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Targeting the mycobacterial envelope for tuberculosis drug development

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Abstract

The bacterium that causes tuberculosis, *Mycobacterium tuberculosis*, possesses a rather unique outer membrane composed largely of lipids that possess long-chain and branched fatty acids, called mycolic acids. These lipids form a permeability barrier that prevents entry of many environmental solutes, thereby making these bacteria acid-fast and able to survive extremely hostile surroundings. Antitubercular drugs must penetrate this layer to reach their target. This review highlights drug development efforts that have added to the slowly growing tuberculosis drug pipeline, identified new enzyme activities to target with drugs and increased the understanding of important biosynthetic pathways for mycobacterial outer membrane and cell wall core assembly. In addition, a portion of this review looks at discovery efforts aimed at weakening this barrier to decrease mycobacterial virulence, decrease fitness in the host or enhance the efficacy of the current drug repertoire by disrupting the permeability barrier.

Keywords

7-amino-4-methylcoumarin; arabinogalactan; BTZ043; cell wall; CD39; CD117; clavulanic acid; DNB; ethambutol; ethionamide; isoniazid; meropenem; mycobacterial outer membrane; mycolic acids; OPC-67683; PA-824; potentiation; pyrazinamide; rifampicin; SQ109; triclosan; tuberculosis

Since the characterization of the mechanism of action of β -lactam antibiotics, it has been known that inhibiting the biosynthesis of the outermost layers of bacterial cells promotes rapid killing of these resilient organisms. Since the targets of the first-line tuberculosis (TB) drugs isoniazid (INH) and ethambutol (EMB) are the enzymes necessary for the biosynthesis of molecular building blocks and assembly of the mycobacterial cell wall core and mycobacterial outer membrane (MOM), research has focused on targeting the enzymes important for mycobacterial cell wall biosynthesis [1,2].

The *de facto* standard for TB treatment, directly observed therapy short course, combines INH, EMB, rifampicin (RMP) and pyrazinamide (PZA) to minimize both treatment time and drug resistance. For those with access to these drugs, this regimen has been for the most part

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successful, but the number of active TB infections caused by drug-resistant strains continues to increase.

A compelling strategy that offers potential for improving TB therapy is the identification of potentiators that either increase the efficacy of current second or third-line TB drugs, or convert an otherwise inactive compound (when used as a monotherapy) into a drug that quickly kills mycobacteria when coadministered with a potentiator. In the first case, by increasing the anti-tubercular activity of relatively poor TB drugs, the drug toxicity diminishes because a lower dose is required. The second scenario is exemplified by the combination of clavulanic acid and Meropenem [3,4]. Neither molecule possesses impressive anti-tubercular activity when administered alone but quickly kills extensively drug resistant (XDR) TB when applied as a cocktail.

This review briefly describes the general structure of the mycobacterial permeation barrier composed of the mycobacterial cell wall and MOM, as well as some of the essential components and the enzymatic activities necessary for biosynthesis. Of particular interest are the various studies focusing on genes involved in biosynthesis of the permeation barrier or drug discovery efforts where those compounds are shown to target biosynthesis of the permeation barrier. In addition, a subset of the discussion focuses on discoveries where the application of a particular compound or gene knockout is permissive for growth in culture but increases the efficacy of known anti-tubercular drugs. This is especially relevant when considering potentiators that affect cell wall and MOM biosynthesis, as disruption of the mycobacterial permeability layer may enhance mycobacterial killing by different classes of anti-tubercular drugs.

The mycobacterial cell wall

The mycobacterial cell envelope contains three covalently linked macromolecules (peptidoglycan, arabinogalactan and mycolic acid) referred to as mycolyl-arabinogalactan-peptidoglycan complex (mAGP), as well as free glycolipids that are not covalently attached to the mycobacterial cell wall but associate with, or are a part of, the MOM. The peptidoglycan (PG) layer is formed of repeating *N*-acetyl- β -D-glucosaminyl-(1 $\rightarrow$ 4)-*N*-glycolylmuramic acid [5–8]. The PG is cross-linked via an L-alanyl-D-isoglutaminyl-*meso*-diaminopymethyl-D-alanine tetrapeptide attached to the muranic acid residues. The arabinogalactan (AG) consists of covalently coupled homogalactan and homoarabinan polymers [9]. In the homogalactan, β -D-galactofuranose units are linked alternatively through C-5 or C-6. The reducing residue of the galactan is linked to the PG through a phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage. The branched arabinan chains containing α -D-arabinofuranosyl (Ara β) are attached through the reducing end to C-5 of the β -D-galactofuranose units. The arabinan domain is arranged with different α (1 $\rightarrow$ 5), α (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 2) Ara β linkages. A branched hexa-arabinofuranosyl structure is located at the nonreducing terminus of the arabinan polymer: two-thirds of the Ara β hexasaccharide residues are esterified to mycolic acid, which are α -alkyl, β -hydroxy fatty acids that consist of a saturated 20–26 carbon α -branch and a meromycolic acid moiety (a chain of up to 60 carbon atoms) that contain different functional groups such as ethoxy, methoxy, keto groups (methoxymycolates or ketomycolates) or cyclopropane rings (α -mycolates) [7]. The α -mycolates are the most abundant type found in *Mycobacterium tuberculosis*. The mycolates esterified to the arabinan chain form the inner leaflet of the MOM.

