

Recent Progress in the Development of Novel Mycobacterium Cell Wall Inhibitor to Combat Drug-Resistant Tuberculosis

Microbiology Insights
Volume 15: 1–13
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DOI: 10.1177/11786361221099878



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ABSTRACT: Despite decades of research in drug development against TB, it is still the leading cause of death due to infectious diseases. The long treatment duration, patient noncompliance coupled with the ability of the tuberculosis bacilli to resist the current drugs increases multidrug-resistant tuberculosis that exacerbates the situation. Identification of novel drug targets is important for the advancement of drug development against *Mycobacterium tuberculosis*. The development of an effective treatment course that could help us eradicate TB. Hence, we require drugs that could eliminate the bacteria and shorten the treatment duration. This review briefly describes the available data on the peptidoglycan component structural characterization, identification of the metabolic pathway, and the key enzymes involved in the peptidoglycan synthesis, like *N*-Acetylglucosamine-1-phosphate uridylyltransferase, mur enzyme, alanine racemase as well as their inhibition. Besides, this paper also provides studies on mycolic acid and arabinogalactan synthesis and the transport mechanisms that show considerable promise as new targets to develop a new product with their inhibitor.

KEYWORDS: Tuberculosis drugs, drug regimens, *Mycobacterium tuberculosis*, drug discovery

RECEIVED: November 26, 2021. **ACCEPTED:** April 21, 2022.

TYPE: Review

FUNDING: The author received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Background

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), is an intracellular pathogen. WHO reported 10 million people developed active TB in 2019 and 1.4 million deaths; of these, 208 000 deaths occurred among people co-infected with HIV.¹ TB is now the leading killer from a single infectious agent.^{1,2} The steady rise in drug resistance has added an ominous new dimension to this global health crisis with an estimated 465 000 cases of multidrug-resistant (MDR) TB, defined as TB that is resistant to isoniazid and rifampicin, with or without resistance to other first-line anti-tubercular drugs. Of these cases, 6.2% had extensively drug-resistant (XDR) TB that is resistant to INH and RIF in addition to any fluoroquinolones and injectable second-line drugs like kanamycin, amikacin, or capreomycin.³

Treatment of TB caused by strains resistant to at least isoniazid and rifampin requires additional drugs and is often less effective and less tolerated.⁴ Additionally, treatment of MDR-TB is more expensive than the standard treatment outcomes are several times worse with a high mortality rate (50%–80%) within 4 months of diagnosis, and patients with MDR-TB have a high risk of relapse after treatment completion. TB treatments caused by an MDR strain require 2 years of treatment that is too expensive with a success rate of 52% for MDR-TB and 28% for extensively drug-resistant TB (XDR-TB).^{5,6} Prolonged management with severe adverse effects reduces the physical and mental endurance of the patient during treatment. Such a situation facilitates the microbe to develop resistance and promote relapse among patients.

This effect makes many research companies to develop a new anti-tuberculosis drug that can effectively cure both the MDR and XDR tuberculosis, reduce treatment duration, and increase patient compliance. Therefore, it is important to develop new anti-tuberculosis drugs that can inhibit both actively multiplying bacilli and a non-growing persistent population of MTB to prevent reactivation of the infection.^{6,7}

Over the past 50 years 2 drugs approved by the Food and Drug Administration (FDA) for the treatment of MDR-TB. Bedaquiline is the first drug approved for the treatment of multidrug-resistant TB in 2012 and pretomanid approved in 2019 as part of a 3-drug regimen (bedaquiline, pretomanid, and linezolid (BPaL)) for the management of drug-resistant TB. Delamanid is another important nitroimidazole compound approved by EMA for the treatment of MDR-TB.^{7,8} This review briefly describes data on the mycolic acid, peptidoglycan (PG), arabinogalactan, and the key enzymes involved in these cell wall component synthesis, as well as their inhibition. This review also provides inspiring target-based approaches that are used to develop an effective inhibitor against selected enzymes from MTB.

History of Development of Tuberculosis Chemotherapy

Streptomycin was the first antibiotic isolated in 1943 with significant activity against MTB, thereby providing hope for TB management. Effective TB chemotherapy began in 1952. MTB has developed resistance to different drugs minimizing both treatment and control.⁹ Between 1940 and 1980, several drugs



have been developed like Isoniazid, p-Aminosalicylic acid, Ethambutol, Rifampicin, and Ofloxacin. The discovery of Rifampicin in the 1960s with a new mechanism shortened treatment duration to 9 months. When pyrazinamide was included in the treatment regimen, the management duration decreased to 6 months. Nowadays, TB management has 4 first-line drugs: Isoniazid, Pyrazinamide, Rifampin, and Ethambutol for the first 2 months followed by Isoniazid, and Rifampin for the next 4 months.¹⁰ Antimycobacterial treatment success rates were 85%, and between 2000 and 2018, 58 million lives were saved.¹¹ The second-line drugs used for the management of MDR-TB include fluoroquinolones (moxifloxacin, levofloxacin, ofloxacin), aminoglycosides (kanamycin, amikacin), capreomycin, cycloserine, para-aminosalicylic acid, and thioamides (ethionamide, prothionamide) as shown in Table 1. Suitable drugs regime are chosen based on efficacy, susceptibility, safety, and cost. The treatment duration of the intensive phase is 6 months when an injectable drug is added to the regime. The continuation phase without the injectable drug prolongs until 18 months after culture conversion. These drugs require a longer duration of treatment than first-line drugs resulting in toxic side effects and loss of adherence. As such, it is needed to develop new drugs that could solve the current challenges associated with the treatment of TB.^{12,13}

The Search for Novel Drugs and the Era of Target-Based Approaches

The problem of multidrug resistance and persistent infection will be solved with the identification of new drugs with novel modes of action. The target-based screening was rendered possible by advances in the genomics and molecular genetics of mycobacteria enabled the identification, functional characterization, and genetic validation of potential targets.³⁹ The advantage of the molecular target-based approach is compound isolated as inhibitors modified to improve effectiveness and selectivity while decreasing side effects.⁴⁰ Because of their mycobacterial-specific metabolic processes, several cell wall synthetic enzymes appeared to be promising candidates agents. Most candidates recently identified and progressed to trials identified through high-throughput screening followed by a drug-to-target approach (eg, bedaquiline and PBTZ169); however, target-based approaches are extensively used in TB drug discovery programs. Now a day, several *in silico* approaches proposed novel compounds as promising anti-TB agents.⁴¹ Ideal targets for TB drug discovery programs are proteins involved in crucial pathways of MTB that are either absent in humans or have low sequence identity to human homologs. Genomic and system biology studies are vital for novel target discovery and validation to increase the probability of successful drug discovery.^{6,41}

Cell Wall Synthesis Inhibitor

Mycobacterial tuberculosis (MTB) cell wall is essential to the survival of microorganisms by providing inherent resistance.

