Published in final edited form as:

Curr Pharm Des. 2014 ; 20(27): 4357–4378.

New Approaches to Target the Mycolic Acid Biosynthesis Pathway for the Development of Tuberculosis Therapeutics

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Abstract

Mycolic acids are the major lipid component of the unique mycobacterial cell wall responsible for the protection of the tuberculosis bacilli from many outside threats. Mycolic acids are synthesized in the cytoplasm and transported to the outer membrane as trehalose-containing glycolipids before being esterified to the arabinogalactan portion of the cell wall and outer membrane glycolipids. The large size of these unique fatty acids is a result of a huge metabolic investment that has been evolutionarily conserved, indicating the importance of these lipids to the mycobacterial cellular survival. There are many key enzymes involved in the mycolic acid biosynthetic pathway, including fatty acid synthesis (KasA, KasB, MabA, InhA, HadABC), mycolic acid modifying enzymes (SAM-dependent methyltransferases, aNAT), fatty acid activating and condensing enzymes (FadD32, Acc, Pks13), transporters (MmpL3) and transferases (Antigen 85A-C) all of which are excellent potential drug targets. Not surprisingly, in recent years many new compounds have been reported to inhibit specific portions of this pathway, discovered through both phenotypic screening and target enzyme screening. In this review, we analyze the new and emerging inhibitors of this pathway discovered in the post-genomic era of tuberculosis drug discovery, several of which show great promise as selective tuberculosis therapeutics.

Keywords

Tuberculosis; Antituberculosis agents; Mycolic acid; Fatty acid synthase; KasA; KasB; MabA; HadABC; InhA; FadD32; Pks13; MmpL3; Antigen 85

Introduction

Mycobacterium tuberculosis (*M. tb*) is the bacillus responsible for tuberculosis (TB) in humans. According to the World Health Organization, approximately one-third of the global population is infected with TB and an estimated 1.4 million died from this disease in 2011[1]. In addition, one-third of all AIDS deaths are attributed to TB [1]. *M. tb* is a slow

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growing bacterium, requiring a six month minimum treatment with the first two month “intensive phase” administration of four first-line drugs: isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin [2]. The later four-month “continuation phase” treatment kills the dormant bacteria and consists of the two most effective anti-TB drugs isoniazid and rifampicin. One primary reason why drug resistant TB develops is due to poor patient compliance with the current lengthy treatment regimen, resulting in the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains [3]. Thus, new anti-TB compounds with novel mechanisms of action, that can potentially shorten treatment duration and have activity against drug resistant strains are urgently needed [4-6].

In recent years, the fight against tuberculosis has been greatly aided by the sequencing of the *M. tb* genome [7], which revealed many potential therapeutic targets involved in lipid biosynthesis and metabolism. High throughput screening (both phenotypic and target based) of large chemical libraries is now routine and has produced many novel antitubercular chemotypes, including many that target the mycolic acid pathway [8]. Modern molecular biology technologies including rapid sequencing of whole genomes allow for rapid identification and confirmation of drug mechanisms of action. Thus, tuberculosis drug discovery efforts have greatly accelerated in the past 10 years and have been successfully applied to therapeutic targets in the unique mycobacterial wall [9].

Mycolic acids

The mycobacterial cell wall is unusual in that it contains extremely large α -alkylated β -hydroxylated fatty acids called mycolic acids (Figure 1) [14]. Mycolic acids are the primary constituent of the mycobacterial cell wall and contribute to outer membrane permeability and integrity as well as virulence [15, 16]. The biosynthesis for the incorporation of mycolic acids on the mycobacterial cell wall is shown schematically in figure 2. For an exhaustive review and analysis of the mycolic acid biosynthetic pathway, please see the following reviews by Takayama *et al* [17] and Raman *et al* [18]. The saturated α -alkyl chain ($C_{22} - C_{26}$) and the long meromycoloyl chain ($C_{40} - C_{60}$) are synthesized by the fatty-acid synthase-I (FAS-I) and fatty-acid synthase-II (FAS-II) complexes, respectively. Desaturases or dehydratases/isomerases and methyl transferases modify the proximal and distal ends of the meromycoloyl chain introducing double bonds, cyclopropyl, methoxy and keto functionalities [19]. After the α -chain is carboxylated by acyl-CoA carboxylases (Acc), the α - and meromycoloyl chains are coupled together via Claisen condensation by acyl-AMP ligase FadD32 and polyketide synthase Pks13 [14, 17, 20]. Upon release from Pks13, reduction by CmrA (Rv2509, Corynebacterineae mycolate reductase A) yields mycolic acid [21, 22]. The intact mycolic acid is then shuttled to the periplasm as a trehalose ester by the membrane transporter, MmpL3, and attached to arabinogalactan or another molecule of trehalose monomycolate (TMM) to form the free lipid trehalose dimycolate (TDM, also known as cord factor) by the antigen 85 complex [23-27]. The resulting mycolic acid rich layer is believed to form a pseudo outer lipid membrane that protects the cell [28]. Thus, it is not surprising that since the advent of tuberculosis chemotherapy, inhibition of mycolic acid biosynthesis has been one of the most widely exploited and successful drug targets [29, 30].

Validation of the mycolic acid pathway as a drug target for the treatment of *M. tuberculosis*

Disrupting the mycolic acid biosynthetic pathway is a well proven and effective therapeutic target for chemical intervention as the approved drugs isoniazid (INH), ethionamide (ETH), isoxyl (ISO), and thiacetazone (TAC) act against this pathway albeit with different mechanisms [31-33]. Interestingly, all of these drugs were developed without prior knowledge of their ultimate molecular target and are prodrugs that require metabolic activation by tuberculosis specific activating enzymes. Mutations in the activating enzymes are the major reasons for the development of clinical resistance [34-38]. The structures for INH, ETH, TAC and ISO are shown in figure 3.

Isoniazid (INH)

INH is a front-line anti-TB drug, and is one of the two most powerful TB chemotherapeutics available which primarily targets enoyl-ACP reductase, InhA. INH is a prodrug and must be activated by the catalase/oxidase KatG to a reactive free radical form, which then covalently binds NAD⁺ to form the isonicotinyl-NAD complex (Figure 4). This complex is a potent non-covalent inhibitor of the reductase, InhA [39].

Ethionamide (ETH)

Ethionamide, an analog of INH, is an approved second line anti-TB chemotherapeutic and exhibits a similar mechanism of action to INH. Ethionamide requires oxidative activation by EthA before inhibiting InhA. Upon activation, ethionamide reacts with NAD⁺ yielding an ethionamide-NAD adduct, which binds and inhibits InhA. EthA is a flavin adenine dinucleotide (FAD)-containing Baeyer-Villiger monooxygenase [34, 35, 37].

Isoxyl (ISO)

ISO (thiocarlide) is a thiourea that was used to treat TB in the 1960's. ISO has been shown to inhibit the biosynthesis of both mycolic acids and fatty acids in *M. bovis* BCG, *M. tb* and *M. aurum* [40-42]. ISO analogs have also been synthesized and tested for inhibition of whole cell *M. tb* growth [43]. It was shown that modification of the alkyl chain on ISO were well tolerated with regards to anti-TB activity [43]. Modifications included n-alkyl, branched-alkyl, thioether and aromatic as represented by compounds **5-7** (Figure 5) [43]. Efforts have also been employed to decrease the hydrophobicity of ISO by introducing an arabinofuranosyl (and analogs) group (**8**), however, to minimal success (Figure 5) [44, 45]. The main effect of ISO in fatty acid metabolism is in the inhibition of the synthesis of oleic and tuberculostearic acids; this was found to be directly attributable to the inhibitory effect of the drug on the membrane-associated stearyl-CoA (9) desaturase DesA3 [40]. Analysis of mycolic acid precursors upon exposure of *M. tb* to ISO revealed a dramatic build-up of 3-hydroxy C18, C20 and C22 fatty acids in the cells suggestive of an inhibition of the dehydratase step of the FAS-II elongation cycle [46]. A similar effect was observed upon exposure to thiacetazone (TAC) suggesting that ISO and TAC may share a common target in the mycolic acid biosynthetic pathway. Consistently, overexpression of the *hadABC* genes encoding the (3*R*)-hydroxyacyl-ACP dehydratases increased more than a 16- and 80-fold the

MIC of ISO and TAC, respectively, against *M. tb*. A missense mutation in the *hadA* gene of spontaneous ISO and TAC-resistant mutants conferred upon *M. tb* high-level resistance to both drugs [46]. Other mutations found in hyper-susceptible or resistant *M. tb* and *M. kansasii* isolates mapped to *hadC* [46]. Genetic analysis of other ISO-resistant mutants, which were generated at a frequency of 10^{-8} in the presence of 4-5× the MIC, revealed a nucleotide mutation at position 1218 in the *ethA* gene confirming the requirement of this enzyme for the bioactivation of ISO [47]. *M. tb* overexpressing the *ethA* gene increases ISO's potency by two- to fourfold [47].