Free glycolipids are important components of the mycobacterial cell wall. Among them can be found trehalose dimycolate (TDM), often referred as cord factor, trehalose monomycolate (TMM), phosphatidylinositol mannosides, sulfolipids, phenolic glycolipids (PGLs) and phthiocerol dimycocerosate (PDIM) [10]. These lipids interact with the mycolyl moiety of

the mAGP skeleton forming a bilayer that functions as a formidable permeation barrier. Another major glycolipid is the lipoarabinomannan (LAM). LAM is a polysaccharide chain containing Araf and mannopyranosyl residues. It spans the MOM and the mAGP, terminating in a phosphatidylinositol-diacylglycerol moiety that is embedded within the cytoplasmic membrane. At the nonreducing end of LAM is a hexasaccharide moiety that, contrary to the AG, lacks any mycolic acid elaboration. Mannose-capped lipoarabinomannan is a variant of LAM that appears to be involved in the host immune response [11]. The merging of the MOM and the mAGP creates an extremely efficient permeability layer embedded with a growing list of possible porin proteins and other predicted outer membrane proteins [12]. A schematic representation of the mycobacterial cell wall is given in Figure 1.

The low permeability of the cell wall appears to be essential for mycobacterial survival in the host. Therefore, enzymes involved in the biosynthesis of these different mycobacterial cell wall components, as well as the enzymes necessary for the attachment of those components, are potentially excellent drug targets. A voluminous number of research articles have been published over the past two decades that describe the efforts to identify these enzymes and to understand their mechanism of action, as well as to discover compounds capable of inhibiting them [13].

Several current anti-tubercular drugs inhibit enzymes involved in cell wall biosynthesis. For instance, INH is a prodrug activated by KatG, a catalase-peroxidase enzyme. Once activated, INH inhibits the InhA enzyme, an NADH-dependent, enoyl-acyl carrier protein reductase of fatty acid synthase-II (FAS-II) that is involved in the mycolic acid biosynthesis [1]. Ethionamide targets the same pathway as INH; in addition, it is also a prodrug that is activated by EthA and is metabolized to the corresponding S-oxide [14,15]. Hence, mutations in the enzyme InhA confer resistance to both ethionamide and INH [16]. However, mutations in the enzymes implicated in the metabolism of these prodrugs are more commonly observed and decrease or abolish the sensitivity to one or the other [17].

Inhibiting mycolic acid biosynthesis

Researchers continue to search for alternatives to INH. Recently, Sacchetti and colleagues developed a series of 5-substituted triclosan derivatives [18]. Triclosan is a commercially available molecule that displays antifungal and antibacterial properties. These compounds were tested against INH-resistant *M. tuberculosis* strains and some derivatives with aryl and alkyl substituents demonstrated higher potency than INH (Figure 2). The most potent inhibitor *in vitro* was a compound containing a 2-phenylethyl moiety at the 5 position on triclosan, which exhibited an IC₅₀ against *M. tuberculosis* InhA of 21 nM (triclosan IC₅₀ = 1100 nM) while the most efficient derivative *in vivo* included a 3-phenylpropyl substituent and displayed a MIC of 4.7 µg/ml against H37Rv (eightfold improvement compared with triclosan) and 9.4 µg/ml against H37Rv $\Delta katG$ (20-fold improvement compared with INH). In another study, Vilchèze *et al.* screened a library of small molecules that were tested against the *Plasmodium falciparum* enoyl reductase, which is an InhA ortholog [19]. The aim of this screening was to identify new InhA inhibitors that do not require activation by KatG, as INH-resistant strains are more commonly due to mutations in *katG* than in *inhA*. They identified two compounds, CD39 and CD117, which demonstrate promising bactericidal activity against *M. tuberculosis* (Figure 2). Both molecules lead to a 4-log decrease in CFU titers for H37Rv and appeared more active against INH-resistant strains. These compounds act synergistically with first-line anti-TB drugs, where treatment of *M. tuberculosis* H37Rv with INH and either CD39 or CD117 causes a 6-log increase in killing, while the combination of RMP with CD117 results in a 4-log decrease of CFU titers. Both CD117 and CD39 inhibit mycolic acid biosynthesis and exhibit bactericidal activity even

under anaerobic conditions. Genetic experiments confirm that InhA is the target of CD39 and CD117, but these compounds may inhibit FAS-I as well, since CD39 exhibited toxicity in eukaryotic cells. Structure–activity relationship studies using CD117 showed that substitutions at the 2 and 4 positions of the pyrimidine moiety provoke the most significant changes in MIC values.

7-amino-4-methylcoumarin (NA5) and its acyl derivatives have been explored as potential anti-tubercular agents (Figure 3) [20]. A screen carried out by Virsdoia *et al.* indicated that a few (arylamino) coumarins exhibit activity against H37Rv with MICs superior to 6.2 mg/l [21]. Tandon *et al.* tested NA5 and its acyl derivatives against H37Rv, as well as drug-susceptible and multi-drug resistant (MDR) clinical strains. They presented MICs values between 1 and 3.5 mg/l against all the strains tested, values comparable with EMB (2 mg/l against H37Rv) or streptomycin (0.25 mg/l against H37Rv or 15 mg/l against MDR clinical isolate) but slightly higher than those for INH (0.03 mg/l against H37Rv) or RMP (0.015 mg/l against H37Rv).