This intrinsic resistance is due to the presence of thick, waxy tightly packed cell walls, and drug degrading enzymes. Cell wall synthesis gives several novel targets because synthetic enzymes do not possess homolog in humans. The current first- and second-line drugs inhibit enzymes involved in cell wall synthesis. Resistance to these drugs develops with mutations in the target enzymes or the activation pathways of the drugs, paving the way for new resistant strains. Resistance against these drugs makes the development of new drugs against the MTB cell wall.^{42,43} The MTB cell wall has 3 layers: PG, mycolic acids, and arabinogalactan covered with a capsule. The capsule consists of protein, polysaccharides, and several types of lipids (phthiocerol dimycocerosate, diacyl trehaloses, and phosphatidylethanolamine).⁴⁴

Besides these parts, different lipids components are available including non-covalently attached waxes and glycerophospholipids that act as a permeability barrier against drugs and play a key role in the survival and pathogenesis of MTB. Several enzymes facilitate the formation of mycobacterial cell wall precursors in the cytoplasm and subsequent transportation into the periplasmic space and polymerization is used as a promising target to develop a new anti-mycobacterial drug.⁴⁵ These enzymes are mycobacterial-specific and do not have homologs in humans. The current available anti-TB drugs inhibit the synthesis of mycolic acids (Isoniazid and Ethionamide), arabinogalactan (Ethambutol), and PG (Cycloserine).^{39,45}

PG Synthesis Inhibitor

PG (murein) is an essential part of the cell wall found on the outside of the cell membrane of MTB that keeps the integrity of the cell by enduring pressure and maintains cell shape. PG also provides support to several cell components (proteins, teichoic acids), hence, it is essential for bacterial survival.⁴⁶ Enzymes that involve during PG synthesis provide a promising target to develop new drugs against TB. PG has linear glycan chains of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) moiety, cross-linked by trans peptide bridges.^{39,46} A set of recently identified PG synthesis pathways are summarized in Figure 1.

N-Acetylglucosamine-1-phosphate uridylyltransferase

PG synthesis starts with the conversion of fructose-6-phosphate to UDP-N-acetylglucosamine (UDP-GlcNAc) by acetyltransferase and uridylyltransferase activity of GlmU, a bifunctional enzyme that has 2 active sites. GlmU catalyzes the acetylation of glucosamine-1-phosphate by its acetyltransferase which further nucleotide activated through uridylyltransferase, thus facilitating the synthesis of UDP-GlcNAc that is the starting material for PG synthesis.⁴⁷ The similarity of the GlmU uridylyltransferase with human analog makes this enzyme, not a potential target. But, the lack of GlcN-1-P in humans makes the acetyltransferase domain the most promising drug target to develop a new anti-TB.⁴⁸ Currently,

Table 1. Clinically important antitubercular drugs and their mechanism of resistance.

DRUG NAME (YEAR OF DEVELOPMENT)	MECHANISM OF ACTION	MECHANISM OF RESISTANCE	ADVERSE EFFECT	REFERENCES
<i>First-line drugs</i>				
Rifampicin (1957)	Inhibit β subunit of DNA-dependent RNA polymerase	Mutation of rpoB induces a change at β -subunit of RNA polymerase, causing decrease in binding affinity	Epigastric distress, Thrombocytopenia, Leukopenia, hemolytic anemia, Menstrual disturbances	Goldstein, ¹⁴ Combrink et al ¹⁵
Isoniazid (1951)	Block mycolic acid synthesis by inhibiting NADH-dependent enoyl acyl carrier protein reductase	KatG suppression causing decreased prodrug activation, and a mutation in the promoter region of InhA causing an overexpression of InhA	Hepatotoxicity, Peripheral neuritis dermatological, gastrointestinal, hypersensitivity, hematological and renal reaction	Combrink et al, ¹⁵ Hsu et al ¹⁶
Pyrazinamide (1952)	Not known, may disruption membrane potential	Mutations in pncA reducing conversion to active acid	Hepatotoxicity, Hyperuricaemia arthralgia, GI disturbances, Thrombocytopenia, sideroblastic anemia	Sun et al, ¹⁷ Kwon et al ¹⁸
Ethambutol (1962)	Arabinogalactan synthesis inhibition	Mutations in embB at codon embB306	Optic neuritis, nausea, rashes, gastrointestinal disturbance	Saroha et al, ¹⁹ Li et al ²⁰
<i>Injectable anti-TB drugs</i>				
Streptomycin (1946)	Protein synthesis inhibition	Mutations in rpsL and rrs confer binding site modulation	Itching, numbness, Ototoxicity, nephrotoxicity	Shrestha et al ²¹
Amikacin (1972)/ Kanamycin (1957)	Protein synthesis inhibition	16S rRNA target site modulation (1400 and 1401 rrs gene) Increased drug inactivation via over expression of aminoglycoside acetyltransferase	Pain or irritation diarrhea, hearing loss, nephrotoxicity	Islam et al, ²² Sowajassatakul et al ²³
Capreomycin	Protein synthesis inhibition	Cross-resistance with aminoglycoside plus mutation of tlyA which decreases rRNA methyltransferase activity	Ototoxicity nephrotoxicity eosinophilia, rashes, fever, and injection site pain	Sowajassatakul et al ²³
<i>Fluroquinol drugs</i>				
Ofloxacin (1982) Levofloxacin (1992) Gatifloxacin Moxifloxacin	DNA gyrase and topoisomerase IV inhibitor	Mutations in gyrA and gyrB causing alteration to DNA Gyrase A/B binding site (later generations not always cross-resistant with first generation) and increased ABC-type efflux pump expression	Tendonitis and tendon rupture, photosensitivity, seizure, and QT prolongation Nausea, diarrhea, constipation, gas, vomiting, skin disturbances	Shi et al, ²⁴ Ginsburg et al ²⁵
<i>Second-line drugs: less effective or more toxic than first-line agents</i>				
Ethionamide (1956)	Mycolic acid biosynthesis inhibition	Mutations in ethA and inhA causes decreased prodrug activation and InhA overexpression (cross-resistance with Isoniazid)	Nausea and vomiting	Vilchèze and Jacobs, ²⁶ Narmandakh et al ²⁷
Prothionamide			Depression and hallucinations	Tan et al ²⁸
Cycloserine (1955)	Peptidoglycan synthesis inhibition	Overexpression of alrA decreasing drug efficiency	Headache, drowsiness, depression	Chen et al ²⁹
Para-aminosalicylic acid (1946)	Folic acid and iron metabolism inhibition	Mutations in the thyA causing a decrease in activated drug concentrations and folC mutations which cause binding site mutations	GI intolerance, lupuslike reactions	Minato et al ³⁰

(Continued)

Table 1. (Continued)

