# Chapter 5

## Strategies for Anti-Tubercular Drug Development

Identification of a potential drug target is the most important step towards successful development of a therapeutic candidate. Desirable targets should be involved in vital aspects of bacterial growth and must ensure killing of the microbe without leaving any chance for persistence or the development of resistance. A thorough understanding on biochemistry of the pathogen immensely helps identify targets to achieve required selective toxicity towards the pathogen. The targets for anti TB drugs are based on enzymes or proteins involved in

- Cell wall component synthesis and assemblage
- Biosynthesis of material and cell division
- Cofactors and essential amino acids
- Protein biosynthesis
- Cellular respiratory apparatus and energy production

The recent advances in genetic engineering of *M. tuberculosis* have now presented many targets to be validated and subjected to high throughput screening. Discovery of new drugs acting on novel protein targets help treat resistant infections. Protein expression and x-ray crystallography studies revealed structures of several essential enzymes, which may offer valuable resource for structure based drug design and discovery.

## 5.1 Cell Wall Components Synthesis and Assemblage

Unlike other microbes *Mycobacterium tuberculosis* has acquired several unique properties due to it's highly lipid rich cell wall. The more important characteristics conferred by this structure include properties such as resistance to chemical injury, low permeability to antibiotic substances, resistance to

dehydration and the ability to persist/thrive within the hostile environment of the macrophage phagolysosome.[1] The cell envelope of mycobacterium consists of three structural components; the plasma membrane, the cell wall, and the capsule. Biosynthesis of these structures offered several important targets to develop new drugs.[2-7]

Mycobacterial plasma membrane appears to be a typical bacterial membrane contributing very little towards the pathological processes. The cell wall in *Mycobacterium* consists of two layers. Beyond the membrane, peptidoglycan (PG) layer is covalently linked to arabinogalactan (AG), which in turn is attached to large mycolic acids to constitute the cell wall core and is a hallmark feature of mycobacteria. A thick layer of extractable lipids containing esters of mycolic acids covers the outer layer forming a capsule.

### 5.1.1 Biosynthesis of Mycolic Acids and Other Lipids

Mycolic acids (MAs) are homogenous long-chain α-alkyl-β-hydroxy fatty acids differing by two-carbon units with the following general structure.

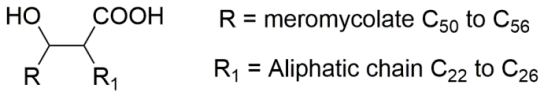


***Fig. 5.1*** *General structure of Mycolic acids.*

The meromycolate chain bears other functional groups like cyclopropyl group (α-mycolate), ketone (ketomycolate), methoxy (methoxymycolate) or hydroxyl group (hydroxymycolate) at proximal (C-16 to C-20) and distal (C-30 to C-34) positions. The MA esters of trehalose/glycerol found in the capsule are extractable with organic solvents. MAs attached to the terminal penta-arabinofuranosyl units of arabinogalactan together with peptidoglycan forms the insoluble cell-wall skeleton. Mycobacterial fatty acids are synthesized by conventional fatty acid

biosynthesis involving fatty acid synthase systems (FAS).

### 5.1.2 Mycobacteria Possessing FAS-I and FAS-II Enzymes

A eukaryote-like multifunctional enzyme FAS-I (*Rv2524c*) performs *de novo* biosynthesis of fatty acyl Co-As (C-16, C-18, C-24 and C-26) which are either used in the synthesis of membrane phospholipids or as primers for the prokaryote-like FAS-II system for meromycolate synthesis. Mycobacterial FAS-II, unlike other bacterial type II FASs, is incapable of *de novo* fatty acid biosynthesis but elongate fatty acids produced by FAS-I to meromycolyl-ACPs (up to C-56), which are direct precursors of mycolic acids. The β-ketoacylACP synthase III (FabH) is an important enzyme which connects FAS-I and FAS-II and performs decarboxylative Claisen condensation of malonyl-CoA produced by acetyl-CoA carboxylase (ACCase) and acyl-CoA produced by the FAS-I system. The resulting 3-ketoacyl-ACP product is reduced to an acyl-ACP (extended by two carbons) and shuffled into the FAS-II cycle.

The succeeding steps of condensation of the elongating chain with malonyl-ACP units are performed by the β-ketoacyl-ACP synthases (KasA and KasB) in the same way as FAS-I except for the fact that FAS-II system consists of harmoniously working individual enzymes. Meromycolic acids are further modified via a series of reactions to introduce cyclopropyl, methyl, hydroxyl, methoxy andketo groups at two positions: One near to proximal end (C18-C24) and other close to distal end (C32-C36) to obtain α-mycolates (*cis, cis*-dicyclopropyl fatty acids), hydroxymycolates, methoxymycolates and to a lesser extent ketomycolates. Finally, the meroacyl-S-ACP is converted to meroacyl-S-AMP by the enzyme FadD32 and the acyl-S-CoA produced via FAS-I is converted to the 2-carboxyl-acyl-S-CoA by acyl-CoA carboxylases (AccD4 and AccD5).