Several of these compounds exhibit bactericidal activity (NA5 exhibits a minimum bacteriocidal concentration of 1.5 mg/l). More importantly, they act in synergy with existing anti-tubercular drugs. For instance, several of them decrease the MIC of INH and RMP against H37Rv 30-fold and 20-fold, respectively. Two of the tested compounds that lack any anti-tubercular activity when applied alone (NA7 and NA38), exhibit synergy with INH and RMP that is comparable with NA5. Electron microscopy revealed that the compounds produce a morphological effect on the mycobacterial cell wall that varies from a twisted cell wall to a completely disrupted cell in some cases. This suggests that NA5 targets activities important for maintaining mycobacterial cell wall integrity, which led the researchers to suggest that mycolic acid biosynthesis could be the target of these compounds.

Inhibitors of mycolic acid biosynthesis in clinical trials

Other compounds have been shown to inhibit mycolic acid biosynthesis. OPC-67683 is a nitro-dihydroimidazooxazole [22] and PA-824 is a nitroimidazo-oxazine (Figure 4) [23]. The screening of a nitro-dihydroimidazooxazole derivative library for inhibition of mycolic acid biosynthesis led to the discovery of OPC-67683 [22]. The production of mycolic acids was detected by ^{14}C -labeling and OPC-67683 appeared to only prevent the production of methoxy mycolic acids and keto mycolic acids in *Mycobacterium bovis* (IC_{50} = 0.021 to 0.036 mg/l), whereas INH inhibits the biosynthesis of all mycolic acid subclasses. The drug exhibited great potency *in vitro* as well as *in vivo* in a mouse model of chronic TB. In addition, OPC-67683 acts synergistically with known TB drugs and even led to a shorter treatment when used in combination therapy with RFP and PZA.

PA-824 was discovered during a screening of substituted nitroimidazopyrans as potential anti-tubercular agents [23]. The library was based on a nitroimidazofuran compound (CGI-17341) that displayed antibacterial activity, but also exhibited mutagenic properties. PA-824 displayed MICs between 0.015 and 0.25 mg/l against H37Rv and MDR-TB strains. The drug was shown to inhibit keto mycolic acids production. Both OPC-67683 and PA-824 are prodrugs that require activation by the deazaflavin-dependent nitroreductase [22,24,25]. Metabolites produced from both drugs lead to the release of reactive nitrogen species [26]. According to Barry *et al.*, the release of nitric oxide and other reactive species allows PA-824 to kill nonreplicating *M. tuberculosis* [27]. OPC-67683 is thought to kill mycobacteria using a similar mechanism. Both drugs show significant potency in a mouse model and act synergistically with first-line TB drugs [22,28–31]. Denny and colleagues explored different analogs of PA-824, in which the $-\text{OCH}_2$ linker is replaced by diverse ether linkers of varying size or flexibility, by testing derivatives containing amide,

carbamate or urea functional groups [32,33]. PA-824 and OPC-67683 are in Phase II and Phase III clinical trials for MDR-TB treatments, respectively [34].

Arabinofuranosyltransferases

The existing first-line drug EMB disrupts the biosynthesis of AG or LAM and promotes the accumulation of β -D-arabinofuranosyl-P-decaprenol, a key intermediate in the biosynthesis of arabinan [35,36]. It targets an arabinosyltransferase encoded by *embB* that belongs to an operon including *embC* and *embA* (*embCAB*) [37]. Between 47 and 65% of the mutations that confer resistance to EMB are observed within this operon [16]. With roughly a third of the EMB-resistant strains possessing an unknown resistance mechanism, other important drug targets may yet be identified by studying these outlying mutant strains.

The Emb proteins participate in the polymerization of Ara α residues from the arabinosyl donor decaprenylphosphoryl- β -D-arabinofuranose (DPA) [37]. EmbC is known to be involved in the biosynthesis of LAM, as a deletion of *embC* disrupts LAM production [38–40]; on the other hand, EmbA and EmbB are essential for the formation of the terminal hexaarabinofuranosyl motif in AG [41,42] and act as $\alpha(1\rightarrow3)$ arabinofuranosyltransferases [43].

Similar to the strategies employed for identifying novel compounds to inhibit mycolic acid biosynthesis, researchers have used EMB as a model scaffold to design better EmbB inhibitors. SQ109, a 1,2-diamine structurally related to EMB, was discovered in 2003 during a screening of EMB analogs [44–47]. SQ109, shown in Figure 4, displays promising pharmacokinetic properties and appeared to be more potent than EMB, even against EMB-drug resistant strains [48]. Contrary to expectations, SQ109 does not inhibit the Emb proteins. Tahlan *et al.* determined that SQ109 inhibits a membrane transporter of TMM, MmpL3 [49]. This inhibitory activity promotes accumulation of TMM in the cytoplasm, thereby preventing incorporation of mycolic acid into the mycobacterial cell wall and inhibiting TDM production [49].

In 2005, Alderwick *et al.* showed that an *emb* deletion mutant in *Corynebacterium glutamicum* still presents an unaffected galactan backbone [50]. Thus, they suggested that a ‘priming’ enzyme catalyzes the transfer of the first Ara α residue to the galactan core before extension by the Emb proteins. The following year, they identified a novel arabinosyltransferase, AftA [51]. The gene encoding AftA, *Rv3792*, is located in the *emb* locus, upstream of *embC*, *embA* and *embB* and downstream of *Rv3790* and *Rv3791*. These last two genes are also involved in biosynthesis of AG and LAM and will be discussed later. AftA contains several transmembrane domains and a glycosyl-transferase motif, characteristic of the Emb proteins. Both the Emb proteins and Ara α are arabinosyltransferases; however, they lack any significant sequence similarity and their activities are sufficiently divergent to be unable to complement each other. This is exemplified by the fact that AftA is insensitive to EMB. AftA appears to be a promising drug target waiting to be characterized.