DRUG NAME (YEAR OF DEVELOPMENT)	MECHANISM OF ACTION	MECHANISM OF RESISTANCE	ADVERSE EFFECT	REFERENCES
<i>Anti-TB drugs with limited data on efficacy and long-term safety in the treatment of drug-resistant TB</i>				
Clofazimine	Release of Reactive Oxygen Species (ROS) and cell membrane disruption	Mutation to Rv0678 causes upregulation of MmpL5, a multisubstrate efflux pump (cross-resistance with Bedaquiline)	Red-brown skin discoloration and ichthyosis acute abdominal pain	Kadura et al ³¹
Linezolid (2000)	Protein synthesis inhibitor (50S subunit)	T460C mutation in rplC, encoding the 50S ribosomal L3 protein and possible efflux mechanisms	Myelosuppression, Neuropathies, lactic acidosis, and rhabdomyolysis diarrhea	Hashemian et al ³²
β -lactam/ β -lactamase inhibitor: Meropenem Imipenem	Cell wall disruption via PG modulation	Overexpression of β -lactamases, (BlaC), point mutations at target site altering deacylation rate and binding affinity, cell permeability, and increased efflux		Jaganath et al, ³³ Kumar et al ³⁴
Thiacetazone	Inhibits methyltransferases in mycolic acid biosynthesis	ethA mutation decrease prodrug activation and mutations to had ABC operon affecting dehydratase activity	GI intolerance, rash, ototoxicity, hypersensitivity reactions, such as hepatitis, marrow aplastic syndromes	Coxon et al ³⁵
Bedaquiline (2012)	Inhibition of mitochondrial ATP synthase	atpE mutations induces binding site modulation. Noted efflux via mmpL5 (cross-resistance with Clofazimine)	QT-prolongation, unexplained excess mortality	Bendre et al ⁶
Delamanid (2013)	Mycolic acid synthesis inhibition	Mutation of reductive activating Rv3547 gene	QT-prolongation	Nguyen et al ^{36,37}
Pretomanid (2019)	Inhibits synthesis of mycolic acid	Mutations in <i>ddn</i> , <i>fgd</i> , and <i>fbtA-C genes</i>	Nerve damage, vomiting, headache, low blood sugar, diarrhea, and liver inflammation	Bendre et al ⁶ , Rifat et al ³⁸

several groups of agents have been reported as promising inhibitors of GlmU, namely, aminoquinazolines, 2-phenylbenzofurans, arylamines, arylsulfonamides, nonspecific thiol reactive agents, GlcN-6-P and GlcN-1-P GlmU analogs, and diterpenoids extracted from medicinal plants. TPSA (2-[5-(2-[[4-(2-thienyl)-2-pyrimidinyl] sulfanyl)acetyl]-2-thienyl]acetic acid) with an IC₅₀ of 5.3 μ M, and 2-amino-2,3-dideoxy-3-fluoro- α -D-glucopyranosyl phosphate possess excellent activity.^{49,50}

The mur enzymes

After the formation of UDP-GlcNAc several Mur enzymes (MurA–F) also contribute to PG synthesis. The first Mur enzyme (MurA) transfers enolpyruvate residues from phosphoenolpyruvate to UDPGlcNAc. Later, MurB catalyzes NADPH-dependent reduction of enolpyruvate residues to D-lactate forming UDPN-acetylmuramic acid (UDP-MurNAc). The muramic acid residues in *MTB* contain *N*-acetyl and *N*-glycolyl derivatizations.⁴⁹ Further, the synthesis of peptide part of the PG monomer unit

(UDP-MurNAc-pentapeptide) is catalyzed by 4 essential Mur ligases MurC, MurD, MurE, and MurF catalyzes the addition of L-Ala, D-Glu, mDAP, and D-Ala-D-Ala to UDP-MurNAc respectively. The whole Mur enzymes are crucial for cell integrity and unique to prokaryotes that make these enzymes the most promising target to develop a new drug.^{51,52} In Gram-negative bacteria, fosfomycin inhibits the MurA enzyme, but mycobacterial MurA has no active site for fosfomycin attachment. Fosfomycin analogs that can inhibit *MTB* MurA are the most promising to develop a new drug. Several MurA inhibitors were identified using an *in silico* approach.⁵³ Peptidomimetic compounds and sulfonoxanthranilic acid derivatives possess a promising inhibitor effect against *MTB* MurA enzyme.^{54,55} MurB enzyme is essential for the viability of bacterial cells and the absence of a homolog in eukaryotic cells makes the MurB enzyme, a potential target for several inhibitors. Several MurB inhibitors were reported using structure-based drug design approaches such as Sulfadoxine, Pyrimethamine, piperazine derivatives, and Chloropicolinate Amides.^{56–59} As there are significant similarities in the structures of MurC and MurD/MurE and

