb	20	34	63	68	82	85	100	100

To date, the only two reported structures of the *bd*-type oxidase are from *Geobacillus thermodenitrificans* and *Escherichia coli* (Safarian et al., 2019; Safarian et al., 2016) (Figure 2). The subunits CydA and CydB possess similar structural moieties except that subunit CydA contains hemes and a menaquinol-binding site (Q-loop), while CydB lacks such features (Safarian et al., 2019; Safarian et al., 2016). Two hemes b and one heme d are arranged in a triangular manner. However, the two structures showed active site rearrangement and different oxygen entry sites. This suggests variability in the mechanism of electron transfer in these organisms. Proton shuffling to the reaction centre is necessary for the reduction of oxygen. It is proposed to occur with the help of the routes on either subunit (*G. thermodenitrificans*) or proton shuffling channel (*E. coli*) (Safarian et al., 2019; Safarian et al., 2016). These structures provided great insight into the molecular mechanism of *bd*-type oxidases. However, given that *M. tuberculosis* shares low sequence identity in CydA and CydB with *E. coli* and *G. thermodenitrificans* (Tables 3 and 4), structural studies of the mycobacterial enzyme are needed for proper enzymatic characterization and rational drug design.

Figure 2. Structures of cyt-*bd* from *G. thermodenitrificans* (PDB ID: 5DOQ) (A, B) and *E. coli* (PDB ID: 6RKO) (C, D) (Safarian et al., 2019; Safarian et al., 2016). For both structures, subunit CydA is presented in blue, while CydB is in cyan. Subunit CydS for *G. thermodenitrificans* and CydH for *E. coli* are presented in red. CydX of *E. coli* is shown in yellow. Pastel orange color depicts ubiquinol-8 bound to CydB in *E. coli*, but not in *G. thermodenitrificans*.

3.2 Biological significance of cyt-*bd* in mycobacteria

In mycobacteria, cyt-*bd* is not essential for viability under axenic conditions, in the mouse model, or during persistence induced by nutrient-starvation (Kalia et al., 2017; Kana et al., 2001; Lu et al., 2015). The hypothesis of its non-strict essentiality is further supported by the absence of a functional cyt-*bd* in the classical strains of *M. ulcerans* (Scherr et al., 2018; Stinear et al., 2007) and in *Mycobacterium leprosy* (Cole et al., 2001). The cyt-*bd* expression in *M. smegmatis* and *M. tuberculosis* are upregulated in response to hypoxia (Berney and Cook, 2010; Kana et al., 2001; Shi et al., 2005), but its role during long-term persistence under conditions of low oxygen tension remains to be demonstrated. Apart from its role in bioenergetics, the cyt-*bd* respiratory chain protects various bacteria from nitrosative stress and cyanide poisoning (Borisov et al., 2011). Consistently, cyt-*bd* protects *M. smegmatis* from H₂O₂ (Lu et al., 2015) and cyanide (Kana et al., 2001). In the *M. tuberculosis* strain deficient in cytochrome *c* maturation, cyt-*bd* expression is approximately 3-fold higher to compensate for the lack of functional cyt-*bcc-aa*₃ (Small et al., 2013). This overexpression of cyt-*bd* in this strain also conferred increased resistance to H₂O₂, however, the strain was surprisingly hypersusceptible to cyanide (Small et al., 2013), a phenotype that is yet to be understood.

3.3 Cyt-*bd* as a drug target

Cyt-*bd* is only present in bacteria (Borisov et al., 2011), which may represent an advantage for anti-TB drug development. However, the cyt-*bd* being not strictly essential for growth, it presents challenges for identification of inhibitors. While deletion of the cyt-*bd*-encoding genes has no obvious phenotype, mycobacteria become hypersusceptible to cyt-*bcc-aa*₃ inhibitors.

This phenotype is thus used as a method of validating the binding target of *cyt-bcc-aa₃* inhibitors (Converse et al., 2019; Phummarin et al., 2016; van der Westhuyzen et al., 2015). Genetic knockout studies of the *cyt-bd* can also shed some light on its interaction with other anti-TB drugs. The *cyt-bd* knockout in *M. tuberculosis* and *M. smegmatis* have an increased sensitivity to bedaquiline (Berney et al., 2014; Lu et al., 2015). In addition, the *M. smegmatis* *cyt-bd* was associated with the conferral of protection against clofazimine, an anti-leprosy drug (Lu et al., 2015). More recently, a strong synthetic lethal interaction between the two terminal oxidases was reported (Kalia et al., 2017). The study demonstrated that the lack of bactericidal activity of *bc₁* inhibitors can be attributed to the presence of a functional *cyt-bd*, which provides an alternative route of electron flow sufficient to maintain viability of the pathogen. These results thus suggest that the *cyt-bd* is an attractive drug target for its synergistic role in potentiating the efficacy of cytochrome *bcc-aa₃* inhibitors.