In 2007, Seidel *et al.* characterized *Rv3805c* in *M. tuberculosis*. This gene appears to be essential and encodes another arabinosyltransferase, AftB [52]. AftB catalyzes the formation of the $\beta(1\rightarrow2)$ -linked Ara α residues, the final step in the biosynthesis of AG before addition of the mycolic acid moieties. More recently, the work by Raad *et al.* confirmed these results and indicated that the cells containing a deletion mutant of *aftB* lacks half of the mycolation sites, as the $\beta(1\rightarrow2)$ -linkages between Ara α residues are missing [53]. Similar to that observed for AftA, EMB does not inhibit the activity of AftB, suggesting that AftB may represent another intriguing target for drug discovery. In 2010, Zhang *et al.* developed a plate-based scintillation proximity assay (SPA) to screen libraries of small molecules and

identify potential inhibitors of AftB [54]. In this cell free assay, AftB allows the addition of DP[³H]A to a biotinylated acceptor, *S*-D-biotinoyl-5-(2-amino-1-thioethyl)-pentyl 5-*O*-(α -D-arabinofuranosyl)- α -D-arabinofuranoside, in order to produce biotinylated trisaccharide products that contain β (1 $\rightarrow$ 2)-linked Araf residues. Then, streptavidin-coated SPA beads are incubated with the reaction mixture to capture the ³H-labeled biotinylated product. The proximity of the ³H to the scintillant impregnated within the beads allows quantification. Steady-state kinetics experiments were carried out for AftB using this SPA technology to form the basis of future screening efforts.

Another arabinofuranosyltransferase was identified and characterized by Birch and coworkers. AftC (Rv2673) catalyzes the formation of the α (1 $\rightarrow$ 3)-linked Araf residues; a deletion mutant of *aftC* in *M. smegmatis* leads to the biosynthesis of truncated AG lacking the α (1 $\rightarrow$ 3)-branching in its arabinan domain [55]. Later, in 2010, they demonstrated that AftC is also responsible for the branching of the LAM arabinan core [56]. This study showed that *M. smegmatis* deficient in AftC leads to a truncated LAM lacking the 3,5-Araf branching and displays an increased proinflammatory activity (LAM is thought to participate in the host immune response [57]). They also confirmed that EmbC acts as an α (1 $\rightarrow$ 5) arabinofuranosyltransferase prior to branching of the arabinan chain by AftC. More recently, Zhang *et al.* expressed, solubilized and purified AftC from *M. smegmatis*, and also developed an enzymatic assay to characterize the activity of AftC and screen for potential inhibitors [58]. They also synthesized a diverse set of DPA analogs, which they used as an artificial donor in their assay. From these studies, AftC appears to only be active when reconstituted with mycobacterial phospholipids to form a proteoliposome.

Finally, Skovierora *et al.* identified and characterized AftD (Rv0236c in *M. tuberculosis*) as an essential polyprenyl-dependent arabinofuranosyltransferase in *M. smegmatis* [59]. AftD is responsible for forming either the α (1 $\rightarrow$ 3)-linkage or the α (1 $\rightarrow$ 5)-Araf *in vitro*. Similar to the arabinofuranosyltransferases described earlier, AftD is insensitive to EMB and appears to be a valuable drug target.

Inhibition of ribose conversion to arabinose by diverse compounds

DPA is the arabinosyl donor in the biosynthesis of the cell-wall arabinans (AG and LAM) and is therefore a key precursor in the formation of the mycobacterial cell wall. In 2005, Mikusova and coworkers described the sequence of events leading to the synthesis of DPA: 5-phosphoribose 1-diphosphate is converted to decaprenylphosphoryl- β -D-5-phosphoribose, which is then dephosphorylated to decaprenylphosphoryl- β -D-ribose (DPR). Finally, DPR is converted to DPA via epimerization [60]. Rv3806c, a decaprenylphosphoryl- β -D-5-phosphoribose synthase identified as UbiA in *C. glutamicum*, was found responsible for the synthesis of DPR in the first step of this pathway [50]. In *M. tuberculosis*, DprE1 (Rv3790) and DprE2 (Rv3791) associate as a heteromeric enzyme called decaprenylphosphoryl- β -D-ribofuranose 2'-epimerase and catalyze the conversion of DPR to DPA in two separate steps [61]. Bioinformatic analysis suggests that DprE1 contains a flavin adenine dinucleotide (FAD) binding domain, while DprE2 likely possesses a FAD/nicotinamide adenine dinucleotide fold [60]. The epimerization is proposed to proceed through a sequential oxidation/reduction reaction. During characterization of the epimerization reaction, an intermediate was observed, decaprenylphosphoryl-2-keto-D-erythro-pentofuranose-X (DPX), but not experimentally confirmed. Hence, it was hypothesized that DprE1 and DprE2 act as decaprenylphosphoryl- β -D-ribose oxidase and decaprenylphosphoryl-D-2-keto erythro pentose reductase, respectively [60,62]. In 2012, Trefzer *et al.* confirmed this hypothesis using recombinant DprE1 and DprE2 from *M. smegmatis* and ¹⁴C-DPR/¹⁴C-DPX substrates [63]. They demonstrated that DprE1 is responsible for the oxidation of ¹⁴C-DPR to ¹⁴C-DPX using FAD as a cofactor, while DprE2 reduces ¹⁴C-DPX to ¹⁴C-DPA in

the presence of NADH (Figure 5). DprE1 has been shown to be essential in both *M. smegmatis* and *M. tuberculosis* [64,65]. While these data suggest a lack of redundancy in the oxidation step, it is possible that *M. tuberculosis* encodes two enzymes capable of catalyzing the reduction step producing DPA, DprE2 and Rv2073. As detailed further, DprE1 appears to be an extremely promising drug target for *M. tuberculosis*.