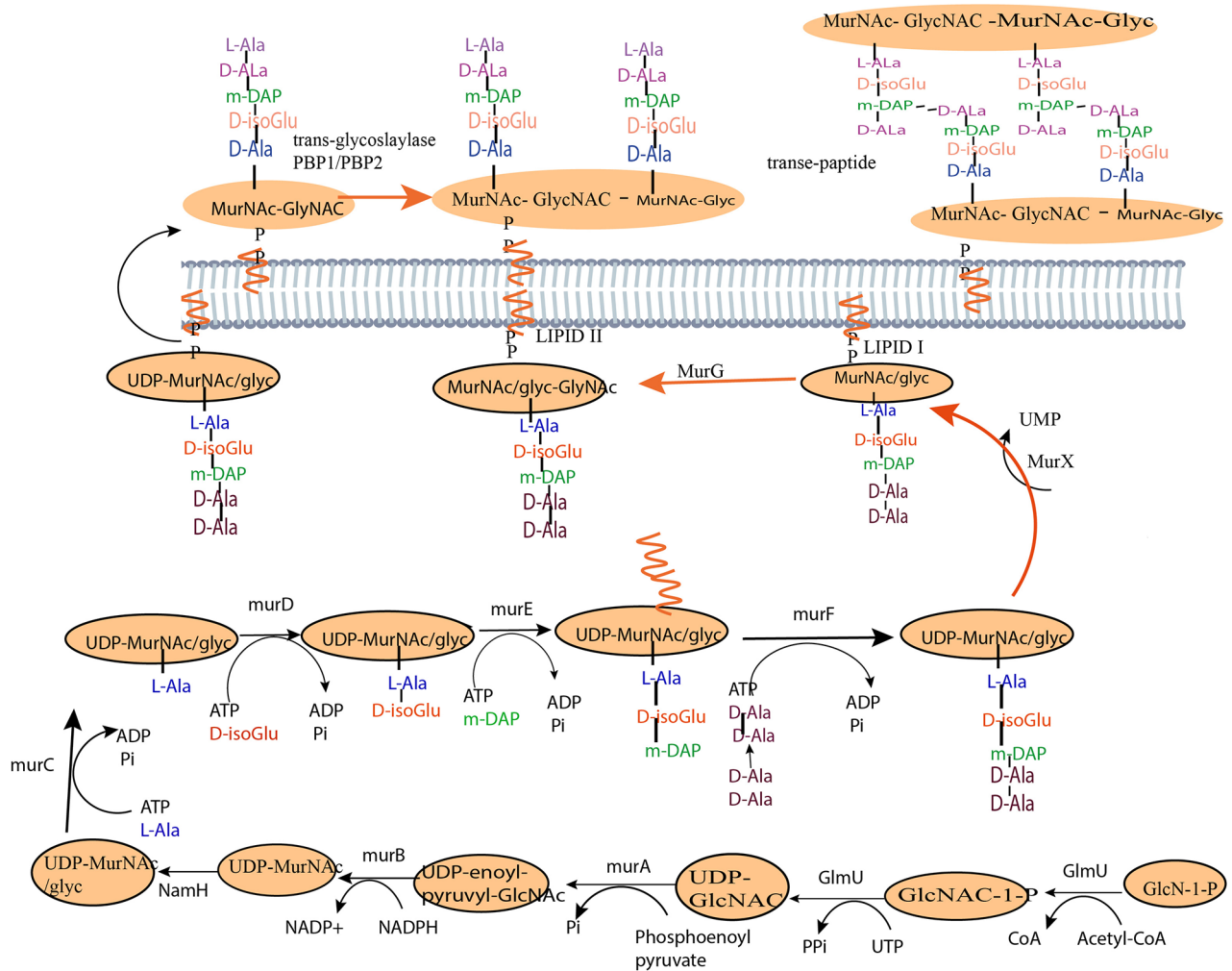


Figure 1. Peptidoglycan synthesis pathway in *M. tuberculosis*.

MurF, it has a potential target to develop a drug that can block the Mur enzymes simultaneously. Glucosamine uridine analogs are inhibitors of MTB MurE enzyme.^{59,60}

Alanine racemase inhibitor

In the cytoplasm, Alanine racemase enzymes catalyze the pyridoxal phosphate-dependent conversion of the L-alanine to D-alanine, which is essential in PG biosynthesis as shown in Figure 2. D-Cycloserine is an analog of d-alanine used as a second-line drug for TB treatment. D-Cycloserine blocks the synthesis of PG by inhibiting Alanine racemase and D-alanine-D-alanine ligase.⁶⁰ Besides, D-cycloserine inhibits several PLP-dependent enzymes, inducing a severe adverse effect, and enters into the central nervous system to induce severe neurological side effects like headaches, drowsiness, depression, dizziness. So that, several studies are undertaken to develop a non-alanine analog inhibitor of Alanine racemase to minimize the off-target effects of D-cycloserine. Thiazolidinediones inhibit Alanine racemase with IC_{50} lower than $1 \mu\text{M}$. But, their inhibition of MTB growth was not related only to Alanine

racemase inhibition, since their effects could not be attenuated by providing exogenous d-alanine.^{61,62}

Mycobacterium L, D-transpeptidase (LdtMt2) inhibitor

Overall, 80% of the peptides are cross-linked in 2 types of linkages to keep the mycobacterial cell envelope during growth and non-replicating conditions. PG structure cross-linking is catalyzed by d,d-transpeptidases (penicillin-binding proteins) and typically by the combined action of non-classical l,d-transpeptidases (Ldts) and d,d-carboxypeptidases. PG structure cross-linked mainly by 4→3 transpeptidase linkages by D, D-transpeptidase enzyme, inhibited by β -lactam antibiotic. But, there are non-classical 3→3 transpeptide linkage predominate in the PG layer of non-replicating *M. tuberculosis*. L,D-transpeptidase (LdtMt2) is responsible for 3 to 3 transpeptide linkages in the PG layer.⁶³ Carbapenem antibiotics inhibit d,d-transpeptidases, l,d-transpeptidases and d,d-carboxypeptidases enzyme. Ldt_{Mt2} is essential for *M. tuberculosis* virulence and intrinsic resistance to β -lactam. Disruption of the *ldt_{Mt2}* gene

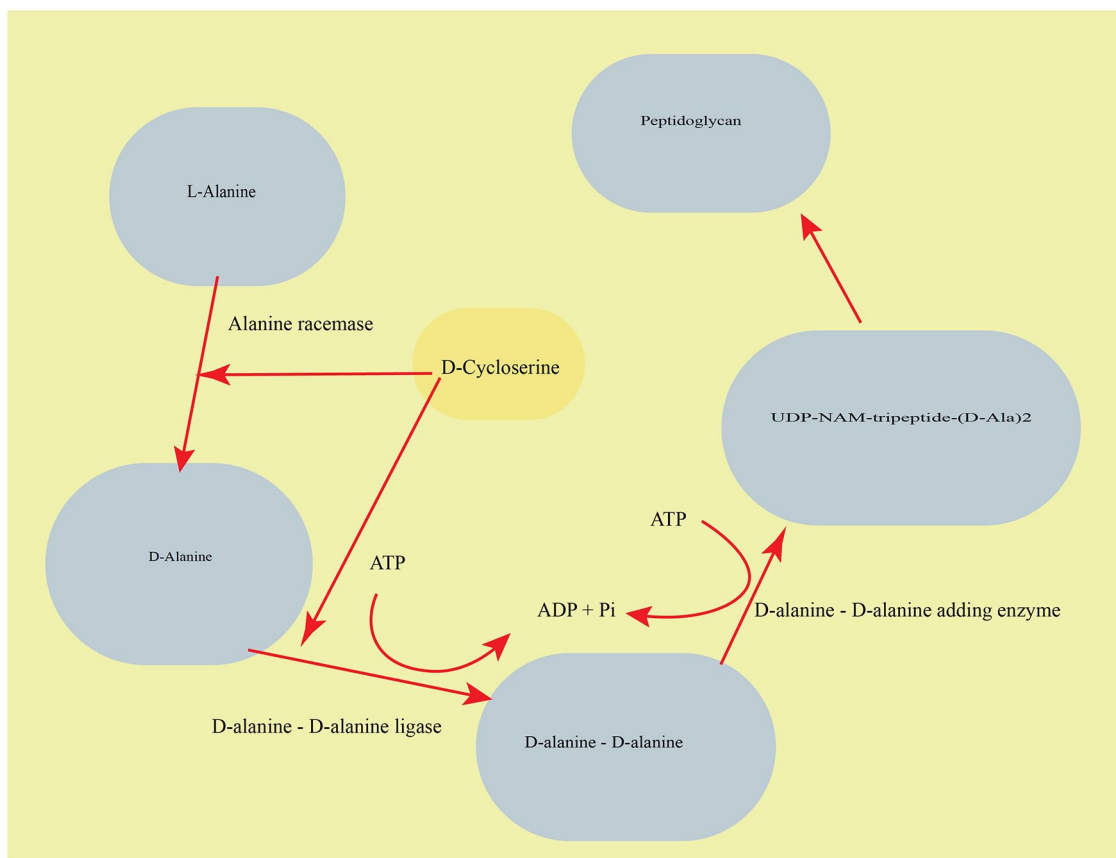


Figure 2. Alanine racemase.

