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Mycobacterial Metabolic Pathways as Drug Targets: A Review

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Abstract: Mycobacterium is acid fast genus of bacteria that include many pathogenic and non pathogenic species. Treatment is made quite difficult by the presence of metabolically silent, persistent or dormant bacteria within host lesions, which are not susceptible to the anti-mycobacteria drugs that usually kill growing bacteria but not persistent mycobacteria. The emergence of antibiotic resistance strains has raised the need towards the development of new antibiotics or drug molecules which can kill or suppress the growth of pathogenic Mycobacterium species. Novel efforts in developing drugs that target the intracellular metabolism of *M. tuberculosis* often focus on metabolic pathways that are specific to mycobacterium. Potential drug targets were also identified from pathways related to lipid metabolism, carbohydrate metabolism, amino acid metabolism, energy metabolism, vitamin and cofactor biosynthetic pathways and nucleotide metabolism. The objective this paper was to review those mycobacterium metabolic pathways as drug target and the problems of current TB drugs. The crucial problems of current TB therapy are development of multi-drug resistance and inefficiency of current TB drug to kill or inhibit non growing mycobacterium. The identification of drug target from that unique metabolism of mycobacterium is crucial to develop new drug for persistent and latent infection of tuberculosis. Despite an urgent need for new therapies targeting persistent bacteria, our knowledge of bacterial metabolism throughout the course of infection remains rudimentary. Therefore, better understanding on the physiology of mycobacterium during the latent period will help in the identification of new drug targets that can act on the persistent mycobacterium. Identification of these targets will to produce new drugs against tuberculosis that will overcome the limitations of existing drugs such as, prolonged chemotherapy, failure against persistent infection and multidrug resistance.

Key words: Anti-tuberculosis agent · Drug targets · Metabolic pathway · Mycobacterium

INTRODUCTION

The genus Mycobacterium comprises non pathogenic and pathogenic species that infect both humans and animals. The genus Mycobacterium encompasses 71 validly named species and 32 species are known to be pathogenic to humans or animals [1]. Tuberculosis is a mycobacterium infection that affects a wide range of mammals including humans. *Mycobacterium tuberculosis* is a tenacious and remarkably successful pathogen that has latently infected one third of the world population. Each year there are eight million of new tuberculosis (TB) cases and two million deaths [2].

M. bovis is the causative agent of bovine tuberculosis, a chronic and occasionally fatal infectious disease primarily infecting cattle and other livestock; but is capable of infecting a wide range of mammals and other vertebrates, including humans [3]. *M. bovis* is very closely related to *M. tuberculosis*, a virulent tubercle bacillus estimated to infect a third of the world's population and cause the deaths of 1.4 million people each year. In an attempt to prevent tuberculosis infections more than 3 billion individuals have been immunized with *M. bovis* BCG, a live attenuated derivative of *M. bovis* [4].

The increasing emergencies of drug resistance tuberculosis and immune compromising disease and allows latent infection to reactivate or render individual more susceptible to TB pose further challenges for effective control of the disease in human [5]. Ethiopia is one of the 27 high MDR-TB countries; it is ranked 15th with more than 5000 estimated MDR-TB patients each year. According to the WHO report, the prevalence of MDR-TB has been 2.8% in newly diagnosed patients; it

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is reportedly even higher in patients who have previously received anti-TB treatment 21% [6]. Published studies on MDR-TB are increasingly available worldwide, but accurate data on drug-resistant TB in Ethiopia is limited [7]. Some recent study shows that a quarter of TB patients were having persistent TB clinical signs after receiving there commended drugs and duration of therapy. Most of the respondents were living in the rural community and they might not report early to health institutes in Ethiopia [8].

Currently, TB chemotherapy is made up of a cocktail of first-line drugs; isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (EMB), given for six months [9, 10]. If the treatment fails as a result of bacterial drug resistance, or intolerance to one or more drugs, second-line drugs are used, such as para-aminosalicylate (PAS), kanamycin (KAN), fluoroquinolones (FQ), capreomycin (CAP), ethionamide (ETA) and cycloserine (CYS), that are generally either less effective or more toxic with serious side effects [9].

Agents with unclear roles in drug-resistant TB treatment are called third-line anti-TB drugs such as clofazimine, linezolid, amoxicillin/clavulanate, thioacetazone and imipenem/cilastatin and high-dose isoniazid. Treatment is made quite difficult by the presence of metabolically silent, persistent or dormant bacteria within host lesions, which are not susceptible to the anti-mycobacterial drugs that usually kill growing bacteria but not persistent bacteria [11]

Using metabolic pathway information as the starting point for the identification of potential targets has its advantages as each step in the pathway is validated as essential function for the survival of the bacterium [12]. It is widely accepted that TB is a dynamic disease that results from combination of phenotypically diverse population of bacilli in continually changing host environment. The release of complete genome sequence of *Mycobacterium* has facilitated the development of more rational and specific methods to search for new drug targets and vaccine candidates [13].