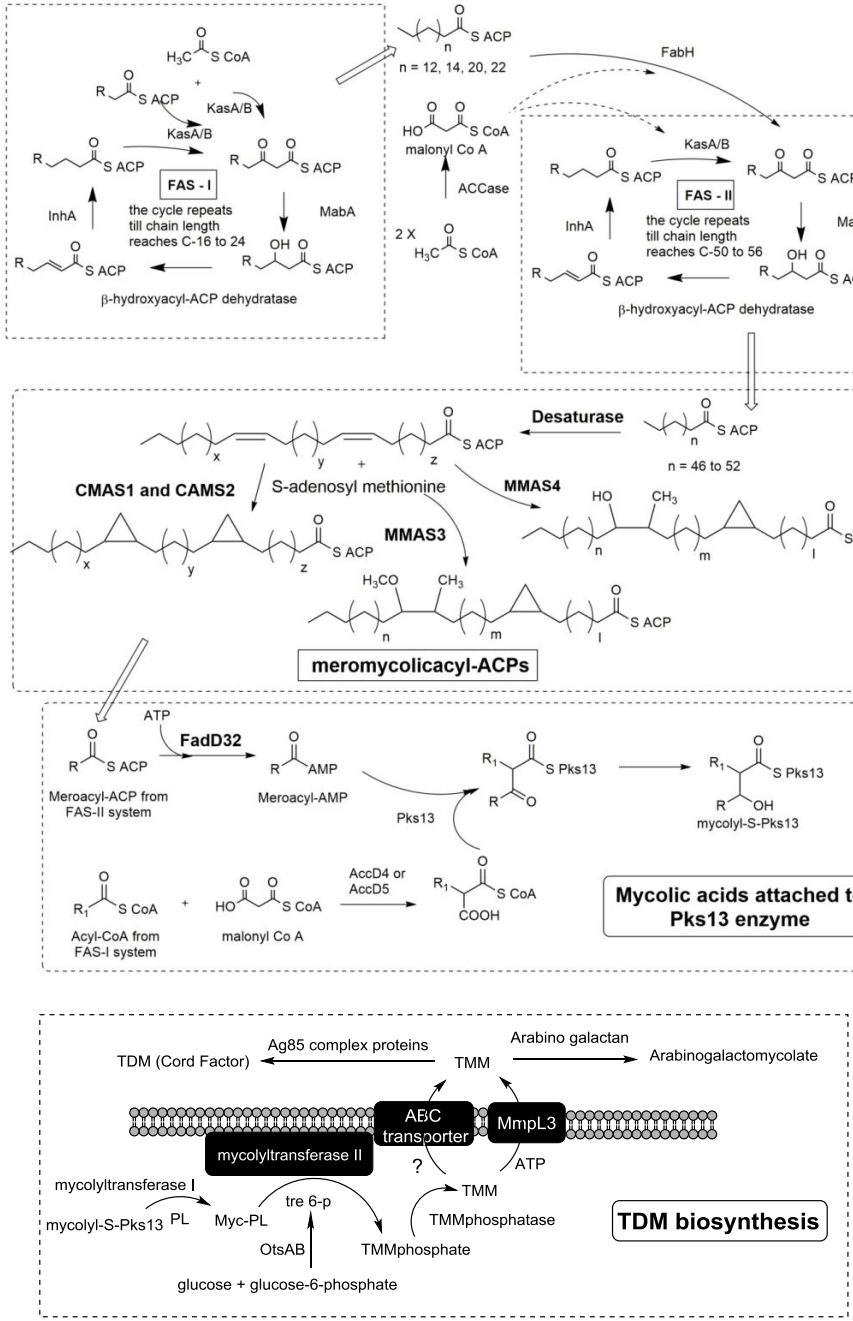


***Fig. 5.2*** *Biosynthesis of mycolic acids and trehalosedimycolates (TDM).*

These two products will be condensed by the polyketide synthase complex (Pks13) to produce α-alkyl-β-ketoacids, which upon reduction yields mycolic acids. The mycolyl group is first transferred from mycolyl-S-Pks13 (mycolyl-S-PPB) to β-D-mannopyranosyl-1-phosphoheptaprenol (PL) by a proposed cytoplasmic enzyme mycolyltransferase I to yield 6-O mycolyl-β-D-mannopyranosyl -1-phosphoheptaprenol (Myc-PL). Myc-PL migrates to the inner surface of the cell membrane and docks next to an ABC transporter, with its hydrophobic heptaprenol tail. The mycolyl group is transferred to trehalose 6-phosphate (Tre-6-p) by a proposed membrane-associated mycolyltransferase II to form 6-O-mycolyl-trehalose-6'-phosphate (TMMphosphate), and the phosphate group is removed by the membrane-associated trehalose 6-phosphate phosphatase, yielding TMM (trehalosemonomycolate). TMM is immediately transported outside the cell by a proposed TMM transporter to avoid accumulation and degradation of TMM by the ubiquitously present Ag85/Fbp.[8]

The antigen 85 complex consists of Ag85A (FbpA), Ag85B (FbpB), and Ag85C (FbpC) as the principle secreted proteins in *M. tuberculosis*. The related genes are *fbpA* (*Rv3804c*), *fbpB* (*Rv1886c*), and *fbpC* (*Rv0129c*). The Ag85 complex proteins share 68–80% sequence identity. The mycolyltransferases of the Ag85 complex are located outside the cell membrane and transfer the lipid moiety of the glycolipid TMM to another molecule of TMM yielding trehalosedimycolate or to arabinogalactan to form cell wall arabinogalactan-mycolate.[9] TDM *aka* "Cord Factor" is thought to be synthesized exclusively outside the cell from its precursor TMM.[10] In Mtb, mycolic acids and other macromolecules are transported across cell membrane via a number of transporter proteins belonging to the family MmpL (mycobacterial membrane protein large). Amongst these, an essential MmpL3 was recently identified as a potential target.[18] The compound BM212 and several other chemically unrelated

anti TB agents were found to elicit their activity via MmpL3 blockade.

## 5.2 Targets in Mycolic Acid Biosynthesis

Mycolic acid synthesis is closely related to cell division and represents a very rich reservoir of drug targets effective in the combat against mycobacterial infection.

### 5.2.1 INH A and Maba

NAD-dependent enoyl-acyl carrier protein reductase (enoyl-ACP reductase, InhA) and NAD(P)-dependent β-keto-ACP reductase (mycolic acid biosynthesis A, MabA) are two very thoroughly studied important targets. Differentiating from the known homologous proteins, MabA preferentially metabolizes long-chain substrates (C8–C20) and has very less affinity for the C4 substrate, in agreement with FAS-II specificities. Isoniazid, a first line anti tubercular drug, acts via inhibition of these enzymes. In fact, INH is a prodrug which requires metabolic oxidation by the *M. tuberculosis* enzyme, catalase-peroxidase katG, to an isonicotinoyl radical which binds covalently to the position 4 of NAD(P) cofactor. The INH-NAD adduct primarily inhibits InhA and also interferes with several other enzymes including Mab A.