Thioacetazone (TAC)

TAC, a thiosemicarbazone, is an old anti-TB compound that is primarily used in developing countries due to its low cost. However, TAC is rarely used in Africa due to potential serious cutaneous hypersensitivity reactions in HIV-positive patients [48]. TAC is primarily used clinically to help reduce INH resistance. Similarly to ETH and ISO, TAC is a prodrug that undergoes S-oxidation by EthA [49]. The Kremer lab have shown that TAC reduces mycolic acid synthesis, especially α -mycolic acids, in *M. bovis* BCG [31]. Using mass spectrometry and high resolution magic angle spinning NMR analyses by monitoring characteristic vinylic protons, they deduced that unsaturated mycolic acid concentrations were increasing with TAC exposure, suggesting post FAS-II inhibition [31]. However, overexpression of multiple cyclopropyl mycolic acid synthases (CMAS), including CmaA2, MmaA2 and PcaA, were unable to fully restore α -mycolic acid synthesis [31]. This suggests that even though the Kremer lab showed that TAC prevents cyclopropanation of mycolic acid on a macromolecular level, it may not be inhibiting specific cyclopropane synthases. More recently, the Kremer lab synthesized a series of TAC analogs and demonstrated that, along with TAC, two new inhibitors (compounds shown in figure 6) prevent α - and methoxy-mycolic acid biosynthesis in whole cell *M. tb* [50]. Grzegorzewicz and colleagues were able to show that activated TAC (and ISO; see above) inhibit the dehydratase involved in the FAS-II complex in *M. tb* [46]. Point mutations in both the HadA or HadC components of the dehydratases of FAS-II increased the resistance of *M. tb*, *M. kansasii* and *M. bovis* BCG to TAC [46, 50-52].

Potentiation of Activity of Ethionamide, Isoxyl and Thiacetazone through the Inhibition of EthR

EthR (Rv3855), a member of the TetR family of repressors [53], has been shown to bind the *ethA* promoter site and therefore inhibiting its expression and, consequently, its ability to activate the previously discussed prodrugs [34]. *ethR* knockout studies have shown that strains with this gene deleted have much increased sensitivity to ETH [53]. Conversely, EthR overexpression renders ETH quite ineffective [53]. Therefore, EthR inhibitors which antagonize its repressor function have the potential to increase ETH, ISO and TAC antitubercular activity, since they are all prodrugs activated by EthA.

Willand and colleagues utilized a structure-based pharmacophore to screen for drug-like EthR antagonists [54]. They discovered a variety of activators of EthA expression, but most significantly the 1,2,4-oxadiazole-based inhibitor (**11**), which lead to the optimized lead

compound **16** (Figure 7) [54-56]. Compound **11** was shown to have no MIC alone, yet when tested in a radial diffusion assay in concert with ETH, was able to inhibit the growth of *M. tb* H37Rv [54]. In order to improve the pharmacokinetic (PK) profiles of **11**, systematic bioisosteric replacements of both thiophene functional groups on **12** were performed by Flipo and colleagues [55, 56]. These compounds were tested in concert with 0.1 µg/mL ETH for activity against *M. tb* inside macrophages represented by the EC₅₀ (represents the concentration needed to inhibit 50% *M. tb* growth) values in figure 7 [55, 56]. Addition of the 4-trifluoro-1-butanone functional group to the piperidine moiety as seen with **14**, modestly decreased EC₅₀ and solubility, however, replacing the thiophene with a thiazole (**15**) increased potency and solubility [55, 56]. Finally, extending the alkyl chain by one methylene group, as seen with **16**, significantly increased potency and, surprisingly, solubility [56]. Co-administration of **13** and ETH in *M. tb* infected mice was able to alleviate a similar *M. tb* CFU burden as ethionamide at 3× higher dosing [54]. Future *in vivo* efficacy trials are ongoing with optimized compounds [56]. Flipo and colleagues also discovered new N-phenylphenoxyacetamides chemotype, which potentiates ETH activity with sub-micromolar potency against *M. tb* in macrophages, from the optimization of a screening hit (represented by **17** and **18**) [57]. This provides yet a new scaffold for further SAR development and PK optimization. In addition, hydrophilic esters are typical EthA products and are potential negative feedback inhibitors of EthR. Therefore, a series of low molecular weight (MW < 210 g/mol) aromatic esters (represented by **19** and **20**) were designed and synthesized as EthR antagonists. Compound **19** and **20** was shown to antagonize EthR activity with IC₅₀ values of 1 mM and 0.5 mM, respectively [58]. Compound **20** has no MIC alone, however it synergized with ETH against whole cell *M. tb* and MDR-TB and in mouse models [58]. Compound **20** was also shown to synergize with ISO and TAC against whole cell *M. tb* H37Rv [59]. These studies highlight the possibilities for future potentiation studies of current anti-TB prodrug chemotherapy.

New Inhibitors of the Mycolic Acid Biosynthetic Pathway

FAS-II Enzymatic Cycle

Fatty-acid synthase II (FAS-II) is a collection of enzymes responsible for elongating fatty acids in the synthesis of meromycolates in *M. tb* [17]. Figure 8 shows the general cycle used to elongate the fatty acids. Alkyl chains are connected to an acyl-carrier protein (ACP) through a thioester linkage. This is condensed to malonyl-S-ACP via β-ketoacyl-ACP synthase A (KasA) or β-ketoacyl-ACP synthase B (KasB), where carbon dioxide is evolved and thermodynamically driving the synthesis. The β-ketone is then reduced to the alcohol through the NADPH dependant β-ketoacyl-ACP reductase (MabA). The β-hydroxyacyl-ACP dehydratases (HadAB and HadBC) then catalyze a dehydration forming a *trans* double bond. Finally, the enonyl reductase InhA reduces the double bond yielding a new fatty acid that has been extended by two carbons. This product then can reenter the FAS-II cycle to further elongate the carbon chain to the required length.

KasA inhibitors

The FAS-II complex comprises a multitude of enzymes responsible for elongating the C₂₄-C₂₆ fatty acids produced by the FAS-I complex. Within the FAS-II complex, there are two

β -ketoacyl synthase (Kas) enzymes, KasA and KasB. These enzymes are part of the thiolase superfamily and have been shown to be essential for *M. tb* survival [60]. The Kas enzymes catalyze the Claisen condensation between malonyl-ACP and acyl-ACP [61, 62]. Kas enzymes share a similar catalytic core comprising of one cysteine (C171) and two histidines (H311 and H345) [61, 62]. A ping-pong catalytic mechanism for KasA has been proposed by the Tonge laboratory [63]. The catalytic residue, C171, initially attacks the carbonyl of the acyl-ACP substrate, yielding an acylated Kas and ACP. Subsequently, malonyl-ACP is activated by the two histidine residues and decarboxylation occurs yielding an ACP-bound enolate which, in turn, attacks the carbonyl of the acylated-Kas complex, releasing unbound enzyme and product, β -ketoacyl-ACP. Since Kas has been shown to be an essential enzyme for *M. tb*, inhibitors of this enzyme may provide new avenues for TB intervention.

Thiolactomycin (TLM, **21**) is a low molecular weight natural product originally isolated from *Nocardia* [64-67] (Figure 9), and a known inhibitor of mycolic acid biosynthesis [68]. TLM is a reversible KasA/B inhibitor that also possesses activity against gram-positive and gram-negative bacteria [64-67, 69-72]. Due to its good pharmaceutical properties, including low cytotoxicity, favorable bioavailability and *in vivo* efficacy, it is an ideal lead compound for further optimization [73, 74]. Machutta *et al* showed that TLM is a slow onset binder to both KasA and KasB [63]. The 4-hydroxythiolactone portion of TLM is an isostere of the malonyl functional group of malonyl-ACP, therefore TLM binds more tightly to the acylated Kas [63]. This accounts for a large difference in IC₅₀ values for the TLM inhibition of KasA, from 4 μ M (acylated) to 242 μ M (unacylated) [63, 74]. TLM effectively blocks the second step of the Kas catalytic mechanism, which is the transfer of enoyl-S-ACP to the acylated-Kas intermediate [63]. Structure activity relationships have been elucidated for TLM as well. Methylation at the three position of the thiolactone confers selectivity for the FAS-II enzymes (KasA and KasB) over the FAS-I enzymes [75]. Kim and colleagues synthesized a small library of compounds modifying the alkyl group at the 5-position [74]. Substitutions included alkyl, branched alkyl, unsaturated alkyl and aromatic groups and they found that the isoprenyl group was required for activity [74]. To help further the SAR, Kapilashrami *et al* modified the alkyl group at the 3-position [76]. Substitutions included alkyl, acyl and aromatic functional groups [76]. They were able to increase the K_i by approximately 2- and 3-fold against the C171Q mutant designed to mimic the acylated enzyme intermediate form [77, 78]. The K_i values for TLM (**21**), acetyl- (**22**) and trifluoroacetyl-TLM (**23**) are approximately 175, 8 and 12 respectively [76]. Despite promising inhibitory activity against the enzyme, TLM has modest whole cell growth inhibition, with an antitubercular MIC value of approximately 62.5 μ M [73, 74].