In 2009, Makarov *et al.* synthesized a new class of antimycobacterial compounds, 1,3-benzothiazin-4-ones (BTZs) that are able to block the arabinan synthesis (LAM and AG [61]). One member of this class, BTZ038, appeared the most promising and was used in further studies. BTZ038 contains a single chiral center and a nitro group. Both S and R enantiomers, respectively BTZ043 and BTZ044, inhibit equally well *in vitro* (Figure 6). Since nitro groups can be reduced to amines via a hydroxylamine intermediate in biological systems, the amine derivatives (R and S, BTZ045) and the hydroxylamine intermediate were also tested *in vitro*. Of those tested, BTZ043 showed the best MIC against *M. smegmatis* (2.3 nM) and *M. tuberculosis* H37Rv (9.2 nM); the viability of *M. tuberculosis* H37Rv decreases more than 1000-fold in the presence of BTZ043. The MICs obtained are lower than first-line TB drugs such as INH or EMB. BTZ043 was also effective against certain MDR and XDR-TB strains. Further studies using macrophages infected with *M. tuberculosis* and mice models of TB indicated that BTZ043 was also potent *in vivo* and no cytotoxic effect was observed. For instance, BTZ043 displayed a MIC of 10 µg/l, which is more potent than INH (100 µg/l) or RMP (1 mg/l). Genome sequencing of *M. tuberculosis* strains resistant to BTZ043 identified DprE1 as the target. The only mutations in DprE1 that confer resistance to BTZ043 are C387G and C387S. Recently, Trefzer *et al.* identified the mechanism of action of BTZ using mass spectrometry [63,66]. The compound is reduced to the corresponding nitrosoarene that reacts with the thiol in C387 to form a semimercaptal adduct of activated BTZ043 with DprE1. Thus, BTZ043 is a suicide inhibitor of DprE1. This mechanism explains very clearly why this molecule is inefficient against DprE1 mutants, where C387 is mutated to amino acid residues that will not readily react with nitrosoarenes.

The same year, Christophe *et al.* identified another class of DprE1 inhibitors, dinitrobenzamide (DNB), using a high-throughput assay in which the growth of GFP-expressing *M. tuberculosis* H37Rv in macrophages is monitored by fluorescence microscopy [67]. Structure–activity relationship studies of DNB derivatives suggest that the nitro group is important for its potency against *M. tuberculosis*. As observed with the BTZ compounds, the modification of one of the nitro groups to either an amine or a hydroxylamine significantly decreases the level of DprE1 inhibition [68]. Two DNB derivatives show considerable potency with DNB1 and DNB2 exhibiting MICs of 200 nM against *M. tuberculosis* H37Rv *in vitro* and *ex vivo* (Figure 6). These compounds also show activity against MDR and XDR-TB strains with no observed cytotoxic effects. While, these two compounds inhibit the conversion of DPR to DPA by inhibiting DprE1, resistance to DNB1 or DNB2 was observed in DprE1-C387S and DprE1-C387G strains, so the mechanism of inhibition is thought to be identical to BTZ [63,67].

Inhibiting phthiocerol dimycocerosate biosynthesis

PDIMs and phenolphthiocerol dimycocerosates are found within the MOM of *M. tuberculosis*. In addition, PGLs derived from phenolphthiocerol dimycocerosates in some strains affect virulence by modulating the host immune response through diverse mechanisms [69–72]. Other studies suggest that inhibiting PGL or PDIM synthesis makes *M. tuberculosis* more susceptible to current TB drugs. Hence, compounds inhibiting enzymes important for PGL and PDIM biosynthesis could synergize with known antibiotics or potentiate second-line anti-tubercular drugs to improve treatment of TB.

For instance, the loss of PGLs is associated with an augmentation in the release of proinflammatory cytokine by infected macrophages [73]. Reed *et al.* show that production of PGLs leads to a 'hyperlethality' phenotype in murine disease models [73]. Inhibiting the production of these two glycolipids has been explored in drug discovery efforts. To this end, Quadri and colleagues have focused on PDIM and PGL [74–76] but the pathway is not yet fully understood [77]. However, it is known that PGLs include a *p*-hydroxyphenyl moiety derived from *p*-hydroxyphenyl acid. The enzyme FadD22 activates *p*-hydroxyphenyl acid by catalyzing the formation of hydroxybenzoyl-AMP (pHB-AMP) in a coenzyme A-dependent manner and promotes initiation of phenolphthiocerol biosynthesis. Further studies describe the development of a small molecule inhibitor of PGL biosynthesis, 5'-*O*-[*N*-(4-hydroxybenzoyl)sulfamoyl]-adenosine, which is an analog of pHB-AMP [78]. The compound inhibited the formation of pHB-AMP with an IC₅₀ of 6 μM. Furthermore, the compound was tested in *M. tuberculosis*, *M. bovis*, *Mycobacterium marinum* and *Mycobacterium kansasii* in which the production of PGL was reduced from 14-fold to 48-fold.