### 5.2.2 Kas A and Kas B

The mycobacterial β-ketoacyl ACP synthases, KasA and Kas B catalyzes the condensation between malonyl-AcpM and the growing acyl chain in the FAS-II system. Several studies proved the essentiality of KasA genes in *M. tuberculosis*.[11] Reduction in the levels of KasA causes a rapid decrease in mycolic acid biosynthesis and leads to bacterial lysis. These attributes of KasA highlights its

potential as a candidate drug target. Even though KasB is not essential in *M. smegmatis*, *M. marinum*or, *M. tuberculosis*, it is concerned in virulence of the bacteria. In another study, *kas B* mutant was found to be more susceptible to lipophilic antibiotics, which means that inhibiting KasB would lead to improvedvulnerability to antibiotics like rifampicin. Thus, inhibitors of KasB might be projected as inhibitors of full-length meromycolates synthesis, which would attenuate *M. tuberculosis*. Cerulenin, produced by *Cephalosporium caerulens*, inhibits both KasA and KasB activity. Another potent inhibitor of KasA is thiolactomycin produced by *Nocardia*.[12, 13]

### 5.2.3 B-Ketoacyl-ACP Synthaseinhibitors

The β-Sulfonylcarboxamide compounds were designed as potential inhibitors of β-ketoacyl-ACP synthases of pathogenic mycobacteria by acting as mimics of the putative transition state in the condensation reaction.[14] These compounds are of important specificity as they show no activity against other bacteria or even non-pathogenic fast-growing mycobacteria. One of them, *n*-octanesulfonyl-acetamide inhibits the growth of a range of slow-growing pathogenic and multidrug resistant *M. tuberculosis* strains.[15] Mycobacterial lipid analysis reveals a marked reduction of all mycolic acid subtypes without affecting the panoply of polar or non-polar extractable lipids. Moreover, the drug-treated bacteria are characterized by a dysfunction in cell wall biosynthesis and incomplete septationas shown by electron microscopy.

### 5.2.4 FadD32 – AccD4 System

FadD32 belongs to a specific subclass of the FadD (fatty acid activating) family of enzymes, whichestablishes the crosstalk between FASs and PKSs by providing the activated fatty acyl adenylates to their cognate PKSs. FadD32 acts

in concert with Pks13 and activates the very long meromycolic acid (C50–C60) prior to its condensation with a C24–C26 fatty acid, which itself is activated by the AccD4-containing acyl-CoA carboxylase ACCase, to yield, upon reduction, mycolic acids (Fig. 5.2). The operon fadD32-pks13-accD4, present in all the mycobacterial specieswas proved to be essential for the viability of mycobacteria. Hence, FadD32 and AccD4 represent an attractive drug target.

### 5.2.5 Methyltransferases

Four methyl transferase enzymes were identified in the conversion of meromycolates to mycolates. A mutant of one of these methyltranferases, mmaA4, was shown to be attenuated in a mouse model of infection. All four enzymes are closely related and share a common cofactor, S-adenosyl methionine. Analogues of S-adenosyl methionine have been successfully synthesized and are effective inhibitors of bacterial and fungal methyltransferases.

### 5.2.6 Polyketide Synthase System (Pks)

Pks13 is a type I polyketide synthase, involved in the final biosynthesis step of mycolic acids, virulence factors, and crucial components of the *Mycobacterium tuberculosis* envelope. Functions and essentiality of Pks13 system was thoroughly studied and important structural information on all the five components of this system; a keto synthase domain, acyltransferase domain, two acyl-carrier protein (ACP) domains and a thioesterase domain were elucidated using X-ray crystallography.

### 5.2.7 Mmpl3 Transporter Protein

MmpL3 is a membrane transporter in the RND (Resistance-Nodulation-Cell

Division) family and is predicted by transposeon mutagenesis as well as targeted inactivation by recombination to be the only essential member of the eleven MmpL gene family of *Mtb*. In recent times MmpL3 was shown to be the target of several small molecules with diverse chemical structures, including BM212, SQ109 and AU1234. MmpL3 is predicted to have two extracellular domains and a central cytosolic domain, each was separated by multiple membrane spanning alpha-helices. Presently, the biological role of MmpL3 in the cell is unclear. It was implicated in the transport of iron, mycolic acids and TMM.

### 5.2.8 Biosynthesis of Mycolyl-Arabinogalactan-Peptidoglycan Complex

Mycobacterial cell wall contains arabinose and mannose polysaccharides in the form of lipoarabinomannan (LAM), lipoarabinogalactan (LAG), lipomannan (LM) and phosphatidyl-*myo*-inositol mannoside (PIM). Branched chain arabinogalactan plays a very important role as a connecting unit to anchor mycolic acids to peptidoglycan layer. Because mAGP is vital for cell wall integrity and mycobacterial survival, its related biosynthetic enzymes represent promising drug targets for new anti-TB therapeutics.[19]

The biosynthesis (Fig. 5.3) of arabinogalactan starts on the cytoplasmic side of the plasma membrane with transfer of GlcNAc-i-phosphate from UDP-GlcNAc onto polyprenylphosphate. Subsequent transfer of Rha from UDP-Rha*f* completes this "linker region" which helps to attach the galactan units transferred from UDP-Gal*f*. The complete polysacharide chain contains as many as 30 mannose units. This product is shifted to the extracytoplasmic side of the membrane via an active mediated pathway catalyzed by "flippases" (wzt and wzm or Rv3781/Rv3783). These enzymes work in harmony with AftA, a priming enzyme that adds single Ara*f*residues to the galactan chain. EmbA,

EmbB are two other enzymes which instigate further growth and branching of arabinan chain. AraT is another proposed enzyme involved in the completion of arabinan synthesis. The matured arabinomanan unit is connected via a linker disaccharide containing a rhamnosyl residue to a muramic acid moiety of the peptidoglycan by a ligase enzyme. Finally, the mycolic acids from TMM are transferred to arabinan units with the help of Ag85 enzymes.