β -hydroxyacyl-ACP Dehydratase (Had) Inhibitors

HadAB and HadBC catalyze the conversion of β -hydroxyacyl-ACP to the α,β -unsaturated carbonyl, *trans*-R-CH=CH-CO-S-ACP, which is the substrate for InhA. Recently, NAS-21 (**24**) and NAS-91 (**26**), which were formerly identified as anti-malarial agents that target the β -hydroxyacyl-ACP dehydratase, FabZ, in *P. falciparum*, were shown to potentially target the same enzyme in whole cell *M. bovis* BCG and *M. smegmatis* cell-free extracts (Table 1) [79, 80]. Compounds **24**, **25** and **27** all had marked increase in MIC value against *M. bovis* BCG overexpressing the Rv0636 (*hadB*) gene, which encodes the catalytic subunit of the β -

hydroxacyl dehydratase heterodimers HadAB and HadBC [81]. However, **26** did not have any activity against whole cell *M. bovis* BCG but did have activity against *M. smegmatis* cell free extracts suggesting it is an inhibitor of the dehydratase(s) but cannot permeate whole cell mycobacteria. Compounds **24**, **25** and **27** were also shown to inhibit α - and keto-mycolic acid biosynthesis in *M. bovis* BCG in a dose-dependent manner. The mechanism of action for these classes of compounds is relatively clear, however, optimization will be required to increase potency.

InhA Inhibitors

InhA is the *M. tb* gene that codes for the enoyl acyl carrier protein (ACP) reductase, which is responsible for the reduction of the α -olefin ACP intermediate in the FAS-II biosynthetic cycle. As already mentioned, InhA is the target for INH and ETH, making it a well validated target for anti-TB chemotherapeutics [82-85] and the most exploited enzyme for drug intervention in the FAS-II complex [30]. INH and ETH are prodrugs and need activation by KatG and EthA, respectively. Therefore, a new generation of non-prodrug non-covalent substrate competitive inhibitors of InhA are being developed. Since most of the clinical resistance to INH is primarily due to mutations in the activating enzyme KatG, new inhibitors that directly target InhA have potential to be active against INH-resistant strains. For a comprehensive review of InhA inhibitors, please see the recent review from the Tonge laboratory [86].

The suitability of enoyl-ACP reductases (FabI, InhA) as drug targets was also boosted by the discovery that the antiseptic triclosan is a competitive inhibitor of this class of bacterial enzymes [87]. Subsequently, the Sacchettini lab developed a variety of triclosan analogs with anti-TB activity [88]. Target-based drug design was utilized as these compounds are active against the enoyl-ACP reductase InhA. Many compounds tested had potent submicromolar IC₅₀ values against purified *M. tb* InhA (Table 2). The butyl substituted triclosan (**28**) had an IC₅₀ value of 55 $\pm$ 20 nM. The potency dropped as saturated hydrophobicity increased as shown by **29** and **30**. However, potency doubles when the cyclohexyl ring is aromatized as seen with compound **31**. An ethylene linker between the triclosan core and the phenyl group was optimal for InhA potency. The ethyl linked compound **32**, achieved the best anti-InhA potency, however, the propyl linked **33**, had the best anti-TB whole cell activity with an MIC of 4.7 μ g/mL.

The Tonge lab has well studied the interaction of triclosan analogs against enoyl-ACP reductases (see [86] for a recent review). They had noticed that InhA has a larger binding domain than the non-mycobacterial FabI domain, and they hypothesized that substituting the A-ring chlorine with bulkier substituents may result in increased favorable interactions with InhA [89]. This, in turn, could potentially increase inhibitory activity of triclosan analogs. They also hypothesized that the B-ring chlorine atoms were non-essential and ultimately were removed. The A-ring chlorine atom was replaced with saturated straight-chain alkyl groups as depicted by compounds **34-39** (Table 2). InhA potency increased as chain length increased, with optimal activity occurring with the n-octyl substituted compound **38**. All triclosan analogs were able to inhibit *M. tb* growth *in vitro* (1.0 < MIC < 3.8 μ g/mL) except the tetradecyl substituted compound **39**. Interestingly, **34** poorly inhibits InhA, however it

can inhibit *M. tb* whole cell growth at a concentration that is on par with the rest of this small series of compounds. This suggests that **34** is either highly permeable and intracellular concentrations are much higher than other compounds in this series or it has an alternative mechanism of action. Removal of the B-ring chlorine atoms resulted in an overall less hydrophobic compound, however this class is quite non-polar and will most likely have poor solubility and hence poor bioavailability. Interestingly, removal of the B-ring chlorine atoms allowed for increasing hydrophobicity at the 5-position on the A-ring, which is opposite to the previously discussed series developed by Freundlich and colleagues. They retained the chlorine atoms on the B-ring and increased hydrophobicity at the 5-position on the A-ring which resulted in decreased activity against InhA.

In order to increase the pharmacological properties of the n-alkyl substituted triclosan analogs (compounds **34-39**), the Tonge laboratory performed bioisosteric replacements on the B-ring to increase polarity [90]. These bioisosteric replacements include heteroaromatics such as pyridine, pyrimidine, pyrazine as well as substituted phenyl groups with substitutions including acetamide, oxalylic acid, isoxyl and piperazine. They modified the B-ring of the n-hexyl substituted compound **37**. Pyridyl replacements introduce a nitrogen heteroatom, resulting in a more polar compound and could potentially decrease cytochrome mediated metabolism [91, 92]. In addition to pyridine-substituted analogs (**40-42**), pyrimidine (**43**) and pyrazine (**44**) analogs were also synthesized and evaluated. Optimal enzymatic inhibitory activity was achieved with the 4-pyridyl (**42**), which had an IC₅₀ value of 160 ± 16 nM. However, none of the pyridyl and pyridyl analogs could inhibit InhA as well as the parent triclosan analog, compound **37**. Interestingly, **41** and **42** achieved similar anti-TB whole cell activity as **37**. The Tonge lab then evaluated the role of nitrophenyls and aniline analogs (compounds **45-50**). Compound **46**, which has the electron withdrawing nitro group in the *meta*-position, was most active against InhA. Conversely, electron donating groups in the *meta*-position yields an inactive compound (as seen with **49**). However, when an amine was in the *ortho*-position (**48**), *M. tb* whole cell growth was well inhibited with an MIC value of 3.13 µg/mL. Acetamide-substituted phenyls were weakly active against InhA with the *para*-substituted (**53**) most active (IC₅₀ ~ 1.3 µM). Compound **53** was able to inhibit *M. tb* whole cell growth with a MIC = 50 µg/mL. Increasing polarity of the acetamide by an oxalylic acid-like substituent was weakly active with *ortho*- and *para*-substituted (**54** and **56**, respectively) phenyls and moderately active at the *meta*-position (**55**). The oxalylic acid-like substituted phenyls were generally inactive at inhibiting *M. tb* whole cell growth. Isoxazole analogs (compounds **57-59**) were completely inactive against whole cell bacteria, yet the *para*-substituted compound (**59**) was a low nanomolar inhibitor of InhA (IC₅₀ = 130 nM). Finally, they evaluated piperazine analogs (compounds **60** and **61**), where the *para*-substituted compound was most active against InhA. However, anti-TB whole cell activity was poor.

The general SAR observed was that *para*-substituted electron donating groups were preferred and so were *meta*-substituted electron withdrawing groups. The pyridyl analogs were most active against whole cell *M. tb*, however, nitro- and amine-substituted phenyls had adequate whole cell *M. tb* activity. To date there are no reports of the efficacy of these

compounds in animal models of tuberculosis infection, however testing is currently reported to be ongoing in this area [90].