More recently, researchers focused on the role of the *tesA* gene in the production of PDIMs and PGLs [79]. This gene encodes a type II thioesterase that is hypothesized to play a role in PDIM and/or PGL biosynthesis [80]: two-hybrid and pull-down assays give evidence that *M. tuberculosis* TesA interacts with *M. tuberculosis* PpsE, a polyketide synthase involved in the synthesis phthiocerols and phenolphthiocerols. Experiments to determine if TesA is involved in the production of PDIMs have been attempted but were inconclusive, and the significance of TesA in PGL biosynthesis has not yet been investigated [81,82]. Recently, Chavadi *et al.* used *M. marinum*, which is known to synthesize both PGL and PDIM, to construct a *tesA* deletion mutant [79]. Liquid chromatography mass spectrometry analysis showed that the deletion of *tesA* in *M. marinum* leads to a significant loss of these glycolipids; only trace amounts of PGLs and PDIMs are observed. Complementing the deletion mutant with wild type TesA rescues PGL and PDIM biosynthesis. The authors hypothesized that TesA acts as a thioesterase that catalyzes product release from PpsE. Based on structural modeling, the authors predicted that a catalytic triad would form from residues S92, D196 and H224. Creation of an S92A mutant reduces the production of the glycolipids and its use does not complement a ΔTesA mutant strain. Both of those results are consistent with the proposed thioesterase activity of TesA.

Interestingly, the lack of PDIMs and PGLs in the deletion mutant of *tesA* induces a pan-antibiotic hypersensitivity exemplified by the increase in susceptibility to five antibiotics used in the treatment of *M. marinum*. This suggests that inhibiting the activity of TesA disrupts the permeability of the mycobacterial cell wall and increases drug susceptibility.

The antigen 85 complex

The antigen 85 complex (Ag85A/Ag85B/Ag85C) plays an important role in the mycobacterial cell wall biosynthesis. The *fbpA*, *fbpB* and *fbpC* genes encode the three Ag85 proteins, with Ag85B being the major secretory protein in *M. tuberculosis* [83]. Each of these proteins possess a N-terminal signal sequence and are secreted, and all three have very similar sequences, possessing between 68 and 80% identity. In 1997, Belisle *et al.* showed that enzymes of the antigen 85 complex catalyze the formation of TDM from TMM via a transesterification reaction [84]. Genetic data suggest that each prefers a different mycolic acid acceptor (Figure 7). Jackson *et al.* established that Ag85C is involved in the transfer of mycolic acid to the AG complex as a deletion mutant of *fbpC* leads to a 40% reduction of the cell wall-bound mycolic acids [85]. On the other hand, knockout studies of *fbpA* and *fbpB* demonstrated that the production of TDM is affected in these mutants [86,87]. More recently, Matsunaga *et al.* showed that Ag85A may be responsible for the glucose

monomycolate biosynthesis [88]. The crystal structure of Ag85C showed that the enzyme possesses an α/β -hydrolase polypeptide fold and revealed that the active site possesses a catalytic triad including S124, E228 and H260; these three residues are conserved in all three functional Ag85s [89].

All known α/β -hydrolases catalyze a two-step reaction using a ping-pong mechanism. The first step uses the conserved serine residue of the catalytic triad to promote nucleophilic attack on the substrate forming an acyl-enzyme intermediate. The second step breaks down the acyl-enzyme intermediate using water or an acyl acceptor molecule as a nucleophile. Activity assays using an S124A mutant confirmed the importance of this residue for catalysis, as the production of TDM or any other acylated trehalose product was not observed [84]. Further structural data suggest that all three enzymes, prior to forming the acyl-enzyme intermediate, have identical active site structures and most likely share the same mycolic acid donor TMM [89–91]. Modification of the active site serine in Ag85C with a covalent inhibitor that mimics a transition state formed during catalysis shows that the enzyme undergoes a conformational change during catalysis, which suggests that this conformational change allows the different Ag85 enzymes to select different mycolyl acceptors.

The Ag85 complex appears as a promising drug target for several reasons. First, all three enzymes share identical active site structure; hence, a single molecule should inhibit all three enzymes [89]. Second, as mentioned previously, the Ag85 proteins are secreted proteins [83]. For this reason, targeting these enzymes can minimize the effect of *M. tuberculosis* defense mechanisms often observed for antimycobacterial drugs, such as efflux by proteins of the *iniBAC* operon. Third, elimination of just one Ag85 enzyme affects the structural integrity of the MOM. The knockout studies of the different *fbp* genes confirm that the three proteins play a significant part in the cell wall biosynthesis and their deleterious effect on *M. tuberculosis* viability [85–88]. Specifically, Nguyen *et al.* showed that disruption of Ag85A results in an increase in sensitivity of *M. tuberculosis* to broad-spectrum antibiotics known to inhibit cell wall biosynthesis [87]. Later, in 2008, Harth *et al.* demonstrated that the reduction in mycolyltransferase expression leads to an eightfold increase in bacterial sensitivity to INH [92]. Finally, attempts to delete any combination of two of the three *fbp* genes have met with failure, suggesting that at least two of the *fbp* genes are necessary for viability. This suggests that the development of resistance to inhibitors of Ag85 would be improbable, since resistant mutants would be required to arise in two *fbp* genes simultaneously.