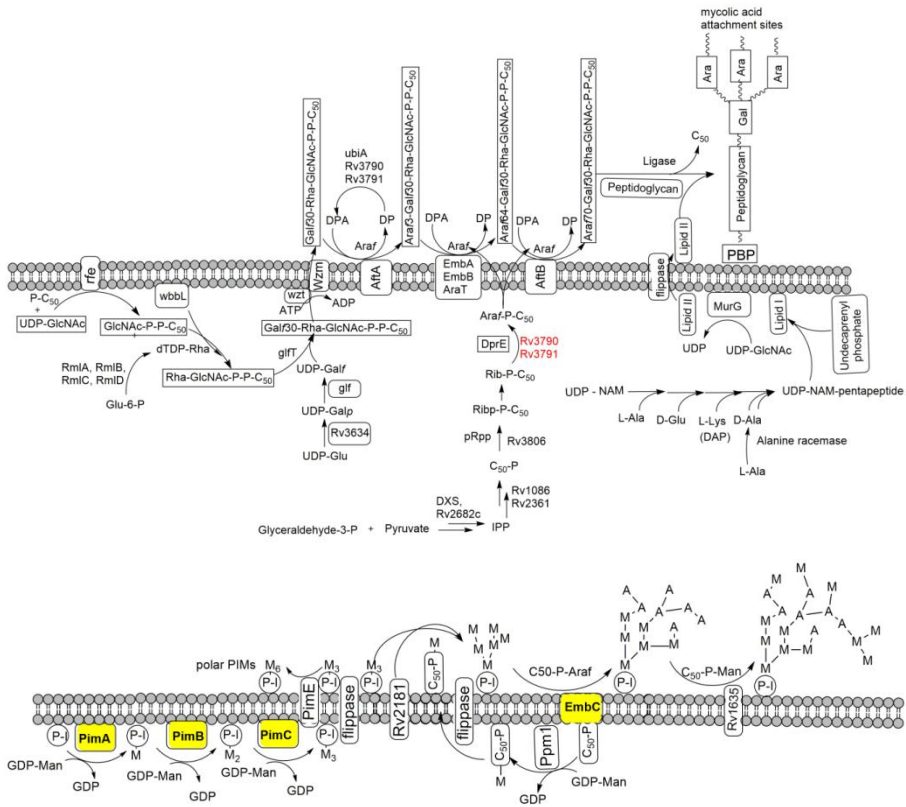


***Fig. 5.3*** *Mycolyl-Arabinogalactan-Peptidoglycan Complex.*[19-21]

The biosynthesis of LM and LAM makes use of a membrane lipid, phosphotidyl-myo-inositol (PI) as a common anchor. The synthesis of PI-trimannoside ($PIM_3$) is achieved in a GDP-Man dependent manner by the

enzymes PimA, PimB and PimC. Then the synthesized $PIM_3$ is translocated to the extracytoplasmic side by "flippases". Further mannosylations most probably happen in a C50-P-Man dependent manner. $PIM_3$ acts as a substrate for PimE towards biosynthesis of the polar PIM6 and also as a precursor in the formation of LM. The mannosyltransferases (ManTs) responsible for synthesis of the mannan backbone of LM is still unknown, but it is plausible that Rv2181, the a1, 2-ManT is responsible for the LM branching. A lipoprotein (LpqW) involved in the regulation of relative amount of polar PIMs and LM/LAM in the mycobacterial cell wall has been identified. The arabinan attachment and branching is assumed to be catalysed by the enzymes AraT, embC and related emb proteins. It would be beyond the scope of this chapter to attempt to describe the complexity of the glycosides present in mycobacterial envelope.[22-24]

## 5.3 Drug Targets for Tuberculosis

The rhamnosyl residue, which is not found in humans, plays important roles in the formation of the mycolic acid-arabinogalactan-peptidoglycan (mAGP) complex.[21, 25] The four Rml enzymes encoded by *rml*genes, including glucose-1-phosphate thymidyltransferase (RmlA), dTDP-glucose dehydratase (RmlB), dTDP-4- dehydrorhamnose 3, 5-epimerase (RmlC) and dTDP-4-dehydrorhamnose reductase (RmlD), that produced TDP-rhamnose from dTDP and glucose-1-phosphate, are smart targets for the progress of new TB therapeutics. Indeed, RmlB and RmlC are essential for the growth of mycobacteria and are considered to be the most promising drug targets in the dTDP-L-rhamnose pathway.

Decaprenylphosphoryl-D–arabinose (DPA) is the only known donor of D-arabinofuranosyl residues for the synthesis of arabinogalactan, a basic precursor for the mycobacterial cell wall core. DPA is biosynthesized in a

sequential oxidation-reduction mechanism by a heteromeric enzyme decaprenyl-phosphoryl-D-ribose oxidase (DprE).[26] DprE is composed of two proteins DprE1 (FAD containing oxidoreductase) and DprE2 (NADH dependent reductase). DprE1 catalyzes the oxidation of decaprenylphosphoryl-D-ribose (DPR) to decaprenylphosphoryl 2-keto-ribose (DPX), which is further reduced to DPA by DprE2 enzyme (Fig. 5.3). In this context, DprE1 was also shown to be essential for cell growth and survival.