abolishes bacterial virulence, alters colony morphology, and increased susceptibility to amoxicillin-clavulanate. Hence, LdtMt2 is considered as a potential anti-tuberculosis drug target that is inhibited by carbapenems and has attracted considerable attention as a potential anti-tuberculosis target.^{64,65} The next step catalyzed by Phospho-MurNAc-pentapeptide translocase (MurX/MraY, translocase I) that links the UDP-MurNAc/Glyc-L-Ala-D-Glu-meso-DAP-D-Ala- D-Ala to a decaprenyl phosphate (C50-P) to form lipid I. MraY is a promising target because it is vital in MTB survival. Several MraY inhibitor including Capuramycin, tunicamycin, and muraymycin D2 have promising effects to design new inhibitors.⁶⁶ Sansanmycin and its analogs have been reported to inhibit mraY and murX.⁶⁷

MurG catalyzes the transfer of GlcNAc from UDP-GlcNAc to MurNAc or MurNGlyc of Lipid I to produce Lipid II. MurG activity is blocked by a lipoglycopeptide antibiotic like ramoplanin and enduracidin that binds the lipid component.⁶⁸ Then, the MurT-GatD complex and AsnB amidate the α -carboxyl group of D-glutamate and the D-carboxyl group of meso-DAP to synthesize amidated Lipid II, respectively. These amidations are essential for PG cross-linking, and as such, the MurT-GatD complex and AsnB are the most promising

targets to develop PG targeting antibiotics.⁶⁹ The soluble UDP-MurNAc-pentapeptide translocate into the periplasmic space. The penicillin-binding proteins (PBPs) carry out transglycosylation and transpeptidation reactions to form mature PG in the periplasmic space. The MurJ Translocat Lipid II from the cytoplasm into the periplasmic space has a promising target to develop new agents including humimycin, b-lactam potentiators.⁷⁰ But, the MurJ enzyme in MTB must be characterized and further study will help to develop a new MTB MurJ inhibitor. Ramoplanin, teixobactin, malacidin, and nisin bind Lipid II but the glycopeptides teicoplanin and vancomycin bind D-Ala-D-Ala terminus of lipid II blocking lipid II elongation.^{69,71}

Once the lipid II is transported into the periplasmic space transglycosylase enzyme catalyzes the addition of the Lipid II to PG chains.⁶⁹ The moenomycin blocks the transglycosylase activity of PBPs while b-lactam blocks the transpeptidase effect of PBPs. The b-lactam antibiotics have no activity against mycobacteria due to b-lactamase—BlaC that breaks down the b-lactam ring. carbapenem could not be broken down by BlaC and combined with b-lactamase inhibitor are effective in killing Mycobacteria. Due to this effect, meropenem plus clavulanic acid with amoxicillin (Mero/Clv/Amx) WHO endorsed the use of this regimen for the treatment of

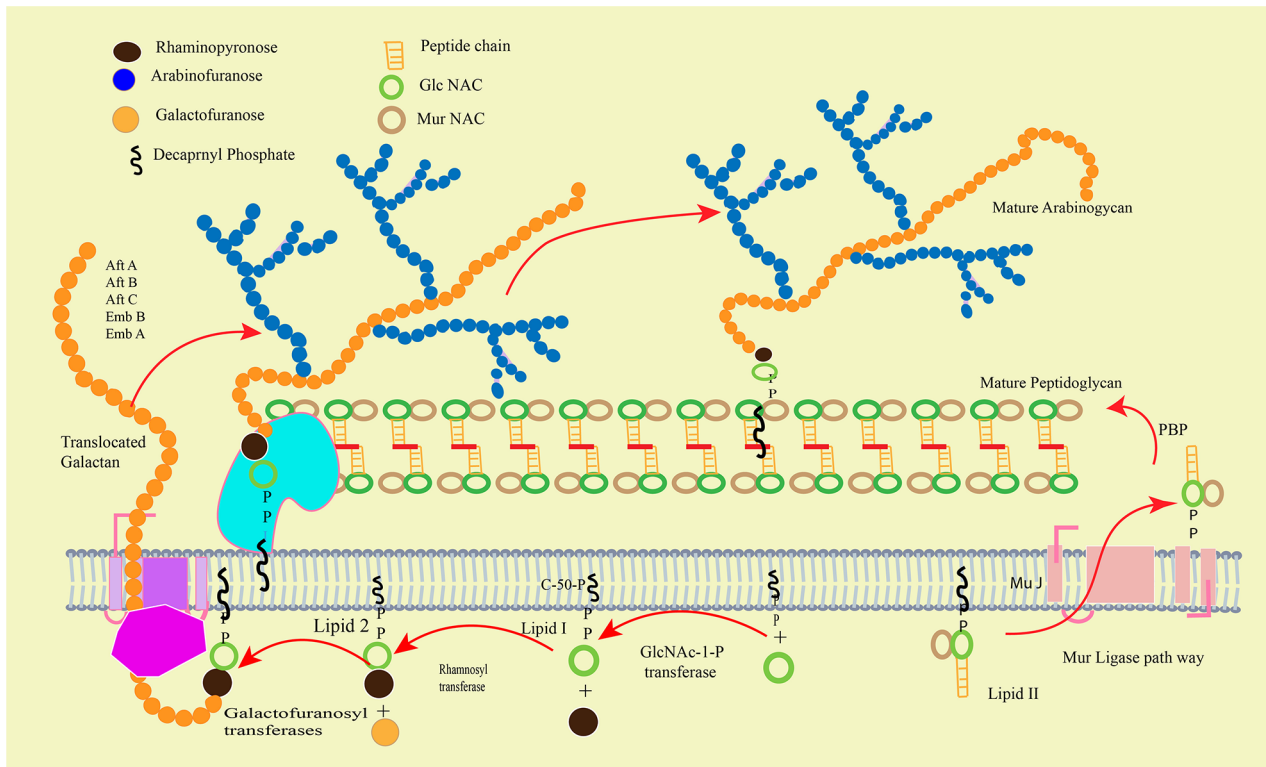


Figure 3. The enzymes involved in the mycolic acid biosynthetic pathway and their inhibitor.

DR-TB. Therefore, developing effective anti-tuberculosis carbapenem agent continued.^{33,72}

Arabinogalactan synthesis inhibitor

Arabinogalactan (AG) is a heteropolysaccharide that is covalently tethered to ~10% of the muramic acid residues of PG. AG consist of galactose and arabinose that constitute ~35% of the cell wall. Both sugars exist as d-arabinofuranose (Araf) and d-galactofuranose (GalF) that are scarce in nature. AG has 3 parts: the linker unit (LU), the galactan chain, and the arabinan chain.⁷³ The d-galactofuranose possesses 30 linear GalF residues linked to the rhamnosyl residue of the LU. The arabinofuranose is a branched polysaccharide with 3 tricosamer domains, each contains 23 Araf residues, linked to 8th, 10th, and 12th GalF residues of the galactan domain as shown in Figure 3.^{73,74}

GlcNAc-1-P transferase

AG synthesis starts in the cytoplasm by forming the linker unit (LU) that connects PG to AG.