The recent rise in TB cases and especially the increase of drug resistant mycobacterium indicate an urgent need to develop new anti-TB drugs. The long duration of TB therapy is a consequence of persistent *M. tuberculosis*, not effectively killed by current anti-TB agents. Recent advances in the knowledge of the biology of the organism and the availability of the genome sequence give an opportunity to explore a wide range of novel targets for drug design. Metabolic studies on mycobacterium have been important areas of the investigation to identify that metabolic pathway as drug target to design more effective [14]. The objective of this paper was to review on mycobacterium metabolic pathways as drug targets and problems of current TB drug.

Metabolic Pathways Used as Drug Targets: Mycobacterium metabolic pathways which do not appear in the host but present in the pathogen are identified as pathways unique to mycobacterium as compared to the host. Enzymes in these unique pathways as well as enzymes involved in other metabolic pathways under carbohydrate metabolism, amino acid metabolism, lipid metabolism, energy metabolism, vitamin and cofactor biosynthesis and nucleotide metabolism are important to identify novel drug targets [15]. An important question to be addressed while choosing potential drug targets is whether the biochemical pathway to be targeted is unique to bacteria. These biochemical pathways which are; Peptidoglycan biosynthesis, Mycobactin biosynthesis, d-alanine metabolism, thiamine metabolism and polyketide sugar unit biosynthesis, all absent in the host and therefore unique to the pathogen [16]. Design and targeting inhibitors against these nonhomologous sequences could be the better approach for generation of new drugs. Thus total five unique metabolic pathways have been taken in *M. tuberculosis* [17].

Mycobactin Biosynthesis: One of the key host defense mechanism is the production of siderocalins that sequester iron-laden siderophore and Mycobacterium replicates poorly in the absence of these siderophores [18]. To overcome iron deficiency imposed by the host defensive system, bacteria have evolved iron acquisition systems where small molecules called siderophores, which bind extracellular iron, are secreted. These get reabsorbed along with the bound iron through specific cell surface receptors. The Mtb siderophore pathway is well studied and consists of secreted siderophore termed Mycobactin [19]. The importance of Mtb iron uptake was highlighted by evidence that the mode of action of one of the earliest anti-TB drugs, p-amino salicylic acid, was inhibition of siderophores biosynthesis [20].

Pathogenic *Mycobacterium* species produces the mycobactin class of siderophore, which contains a salicylic acid derived moiety. Therefore, these siderophores used for mycobactin biosynthesis, d-alanine metabolism and Peptidoglycan biosynthetic pathways [18]. Mycobactin G (Mycobactin lysine-*N*6-hydroxylase), which catalyzes the hydroxylation of lysine moiety in mycobactin synthesis, is the potential target in this pathway. It has been shown that there is no possibility of

bacterial survival on more than a few generations if it is deprived of iron. So to acquire iron from host, it relies on a siderophores mediated pathway [21].

Disruption of mycobactin biosynthetic pathway may affect the survival of the bacterium under these conditions of iron limitation. It has been shown that siderophore production is also important for the virulence of *M. tuberculosis*. But some very recent research showed that even though siderophore are unique they are not the only machinery employed by the mycobacterium to acquire iron from vicinity. Besides non-heme iron uptake, mycobacteria also have a heme iron uptake pathway. Initial evidence for this pathway was based on the observation that a recombinant bacillus calmette-guerin harboring a defective siderophore biosynthetic pathway, replicated slowly in mice, suggesting it acquires heme iron [22]. The mycobacterium can utilize heme as an Iron source [23].

An *Mycobacterium tuberculosis* heme-uptake system has been defined that consists of the secreted protein known as hypothetical protein (Rv0203) and the trans membrane proteins called possible membrane transport protein (MmpL3 and MmpL11) [24]. Some recent experiments showed that hypothetical protein transfers heme to both MmpL3 and MmpL11 during *Mycobacterium tuberculosis* heme uptake making these proteins potential targets for TB drugs [25].

Peptidoglycan Biosynthesis: The cell envelope of mycobacterium is made up of three major components: plasma membrane, cell wall (MAPc) and polysaccharide rich capsule like materials. The cell wall of mycobacterium is made up of cross-linked peptidoglycan which is covalently linked to Arabinogalactan chain via polyketide linkage unit. Arabinogalactan in turn is esterified to variety of alpha-alkyl, beta-hydroxyl mycolic acid. The unique nature of the MAPc leads to the conclusion that enzyme that synthesizes these structures yield many number potential new drug targets [26]

Mycobacterium is surrounded by a lipid-rich outer capsule that protects it from the toxic radicals and hydrolytic enzymes produced as defense by macrophages [22]. The peptidoglycan layer of the cell wall serves as a base for the lipid-rich capsule. Peptidoglycan is the polymeric mesh of the bacterial cell wall, which plays a critical role in protecting the bacteria against osmotic lysis. The currently used anti-mycobacterial drugs are isoniazid (INH) and ethambutol (EMB) were target cell wall biosynthesis. Isoniazid is known to inhibit mycolic acid synthesis [26], where as ethambutol inhibits the polymerization step of arabinan biosynthesis of Arabinogalactan. Arabinosyl transferase, encoded by embB, an enzyme involved in synthesis of Arabinogalactan, has been proposed as the target of ethambutol [27]

The primary target of inhibition is the cell wall mycolic acid synthesis pathway, where enoyl ACP (acyl carrier protein) reductase (InhA) was identified as the target of INH inhibition. The active species for InhA inhibition has been found to be isonicotinic acyl radical, which reacts with NAD to form INH-NAD adduct and then inhibits the InhA enzyme. The reactive species produced during INH activation could also cause damage to DNA, carbohydrates and lipids and inhibit NAD metabolism. INH is a prodrug that requires activation by *M. tuberculosis* catalase-peroxidase (KatG) to generate a range of reactive oxygen species and reactive organic radicals, which then attack multiple targets in the tubercle bacillus. Mutations in KatG involved in INH activation in the INH target InhA and Ndh II (NADH dehydrogenase II) could all cause INH resistance. KatG mutation is the major mechanism of INH resistance [26].