The design of inhibitors based on the Ag85 mechanism has been explored for the past decade. First, Belisle *et al.* showed that 6-azido-6-deoxy- α,α' -trehalose, an analog of trehalose, inhibited the activity of Ag85C by 60% at a concentration of 100 $\mu\text{g/ml}$ [84]. Then, different libraries of compounds based on trehalose analogs [93,94] or mimics of the tetrahedral transition state [95–97] were screened using the assay developed by Belisle.

More recently, new high-throughput assays were developed to screen potential inhibitors of the Ag85 proteins and to test the effect of different acyl acceptors of the Ag85 enzymes [98–100]. Based on the mechanism of Ag85, Sanki *et al.* synthesized methyl 5-*S*-alkyl-5-thio-D-arabinofuranoside analogs and the compounds were tested for inhibitory activity [101]. Several compounds demonstrated inhibition of growth against *M. smegmatis* ATCC 14468. However, they did not inhibit the activity of Ag85C in the colorimetric assay. These data suggest that the arabinofuranosides were most likely inhibiting enzymes other than the Ag85 complex. Pursuing mutants resistant to these compounds may identify new drug targets.

Later, Sanki *et al.* synthesized a library of arabinose and trehalose-based molecules elaborated with esters, α -ketoesters and α -ketoamides [102]. The compounds were again tested against Ag85C *in vitro*. One of the compounds, a methyl ester, showed modest inhibition of Ag85C in the millimolar range, but did not exhibit growth inhibition of *M. smegmatis*. Significant improvement of the inhibitory activity *in vitro* will be necessary before any growth inhibition is observed.

More recently, Barry *et al.* developed a label-free assay based on electrospray ionization mass spectrometry to determine the kinetic parameters and confirm the previously proposed enzymatic mechanism of the Ag85 enzymes [103]. They used 6,6'-di-hexanoyltrehalose (TDH) and trehalose as the substrates (acyl donor and acyl acceptor, respectively) and monitored the formation of 6-hexanoyltrehalose and the decrease in TDH concentration based on total ion counts. In these studies, the authors examined the relationship of the sugarring conformation to substrate specificity by testing a series of different monosaccharides with the three Ag85 isoforms. It was observed that deviation from trehalose and Araf in the sugar ring significantly decreases the activity of the antigen enzymes. Also, the change in anomeric configuration leads to a difference in activity between the three isoforms. Thus, the differences related to substrate recognition confirmed that the three enzymes play a different role *in vivo* [85–88]. Barry *et al.* used this information to design a potentially selective inhibitor of the three antigen enzymes; one that contains an electrophilic fluorophosphonate that is predicted to form a stable, covalent complex with the Ag85 enzyme following nucleophilic attack on the inhibitor by S124. Unfortunately, this inhibitor (similar to that used to form the covalent adduct necessary to determine the Ag85C-diethylphosphate crystal structure) cannot be used as a drug due to extremely high cross-reactivity with hydrolytic enzymes and its subsequent cytotoxic effects [89].

Very recently, a new inhibitor of Ag85C has been developed: 2-amino-6-propyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile (I3-AG85) [104]. Warrier *et al.* screened a library of 5000 compounds and isolated I1-AG85 that was shown to bind Ag85C by nuclear magnetic resonance. Different analogs were synthesized that demonstrated lower MICs. I3-AG85 appears to be the most active; the compound exhibits a MIC of 100 μ M and is able to reduce the viability of *M. tuberculosis* within macrophages. In addition, I3-AG85 is active against different drug-susceptible strains. The production of TDM is affected by the presence of I3-AG85, but not the cell wall-linked mycolic acids. This is in apparent contradiction with the proposed role of Ag85C, but this result may simply reflect the functional redundancy of the Ag85 enzymes or possibly inefficient transport of I3-AG85 through the MOM [85].

New structural information about DprE1

During editorial review, two papers have extended our understanding of the DprE1 structure and the mechanism of BTZ inhibitory activity. Batt *et al.* published the crystal structures of DprE1 in a ligand-free form, a BTZ-derived nitroso derivative (CT325) form, and in a form bound to an inert form (CT319) as observed in the apparent BTZ043 pro-drug form [105]. The structure confirms the presence of an FAD binding domain and reveals a covalent linkage between residue C387 of DprE1 and the nitroso group of CT325. This result supports the earlier published reports that benzothiazinones act as suicide inhibitors of DprE1. Neres *et al.*, using the *M. smegmatis* encoded form of DprE1, also exhibit the formation of the semimercaptol adduct within the DprE1 active site and assessed the enzymatic consequences of various active site mutants [106]. They observed that the DprE1-C394G mutant (C387G in *M. tuberculosis*), while no longer inhibited by BTZ, possessed only a fourfold decrease in the catalytic rate.

Conclusion

Now, as it has been for the past 50 years, targeting the mycobacterial cell wall and outer membrane biosynthesis is a cornerstone of TB therapy. With the relentless application of forward genetics, as well as target-based discovery methods, new compound classes are being developed that are increasingly efficient at disrupting this protective barrier and are raising hope that the treatment times and the likelihood of drug resistance will both decrease. A summary of all the potential new drugs cited in this review is presented in Table 1, as well as their targets and modes of action. With careful application of these drugs in a multidrug treatment program, this hope may soon become reality.