Enzymes involved in the biosynthesis of LAM**,** such as polyprenolmono-phosphomannose synthase (Ppm1) and mannosyltransferase (PimB, PimF); Enzymes playing central roles in the biosynthesis of PDIM, including PDIM transferase (Papa5), PpsA-E, Mas, Fad26 and FadD28; Membrane transporter needed for the transport of PDIM through the cell membrane to the cell surface (*mmpL7* gene product) are also considered druggable targets.

### 5.3.1 Peptidoglycan Biosynthesis

The PG in bacterial cell wall contains the tetra peptide side chains consisting of L-alanine-D-isoglutaminyl- meso-diaminopimelyl-D-alanine (L-Ala-D-Glu-A2pm-D-Ala), with the Glu being further amidated. The mycobacterial PG differs in two ways from that commonly found in other bacteria; some or all of the muramic acid residues are N-glycolylated with glycolic acid (MurNGly), and the crosslinks include bonds between two residues of diaminopimelic acid as well as between diaminopimelic acid and D-alanine.[28-30]

The mycobacterial peptidoglycancontains D-alanine and differs from other groups of bacteria by the very fact that meso-diaminopimelic acid constitutes the diaminoacid moiety. Moreover, the muramic acid component which, due to experimental artifact, was thought to always bear an N-glycolyl, can actually be N-acetylated as well.[31, 32]

As for other bacteria, a central feature in peptidoglycan synthesis is the cytosolic UDP-muramyl-pentapeptide which can be considered as a building block. From UDP-Glc-NAc, this cofactor requires at least six distinct enzymes (MurA to MurF) for its synthesis.[33] The enzyme responsible for the transformation of the N-acetyl into an N-glycolyl is still the matter of investigations.

*Ethambutol* inhibits the biosynthesis of arabinan in both AG and LAM.[34-36] The ultimate steps in the biosynthesis of mAGP complex and the attachment of mycolic acids and ligation to PG, anticipates further research as it proves to be a remarkable drug target for new generation of anti TB drugs. Cycloserine blocks PG biosynthesis by inhibiting the enzymes D-alanine racemase and D-alaninyl alanine synthetase. Microorganisms treated with cycloserine accumulate a muramic-uridine-nucleotide-peptide, which differs from that produced by mycobacteria in the absence of terminal D-alanine dipeptide.[37, 38]

### 5.3.2 Protein Synthesis as a Target

Protein synthesis is a very important process involving several complex enzymes. The ribosome is one of the nature's largest and most complex enzyme system involved in the translation machinery. Large size and multitude of heterogeneous “mechanistically active” regions made ribosomes as Nature's preferred targets for antibacterial compounds. Nearly 60% of chemical classes of antibiotics target the ribosome, primarily (and often exclusively) by interacting with the ribosomal RNA. Interestingly, these ribosome-targeting antibiotics interfere with conformational changes, optimal arrangement of components, etc., not by competition with binding of cognate ligands hence high affinity is not required. Ribosomal RNA is highly conserved within bacteria, archae and eukaryotes. Hence this mechanism is considered as one of the best target for discovery of broad-spectrum antibiotics.

The aminoglycoside antibiotic, Streptomycin acts by disrupting the protein synthesis in bacteria and is the first antibiotic to be used in the treatment of tuberculosis. The site of action is in the small 30S subunit of the ribosome, specifically at ribosomal protein S12 (*rpsL*) and 16S rRNA (*rrs*) in the protein synthesis.[39] Most of the aminoglycosides act through this mechanism.[40] There aremany different inhibitors of protein synthesis, like tetracycline, chloramphenicol and macrolides (erythromycin) that do not show activity against *M. tuberculosis*. The rigorous efforts put by medicinal chemists in developing anti-tubercular agents based on inhibition of protein synthesis, suggests that, the ribosome may not be a striking target for new anti-TB drugs.

Linezolid is a new drug approved by FDA in the year 2000 as an antimicrobial agent for treating drug-resistant infections and has also shown clinically significant antitubercular activity.[41] Due to its toxicity, the structure optimization studies were conducted to obtain PNU-100480 (Sutezolid) and AZD-5847 (Posizolid), that are found to enhance antibiotic activity.[44] Whereas most of the wide known antibiotics (macrolides, chloramphenicol) hinder bacterial protein synthesis at the peptide chain elongation stage; but linezolid acts early by effective interaction with 50S ribosomal subunit. In the initiation step of bacterial translation, 50S subunit is associated with fMet-tRNA and a complex composed of 30S ribosomal subunit and mRNA to form the functional initiation complex. Linezolid interacts with the peptidyl-tRNA binding P site at the 50S subunit with micro molar affinity and it has no similarity to the 30S subunit. This interaction prevents binding of fMet-tRNA to this site during the formation of the initiation complex.[42]

### 5.3.3 Decaprene Biosynthesis

Decaprenyl phosphate acts as a carrier of activated sugar across cell membrane during the biosynthesis of mycobacterial cell wall. Another essential biochemical

of the electron transport chain, menaquinone, also possess a side chain derived from polyprenyl diphosphate. Hence the biosynthesis of isoprenoidsis essential for cell wall biosynthesis and energy production by MTB organism.[27] Isoprenoids are biosynthesized via mevalonate pathway (human) or methyl-erythritol phosphate (MEP) pathway in *M. tuberculosis*. The MEP pathway has been implicated in the virulence, combating oxidative stress and adaptation to the host environment through enhanced stress resistance or other mechanisms.

The biosynthesis starts with formation of 1-deoxy-D-xylulose 5-phosphate (DOXP) via condensation of pyruvate and glyceraldehyde 3-phosphate. DOXP is converted to MEP through the action of the enzyme DOXP reductoisomerase (DXR/IspC). 4-Diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME) is formed from the reaction of MEP with CTP, catalysed by YghP/IspD. The fourth reaction, catalysed by YchB/IspE, gives 4-diphosphocytidyl- 2C-methyl-D-erythritol-2-phosphate (CDP-ME2P). The final reactions are catalysed by YghB/IspF, GcpE/IspG and LytB/IspH to produce IPP (Fig. 5.4). The isomerase enzyme which catalyses the interconversion of IPP and DMAPP can be considered as common to both the MEP and the mevalonate pathways.