D-Alanine Metabolism: D-alanine is a necessary precursor in the bacterial peptidoglycan biosynthetic pathway. The naturally occurring L-isomer is racemized to its D-form through the action of a class of enzymes called alanine racemases. These enzymes are ubiquitous among prokaryotes and are absent in eukaryotes which makes them a logical target for the development of antibiotics. The d-alanine–d-alanine ligase (ddlA) and alanine racemase (alr) from this pathway have no similarity to any of the host proteins. Alanine racemase has been identified as a target as all the bacteria investigated contained either one or two alanine racemase genes [28].

However, in mycobacterium, there is a single alanine racemase gene. These two enzymes catalyze the first and second committed steps in bacterial peptidoglycan biosynthesis. Alanine racemase is a pyridoxal 50-phosphate-containing enzyme that catalyzes the racemization of L-alanine into D-alanine, a major component in the biosynthesis of Peptidoglycan [29]. One alanine racemase inhibitor, the structural d-alanine analogue d-cycloserine has been marketed clinically. Both alanine racemase and D-Ala-D-Ala ligase are targets of D-cycloserine, a second-line anti -TB drug. Although, this is supposed to be an excellent inhibitor of mycobacterium and other pathogenic species bacteria, serious side effects especially CNS toxicity has limited its use [30].

Polyketide Sugar Unit Biosynthesis: AG and PG chains are tethered via a linker region known as Phosphoryl-N-acetyl-glucosaminosyl-rhamnosyl [31]. Arabinogalactan a heteropolysacharide is connected via a linker disaccharide called polyketide (a-L-Rha--a-D-Glc-NAc-1 -phosphate) to the sixth position of a muramic acid residue in the peptidoglycan. The reaction is catalyzed: by the enzyme rhamnosyl transferase [32]. Rhamnose residue and large portion of arabinogalactan polysaccharide are synthesized on GIcNAc-P-P-decaprenyl carrier lipid [27].

The eventual transfer of the arabinogalactan-Rha-GlcNac-phosphate unit to the O-sixth of a muramic acid places the polysaccharide in mass on to the peptidoglycan. The biosynthesis of arabinogalactan in *M. tuberculosis* begins with the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetyl glucosamine to prenylphosphate followed by an addition of rhamnose (Rha) from dTDP-Rha, forming a linker region of the arabinogalactan [33]. L-rhamnose transferase (WbbL) is an enzyme that utilizes dTDP-Rha as a substrate for the formation of final product L-rhamnose which plays a crucial role in the linkage of cell wall. The biosynthesis of dTDP-rhamnose is catalysed by four enzymes coded by the genes; RmlA (Rv0334), RmlB (Rv03464), RmlC (Rv3465) and RmlD (Rv3266) and ultimately synthesizes dTDP rhamnose from glucose-1-phosphate. Among these genes RmlC has no human homologue. RmlC codes for dTDPd-glucose-3, 5-epimerase which is involved in the arabinogalactan biosynthesis [32].

Targets from Other Pathways: Even amongst the pathways shared by the host and the pathogen, there are several proteins from pathways involved in lipid metabolism, carbohydrate metabolism, amino acid metabolism, energy metabolism, vitamin and cofactor biosynthetic pathways and nucleotide metabolism which do not bear similarity to host proteins. While some of them are known to be associated with virulence or important for persistence or vital for mycobacterial metabolism, others should further be investigated for their potential to be drug targets [34].

There are many enzymes from the glyoxylate by pass, which is important for mycobacterial persistence. Among these enzymes the most important are Isocitrate Lyase and Malate Dehydrogenase. It has been proposed by Waynes and Lin [35] that the enzymes of the glyoxylate cycle are activated during adaptation to the low oxygen environment of the granuloma. The glyoxylate by pass allows the bacterium to synthesize carbohydrates from fatty acids. Succinate and glyoxylate produced by this cycle are supplied to the TCA cycle and gluconeogenesis. Disrupting this pathway by targeting these enzymes has a potential in the treatment of latent tuberculosis infections.

Strikingly, a functional glyoxylate cycle appears to be required for the intracellular survival (persistence) of Mycobacterium tuberculosis. Despite past claims for enzymatic activity in vertebrates and the human genome have no apparent genes for key glyoxylate cycle enzymes. The absence of these enzymes provides potential targets for drugs directed against bacterial and fungal pathogens or parasites [36].