LU synthesis begins on decaprenyl-phosphate (C₅₀-P) where GlcNAc-1-P transferase transfers GlcNAc-1-P to C50-P to form C₅₀-P-P-GlcNAc referred to as glycolipid 1 (GL-1). GlcNAc-1-P transferase (WecA) enzyme is a promising target to develop a new drug. It is inhibited by tunicamycin and caprazamycin derivatives, such as CPZEN-45.^{39,75}

Several In-vitro studies revealed that CPZEN-45 is active in replicating and non-replicating bacteria with potent MIC (3 µg/mL).⁷⁶

Rhamnosyltransferase (WbbL)

Then rhamnosyltransferase (WbbL) catalyzes the transfer of L-rhamnose (L-Rhap) from dTDP-L-Rha to 3-position of the GlcNAc of GL-1 forming glycolipid 2 (GL-2) or the linker unit (C50-P-P-GlcNAc-L-Rha). The formation of GL-2 by using rhamnosyltransferase is a promising novel target to develop a new drug.⁷⁷

Galactofuranosyltransferases

The linker unit provides an attachment site for the polymerization of the galactan chain in the cytoplasm. The bifunctional galactofuranosyltransferases (GlfT1 and GlfT2) catalyze the synthesis of the galactan chain. Initially, GlfT1 transfers GalF from UDP-Galf to the C-4 position of L-Rha, and then adds a second GalF residue to the C-5 position of the primary GalF, generating C50-P-P-GlcNAc-L-Rha-Galf1-Galf2. GlfT2 add more GalF residues to the growing galactan chain with alternating β (1→5) and β (1→6) glycosidic linkages.⁷⁸ The galactan chains form C50-PP-GlcNAc-L-Rha-Galf. GlfT1 and GlfT2 are promising targets to develop a new drug. UDP-Galf derivatives are suitable inhibitors of these enzymes, whereby they cause premature galactan chain termination.⁷⁹

Table 2. Arabinogalactan biosynthesis pathway inhibitors in *M. tuberculosis*.

INHIBITORS	TARGET	MIC	DEVELOPMENTAL PHASE (COMPANY)	REFERENCES
Caprazamycin	WecA	3 mg/mL	Preclinical	Xiong et al ⁸⁵
CPZEN-45 (Caprazene nucleoside)	DprE1 (Covalent inhibitors)	1.56 mg/mL	—	Xiong et al ⁸⁵
BTZ-043 (Nitrobenzothiazinone)	DprE1 (Covalent inhibitors)	1 ng/mL	Phase1b/IIa((University of Munich; Hans Knoll Institute, Jena; German Center for Infection Research)	Mariandyshev et al ⁸⁶
PBTZ169 (Mecozinone) (piperazinobenzothiazinone)	DprE1 (Covalent inhibitors)	<0.19 ng/mL	Phase II ((Innovative Medicines for Tuberculosis Foundation)	Hariguchi et al ⁸⁷
OPC-167832 (3,4-dihydrocarbostyryl derivative)	DprE1 (Covalent inhibitors)	0.24_2 ng/mL	Phase I/II (Otsuka)	Makarov and Mikušová ⁸⁸
TBA-7371	DprE1 (Covalent inhibitors)	0.78_3.12 mM	Phase I (TB Alliance, Bill & Melinda Gates Medical Research Institute, Foundation for Neglected Disease Research)	Gawad and Bonde ⁸⁰
TBI-1665	(Clofazimine-analog)		Phase1(Institute of Materia Medica, TB Alliance, Chinese Academy of Medical Sciences & Peking Union Medical College)	Gawad and Bonde ⁸⁰
Pyrrrole-benzothiazinone (PyrBTZ01)	DprE1	0.16 µg/mL	Preclinical	Boeree et al ⁸⁹
SQ109 (1,2-ethylene diamine)	MmpL3 (Ethambutol derivative)	<0.39 µg/mL	Phase II/III	Furin et al ⁹⁰
AZD-5847	Inhibit protein synthesis	1.0 µg/mL	Phase II	Pool ⁹¹
Sutezolid	Inhibit protein synthesis		Phase 4	Ntshangase et al ⁹²
TBA-354			Phase 1	Giraud et al ⁹³

Decaprenylphosphoribose-2-epimerase (*DprE1* and *DprE2*)

Arabinofuranosyl transferase catalyzes the addition of the first Araf residues into galactan core (C50-P-P-GlcNAc-L-Rha-Galf) to form decaprenylphosphoryl-D-arabinose (DPA). DPA synthesis occurs from phospho- α -D-ribose-1-pyrophosphate (pRpp). The pRpp synthetase transfers pyrophosphate from ATP into C-1 of ribose-5-phosphate to produce pRpp. A decaprenyl moiety is added, catalyzed by UbiA (decaprenol-1-phosphate 5-phosphoribosyltransferase) to form decaprenol-1- monophosphate 5-phosphoribose. Then phospholipid phosphatase catalyzes C-5 dephosphorylation to form decaprenol-1- phosphoribose (DPR). Finally, DPA is formed by epimerization of the ribose C-2 hydroxyl, catalyzed by decaprenylphosphoribose-2-epimerase consisting of subunits DprE1 and DprE2.^{80,81} Several compounds effectively inhibit epimerase activity of MTB'S DprE1 as shown in Table 2. Nitrobenzothiazinone (BTZ-043) has a MIC of 1 ng/mL (0.23 nM) against replicating and non-replicating MTB and entered Phase1b/IIa clinical trials. BTZ-043 showed selective effect against *MTB* activity against both MDR and XDR-MTB strains, synergy with drug bedaquilin, and safety profile.

The derivative of BTZ-043, PBTZ169 is in Phase II with MIC 0.19 ng/mL. PBTZ169 has better advantages over its predecessor including, a simple synthetic route and better pharmacodynamics.^{82,83} Recent studies identified the Rv3789 gene that transports DPA to the periplasmic space to donate arabinose for the synthesis of AG. Several *Araf*Ts contribute to the assembly of arabinan into AG. AftA transfers 3 single *Araf* residues to the 8th, 10th, and 12th *Galf* residues of AG. Further α (1 \rightarrow 5) polymerization of the arabinan domain catalyzed by EmbA and EmbB. The AftC and AftD responsible for the α (1 \rightarrow 3) branching of the internal arabinan domain of AG. AftB catalyzes the transfer of β (1 \rightarrow 2) *Araf* residues from DPA to the hexa-arabinofuranoside motif of AG. Ethambutol is the first line of anti-TB drug that inhibits AG synthesis by blocking the effect of arabinosyl transferase.⁸⁴