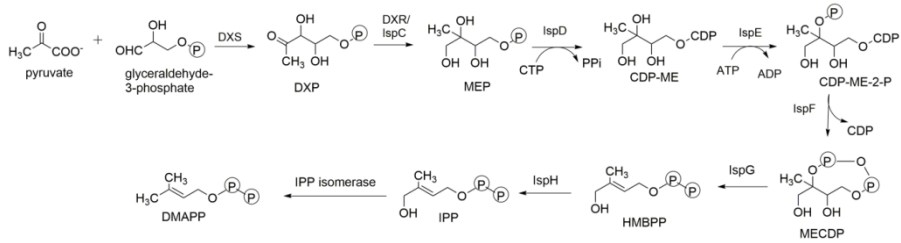


***Fig. 5.4*** *Biosynthesis of Decaprene.*

## 5.3.4 The MEP Pathway as a Drug Target

All enzymes involved in the MEP pathways which are essential for Mtb and several other human pathogens are considered as metabolic chokepoints. Absence

of MEP enzyme homologues in human proteome made them a very potential drug target for novel anti TB drug design and development. YchP, YchB and YghB have all been shown to be essential in *Mtb* and several other microbes. The IspD protein has no ortholog and considered a promising drug target for anti-tuberculosis drugs. The MEP pathway has been proven as a viable target for drug development since it was shown that the antimicrobial compound fosmidomycin and its derivative FR-900098 both inhibit DXR, the enzyme catalysing the second step of the pathway. These compounds have also proven to be effective against the malaria parasite and are currently in clinical trials.

## 5.4 Enzymes Involved in Amino Acids or Co-Factor Biosynthesis

### 5.4.1 Pantothenatesynthetase

Pantothenate (vitamin B5) is a key precursor of the 4-phosphopantetheine moiety of coenzyme A (CoA) and the acyl carrier protein (ACP). Both CoA and ACP are necessary cofactors for cell growth and are involved in essential biosynthetic pathways. Pantothenate is produced in micro-organisms, plants, and fungi, but not in animals.[43] The four enzymes in pantothenate biosynthesis (Pan B–E) and lumazine synthase (LS) that catalyzes vitamin B5 and B2 synthesis, are considered as attractive targets for anti *M. tuberculosis* drug discovery. The pathway to pantothenate is best understood in *Escherichia coli*, where it comprises four enzymatic reactions.[44, 45] The final transformation to produce pantothenate is catalyzed by pantothenate synthetase, encoded by the *panC*gene. Pantothenate is biosynthesized by the condensation of D-pantoate and β-alanine. One equivalent of ATP is used, resulting in the formation of AMP and pyrophosphate (PPi). The $Mg^{2+}$-dependent reaction consists of two

sequential steps, initial pantoyladenylate formation, followed by subsequent nucleophilic attack on the activated carbonyl by β-alanine. The kinetic mechanism of *Mycobacterium tuberculosis* pantothenatesynthetase has been shown to be a BiUniUniBiPingPong mechanism.[46, 47] Pantothenatesynthetase is a member of the aminoacyl-tRNAsynthetase superfamily and the mechanism for formation of the pantoyladenylate is similar to that for formation of the acyl adenylate intermediate. Quite a lot of aminoacyl-tRNA synthetase inhibitors are known which mimic the aminoacyladenylate intermediate.[48-50]

### 5.4.2 Quinolinatephosphoribosyltransferase (QAPRTase)

Quinolinic acid phosphoribosyltransferase (QAPRTase) encoded by the *nadC* gene, is an important enzyme in *de novo* biosynthesis of nicotinic acid dinucleotide (NAD). The enzyme carries out the $Mg^{2+}$- dependent transfer of the phosphoribosyl moiety from 5-phosphoribosyl-1-pyrophosphate (PRPP) to quinolinic acid (QA) yielding nicotinic acid mononucleotide (NAMN), pyrophosphate and $CO_2$. In eukaryotes, QA is mostly formed by tryptophan degradation, while in prokaryotes it is produced from L-aspartate and dihydroxyacetone phosphate by products of the *nadB* (L-aspartate oxidase) and *nadA* (quinolinate synthase) genes.[51] In *Mtb*, the three genes encoding the enzymes involved in the *de novo* biosynthesis of NAMN are part of a single operon (*nadABC*).[52] In bacteria, the *nad* operon is transcriptionally regulated by a repressor encoded by the *nadR* gene in response to intracellular levels of nicotinamide mononucleotide (NMN).[53] Alternatively, NAMN can be produced by a salvage pathway that proceeds via the phosphoribosylation of nicotinic acid (NA), generated by the degradation of NAD; this reaction is catalyzed by the enzyme nicotinatephosphoribosyltransferase (NAPRTase).[54] In spite of the similarity between their enzymatic reactions, QAPRTase and NAPRTase show exclusive specificity for their respective substrates.[55, 56] In *Mtb*, unlike most

organisms, the salvage pathway appears to be interrupted. This is proposed to be a result of the lack of detectable NAPRTase activity and results in secretion of NA produced by degradation of NAD.[57] Relying entirely on the *de novo* pathway for its NAD requirements, *Mtb* should be extremely vulnerable to drugs targeted against QAPRTase.