Identification of Unique Pathways and Potential Drug Target: So far, more than 100 bacterial genomes have been sequenced. As bacterial genome sequences become available, there is increasing interest in developing new antibacterial agents using genomics-based approaches [14]. No new anti-tuberculosis drugs have been developed for well over 20 years. In view of the increasing development of resistance to the current leading anti-tuberculosis drugs, novel strategies are desperately needed to avert the “global catastrophe” forecast by the WHO. The first bacterial genome was sequenced by Fleischmann and colleagues at the Institute for Genomic Research (IGR) in 1995 [37].

The recent developments in microarray technology, signature tag mutagenesis, mycobacterial transposon mutagenesis and gene knock-out technology provide important tools to identify new drug targets. Microarray has been used to identify *M. tuberculosis* genes that are induced by INH and ETH and by INH [39]. Microarray was also used to identify genes that are switched on in the Wayne “dormancy” model under hypoxic and nitric oxide stress conditions a discovery that led to the identification of a 48-gene “dormancy regulon” controlled by DosR [40].

Identification of Essential Genes: Essential genes are those indispensable for the survival of an organism and their functions are considered as foundation of life. Total 55 enzymes out of all were found to be essential for *M. tuberculosis* life cycle. These targets were found to be potential targets and could be considered for rational drug design. Using metabolic pathway information as the starting point for the identification of potential targets has its advantages as each step in the pathway is validated as the essential function for the survival of the bacterium [41].

Identification of Drug Target's Functions Using Uniprot (Universal Protein Resource): The sub cellular localization analysis of all supposed essential and unique enzymes of *M. tuberculosis* were evaluated by UniProt server. As it was suggested that, membrane associated protein could be the better target for developing vaccines. After functional analysis unique enzymes involved in cellular components like cell wall, cytoplasm, extra cellular region, plasma membrane and so forth, their biological processes and their functions have been retrieved. Further, the functional analysis using Uniprot showed involvement of all the unique enzymes in the different cellular components [17].

The complete genome sequence of *M. tuberculosis* provides an opportunity for a more focused and planned approach towards the identification of new drug targets [13]. The availability of the *M. tuberculosis* genome sequence opens up a new opportunity to understand the biology of the organism and provides a range of potential drug targets [12].

Possible Drug Targets: Desirable targets should be involved in vital aspects of bacterial growth, metabolism and viability, whose inactivation will lead to bacterial death or inability to persist [42]. In recent years, a number of new genes and their products in *M. tuberculosis* have been identified, which can be possible drug targets for mycobacterium. The gene products that control vital aspects of mycobacterial physiology like, metabolism, persistence, virulence, two component system and cell wall synthesis would be attractive targets for new drugs. A large number of genes are being studied in the search for new drug targets using various approaches [13].

Recent developments in mycobacterial molecular genetics tools such as transposon mutagenesis, signature-tagged mutagenesis, gene knockout and gene transfer will facilitate the identification and validation of new drug targets essential for the survival and persistence of tubercle bacilli not only *in vitro* but also *in vivo*. Below is a list of potential targets where by new drugs may be developed for improved treatment of TB [14].

Because of the drug-resistant TB problem, it is important to develop new drugs that inhibit novel targets that are different from those of currently used drugs. To avoid significant toxicity, the targets of inhibition should be present in bacteria but not in the human host. Although modification of existing drugs for improved half-life, bioavailability, or drug delivery may be of some use, agents obtained by this approach may have a cross-resistance problem. Similarly, targeting existing TB drug targets for drug development may be limited value because of potential cross-resistance [13]

Targeting Mycobacterial Persistence: Mycobacterial persistence refers to the ability of tubercle bacillus to survive in the face of chemotherapy and/or immunity [43]. The nature of the persistent bacteria is unclear but might consist of stationary phase bacteria, post-chemotherapy residual survivors or dormant bacteria that do not form colonies upon plating [11]. The presence of such persistent bacteria is considered to be the major reason for lengthy therapy. A lot of research activity is currently aimed at understanding the biology of persistence of the tubercle bacillus and developing new drugs that target the persistent bacteria [44].

The glyoxylate cycle was described by Kornberg and Madsen as a "modified tricarboxylic acid (TCA) cycle", with which it shares malate dehydrogenase, citrate synthase and aconitase activities. However, instead of the two decarboxylation steps of the TCA cycle the key enzymes of the glyoxylate cycle, namely isocitrate lyase and malate synthase, convert isocitrate and acetyl-CoA into succinate and malate. Isocitrate lyase splits the C_6-unit into succinate and glyoxylate, which in turn is condensed by malate synthase with acetyl-CoA generating free CoA-SH and malate. The latter is used by malate dehydrogenase to continue the cycle and succinate is released as net product. The intermediate glyoxylate provides the name for this metabolic pathway, which allows cells to convert two acetyl-CoA units generated by various catabolic processes into C_4-units (succinate) which can be used to replenish the TCA cycle or to function as precursors for amino acid biosynthesis carbohydrate biosynthesis. Thus, the glyoxylate cycle serves as a link between catabolic activities and biosynthetic capacities and enables cells to utilize fatty acids or C_2-units such as ethanol or acetate as sole carbon source [36]. ICL catalyzes the conversion of isocitrate to glyoxylate and succinate and is an essential enzyme for fatty acid metabolism in the glyoxylate shunt pathway [45]. Survival of *M. tuberculosis* in the adverse in vivo environment requires utilization of C2 substrates generated by *â*-oxidation of fatty acids as the carbon source [46]. ICL was induced in the Wayne "dormancy" model, inside macrophages and in the lesions of the human lung. ICL is not essential for the viability of tubercle bacilli in normal culture or in hypoxic conditions, but it is needed for long-term persistence in mice. The phenotype of the isocitrate lyase mutant a pronounced

defect in long-term persistence, suggests that a drug targeting the enzymes of the glyoxylate cycle might be useful in treating the latent infection that affecting perhaps one-third of the planet as well [47].