Pyridomycin (**62**, Figure 10) is a natural product possessing potent anti-TB activity that is produced by *Streptomyces pyridomyceticus* and *Dactylosporangium fulvum* [93-97] and inhibits InhA (Figure 8) [98]. Pyridomycin seems to be selective for mycobacteria with MIC values of 0.39, 0.78 and 3.13 $\mu\text{g/mL}$ for *M. tb*, *M. smegmatis* and *M. marinum*, respectively [98]. Pyridomycin has poor MIC values of $>100 \mu\text{g/mL}$ against gram-positive and gram-negative bacteria [50]. Pyridomycin lacks activity against latent *M. tb*, which is often suggestive of cell wall inhibitors, such as INH, that require active growth to be effective [98]. Pyridomycin has low cytotoxicity against a human hepatic cell line (HepG2 $\text{IC}_{50} = 100 \mu\text{g/mL}$) and a human lung epithelium cell line (A549 $\text{IC}_{50} = 50 \mu\text{g/mL}$) [98]. The mechanism of action of pyridomycin was determined by analyzing genetic mutations of pyridomycin-resistant mutants (mutants arose at 10^{-6} on medium containing $10\times$ the MIC), where mutations were found in the gene coding for InhA [98]. The mutation translates to the D148G point mutation in InhA [98]. Pyridomycin was shown to deplete α -, methoxy- and keto-mycolic acid synthesis in a concentration dependent fashion similar to INH [98]. Purified InhA (D148G) was catalytically active albeit with a 14-fold reduction in the binding constant compared to wild-type InhA (wild-type $K_m = 13.5 \pm 2.3 \mu\text{M}$, D148G $K_m = 190 \pm 16 \mu\text{M}$) [98]. Structural analysis of NADH bound to wild type and D148G mutated InhA showed that F149 makes a 90° rotation in the mutant, reducing NADH binding affinity and reflected in the K_m . Interestingly, pyridomycin-resistant mutants were not cross-resistant with INH [98]. This was structurally rationalized with the INH-NAD complex bound to the pyridomycin-resistant InhA (D148G) mutant where the rotated phenylalanine makes a π - π interaction with the INH portion of the INH-NAD complex [98]. Pyridomycin is a potent anti-mycobacterial agent with low cytotoxicity and resultant resistant mutants are susceptible to INH making it a promising candidate for further studies and SAR development. However, despite the fact that pyridomycin is easily produced through fermentation, the chemical synthesis of pyridomycin analogs is likely to be complex and therefore future tasks of SAR development and optimizing its biological and pharmacological properties may prove to be challenging.

Methyl Transferase Inhibitors

Methyl transferases are responsible for modifying the meromycoloyl chain including cyclopropanation and methylation. These modifications are crucial for *M. tb* to retain its pathogenicity, virulence and persistence [15]. Methyl transferase enzymes also play roles in oxygenating the meromycoloyl chain, which are also essential for *M. tb* virulence [16, 99-101]. Therefore, mycolic acid methyl transferases may make good targets for the discovery and development of new anti-TB agents [19].

S-adenosyl-L-homocysteine (AdoHcy, **64**) and sinefungin (**65**) are substrate mimics for S-adenosyl-methionine-dependent methyltransferases (AdoMet), which catalyzes the transfer of a methyl or methylene group from S-adenosyl-L-methionine (**63**) to the meromycoloyl chain (Figure 11). AdoHcy (**64**) and sinefungin (**65**) have been shown to have weak activity against MmaA2 [102], although they have strong activity against cyclopropane fatty-acid

synthases (CFAS) [103, 104]. *S*-adenosyl-N-decyl-aminoethane (SADAE, **66**, Figure 11), an analog of AdoHcy (**64**) wherein the amino-acid portion is replaced with a saturated 10 carbon chain, was shown to have activity against *E. coli* CFAS *in vitro* ($K_i = 6\text{--}16\text{ }\mu\text{M}$ depending on substrate) [105]. Recently, **66** was shown to have an alternative mode of action pertaining to the oxygenation of the meromycoloyl chain [106]. Compound **66** was evaluated for its ability to block mycolic acid biosynthesis in cell extracts prepared from control *Mycobacterium smegmatis* (*M. smegmatis*) and *M. smegmatis* cells expressing *mmaA4* (*hma*) to allow for the production of hydroxyl- and keto-mycolates in this species [106]. At $10\text{ }\mu\text{M}$, **66** was able to block epoxy-, keto- and hydroxyl-mycolic acid biosynthesis whereas compounds **64** and **65** had no effect at much higher concentrations [106]. Compound **66** was able to block keto- and epoxy-mycolic acid synthesis with IC_{50} values of $31 \pm 6\text{ ng/mL}$ and $333 \pm 17\text{ ng/mL}$, respectively. Despite the highly potent activity of **66** in cell extracts, it has relatively low whole cell activity against *M. tb* and *M. smegmatis* with an MIC value of $47\text{ }\mu\text{g/mL}$ presumably due to poor intracellular accumulation and/or intracellular metabolism of **66**. Analysis of cells treated with **66** at the MIC concentration, showed the biosynthesis of epoxy-, keto- and hydroxyl-MA were nearly completely inhibited indicating on target mode of action. Interestingly, the biosynthesis of α -mycolic acids were unaffected. These substrate mimics may have off target effects that are based on similar substrates found in human metabolism.

Arylamine N-Acetyltransferase Inhibitors

Arylamine *N*-acetyltransferase (aNAT) is a cytosolic enzyme responsible for the transfer of an acetyl group to an arylamine, via the hydrolysis of acetyl-CoA [107-109]. How these enzymes exactly function in mycolic acid biosynthesis cascade is not known, however aNAT (Rv3566c) knockouts in *M. bovis* BCG are defective in mycolic acid biosynthesis and consequently unable to survive inside macrophages [110]. Humans also possess aNAT enzymes, which are important in the metabolism of many drugs including the inactivation of INH [111, 112]. Therefore, the design of inhibitors with selectivity for mycobacterial aNAT over human enzymes is a key design premise for this compound class to minimize the potential for drug-drug interactions.

The Sim lab performed an extensive screen for selective prokaryotic aNAT inhibitors against five purified aNAT isoforms including hamster, human, *M. smegmatis*, *S. typhimurium* and *P. aeruginosa* [113]. Compounds, tested at $30\text{ }\mu\text{M}$, were deemed active and selective if they achieved 50% inhibition of no less than two bacterial aNAT enzymes and 20% inhibition of both eukaryotic aNAT enzymes [113]. From this screen they identified a variety of inhibitors and have followed up on three unique scaffolds. First, the 1,2,4-triazole compound **67** (Figure 12) was identified from the initial screen with a *P. aeruginosa* aNAT IC_{50} of $6.4 \pm 0.2\text{ }\mu\text{M}$ and *M. tb* MIC value of $<10\text{ }\mu\text{g/mL}$. To expand the SAR, they identified a further six compounds with increased potency against aNAT [113]. Modifying the methyl propionate functional group on **67** to a biphenyl (**68**) or n-heptyl (**69**) chain only slightly increased potency [113]. However, removal of the alkyl chain on the sulfur, giving the free thiol significantly increased potency (compound **70-73**) [113]. Ultimately, the 3-chloro substituted phenyl (compound **73**) achieved maximum potency at approximately 240 nM [113]. Compounds **68** and **69** were able to inhibit whole cell *M. tb*

growth with MIC values no less than 10 µg/mL. Despite increased potency against purified aNAT from *P. aeruginosa*, compounds **70-73** failed to inhibit whole cell growth of *M. tb* [113], which may indicate that the MIC activity of **67** is through an alternative mechanism.

A second structurally similar pyrazole-based chemotype (**74**) was also identified in the screen described above with IC₅₀ values of 9.7 ± 0.3 µM and 18.5 ± 1.0 µM against *M. marinum* and *P. aeruginosa* aNAT enzymes, respectively (Figure 13) [113, 114]. Compound **74** was also determined to have a K_i value of 23 µM against *M. marinum* aNAT [114]. Optimization of compound **74** was carried out by modifying the substitutions on each of the two phenyl rings [114]. Potency was increased with **76** and **77**, where the phenyl rings at the 3-position on the pyrazole ring are both unsubstituted (Figure 13). Alkylation of the phenolic hydroxyl group was negligible. Aside from compound **74** (*M. tb* MIC < 10 µg/mL), whole cell anti-TB activity was not reported for this compound series [113, 114]. These compounds in general may have limited metabolic stability due to readily available or produced phenolic hydroxyl groups that may easily undergo phase II metabolism. Therefore, new compounds lacking phenolic hydroxyl groups and installing functional groups that could block phase I metabolism may be worth synthesizing and testing.