### 5.4.3 Shikimate Kinase (SK)

De novo synthesis of essential amino acids and cofactors is necessary for the survival of mycobacteria, especially in starvation/stressful conditions. The shikimate pathway is the biosynthetic route that converts erythrose-4-phosphate to chorismic acid in seven steps. Chorismic acid is an essential intermediate for the synthesis of aromatic compounds, such as aromatic amino acids, p-aminobenzoic acid, folate and ubiquinone. The shikimate pathway is essential for algae, higher plants, bacteria, and fungi whereas it is absent from mammals.[57, 58] Shikimate kinase (SK) and other enzymes (AroB, AroC, AroE, AroG, AroK, AroK and AroQ), in the shikimate pathway are potential targets for developing non-toxic antimicrobial agents, herbicides, and anti-parasite drugs. Shikimate kinase (SK), the fifth enzyme in the shikimate biosynthetic pathway, from *M. tuberculosis*is obviously an excellent target for developing novel anti *M. tuberculosis* agents. The three-dimensional structure of MtSK will provide crucial information for elucidating the mechanism of SK-catalyzed reaction and structure-based drug design. Therefore, the inhibitors to target the enzymes in shikimate pathway are hypersensitive.

### 5.4.4 Thymidylate Kinase

Biosynthesis of nucleotides has recently been reported to be a worthy target particularly for TB in HIV cases. Very recently, thymidine monophosphate kinase

(TMK)[60] has been recommended as a validated target to develop new antitubercular agents, particularly for the treatment of MDR-TB and TB-HIVco-infected patients. This is an essential enzyme of nucleotide metabolism that catalyzes the reversible phosphorylation of thymidine monophosphate (dTMP) to thymidine diphosphate (dTDP). TMK from *M. tuberculosis* is a homodimer with 214 amino acids per monomer.[61] The x-ray three-dimensional structure has been newly solved at 1.95Å resolution [62, 63] as a complex with TMP, thus making it possible to start structure based drug design studies.[64]

## 5.5 Targets in DNA Biosynthesis and Metabolism

The anti TB drug *p*-aminosalicylic acid (PAS), initially designed as a competitive inhibitor of salicylic acid, has been reported to act on the tetrahydrofolate pathway as well as salicylate dependent biosynthesis of mycobactins, essential for iron transport. Efforts have been made to enrich the efficacy of sulphonamides in combination with other drugs (trimethoprim) which helps in inhibiting subsequent steps of tetrahydrofolate pathway, catalyzed by the enzyme dihydrofolate reductase. A comprehensive study of enzymes involved in tetrahydrofolate biosynthesis may lead to a rational design of new and novel anti TB drugs.[65, 66]

### 5.5.1 Ribonucleotide Reductases

Deoxyribonucleotides were biosynthesized from ribonucleotides by ribonucleotide reductase (RNR). RNRs are iron-dependant enzymes constructed from two large subunits (R1) and two small (R2) to form a heterodimeric bioactive structure. Although genes R1 and R2 are found to be essential for Mtb, the gene encoding R2 is generally believed to express the enzymatically active region.[67] Discovery of significant inhibitory activity observed for hydroxy urea

on RNR and crystallographic studies helped derive in-depth knowledge on the structure and function of MtbRNR and provides a new direction for design and discovery of newer inhibitors.[67-69]

### 5.5.2 DNA Ligase

This enzyme can link together two DNA strands that have double-strand break. DNA ligases are classified depending upon their cofactor specificity as either NAD+ or ATP-dependent.[70] In comparision with universal ATP-dependent ligases, NAD+-dependent ligases (LigA) are only found in certain viruses and bacteria, including Mtb. DNA ligase is an essential enzyme. The crystal structure of MtbLigA with bound AMP was recently reported and proved to be a potential novel target for anti-*M. tuberculosis* drug discovery. Several nucleoside analogues and other compounds are found to be effective LigA inhibitors.[71-73]

### 5.5.3 DNA Topoisomerase

Another promising target is DNA gyrase, a type II topoisomerase. DNA gyrase is involved in many critical steps involved in the DNA replication, including ATP-dependent negative supercoiling of closed circular double stranded DNA; ATP-independent or nucleotide dependent relaxation of negatively supercoiled DNA; formation and resolution of catenated DNA; resolution ofknotted DNA; quinoline or calcium ion induced double stranded breakage of DNA and DNA dependent ATP hydrolysis.[74] Many fluoroquinolone antibiotics act by inhibiting DNA gyrase. Recently gyrA and gyr B have been cloned from *M. tuberculosis* and *M. smegmatis*. The Topo-IV enzyme is responsible for resolution of daughter molecules after chromosomal replication. In some organisms, like *E. coli*, DNA gyrase is the key target, whereas in other organisms, particularly gram positive

cocci, DNA-topo IV will be a prime target.[75] Quinolone drugs such as ofloxacin and levofloxacin are in use, whereas Moxifloxacin and gatifloxacin, being developed currently for drug sensitive-TB.

### 5.5.4 Respiratory Chain Inhibitors

All bacteria require energy to remain viable. Even if the energy production pathways in *M. tuberculosis* are not well regarded as, but their importance as drug targets is demonstrated by the recent finding, that PZA (a frontline TB drug that is more active against non-growing persistent bacilli than growing bacilli and shortens TB therapy) acts by disrupting membrane potential and depleting energy in *M. tuberculosis*. The electron transport chain of Mtb needs an essential chemical menaquinone, especially during persistent stage. Menaquinone biosynthesis pathway is absent in humans. Menaquinone biosynthesis is extensively studied in *E. coli*. Among the enzymes MenA–F present in Mycobacterium, Men A, B, D and E are validated as potential targets for structure-based anti TB drug discovery. The type II NADH: menaquinoneoxidoreductase was also identified as a unique and interesting antimicrobial target.

A recently approved Anti-TB drug TMC207 (Bedaquiline), a diarylquinoline inhibits ATP synthesis by targeting F0 subunit of ATP synthase. Along with bactericidal characteristic against dormant (non-replicating) tubercle bacilli, it also got the potential to reduce the duration of treatment.