Using a transposon mutagenesis approach based on changes in colony morphology, a gene called *pcaA* encoding a novel methyl transferase involved in the modification of mycolic acids in mycobacterial cell wall was identified [14]. Although the PcaA knockout mutant grew normally in vitro and replicated in mice initially like the parent strain, the mutant was defective in persisting in mice and could be a target for drug design against persistent bacilli [48].

PcaA is required for cording and mycolic acid cyclopropane ring synthesis in the cell wall of both BCG and MTB. The site-specific cyclopropane modification of mycolic acids could be an important determinant of the interaction between M. tuberculosis and the host. Since this modification of mycolic acids is absent in non-pathogenic mycobacteria, the phenotypes of the ΔpcaA strain suggest that the cyclopropyl modification system evolved to mediate principal virulence functions such as interaction with host innate immune receptors recognition [49] also have a profound effect on the function of these lipids as important virulence factors of the bacteria.

RelA (ppGpp synthase) is critical for the successful establishment of persistent infection in mice by altering the expression of antigenic and enzymatic factors that may contribute to successful latent infection. A recent microarray study has found that DosR controls the expression of a 48-gene “dormancy regulon, ” which is induced under hypoxic conditions and by nitric oxide [50]. DosR is a transcription factor of the two component response regulator class and the primary mediator of a hypoxic signal within MTB, used to control a 48-gene regulon involved in MTB survival under hypoxic conditions have been identified and could be good targets for development of drugs that target persistent bacilli [51].

Rv2421c transfers phosphorous groups in nicotinate/nicotinamide salvage and de novo synthesis to convert necotinamide to nicotinic acid. Rv2043c of this pathway is the target of the highly effective drug PZA that kills persistent bacilli in the initial phase of TB therapy. Mutations in the encoding gene pncA confer resistance to PZA. Successful inhibition of Rv2421c could thus help to eradicate slowly growing persistent bacilli in TB infection [52].

Genes encoding, NadD and NadE enzymes, conserved among bacterial species and proven to be essential in M. *Tuberculosis* are attractive and actively pursued drug targets [53]. The disruption of NAD production in the cell via genetic suppression of the essential enzymes (NadD and NadE) involved in the last two steps of NAD biogenesis would lead to cell death, even under dormancy conditions results suggest that targeting NAD biosynthesis could lead to production of highly effective bactericidal antituberculosis compounds [54].

Targeting Essential Genes: Essential genes are genes whose inactivation leads to non-viability or death of the bacteria. Transposon mutagenesis and signature-tagged mutagenesis have been used to identify genes essential for M. tuberculosis growth in vitro and survival in vivo [41]. Transposon mutagenesis is a biological process that allows genes to be transferred to a host organism's chromosome, interrupting or modifying the function of an extant gene on the chromosome and causing mutation. Signature tagged mutagenesis is a genetic approach that was developed to identify novel bacterial virulence factors. In a recent study, 614 genes, about one-sixth of the total number of genes in *M. tuberculosis*, were found to be essential for *in vitro* growth, whereas 194 genes were demonstrated to be essential for *in vivo* survival in mice [55].

The genes that are essential for survival *in vitro* and *in vivo* are grouped into the following categories: lipid metabolism; carbohydrate and amino acid transport and metabolism; inorganic ion transport and metabolism; nucleotide transport and metabolism; energy production and conversion; secretion; cell envelope biogenesis; cell division; DNA replication; recombination and repair; transcription and translation; post-translational modification; chaperones; coenzyme metabolism; and signal transduction [41].

However, the function of a significant number of essential genes is unknown. Targeted knockout of specific genes is also a valuable approach to identifying essential genes, in other words, those whose disruption leads to non-viability of the bacilli. These essential mycobacterial genes should be good targets for TB drug development [56].

Targeting Energy Production Pathway: All bacteria require energy to remain viable. Although the energy production pathways in *M. tuberculosis* are not well characterized, their importance as drug targets is demonstrated by the recent finding that PZA acts by disrupting membrane potential and depleting energy in

M. tuberculosis. PZA is a frontline TB drug that is more active against non-growing persistent bacilli than growing bacilli and shortens TB therapy. That energy production or maintenance is important for the viability of persistent non-growing tubercle bacilli *in vivo* [51].

The recent discovery of the highly effective TB drug diarylquinoline also highlights the importance of energy production pathways for mycobacteria. The target for diarylquinoline was proposed to be the mycobacterial F1F0 proton ATP synthase, which is a new drug target in mycobacteria. It is likely that energy production pathways, such as the electron transport chain, glycolytic pathways and fermentation pathways, could be good targets for TB drug development [56].