Finally, the screen revealed a novel mechanism-based irreversible class of piperidinol aNAT inhibitors **78-82** with good potency against *M. tb* and *M. marinum* aNAT enzymes (Table 3) [113, 115]. Compound **78** has IC₅₀ values of 7.7 ± 0.9 and 1.3 ± 0.0 µM against *M. tb* aNAT (TBaNaT) and *M. marinum* aNAT (MMAaNaT), respectively. The addition of chlorines in the 4-position of both phenyl rings as seen with **79**, increased potency 5- and 8-fold against TBaNaT and MMAaNaT, respectively. Substituting the *N*-methyl group to a *N*-cyclohexyl group (compound **80**) decreased potency against TBaNaT. The addition of a second basic functionality with compound **81** increased activity significantly, yielding the most potent anti-TB compound both enzymatically and against whole *M. tb* cells. All compounds had MIC values of 2.7-16.9 µM and 14.8-42.3 µM against *M. bovis* BCG and *M. tb*, respectively [115]. Figure 14 shows the plausible mechanism for suicide inhibition of the piperidinols [115]. The ring opening of the piperidine ring allows for a reverse aldol condensation yielding a Michael acceptor which in turn alkylates and inactivates aNAT. Compound **82** is the ring opened version of **78** and is approximately 6-fold more potent against TBaNaT. This helped confirm that an intact piperidine ring is non-essential to activity. The discrepancy of activity between **78** and **82** suggests that piperidine ring opening is the rate-limiting step and that compounds without an intact piperidine can undergo a retro aldol condensation more readily. However, the piperidine ring may be more stable to endogenous plasma proteins and therefore have a long metabolic half-life and may potentially reduce undesirable side-effects.

In summary, the Sim lab have identified and elaborated SAR on three unique scaffolds. Among the three series investigated, the piperidinols achieved the most effective activity against whole bacterial cells (Table 3) [115]. Although, as irreversible inhibitors, the piperidinols may have the potential to react with other cellular nucleophiles, which is a concern for future development efforts. However, encouragingly no cytotoxicity was observed for the piperidinols (**78-81**) in a mammalian or rodent cell lines. The triazole **73**, had the most potent enzymatic activity among the three series with an IC₅₀ of 240 nM.

Coupled with the extensive docking studies the Sim lab has performed and high synthetic ease to which the triazole series possesses, future opportunities for future structure-based drug design is warranted. The low molecular weight free thiol triazoles lacked anti-TB whole cell activity, therefore if co-crystal structures of these compounds bound to aNAT are solved, this then could potentially reveal a new binding pocket not yet exploited by the current compounds. Therefore, one could “grow” these fragment-like compounds into new pockets of aNAT which could increase potency against aNAT and theoretically gain activity against whole cell *M. tb*.

Macromolecular Assembly

Once the products of the FAS-I and FAS-II systems have been synthesized, they need to be coupled together to form the intact mycolic acid [17]. The product of the FAS-I system is C₂₆-S-CoA and is carboxylated by AccD4 and AccD5 forming 2-carboxyl-C₂₆-S-CoA. The product of the FAS-II system is the meroacyl-S-ACP and after modification by the methyltransferases is activated by the fatty acyl-AMP ligase, FadD32, to meroacyl-AMP. Meroacyl-AMP and 2-carboxyl-C₂₆-S-CoA are substrates for the polyketide synthase, Pks13, which catalyzes the Claisen-type condensation of these two substrates forming mycolic acid. There have not been an exhaustive amount of inhibitors identified for these targets (AccD4, AccD5, FadD32 and Pks13), however, there have been recent reports describing new inhibitors against these enzymes. The enzymes provide a relatively unexplored area for antitubercular chemotherapeutics.

Acyl-CoA Carboxylase (Acc) Inhibitors

Acc inhibitors of FAS substrate malonyl-CoA biosynthesis have been exploited in other areas of antibacterial drug discovery and may be considered potential therapeutics in the mycolic acid biosynthesis cascade [116]. Mycobacteria possesses unique Acc enzymes that are required for carboxylating the mycolic acid α -chain precursor producing one of the substrates for Pks13 [17]. Specifically, the Acc enzymes catalyze the carboxylation of C₂₆-S-CoA, yielding 2-carboxyl-C₂₆-S-CoA [17]. There are six Acc enzymes (AccD1-6) encoded in the *M. tb* genome [7]. The Tsai lab crystallized AccD5 and performed docking studies and structure-based drug design to identify AccD5 inhibitors with IC₅₀ values ranging from 10 μ M to 300 μ M (Figure 15) [117]. These compounds are the first reported inhibitors of any *M. tb* Acc enzyme, however no whole-cell activity was reported and the molecules bare similarity to anionic dyes which often interfere with spectrophotometric bioassays.

FadD32 Inhibitors

The Hung lab performed a high-throughput screen of 20,502 compounds against *M. tb* (H37Rv strain) [118]. They identified multiple coumarin-based compounds with activity against *M. tb*. After performing some classic medicinal chemistry on the screening hits, they were able to discover new coumarins with increased potency (Figure 16). The most potent compound was an aniline-substituted coumarin (**90**) with an MIC of 0.24 μ M against *M. tb* H37Rv [118]. Compound **90** was also active against five clinical *M. tb* isolates with an MIC range of 0.33 – 0.57 μ M [118]. Genetic analysis of resistant-mutants from two screening hits

including **90** revealed point mutations (E120A, E120V and F291V) in the *fadD32* gene [118]. All coumarins had $10 \times$ MIC for resistant-mutants compared to the MIC against wild-type [118]. In addition, **90** provided significant protection in a mouse efficacy trial [118]. BALB/C mice were infected with the Erdman strain of *M. tb* and treatment (four i.p. doses at 35 mg/kg every other day) began one week after exposure [118]. Compound **90** was able to provide a 4- and 30-fold reduction in CFU formation after 4 days and 8 days of treatment, respectively [118]. In another recent study, an assay was reported for the identification of FadD32 inhibitors which will undoubtedly aid the development of drugs against this important new target [119].

Pks13 Inhibitors

In an effort to identify novel Pks13 inhibitors, the Alland lab screened 1,113 compounds with known activity against *M. tb* for their ability to induce the *iniBAC* regulon [120]. Induction of the *iniBAC* regulon has been previously shown to directly correlate to mycobacterial cell wall disruption [121]. Mechanism of action for compounds **91** and **92** (Figure 17) was identified by genomic sequencing of resistant-mutants against **91** [120]. Resistant-mutants possessed a T236C single-nucleotide polymorphism in the *pks13* gene [120]. This corresponds to a F79S substitution in the enzyme. *M. bovis* BCG overexpressing *M. tb* Pks13 resulted in a 4- to 6-fold increase in MIC values, further validating that Pks13 is the target for **91** and **92** [120]. These thiophenes also showed efficacy in macrophages, demonstrating >90% intracellular bactericidal activity against *M. bovis* BCG infected macrophages at 12.8 and 6.4 μ M for **91** and **92**, respectively [120]. These thiophenes have been shown to have a novel mechanism of action and help validate Pks13 as a viable target for future antitubercular research. However, these compounds may suffer from metabolic liabilities since endogenous esterases could potentially hydrolyze the esters to the corresponding carboxylic acids, thereby reducing activity.

MmpL3 Inhibitors

Mycobacterial membrane protein large 3 (MmpL3) is a membrane transporter responsible for the transport of mycolic acid to the outer membrane [122]. The *M. tb* genome codes for twelve MmpL proteins and only MmpL3 is essential for TB viability [123]. MmpL3 consists of 12 transmembrane domains and contains 944 amino acids. MmpL proteins in general belong to the large class of RND proteins (resistance, nodulation and cell division) which mediate the translocation/extrusion of a variety of physiological compounds [124, 125].

AU1235 (**93**), 1-(2-adamantyl)-3-(2,3,4-trifluorophenyl)urea, is a potent bactericidal anti-TB agent with an MIC of 0.01 μ g/mL [122, 126]. It retains high activity against strains resistant to front line anti-tubercular agents and lacks MIC potency against other bacteria, suggesting a novel and specific mechanism of action [122]. The mechanism of action of this compound was demonstrated in a series of elegant experiments that demonstrated that exposure of *M. tb* to AU1235 decreased trehalose dimycolate (TDM) formation and cell wall-bound α -, methoxy- and keto-mycolic acids in a dose dependent fashion [122]. Intracellular TMM concentrations also rose as AU1235 concentrations increased indicating that TMM was being prevented from translocating to the cell surface [122]. The actual target of AU1235

was determined upon genetic analysis of spontaneous-resistant mutants, which revealed a single nucleotide polymorphism (G758A) in the gene coding for the protein MmpL3 [122].