To date, aurachin D remains the only known *cyt-bd* inhibitor (Kunze et al., 1987; Li et al., 2013; Meunier et al., 1995) (Figure 3). While the binding target of aurachin D was confirmed based on its effect on *cyt-bd*-specific activities – either through the measurement of its function, *i.e.* oxygen consumption (Meunier et al., 1995), or through its heme's oxidation-reduction signature by spectral analysis (Lu et al., 2015; Mogi and Miyoshi, 2009) – its exact binding site on the cytochrome was not established. More recently, Lu *et al.* reported that addition of aurachin D to Q203 treatment potentiated the bactericidal activity of the *bcc* inhibitor (Lu et al., 2018). While this finding is consistent with the synthetic lethal interaction between the two terminal oxidases, it is important to note that aurachin D is a structural analogue of ubiquinol (Li et al., 2013). Since ubiquinol binding sites can be also found on other ETC complexes such as NADH dehydrogenases (Complex I), it is also possible that the synergism observed in the study is due to aurachin D's non-specific effect on other targets. Indeed, the ability of inhibitors not targeting the *cyt-bd* to synergize with Q203 was reported (Flentie et al., 2019). In this study, the C10 compound displayed traits consistent with a *cyt-bd* inhibitor: inactive as a standalone treatment, while combination with Q203 resulted in bacterial death (Flentie et al., 2019). C10 however is evidently not a *cyt-bd* inhibitor as it retained potency in a *cyt-bd* knockout background (Flentie et al., 2019). This serves as a word of caution that potential *cyt-bd* inhibitors should not be validated solely by their capability in potentiating Q203 but should be reinforced with biophysical and biochemical evidences. Moreover, to fully harness the therapeutic potential of *cyt-bd* inhibition, fundamental understanding of its enzymatic structure and function is imperative. The atomic structure of the *G. thermodenitrificans* *cyt-bd* can provide a basis for structure-activity research that can aid in the development of potent inhibitors. However, differences in genetic sequences between species can lead to variation in protein structure and function, therefore it is essential to study the structure and function of mycobacterial *cyt-bd*.

Figure 3. Chemical structure of Aurachin D.

4. Targeting terminal oxidases in mycobacteria: prospects and challenges

In addition to examining the potency of their inhibition in *M. tuberculosis*, other factors about the terminal oxidases should be taken into account when evaluating their suitability as targets for drug development. Firstly, understanding the interaction between inhibitors of these targets with classical anti-TB drugs is important. *M. tuberculosis* can enter dormant state and exhibit antibiotic-resistant phenotypes (Betts et al., 2002; McDermott, 1959; Rao et al., 2008; Wayne and Sohaskey, 2001), and successful treatment of TB relies on a favourable combination of drugs. One study reported that *cyt-bc₁:aa₃* inhibitor Q203 improved the bactericidal activity of MenA inhibitors (Berube et al., 2019), which interfere with menaquinone biosynthesis, demonstrating that chemical inhibition of *cyt-bcc-aa₃* can improve the potency of other ETC inhibitors. However, since the terminal oxidases are relatively novel targets, their effects on the potencies of other drugs are not fully resolved. Secondly, deciphering the terminal oxidases' interaction with other cellular pathways may also be critical. The function of the ETC is intricately related to the central carbon metabolism: some complexes from the ETC are also involved in the TCA cycle (Cecchini, 2013; Hartman et al., 2014). Additionally, the exchange of electron carriers between the two pathways are essential for their functions, revealing their functional interdependency. Indeed, the treatment of AX-35, a QcrB inhibitor, triggered the remodelling of the bacteria's central carbon metabolism toward lipid synthesis and utilisation (Foo et al., 2018). The recent report that demonstrated glycerol metabolism affects the potency of *cyt-bcc-aa₃* inhibitors reaffirmed the relationship between the terminal oxidases and central carbon metabolism (Kalia et al., 2019). Besides central carbon metabolism, the terminal oxidases are also implicated in antibiotic-induced bacterial cell death. This interaction was first reported in *E. coli* and *S. aureus* in 2007 (Kohanski et al., 2007), and recently confirmed in mycobacteria (Lee et al., 2019; Shetty and Dick, 2018; Zeng et al., 2019). Partial oxidative phosphorylation inhibition, including at the *cyt-bcc-aa₃* level, affected the early bactericidal activity of isoniazid and moxifloxacin *in vitro*. Knowledge about the drug interactions and involvement in other cellular pathways could thus impact the decision in formulating therapeutics combinations for the treatment of TB. Deciphering their connections to other cellular pathway may also reveal potential targets that are synergistic with the inhibition of the *cyt-bcc-aa₃* or *cyt-bd*.

5. Conclusions

The terminal oxidases of *M. tuberculosis* have emerged as attractive drug targets in the recent years. High incidence rates of multi- and extensively- drug resistant tuberculosis is a rising concern. Newly approved anti-TB drugs such as bedaquiline and delamanid are used exclusively for the treatment of drug resistant tuberculosis. The rapid emergence of resistance to those drugs is all the more a worrying sign (Gler et al., 2012; Hartkoorn et al., 2014; Pym et al., 2016; Xu et al., 2012). This observation stresses the importance of considering drug candidates has part of a rational drug combination to avoid rapid emergence of resistance when evaluating their suitability for further development. Despite the time-sensitive quest for novel drugs, collective effort should be placed on understanding the interaction between various antibiotic targets. The formulation of drug regimens supported by such evidence should be efficacious and reliable in clearing infection. In the case of drugs targeting the terminal oxidases, extensive research should be carried out to understand their interaction with other antibacterials. The implications of their chemical inhibition on the overall metabolism of *M. tuberculosis* should be also taken into account. If the development stages are approached with mindful consideration, inhibitors of *cyt-bcc-aa₃* and *cyt-bd* have the potential

358 to be incorporated in the panel of anti-TB drugs and improve the treatment outcomes of drug
359 resistant tuberculosis, and probably other mycobacterial diseases.

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