Expert commentary

Over the past 5 years, culture-based screening and forward genetics has significantly expanded the pipeline of new lead compounds for treating TB, and has expanded the number of targets for further drug discovery efforts. Many of these compounds target the production of components essential for mycobacterial outer membrane maintenance, such as mycolic acids, arabinose and the arabinan of the cell wall, in novel ways. In particular, the DprE1 enzyme and the MmpL3 transporter are validated targets inhibited by multiple new compounds, and their further study will expand the repertoire of potent anti-TB compounds. If PA-824 and OPC-67683 clear clinical trials, these drugs will revolutionize TB treatment because of their efficacy against both actively dividing and dormant mycobacteria, but only if used in combination with other TB drugs.

Five-year view

Looking forward, the TB lessons of the past will be remembered. Fifty years ago, physicians had new drugs at their disposal to treat TB and selection for resistant mutants through the use of monotherapy created some of today's drug resistance problems. The fact that mutants resistant to all of the newly identified leads have been observed in the laboratory reinforces the essentiality of multidrug therapy. With the specter of drug-resistance in mind, the current enthusiasm surrounding the potential new TB drugs should be tempered with calm rigor in assessing the mechanism of drug activity in detail, in order to improve activity and minimize resistance. As one example, characterization of DprE1 epimerase inhibitor mechanisms clearly exhibits the necessity of forming a covalent complex with a cysteine residue in the enzyme active site, and that mutations at this site promote resistance to both BTZ043 and DNBs. With this information in hand, researchers can now focus on developing noncovalent inhibitors of DprE1, whose activity is insensitive to this apparently common mutation in the *dprE1* gene. Indeed, one could argue that drug discovery efforts toward inhibiting DprE1 should target the mutant forms resistant to BTZs and DNBs to ensure that inhibitory activity is not dependent on covalent modification of the enzyme.

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- of interest
- of considerable interest

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Key issues

- Standard therapy for tuberculosis, directly observed therapy short course, is at least 6 months and typically longer.
- More effective drugs are needed to decrease treatment time.
- Drugs must be identified that quickly and effectively kill dormant bacteria.
- The mycobacterial permeation barrier limits the efficacy of many drugs. Drugs enter the bacterial cytoplasm very slowly, but drug efflux systems expel many classes of drugs very quickly.
- Combination therapy is essential for limiting drug resistance, as drugs administered as monotherapy select for resistant mutants within 2–3 years.
- Drug potentiation may aid in accelerating drug discovery efforts. Many second and third-line drugs require doses that are toxic. Potentiation could significantly decrease the required dose, thereby rapidly expanding the pool of first-line drugs.
- Continued expansion of culture-based screening is essential for identifying new targets.
- Target-based structure activity relationship studies are important for improving on original leads.

Figure 1. Schematic representation of the mycobacterial cell wall

LAM: Lipoarabinomannan; P: Phosphate; PDIM: Phthiocerol dimycocerosate; PGL: Phenolic glycolipids; TDM: Trehalose dimycolate.

Figure 2.
Potential inhibitors of InhA.

Figure 3.
7-amino-4-methylcoumarin (NA5) and its derivatives.

Figure 4. Chemical structures of PA-824, OPC-67638 and SQ109 in clinical trials

In addition to inhibiting mycolic acid biosynthesis, the nitro groups shown on the left of PA-824 and OPC-67638 are proposed to be the source of the nitric oxide released by metabolism of these compounds. The diamino moiety of SQ109 is meant to mimic ethambutol.

Figure 5. Enzymatic activity of DprE1 and DprE2

DPA: Decaprenylphosphoryl- β -D-arabinofuranose; DPR: Decaprenylphosphoryl- β -D-ribose; DPX: Decaprenylphosphoryl-2-keto-D-erythro-pentofuranose-X; FAD: Flavin adenine dinucleotide; NAD: Nicotinamide adenine dinucleotide; R: Decaprenylphosphate.

Figure 6.
Chemical structure of BTZ043, DNB1 and DNB2.

Figure 7. Antigen 85 pathway

The three enzymes have been shown to use the same mycolic acid donor but they prefer different acyl acceptors. Ser represents the serine nucleophile that attacks the TMM substrate, thereby forming the acyl-enzyme intermediate.

AG: Arabinogalactan; mAG: Mycolyl-arabinogalactan; TDM: Trehalose dimycolate; TMM: Trehalose monomycolate.

Table 1

Summary of the drugs cited in this review, their targets and mode of action.

Name	Target protein	Target biosynthetic pathway	Ref.
BTZ043	DprE1	Arabinan biosynthesis	[61,63]
CD39/CD117	InhA	Mycolic acid biosynthesis	[19]
DNB1/DNB2	DprE1	Arabinan biosynthesis	[67]
Ethambutol	EmbB	Arabinan biosynthesis	[35,36]
Ethionamide	InhA	Mycolic acid biosynthesis	[1]
Isoniazid	InhA	Mycolic acid biosynthesis	[1]
OPC-67683 (in clinical trials: Phase III)	ND	Mycolic acid biosynthesis	[22]
PA-824 (in clinical trials: Phase II)	ND	Mycolic acid biosynthesis	[23]
SQ109	MmpL3	TMM transport	[44,45,49]
Triclosan and derivatives	InhA	Mycolic acid biosynthesis	[18]
7-amino-4-methylcoumarin (NA5) and derivatives	ND	Mycolic acid biosynthesis (proposed)	[20]

ND: Not determined; TMM: Trehalose monomycolate.