Isocitrate lyase and malate dehydrogenase are an important enzyme in this category and also an important drug target. ICL is involved in energy production via the metabolism of acetyl-CoA and propionial CoA of the glyoxilate pathway. Inactivation of the *icl* gene leads to attenuation of both persistent and virulent strains of *M. tuberculosis* [45].

Five candidates, Rv2984, Rv2194, Rv1311, Rv1305 and Rv2195, map to the oxidative phosphorylation pathway. The target Rv1854c (gene ndh) in this pathway is the target for INH and several mutations in this gene account for INH resistant cases. Inhibiting any of the five proposed targets could disrupt the pathway and eliminate M. tuberculosis by reducing its limited ATP availability during dormancy [52]

Targeting Virulence Factors: Despite intensive research efforts, there is little information about the molecular basis of mycobacterial virulence. Undoubtedly, one requisite to classify a gene as a virulence factor is that its absence attenuates the virulence of the microorganism in an in vivo model [48]. A number of genes have been identified, using different techniques like allelic exchange, signature tagged mutagenesis and anti-sense RNA, that show a role in the virulence of *M. Tuberculosis*. Some of these genes include Cell Envelope Protein erp (Rv3810) .Exported repetitive protein (erp) which has been shown to be essential for the multiplication of mycobacteria during the acute phase of infection in the mouse model [49].

Recently, two gene clusters were identified and shown to be important for the growth of mycobacteria in the lungs during the early phase of infection. This gene cluster is involved in the synthesis (fadD28) and export (mmpL7) of a complex cell wall associated lipid, phthiocerol dimycocerosate [57]. Among the secreted proteins of *M. tuberculosis* which could act as virulence factors are a series of phospholipases C, lipases and esterase which might attack cellular or vacuolar membranes as well as several proteases [35]. Notable amongst these are phospholipases plcA, plcB, plcC and serine esterase [33]

But, inhibition of virulence factors may not be lethal because they may not essential to the pathogen. The other very important hurdle in this approach is that drugs that target virulence factors may be of very little use if the disease has already been established. However, inhibitors of these virulence gene products may be used in combination with existing drugs to improve the regime of chemotherapy [58].

Targeting Two-Component Systems: Mycobacterial disease is characterized by the lack of involvement of classical virulence factors; rather a dynamic balance between host and pathogen defines the outcome of an infection. Therefore those mycobacterial genes that confer an advantage to the organism in this ongoing battle would qualify as virulence factors. Infection of macrophages constitutes an early stage in the host pathogen encounter. Obvious candidates among mycobacterium genes that can mastermind the intracellular survival and multiplication within macrophages as also the shutdowns of mycobacterium during persistence are signal transduction systems, in particular TCS. Therefore in vitro infection models have been used extensively to delineate the role of TCS during the stage of pathogen macrophage interaction. Animal models have also been used to study the effect of defined mutations in TCS on growth and virulence of the mycobacterial strains [1].

Two-component systems (TCS) are vital components of signal transduction systems in a number of organisms. It consists of a sensor kinase that senses external signals and transmits the signals to the response regulator. The response regulator interacts with transcription factors which in turn will switch on/off a number of genes [59]. The mycobacterial genome encodes several two-component systems, which consist of histidine kinases and their associated response regulators. These control the expression of target genes in response to stimuli that are involved in chemotaxis, phototaxis, osmosis, nitrogen fixation and intracellular survival [60]. The magnesium transporter (MtrA) and histidine kinases (SenX3) that are essential for mycobacterial virulence and persistence in mice, could also be good targets for the development of new drugs for persistent TB bacteria [61].

It has been shown that the inactivation of mtrA component of mtrA-mtrB complex of *M. tuberculosis* H37Rv was possible only in the presence of a functional copy of mtrA, suggesting that this response regulator is essential for the viability of *M. tuberculosis* [62].

Interestingly, another two-component system, devR-devS, was found to be over expressed in a virulent strain, H37Rv [63]. Disruption of the phoP component of the PhoP/PhoR in *M. tuberculosis* resulted in a mutant strain with impaired multiplication in the host. This mutant was also found to be attenuated *in vivo* in a mouse model, suggesting that PhoP is required for intracellular growth of *M. tuberculosis*. These observations collectively suggest that TCS in *M. tuberculosis* could be important drug targets [64].

Targeting Cell Wall Synthesis: The *mycobacteria* cell wall is a complex structure that is required for cell growth, resistance to antibiotics and virulence [65]. The cell wall acts as an exceptional permeable barrier and it requires robust biosynthetic machinery for its formation. Targeting the enzymes of cell wall biosynthetic pathway is a very reasonable strategy because the homologs of these enzymes are absent in mammalian system [66].

It is composed of three distinct macromolecules; peptidoglycan, arabinogalactan and mycolic acids which are surrounded by a non-covalently linked outer capsule of proteins and polysaccharides [67]. The outermost, the mycolic acids are thought to be significant determinant of virulence in Mtb because they prevent attack of *mycobacteria* by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule [27]. The mycolic acids are esterified to the middle component, arabinogalactan, a polymer composed primarily of D -galactofuranosyl and D-arabinofuranosyl residues. AG is connected via a linker disaccharide, a-L-rhamnosyl-a-D-acetyl-glucosaminosyl-1 -phosphate, to the sixth position of a muramic acid residue of peptidoglycan [32], which is the innermost of the three cell wall core macromolecules.