Rhamnose synthesis inhibitor

Rhamnose is a saccharide component essential for the virulence of pathogenic bacteria. The synthesis of rhamnose is vital for mycobacterial cell growth. Inhibiting rhamnose sugar synthesis has potential drug targets because rhamnose sugar synthesis is absent in humans. l-Rhamnose synthesis comes from

glucose-1-phosphate and dTTP (deoxythymidine triphosphate) by 4 different enzymes.⁹³ Glucose-1-phosphate thymidyl transferase (RmlA), couples the glucose-1-phosphate moiety to deoxythymidine triphosphate. dTDP-d glucose 4,6-dehydratase (RmlB), then oxidizes the 4 hydroxyls and dehydrates the 6 hydroxyls. dTDP-6-deoxy-d-xylo-4-hexulose 3,5-epimerase (RmlC), inverts the 3 and 5 hydroxyls, creating an unstable ring structure that flips. Finally, dTDP-6-deoxy-d-xylo-4-hexulose reductase (RmlD) reduces the 4 ketones to form dTDP-rhamnose. Currently, the RmlC is the best promising drug target, because RmlC is specific, unique, and does not need cofactor binding.⁹⁴ Triazinoindol-benzimidazolones inhibit RmlC enzyme.⁹⁵

Mycolic acids synthesis

Mycolic acids (MAs), 2-alkyl, 3-hydroxy long-chain fatty acids (FAs), are the hallmark of the cell envelope of *Mycobacterium tuberculosis*. MAs are characterized by very hydrophobic C54 to C63 fatty acids with C22 to C24 α side chain. MA in *M. tuberculosis* exists as α , methoxy, and keto-mycolic acids. The α -mycolic acid is the most abundant (70%), whereas methoxy and ketone mycolic acids are the minor components (10%-15%). MA synthesis inhibitor has a promising anti-tubercular effect because it enhances the permeability and sensitivity of the organism to antibiotics.^{96,97} The synthesis of mycolic acids occurs in different stages as shown in Figure 4. The first is the de novo fatty acids (C16–26) that occur in the FAS-I pathway. These fatty acids help to form the α -alkyl branch (C24) or further elongated by the FAS II pathway to give fatty acids up to C56 to produce mycolic acids that are essential for the growth and survival of *Mycobacterium tuberculosis*. The mammalian fatty acid synthesis is similar to the FAS I pathway, making the FAS II pathway unique to microbes.⁹⁸ FAS-II pathway begins with a condensation reaction catalyzed by FabH (β -ketoacyl-ACP synthase III) using malonyl-AcpM and palmitoyl-CoA to produce a β -ketoacyl-AcpM intermediate. FabH enzyme links the FAS-I and FAS-II pathways. Then β -ketoacyl-AcpM is reduced by MabA (β -ketoacyl-ACP reductase), yielding β -hydroxy acyl-AcpM that is dehydrated by HadAB/BC (β -hydroxy acyl-ACP dehydratase) to produce a *trans*-2-enoyl-AcpM intermediate. This is then reduced by InhA (enoyl-ACP reductase) to produce an acyl-AcpM elongated by 2 carbons. The FAS-II cycle continues with further condensation, catalyzed by KasA/B (β -ketoacyl- ACP synthase), repeating the above cycle via MabA, HadAB/BC, and InhA and continues until the acyl chain reaches C42–62, producing the saturated long-chain meromycolate.^{97,98}

Modification of long-chain meromycolate introduces functional groups including *cis*-/*trans*-cyclopropanation, keto, and methoxy groups by CmaA1-2-, MmaA1-4-, and PcaA-type enzymes. The meromycolic acid chain (C42–62) is activated for condensation by generation of a meromycolyl-AMP by fatty

acid adenylating enzymes (FadD32). The meromycolyl-AMP linked to α -alkyl short-chain (C22–24) in a reaction catalyzed by polyketide synthase (PKS13) to produce α -alkyl- β -keto-mycolic acid that subsequently reduced. The mycolates in the cytoplasm are converted to trehalose monomycolate (TMM), which are effluxed out through mycobacterial membrane proteins large (MmpL3). The mycolate on the translocated TMM is then coupled to AG through the mycolyltransferase Antigen 85 complex completing the mAGP assembly. Antigen 85 complex also catalyzes the synthesis of membrane unbound mycolates such as trehalose dimycolate (TDM) from TMM.^{99,100}

Mycolic acids targets and their inhibitors

β -Ketoacyl-acyl carrier protein synthase III (KAS III, FabH) inhibitor. FabH controls the initial step of FAS II by catalyzing the condensation of acetyl-CoA with a malonyl-acyl carrier protein (ACP) to form β -ketoacyl-ACP. The significance of FabH for bacterial viability makes it an ideal target for the development of novel bactericides.¹⁰¹ In the past decades, several FabH inhibitors were reported such as platensimycin, platencin, thiazole, and pyrazol-benzimidazole amide derivatives.^{102,103}

Enoyl-ACP reductase (InhA) inhibitors. InhA is a clinically validated MTB target, as highlighted by the success of Isoniazid in treating patients with TB. Triclosan is one of the first InhA inhibitors identified, had a submicromolar (0.2 μ M) IC_{50} toward the target, but its antimycobacterial activity was weak with a MIC of 64 μ M.¹⁰² Isoniazid and Ethionamide inhibit enoyl-ACP reductase (InhA) that is involved in the synthesis of mycolic acids that is crucial for growth and virulence. Isoniazid is prodrugs that are activated by catalase-peroxidase KatG enzyme- producing reactive oxygen species that inhibit InhA and this bioactivation step is where MTB primarily develops resistance by inactivating this enzyme. Therefore, direct InhA inhibitors targeting MDR-TB would solve the risk of cross-resistance.^{104,105} Currently, several direct InhA inhibitors have been identified such as thiadiazoles-based chemical inhibitors (GSK693), Pyridomycin, Pyrrolidine, Carboxamides, Pyrroles, Acetamides, Thiadiazoles, and Triazole act without prodrug activation. Pyrrolidine and carboxamides were the best in inhibiting InhA with good bioavailability and better MIC.¹⁰⁶

KasA and KasB inhibitor. The enzyme β -Ketoacyl ACP synthases (KasA and KasB) is a promising drug target in the mycolic acid pathway of *Mycobacterium tuberculosis* (MTB).⁹⁸ KasA inhibitor causes cell lysis signifying this enzyme plays a vital role in the MA pathway.¹⁰⁷

MmpL3 inhibitors. MmpL3 export trehalose-monomycolates (TMM), precursors for trehalose-dimycolates and mycolic acids that build the mycobacterial cell wall. MmpL3 inhibitors weaken the mycobacterial cell wall and causes cell death. This highlights the promising novel target of MmpL3.¹⁰⁸