The Lee lab explored the anti-tubercular SAR surrounding 1-adamantyl-3-phenyl urea pharmacophore of AU1235 (Table 4) [126, 127]. Modifications evaluated on AU1235 were used to determine the role of adamantane, urea and the phenyl groups. The SAR generated was as follows: 1) bulkier substituents such as adamantane or cyclooctyl were optimal, 2) *para*- and *meta*-substituted phenyl groups were optimal, 3) intact urea was required. Highly potent anti-TB compounds were identified, however, these compounds suffered from poor PK properties, namely solubility and human plasma protein binding [127].

Adamantyl ureas have also been long studied as human soluble epoxide hydrolase (sEH) inhibitors as a target for potential anti-inflammatory agents [128]. The *M. tb* genome codes for six putative epoxide hydrolases [7], which are enzymes involved in the removal of reactive oxygen species and play roles in lipid metabolism. In the next generation of AU1235 optimization, the Lee lab optimized the sEH selectivity and PK properties of AU1235 by replacing the 3- phenyl group with heteroaromatic rings [92]. Lead compounds from that series improved PK profiles, namely solubility, and selectivity for anti-TB activity over human sEH inhibition, thereby decreasing potential side effects [92]. Compounds **96** and **97** also showed a decrease of TDM and increase of TMM in whole cell *M. tb* in a dose dependent manner confirming that they also were inhibitors of MmpL3 [127]. The McNeil lab evaluated the intracellular concentrations of adamantyl ureas that had poor MIC values against *M. tb* yet could inhibit sEH [129]. Compound **94** had a poor MIC yet was a potent inhibitor of *M. tb*'s putative epoxide hydrolase E (EphE), and since intracellular concentrations of **94** were greater than AU1235, it was concluded that loss of TB whole cell activity was due to the inability to inhibit MmpL3 [122]. Compound **95** yielded a similar result as **94**, however it was not a potent inhibitor of *M. tb* EphE [129].

Similar to the situation with AU1235, spontaneous mutants of *M. tb*, *M. smegmatis* and *M. bovis* BCG to the pyrrole derivative, BM212 (**98**, Figure 18), and analogs have recently been shown to harbor mutations in the membrane transporter MmpL3 [130, 131]. BM212 has a MIC of 1.5 µg/mL against *M. tb* and has potent activity against many other mycobacteria, including *M. smegmatis* (MIC = 3.125 µg/mL) [130, 132]. Genetic analysis of spontaneous BM212-resistant mutants in *M. smegmatis* revealed alterations in three genes, MSMEG_0249 (integral membrane protein), MSMEG_0250 (MmpL3) and MSMEG_0251 (hypothetical protein). Upon further evaluation, MSMEG_0249 and MSMEG_0251 were identical to wild-type, but MSMEG_0250 contained two non-synonymous mutations translating to A254V and I296L amino acid changes in MmpL3 [130]. The MmpL3 mutation observed in *M. tb* is L215S, which is located in the third transmembrane. RND proteins have classically been associated with drug resistance in gram-negative bacteria [133]. Thus, MmpL3 and mutants were evaluated for their ability to confer bacterial resistance through an efflux mechanism [130, 133]. If MmpL3 mutations confer an efflux mechanism, then intracellular concentrations of inhibitor would be considerably less for mutant cell lines than wild-type. Intracellular concentrations of BM212 were identical for wild-type and BM212-resistant *M. smegmatis* [130]. This is consistent with the work of

Jackson and colleagues which show MmpL3 to be the membrane transporter of the mycolates [122].

The biochemical function and druggability of MmpL3 was further confirmed by studies on SQ109 (**99**, Figure 18), which is an *N*-adamantyl-*N*-geranyl-ethylidiamine that is currently undergoing phase II clinical trials for tuberculosis. SQ109 was originally designed as an ethambutol (EMB) analog [134]. However, even though SQ109 and EMB are structurally similar diamine compounds, they have different modes of action. EMB inhibits the biosynthesis of arabinogalactan and SQ109 inhibits the membrane transporter MmpL3. Macromolecular effects of SQ109 show that it inhibits mycolic acid attachment to the cell wall rather than their biosynthesis [135]. Other observed effects include accumulation of trehalose monomycolate (TMM) and depletion of trehalose dimycolate (TDM) [135]. SQ109 resistant mutants were generated by SQ109 analogs and were found to have an altered gene coding for MmpL3 [135]. The gene mutations coded for the amino acid mutations Q40R, L567P and A700T [135]. The Q40R mutation resides in the first extracytoplasmic loop, L567P resides in the fourth extracytoplasmic loop and A700T resides at the base of the 12th transmembrane.

Stanley *et al* recently identified a new MmpL3 benzimidazole inhibitor (C215, **100**, Figure 18) from a whole cell phenotypic MIC-based high-throughput screen against the *M. tb* H37Rv strain [136]. C215 has an IC₉₀ of 16 µM against *M. tb* and has efficacy with *M. tb* inside macrophages [136]. To elucidate mechanism of action, C215 resistant mutants were isolated and sequenced [136]. All of the resistant strains had a mutation found in the Rv0206c gene, which codes for the MmpL3 protein [136]. The resistant strains had only a modest increase in IC₉₀ of approximately 2-fold [136]. The MmpL3 mutants contained four discernible point mutations, which are V51A, L320P, T667A and V684A [136]. The V51A mutation can be found in the first extracytoplasmic loop, L320P is in the 6th transmembrane domain, T667A is in the 5th intracellular loop and V684A is in the 11th transmembrane domain.

GlaxoSmithKline (GSK) has launched an open-source drug discovery campaign to identify new anti-TB scaffolds with an interest in stimulating collaborative efforts for developing novel TB chemotherapeutics [137]. Their screening paradigm consists of a primary high-throughput screen against *M. bovis* BCG followed by a chemoinformatic and cytotoxicity assessment [137]. Compounds not triaged by poor physicochemical and/or cytotoxic properties are subsequently tested against the *M. tb* H37Rv cell line [137]. GSK was able to identify 177 small-molecule antitubercular lead compounds [137]. Recently, GSK further characterized two compounds identified from their screen, the tetrahydropyrazolopyrimidine (**101**) and the spiro-piperidine (**103**) [137, 138]. The structures for **101** and **103** are shown in figure 19. The compounds possessed potent MIC values and were non-cytotoxic [138]. Compound **101** and **103** were tested against HepG2 cells and had IC₅₀ values of >25 and 36 µM, respectively [138]. Genetic analysis of resistant mutants generated from compounds **101** and **103** resulted in mutations in the *mmpL3* gene [138]. These compounds were shown to have similar macromolecular effects to previously identified MmpL3 inhibitors indicative of MmpL3 inhibition. Medicinal chemistry was performed on compounds **101** and **103** to overcome metabolic and pharmacokinetic liabilities, yielding **102** and **104** (Figure 19) [138].

These compounds were found to be cross-resistant to the **101** and **103** resistant mutants, suggesting similar mode of action [138]. Finally, **102** and **104** were found to be efficacious in an acute TB murine model, reducing bacterial loads by 1.5 (for **102**) and 2.7 (for **104**) log units with oral doses of 100 mg/kg [138].

MmpL3 has emerged as a hot and intriguing target for future anti-TB chemotherapeutics. With the discovery of the mechanism of action of the orphan drug SQ109, MmpL3 has become a clinically validated target for anti-TB compounds. Since MmpL3 is a 12-transmembrane domain transporter, protein crystallization is unlikely in the near future, therefore structure-based drug design is as of yet unavailable. An alternative approach for drug design is a ligand-based approach. However, with the structural diversity of current MmpL3 inhibitors, which may not all bind to the same site, a comprehensive ligand-based pharmacophore model may be difficult to generate. Therefore, this may only be applied to compound classes with large inhibitory data sets of similar compounds such as the adamantyl ureas and diamines of SQ109 [134]. C215 is a synthetically tractable scaffold and therefore new C215 analogs would be useful in establishing more SAR as this scaffold may have some pharmacological advantages over adamantyl ureas and diamines.

Antigen 85 / cell wall transferase inhibition

The antigen 85 (Ag85) complex is a set of enzymes that catalyze the transfer of mycolic acid from TMM to another TMM, forming TDM, or to arabinogalactan [23, 24, 139]. The Ag85 complex consists of three enzymes, Ag85A, B and C. These three enzymes share 70.8 to 77.5% sequence homology among each other [140, 141]. Genetic studies have shown that Ag85C seems to be the most important for the incorporation of mycolic acids in the cell wall [24]. When the Ag85C gene was knocked out, there was a 40% loss in mycolic acid incorporation in the mycobacterial cell wall [24]. The crystal structure of Ag85 showed that the proteins possess an α/β -hydrolase polypeptide fold [141]. The Ag85 protein's catalytic site contains a triad of residues (S124, E228 and H260) crucial for enzymatic activity [141]. The catalytic residue S124, initially attacks the carbonyl of TMM forming a bound enzyme and tetrahedral intermediate. Upon release of trehalose, yielding acylated enzyme, another TMM molecule attacks the carbonyl of the acylated Ag85 complex, ultimately forming TDM. Ag85A knock out studies have shown increased drug sensitivity to vancomycin and imipenem, two drugs that are known to inhibit peptidoglycan synthesis [142]. Therefore, inhibition of all or part of the Ag85 complex prevents assembly of the mycolyl-arabinogalactan-peptidoglycan complex and could provide promising anti-TB chemotherapeutics.