## 5.6 Miscellaneous Targets

### 5.6.1 Isocitratelyase (ICL)

Glyoxylate shunt pathway is a carbon assimilatory pathway that allows the net synthesis of C4 dicarboxylic acids from C2 compounds present in bacteria. The first step of glyoxylate shunt is catalyzed by ICL, an obligate enzyme for metabolism of fatty acids. This shunt bypasses the two decarboxylative steps of the Krebs cycle, allowing organisms to stay alive under nutrient-limiting conditions. The data shows that ICL is an important for survival of *M. tuberculosis* in the lung during the persistent phase of infection. The structure of MtbICL was thoroughly studied and precise inhibitory mechanism of the potent MtbICL inhibitors 3-nitropropionate and 3-bromopyruvate was elucidated. Combination therapy of existing TB drugs with an ICL inhibitor might be expected to expedite sterilization of infected lungs.

### 5.6.2 Mycobacterium Protein Tyrosine Phosphatase B (mPTPB)

The mPTPB is an essential virulence factor possessed by all mycobacterial species that cause TB in humans or animals. It is secreted into the cytosol of infected macrophages to target components of host signalling pathways, thus enabling bacterial survival. Moreover, deletion of the gene encoding mPTPB attenuated growth and virulence of Mtb in interferon-γ (IFN-γ)-stimulated macrophages and in guinea pigs. The greatest advantage of this target is its availability outside the mycobacterium and hence circumvents the permeability problem to cross the mycobacterial cell wall. Not surprisingly, there is increasing interest in targeting mPTPB for therapeutic development.[86, 87]

### 5.6.3 Carbonic Anhydrase

Carbonic anhydrase (CA) catalyses the interconversion between carbon dioxide and bicarbonate, with release of a proton. This enzyme is involved not only in pH homeostasis and regulation but also in biosynthetic reactions, such as gluconeogenesis and urea genesis etc. Biochemical studies revealed presence of two essential CA enzymes namely, mtCA1 (Rv1284) and mtCA2 (Rv3588c) inMtb. Significant structural differences observed between bacterial βCAs and Human αCAs offers valuable opportunity for design of selective MtbCA inhibitors.

### 5.6.4 Mycobacterial Thioredoxin Reductase (MtTrxR)

Thioredoxin reductase enzymes (TrxRs) are essential for Mtb. These enzymes are implicated in (i) the peroxiredoxin-mediated reduction of hydroperoxides and peroxynitrite considered to be pivotal for the pathogen's survival in macrophages and (ii) as in other species, the synthesis of deoxyribonucleotides is indispensable for DNA synthesis and thus, for proliferation. Hence, MtTrxR has become a most promising target for structure-based drug design. MtbTrxRs differ from their human counterparts significantly making structure based design of selective inhibitors possible.

### 5.6.5 Glutamine Synthetase (GS)

GS catalyses the conversion of glutamate to glutamine, ammonia to phosphate and ATP to ADP. MtbGS plays a key role in controlling the ammonia levels within infected host cells and so contributes to the pathogen's capacity to inhibit phagosome acidification and phagosome−lysosome fusion. In addition, MtbGS is thought to be involved in cell wall biosynthesis; It is found

extracellularly in huge quantities, and is related to a role in the production of the poly-L-glutamate−glutamine, which is a major component of the cell wall in pathogenic mycobacteria. Trisubstituted imidazoles were recently identified as potent MtbGS inhibitors.[98]

### 5.6.6 Cysteine Biosynthetic Pathway

CysK1 is a pyridoxalphosphate-dependent O-acetyl sulfhydrylase that catalyses the formation of L-cysteine through O-acetyl serine and hydrogen sulfide. The classical CysK1 dependent pathway to produce cysteine is completely absent in human.[99, 100] In Mtb this enzyme uses hydrogen sulfide derived from the APS−PAPS pathway as sulphur source for the biosynthesis of cysteine. Highly potent inhibitor activity for this enzyme was recently observed in a series of thiazolidine compounds.[101]

### 5.6.7 Acetohydroxyacid Synthase (AHAS)

Itcatalyzes first step in the biosynthesis of branched-chain amino acids (BCAAs). AHAS catalyzes the condensation of two molecules of pyruvate to form 2-acetolactate in the biosynthesis of valine and leucine or the condensation of pyruvate and 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate in the biosynthesis of isoleucine. Therefore, AHAS is an attractive target enzyme for development of herbicides and antimicrobial drugs. Recently, it has been shown that BCAAs in auxotrophic strains of *Mtb* are attenuated in mice because of the inability to use the BCAAs from their host. Herbicidal sulfonylurea AHAS inhibitors effectively inhibited the growth of several Mtbstrains.

## 5.7 Conclusions

*Mycobacterium tuberculosis* is one of the most resilient infectious agents known to man. With a virtually impervious lipid rich cell wall to its fore, this microbe could survive most of the chemical and biological threats for which other microbes would easily succumb. Complete elucidation of gene map for Mtb opened a plethora of opportunities to understand the biology of the microbe and also to differentiate its biochemical organization with other living beings. The availability of the Mtb gene map in the public domains has also catalysed the anti TB drug research. Latest developments in molecular biology techniques including genetic mutation studies immensely helped in target identification which has remained elusive for years. Biosynthetic pathway of mycolic acids, including transmembrane transporting mechanisms is highly essential for Mtb survival. Drugs acting on several key proteins including mmpL3 identified and were found to be less prone to development of resistance. But the challenge of eliminating the hibernating microbe from host and complete sterilization still appears to be a far reaching goal. Recent approval of the drug Delamanid, a nitroimidazo-oxazole derivative, in European region is a significant development.

Exploration of non-mycobacterial targets is also gaining importance as they are innately free from forcing the microbe to develop resistance. Accidental discovery of potential antitubercular activity in thioridazine (an antipsychotic drug) and one of its multiple mechanisms involving macrophase activation deserves further study. It is also enthusing to see “repurposing” non-antibiotics like verapamil and piperineas drug resistance reversal agents to facilitate current therapeutic regimens. With immense progress made in the understanding of biological organization and biochemistry of *Mycobacterium*, the multipronged attack on tuberculosis is more productive and optimistic now.

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