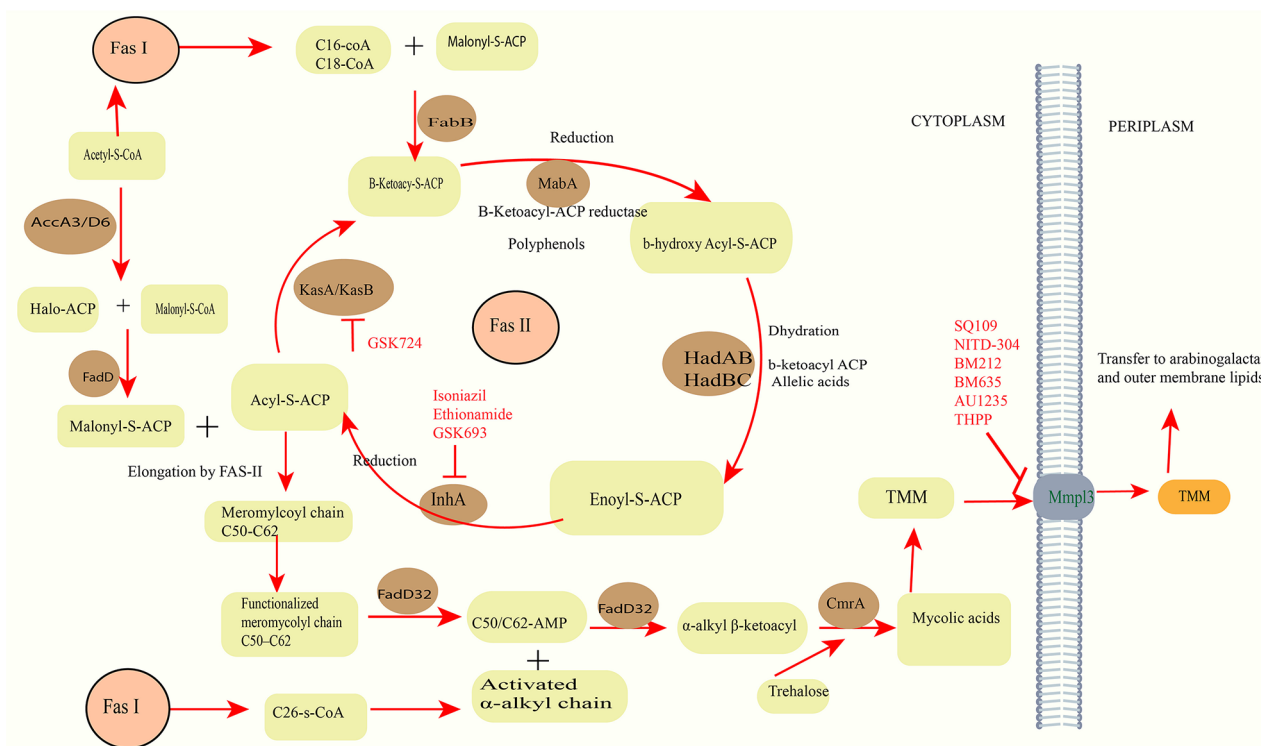


Figure 4. Arabinogalactan synthesis pathway in *M. tuberculosis*.

Several compounds were identified as MmpL3 inhibitors. SQ109 is in Phase 2b-3 trial and reported safe and effective. BM212/ BM635, NITD-304, and NITD-349 showed good preclinical safety.^{109,110}

Polyketide synthase 13. Polyketide synthase 13 (Pks13) plays a key role in the mycolic acid synthesis and is essential in MTB survival. Pks13 condenses 2 fatty acyl chains to produce α -alkyl β -ketoesters that serve as the precursors for the synthesis of mycolic acids.¹⁰² Therefore, Pks13 is a promising target for a new antituberculosis drug. Currently, several Pks13 inhibitors have been reported including Cerulenin, plastensimycin, thiolactomycin, thiophene compounds, β -lactones, benzofuran derivatives (MIC 2.0 μ M) and coumestan compounds.^{61,111,112}

Antigen 85 complex inhibitors. Antigen 85 complex enzyme has 3 homologous (Ag85A, Ag85B, Ag85C). The Ag85C homolog catalyzes the conversion of TMMs to trehalose dimycolates (TDMs) before being attached to AG. Ag85C also helps the mycobacteria to evade the host immune response by inhibiting the formation of phagolysosomes to eradicate the infection. It has a key role in *MTB* virulence.¹¹³ The Ag85C emerged as a promising novel drug target due to its key role in forming major parts of the mycobacterial membrane. Mutation of the Ag85C gene reveals \sim 40% decrease in the mycolic acid amount in the cell wall. Currently, several compounds like CyC (CyC₇ β , CyC₈ β , CyC₁₇), Phosphonic acids, ebselen, Cyclopiostins, and cyclophostin inhibition Ag85C causing the incorporation of MAs into the mycobacterial cell wall.^{114,115}

FadD32 inhibitor. FadD32, a fatty acyl-AMP ligase (FAAL32) involved in the synthesis of mycolic acids. FadD32 catalyzes the conversion of fatty acid to acyl-adenylate (acyl-AMP) in the presence of adenosine triphosphate. It is conserved in all the mycobacterial species. Thus, FadD32 represents a promising target for the development of novel antituberculous drugs.¹¹⁶ Coumarin compounds showed a good antimycobacterial activity (MIC₉₀ = 0.24–5.6 μ M) against drug-susceptible and drug-resistant TB, species that provided a starting point to validate FadD32 as a promising target for the treatment of TB. But, this class of agents has short half-lives. Modification of the metabolically labile coumarin core with a 2-quinolone core achieved high antimycobacterial activity, with MIC value of 0.5 μ g/mL against mycobacterial TB.¹¹⁷

Conclusion

The emerging drug-resistant MTB to the current drugs makes the eradication of TB remains a global challenge. Therefore, it is urgent to develop a new drug with a novel target that does not suffer from cross-resistance with the existing drugs. It is also interesting to identify novel targets that are vital for replicating and non-replicating mycobacteria. Understanding the mechanism of action of drugs may shed light on structural elements that are needed for multi-targeting strategies. Mycobacterial cell wall synthesis inhibitor is a cornerstone for tuberculosis management. Several studies showed that enzymes are involved in cell wall synthesis and proceed to be potential drug targets in antimycobacterial drug discovery. The whole cell phenotypic screening and target-based development approaches facilitate

new groups of drug candidates being identified as mycobacterial cell wall inhibitors with excellent antimycobacterial activity. This increased a ray of hope in the discovery of new drugs that will control the problem of drug resistance.

Future TB drug regimens should include inhibitors having non-canonical targets or binding different regions of the same target or blocking all alternate paths may be more effective, less prone to resistance, and may shorten therapy duration. The scientific community should try attaining a cumulative treatment outcome from different approaches, fill gaps that exist within a strategy, pursue newer treatment strategies, and continuously engage in the socio-political establishment to achieve a holistic control strategy against MTB.

Acknowledgment

I would like to acknowledge Mrs Fasika Abu for editing the paper.

Author Contributions

The author confirms sole responsibility for the design, data collection, Drafting, and critical revision of the article, and final approval of the version to be published.

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Data Sharing Statement

All data are provided in the manuscript or found from published papers as cited.

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