Gobec and colleagues designed alkyl-phenyl-phosphonates as transition-state mimics [143]. The alkyl groups (C₄-C₁₄) were designed to mimic the α -branch of mycolic acid and the phenyl group was designed to mimic trehalose [143]. Optimal activity was achieved with short C₄ alkyl chains (compound **105**, Figure 20) with an IC₅₀ of 2.0 μ M against Ag85C [143]. Anti-Ag85C activity dropped as the alkyl chain was elongated [143]. Whole cell activity was modest with an MIC value of approximately 249 μ g/mL against *M. avium* [143]. Modifying the aromatic portion to a succinamide functional group (compound **106**), Ag85C inhibition was slightly increased (IC₅₀ = 1.5 μ M). Despite the increased enzyme

inhibition capabilities, whole cell activity was abolished [143]. Much optimization is needed with these scaffolds, which at this stage are best considered probe compounds due to their high lipophilicity.

In an effort to establish an assay for direct Ag85 inhibition, a mechanism-based inhibitor was developed [144]. This assay utilized an ESI-MS techniques to directly monitor trehalose and TDM formation [144]. Prior to the MS-based assay, Ag85 inhibition assays were monitoring enzyme coupling or generic mycolyl transferase activity [23, 27, 145]. The mechanism-based inhibitor probe (**107**) was able to reduce enzymatic activity by ~93% at 150 μ M [144] (Figure 21). However, the metabolic stability of **107** will most likely be low due the nascent chemical reactivity of the fluorophosphonate group and consequently further advancement of similar molecules is unlikely.

Fragment-based screening has also been utilized in identifying new leads for Ag85 inhibition [146, 147]. Fragments **109-112** (Figure 22) have MIC values of 1.3 mM, 250 μ M, 100 μ M and 50 μ M, respectively, against whole cell *M. tb* H37Rv [147]. Compounds **110-112** were designed to probe SAR and potentially increase anti-mycobacterial activity. In addition, these compounds also have been shown to have activity against MDR-TB [147]. NMR studies were used to show that **111** was able to bind especially well to Ag85C and weakly to Ag85A [147]. Conversely, **111** does not bind to Ag85B [147]. Compound **111** was the only compound able to kill *M. tb* residing in macrophages [147]. Macromolecular effects include a decrease in TDM formation by 15%, increase in TMM by 200% and increase in free mycolic acid by 200% [147]. These results suggest that the Ag85 complex is being inhibited. The compounds identified are low molecular weight fragments which have room in “drug like” chemical space to be grown out to high affinity inhibitors, which in turn should lead to lower MIC values.

Ibrahim and colleagues developed a series of glycoconjugates based on the fragments above as potential inhibitors of the Ag85 complex [148]. They tested these compounds against Ag85C in the presence of 4-methylumbelliferyl butyrate (4MUB), a synthetic substrate that when Ag85C binds 4MUB, the fluorescent 4-methylumbelliferone is released [148]. Therefore, suppression of a fluorescent signal corresponds to Ag85C inhibition. The compounds were designed to have three portions: mycolyl-like, arabinan-like and a linker [148]. The portion of the glycoconjugate anticipated to bind to the mycolyl binding domain, is the drug-like thiophene portion. The carbohydrate portion of **113** (compound shown in Figure 23) is arabinan-like and was hypothesized to gain selectivity for Ag85. Docking studies suggested replacing the cyano group as in compounds **109-112** with an ethyl ester would bind better to Ag85C. The most active compound (**113**) achieved a K_i value of approximately 18 μ M [148]. However, this compound did not have any whole cell activity against *M. smegmatis* [148].

Conclusions

Mycolic acids comprise a large proportion of the unique mycobacterial cell envelope, thus it is not surprising that their biosynthesis remains a great target for tuberculosis drug discovery. In the past 10 years there have been active efforts towards developing inhibitors

acting on virtually all portions of the mycolic acid biosynthetic pathway. Many techniques have been employed to identify and design new anti-TB chemotherapeutics, however, there are two principle ways to test new chemical entities: 1) target-based screening and 2) phenotypic-MIC-based screening. Each has their strengths and weaknesses. Target-based screening utilizes biochemical and structural approaches to evaluate a compound's activity against a specific molecular target. With target-based screening, one can structurally design compounds to specifically modulate enzymatic/receptor activity, hence potentially decreasing off-target effects. This approach works best for well validated targets for which structural information and already well defined inhibitors are known, which in this case is best typified by the development of novel InhA inhibitors. Phenotypic-based screening utilizes whole cells and the results include cell growth inhibition or cell death. Compounds that are active in a phenotypic or whole cell screen already have the pharmacodynamic properties necessary to penetrate the tubercular cell wall which is not guaranteed for enzyme inhibitors derived from target-based screen. This approach when coupled with modern molecular biology techniques for mechanism of action determination have proved to be highly effective in recent years for the discovery of inhibitors active against many unique targets in the mycolic acid biosynthesis pathway. Many of which that would be impractical to discover through target-based methodologies due to the availability and stability of biochemical substrates, protein expression of integral membrane proteins and suitable chemical matter. This has lead notably to the discovery of MmpL3 inhibitors including SQ109 which is now in Phase II clinical trials [149]. However, active compounds derived from whole cell screen may be more prone to secondary pharmacology as it is likely that the originating scaffold was derived around an already bioactive molecule as we observed with the MmpL3 targeted adamantyl urea series that also had significant human sEH inhibitory activity [126-129].

One general problem associated with the therapeutic targeting of this pathway is that mycolic acids are very large and greasy molecules that form a lipophilic barrier to cell entry. Thus it is not surprising to find many lipophilic hits are often derived from these targeted screens, which have poor physicochemical and PK properties including poor solubility, high protein binding and rapid hepatic metabolism, which requires much medicinal chemical effort to overcome. For example the adamantyl ureas, SQ109 and BM212 are all highly non-polar with high serum binding. Aside from the high hydrophobicity, compounds that target the mycolic acid pathway generally have high selectivity indices for antimycobacterial activity over cytotoxicity and MIC activity against other bacteria. This is due to the fact that the enzymes involved in the synthesis/transport/transfer of mycolic acids are either not present in the human system or other bacteria, thus producing a large therapeutic window, suitable for the long duration of treatment usually required for tuberculosis chemotherapy.

Even though INH is one of the two most effective anti-TB medications available today, it is our belief that prodrugs are unlikely to be the future of TB chemotherapy as the chemical matter for such approaches has been largely exhausted and/or removed from screening libraries. Prodrugs are potentially reactive molecules and are now commonly culled from screening collections. Another problem with this approach is the likelihood of rapid development of clinical resistance that is often due to the low fitness cost associated with inactivation of the prodrug activating enzyme. Though barriers to the discovery and

development of tuberculosis prodrug specific agents that inhibit the mycolic acid biosynthetic pathway are now high, the success of past agents that utilize this approach should not be discounted and still provides a potential opportunity. An example of such is the generation of novel drug-like antagonists of EthR, the repressor of the key prodrug activating enzyme *ethA*, which is clearly an innovative approach to this problem. These studies have produced several interesting lead candidates with clinical potential as partner drugs to classical prodrug agents activated by EthA.

Inhibiting the FAS-II system and MmpL3 are currently the most attractive / druggable targets for tuberculosis drug discovery in this pathway. The advancement of InhA inhibitors is greatly aided by the recent progress in the clinical development of FabI inhibitors such as AFN-1252, which target the analogous enoyl acyl carrier protein (ACP) reductases in other bacteria [150]. Encouragingly, GlaxoSmithKline and the Global TB Alliance have recently reported large efforts to identify novel drugs targeting InhA that do not require prodrug activation. MmpL3 is also an exciting new target for anti-TB intervention; this target seems well matched for current chemical screening libraries which have produced many diverse chemotypes active against this target, despite the fact that direct enzyme assays for testing inhibitors of this protein are not yet available. Establishing a validated assay for the direct measurement of MmpL3 inhibition is a high priority as it could potentially reveal new scaffolds for development and help weed-out development compounds which move off target.