This covalently linked structure is intercalated with numerous glycolipids such as lipoarabinomannan (LAM), the phosphatidyl inositol containing mannosides (PIMs), trehalose dimycolate (cord factor), trehalose-monomycolate (TMM), which play an important role in virulence of Mtb. Lipids such as cord factor have been suggested to play an important role in the virulence of *M. tuberculosis* by inducing cytokine mediated events. LAM is also a major constituent of the mycobacteria cell wall and has been shown to induce TNF release from the macrophages which plays a significant role in bacterial killing [13].

The cell wall is the most common target of antituberculosis drugs and many compounds that are in clinical use or under development target enzymes that synthesize distinct layers of the cell wall [68].

The first committed step in the synthesis of decaprenyl phosphoryl-D-arabinose, the lipid donor of mycobacterial D-arabinofuranosyl residues during AG biosynthesis is the transfer of a 5-phosphoribosyl residue from phosphoribose diphosphate to decaprenyl phosphate to form decaprenylphosphoryl–5-phosphoribose.This step is catalyzed by a ribosyltransferase that has recently been characterized and shown to be essential for growth [69]. Other enzymes essential for arabinogalactan biosynthesis have been identified, including UDP-galactopyranose mutase (glf gene), galactofuranosyl transferase and dTDP-6-deoxy-Llyxo- 4-hexulose reductase, the enzyme that catalyzes the final step in the formation of dTDP-rhamnose. DTDP rhamnose is a product of four enzymes, RmlA–D and a recent report has demonstrated that both RmlB and RmlC are essential for mycobacteria growth [70].

Because of the reasons cited above, genes involved in cell wall synthesis of mycobacteria have been exploited as targets for many anti-mycobacterial drugs. Several important TB drugs such as INH, ETA and EMB target mycobacterial cell wall synthesis. Current anti-TB drugs also include inhibitors of mycolic acid (isoniazid and ethambutol), arabinogalactan (ethambutol) and peptidoglycan (cycloserine). Enzymes involved in this pathway have always been preferred targets in drug development efforts [71].

Recently, a number of new drug candidates that target M. *tuberculosis* cell wall have been identified and they are in Phase II clinical trials and in preclinical phase of development [72]. Thiolactomycin (TLM) targets two â-ketoacyl-acyl-carrier protein synthases, KasA and KasB enzymes that belong to the fatty acid synthase type II system involved in the fatty acid and mycolic acid biosynthesis [13]. TLM has also been shown to be active against MDR-TB clinical isolate. Cerulenin, an inhibitor of fatty acid synthesis, has also been shown to inhibit mycobacterial lipid synthesis and is active against *M. tuberculosis in vitro* with an MIC of 1.5-12.5 mg/ml [61].

Octane sulphonyl acetamide (OSA) has recently been identified as an inhibitor of fatty acid and mycolic acid biosynthesis in mycobacteria. The inhibitor was found to be active against both slow growers such as *M. tuberculosis* and also MDR-TB strains with a MIC of about 6.25-12.5mg/ml. These reports clearly suggest that several genes of the cell wall synthesis pathway and

enzymes involved in fatty acid and mycolic acid synthesis could be good candidates for further drug development [73].

Genes of Other Metabolic Pathways: Genes of some other metabolic pathways can also serve as possible targets for developing drugs against tuberculosis. Some of these genes include, mgtc, which codes for a putative Mg^{+2} transporter protein. This protein has been shown to be essential for the survival of mycobacteria both in macrophages and mice. The ?-mgtc mutant showed *in vitro* growth defects. Similarly ?-mbtB mutant deficient in synthesis of siderophores was unable to replicate within the macrophages. Failure of mycobacteria to survive in the absence of specific iron uptake system suggests the scarcity of this important nutrient in phagosomal environment [74].

The target Rv1712 is central to the phosphorylation of ATP to nucleoside diphosphate in the pyrimidine pathway [74]. The known TB drug target Rv0667 forms part of the purine and pyrimidine pathway and mutations in its gene rpoB lead to RIF's resistance. With Rv1712 sharing this pathway it could be an attractive alternative target to inhibit this pathway [49].

Anti-Tuberculosis Drugs in Current Clinical Practice: Chemotherapy regimens that are used for the treatment of all types of TB are classified as first- and second-line anti-TB drugs1. First-line anti-TB drugs include isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STM). INH and RMP are the two most commonly used drugs for treatment of TB. First-line anti-TB drugs are safe and effective if used correctly [6]. The effective treatment of MDR-TB is critical to reducing the spread of drug-resistant TB in the community. Currently, TB care and treatment has become more complicated due to the emergence of M/XDR-TB and Latent infection. Second-line drugs that are used for the treatment of MDR-TB are listed as aminoglycosides (kanamycin); e.g., amikacin (Am) and Kanamycin (Km); polypeptides: capreomycin (Cm), fluoroquinolones; e.g. ciprofloxacin; thioamides: e.g., ethionamide (Eto), cycloserine (Cs) and P-aminosalicylic acid (PAS). Second-line anti-TB drugs are less potent, need to be administered for a much longer time, are more toxic and are high-cost compared to first-line anti-TB drugs [7].