With the further understanding of how mycolic acids are synthesized and how they are incorporated into the cell envelope coupled with new chemical entities that hit these targets originating from phenotypic screening efforts, new lead anti-TB compounds and targets are likely to continue to emerge in the mycolic acid biosynthesis pathway such as the recently discovered class of coumarin FadD32 inhibitors. These efforts are being greatly enhanced by new technologies for high throughput whole cell screening [136, 151] including for inhibitors that are active against tuberculosis grown in macrophages, which will most likely produce hits with improved physicochemical properties including macrophage penetration. Validated chemotypes may now be rapidly advanced using target-based optimization including structure based drug design which takes advantage of the rapidly increasing numbers of *M. tb* crystal structures available. These structures also open the door to other discovery techniques which are showing great promise including fragment-based drug design techniques as has already been applied to the discovery of novel antigen 85C inhibitors [76]. Fragment-based drug design has many advantages over traditional HTS approaches, including a much better coverage of chemical space which is compressed at lower molecular weights, considerably increasing the random statistical chance of finding true binders. Fragments also are low molecular weight molecules that have excellent solubility profiles, which allows for a battery of biophysical screening technologies to be applied even at high ligand concentrations providing clear structural binding information. Optimization of the fragments can then be accomplished two ways: 1) grow the fragment into new pockets of the target and/or 2) link two fragments together that are known to bind two unique sites on the target. Fragment screening, however, is relatively low throughput as

analytical technologies and the building out of fragment hits often requires a significant investment in chemistry efforts

Thus, we are at an exciting time for tuberculosis drug discovery in the mycolic acid biosynthesis pathway with many productive methods available for inhibitor discovery, several newly discovered druggable targets, such as MmpL3, PKS13 and FadD32, and many novel inhibitory compounds moving forward in development that show promising efficacy in animal models of tuberculosis infection.

Acknowledgments

The authors' research on mycolic acid inhibitors is supported through the National Institutes of Health grants AI062415 and AI063054 and the American Lebanese Syrian Associated Charities (ALSAC), St. Jude Children's Research Hospital. The authors would also like to thank Dr. David F. Bruhn for helping proof this manuscript.

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Figure 1.
Representative structures of mycolic acids [10-13].

Figure 2.

Pictorial representation of key enzymes, transporters and transferases involved in the mycolic acid biosynthetic pathway. β -ketoacyl-ACP synthase A (KasA), β -ketoacyl-ACP synthase B (KasB), β -ketoacyl-ACP reductase (MabA), β -hydroxyacyl-ACP dehydratase (Had), enoyl-ACP reductase (InhA), trehalose monomycolate (TMM).

Figure 3.
Structures of approved drugs that target the mycolic acid biosynthetic pathway.

Figure 4.

Activation of INH by KatG and formation of the isonicotinyl-NAD complex, which is a non-covalent InhA inhibitor.

Figure 5.
Representative ISO analogs and *M. tb* MIC values.

Figure 6.
TAC analogs and MIC values.

Figure 7.
Representation of current EthR inhibitors.

Figure 8.

FAS-II cycle used by *M. tb* to elongate fatty acids in the synthesis of mycolic acids. β-ketoacyl-ACP synthase A (KasA), β-ketoacyl-ACP synthase B (KasB), β-ketoacyl-ACP reductase (MabA), β-hydroxyacyl-ACP dehydratases (HadAB and HadBC), enoyl-ACP reductase (InhA).

Figure 9.
Representative structures of the Kas inhibitor thiolactomycin and its analogs.

Figure 10.
Structure of the InhA inhibitor pyridomycin.

Figure 11.
Structures of methyl transferase inhibitors.

Figure 12. Structures of aNAT triazole-based inhibitors and IC_{50} values against *P. aeruginosa* aNAT [113].

Figure 13. Structures of aNAT pyrazole-based inhibitors and IC₅₀ values against *P. aeruginosa* aNAT [114].

Figure 14.
Proposed mechanism for piperidinol suicide inhibitors [115].

Figure 15. Structures and IC_{50} values of reported AccD5 inhibitors [117].

Figure 16. Structures and MIC values for lead coumarin-based FadD32 inhibitors [118].

Figure 17.
Structures and activity of thiophene-based Pks13 inhibitors [120].

Figure 18.
Structures of MmpL3 inhibitors BM212, SQ109 and C215.

Figure 19. Representative structures and antitubercular data for GSK's MmpL3 inhibitors [137, 138].

Figure 20.
Structures of phosphonate inhibitors of Ag85C [143].

Figure 21.

Structure for the suicide inhibitor of Ag85 and plausible mechanism of action [144].

Figure 22.
Anti-Ag85 thiophenyl and thiazole compounds implicated in altering the TDM and TMM balance.

Figure 23.
Structure of glycoconjugate targeting the Ag85 complex.

Table 1

Structures and biological data for representative dehydratase inhibitors.

Compound #	Structure	<i>M. bovis</i> BCG MIC (µg/mL)				<i>M. smegmatis</i> Cell Free Extracts IC ₅₀ (µg/mL)	
		Empty Vector	<i>hadB</i> Overexpressed	Empty Vector	<i>hadB</i> Overexpressed	Empty Vector	<i>hadB</i> Overexpressed
24 (NAS-21)		63	105	94	174		
25		49	67	69	92		
26 (NAS-91)		>250	>250	154	185		
27		18	100	98	184		

Table 2Triclosan analogs with InhA IC₅₀ and MIC values.

Compound #	Structure	InhA IC ₅₀ (nM)	<i>M. tb</i> H ₃₇ R _v MIC (μg/mL)
Triclosan		1000	12.5
28		55 ± 20	9.4
29		96 ± 46	19
30		110 ± 31	9.4
31		51 ± 6	9.4
32		21 ± 8	19
33		50 ± 14	4.7
34		2000 ± 700	3.8
35		80 ± 15	ND
36		17 ± 5	1.0
37		11 ± 1	2.1
38		5.0 ± 0.3	1.9

Compound #	Structure	InhA IC ₅₀ (nM)	<i>M. tb</i> H ₃₇ R _v MIC (μg/mL)
39		150 ± 24	175
40		11,500 ± 1160	50
41		236 ± 31	3.13
42		160 ± 16	3.13
43		8,200 ± 980	100
44		650 ± 60	6.25
45		180 ± 20	12.5
46		48 ± 6	12.5
47		90 ± 10	25
48		62 ± 5	3.13
49		1,090 ± 90	100
50		55 ± 6	12.5
51		1,550 ± 460	>200
52		5,600 ± 770	100
53		1,300 ± 200	50

Compound #	Structure	InhA IC ₅₀ (nM)	<i>M. tb</i> H ₃₇ R _v MIC (μg/mL)
54		2,360 ± 200	100
55		580 ± 40	130
56		1,930 ± 90	>200
57		3,220 ± 550	>200
58		1,220 ± 60	>200
59		130 ± 34	>200
60		1,315 ± 256	>200
61		306 ± 46	>100

Table 3Structures, IC₅₀ and MIC data for piperidinols [115].

Compound #	Structure	IC ₅₀ (μM)			MIC (μM)	
		<i>a</i> TBaNaT	<i>b</i> MMaNaT	<i>M. tb</i>	<i>M. bovis</i>	BCG
78		7.7 ± 0.9	1.3 ± 0.0	21.3-42.3	3.4-16.9	
79		1.6 ± 0.1	0.16 ± 0.01	17.3-34.3	2.7-13.7	
80		4.4 ± 0.1	ND	17.3-39.4	2.8-13.8	
81		1.1 ± 0.3	2.7 ± 0.4	14.8-29.3	2.3-11.7	

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Compound #	Structure	IC ₅₀ (μM)		MIC (μM)	
		<i>a</i> TBaNaT	<i>b</i> MMaNaT	<i>M. tb</i>	<i>M. bovis</i> BCG
82		1.2 ± 0.1	0.14 ± 0.02	21.3-42.3	3.4-16.9
<i>a</i> <i>M. tb</i> arylamine N-acetyltransferase (TBaNaT)					
<i>b</i> <i>M. marinum</i> arylamine N-acetyltransferase (MMaNaT)					

Table 4
Structures of MmpL3 inhibitors (adamantyl ureas) with biological data [126, 127, 129].

Number	Structure	Human sEH IC ₅₀ (nM)	% EphE Inhibition @ 10 nM	<i>M. tb</i> MIC (µg/mL)	Intracellular <i>M. tb</i> Concentration (ng/50 µL cell pellet)
93 (AU1235)		0.4	68	0.01	75
94		11.4	93	25	128
95		9.4	19	17	103
96		73	ND	0.1	ND
97		382	ND	0.78	ND