Status of Current Tuberculosis Drug Therapy: The current live vaccine Bacillus Calmette Guerin (BCG) attenuated strain of M *bovis* was introduced in 1922. It does not protect all age groups as its efficacy is globally variable and it does not provide protection in most parts of the world where TB is effectively prevalent. It is not suitable to use for immune compromised patients. In addition to this, BCG only reduces dissemination of Mtb to the spleen and other organs, but it does not prevent mycobacterial growth in the lungs [7, 10].

Current TB therapy, also known as DOTS (directly observed treatment, short-course) consists of an initial phase of treatment with 4 drugs, INH, RIF, PZA and EMB, for 2 months daily, followed by treatment with INH and RIF for another 4 months, three times a week [3]. The targets of these drugs are varied. INH inhibits synthesis of mycolic acid, a cell well component (PZA targets cell membrane where as rifampin and streptomycin interferes with the initiation and streptomycin interferes with the initiation of RNA and protein synthesis respectively. EMB blocks biosynthesis of arabinogalactan, a major polysaccharide present in the mycobacterial cell wall and kanamycin and capreomycin, like streptomycin, inhibit protein synthesis through modification of ribosomal structures at the 16S rRNA [70]. Cycloserine prevents the synthesis of peptidoglycan, a constituent of cell wall [13].

Limitation of Current Tuberculosis Therapy: Recent reports indicate that, areas where there is a high incidence of MDR-TB, DOTS is failing to control the disease. In such circumstances, the second line drugs are prescribed in combination with DOTS. However, this combination of drugs is very expensive, has to be administered for a longer duration and has significant side effects. One major drawback of current TB therapy is that the drugs are administered for at least 6 months [71].

The length of therapy makes patient compliance difficult and such patients become potent source of drug-resistant strains. The second major and serious problem of current therapy is that most of the TB drugs available today are ineffective against persistent bacilli, except for RIF and PZA. RIF is active against both actively growing and slow metabolizing non-growing bacilli, whereas PZA is active against semi-dormant non-growing bacilli. However, there are still persistent bacterial populations that are not killed by any of the available TB drugs. Therefore, there is a need to design new drugs that are more active against slowly growing or non-growing persistent bacilli to treat the population at risk [14].

Table 1: Current tuberculosis drugs and their targets

Drugs	MIC (g/ml)	Mechanism of action	Targets	Gene involved in resistance
Isoniazid	0.01-0.20	Inhibition of cell wall (Mycoli acid synthesis)	Enoyl acylcarrier protein, Reductase (InhA)	KatG inhA
Rifampin	0.05-0.50	Inhibition of RNA synthesis	RNA polymerase beta subunit	rpoB
Pyrazinamide	20-100	Depletion of membrane energy	Membrane energy metabolism	pncA
Ethambutol	1-5	Inhibion of cell wall(arabinogalactan synthesis)	Arabinsyltransferase	embCAB
Streptomycin	2-8	Inhibition of protein synthesis	Ribosomal S12 protein & 16s rRNA	rpcl, rrs
Kanamycin	1-8	inhibition of protein synthesis	16s rRNA	Rrs
Capreomycin	4	Inhibition of protein synthesis	16s rRNA, 50s ribosome, rRNA methyltransferase (TlyA	rrs, tlyA
Fluoroquinolones	0.2–4.0	Inhibition of DNA synthesis	DNA gyrase	*gyrA*, *gyrB*
Ethionamide	0.6–2.5	Inhibition of mycolic acid synthesis	Acyl carrier protein reductase (InhA)	*inhA*, *etaA*/*ethA*
PAS	1–8	Inhibition of folate pathway andmycobactin synthesis?	thymidylate synthase (ThyA)?	*thyA*c

KatG, PncA, EtaA/EthA are enzymes involved in the activation of the prodrugs isoniazid, pyrazinamide and ethionamide, respectively.

Source: [75], [76] and Reviewed by [77]

CONCLUSIONS AND RECOMMENDATIONS

Tuberculosis is still a leading infectious disease worldwide. Along with the socio-economic and host factors that underlie this problem, a fundamental problem that hinders more effective TB control is the tenacious ability of *Mycobacterium* bacteria *to* persist in the host and to develop drug resistance, often as a consequence of poor compliance to lengthy therapy. Major obstacle in the cure and prevention of tuberculosis is posed by the latent or persistent *mycobacterium* infection. This is due to the fact that most of the currently available drugs are ineffective against latent infection. A better understanding on the physiology of mycobacteria during the latent period will help in the identification of new drug targets that can act on the persistent mycobacteria. The list of potential drug targets encoded in the genome of *M. tuberculosis* include genes involved in persistence or latency, cell wall synthesis, virulence, signal transduction, genes encoding transcription factors and enzymes of other metabolic pathways. Identification of these targets will to produce new drugs against tuberculosis that will overcome the limitations of existing drugs such as, prolonged chemotherapy, failure against persistent infection and multidrug resistance.

Based on above conclusion the following recommendations are forwarded

- The lists of potential drug targets encoded in the genome of *M. tuberculosis* should be explored to identify new drug against tuberculosis that will overcome the limitation of existing drugs.
- Research should involve testing new or reformulated drug, combination of different drugs to shorten therapy, supplementation and enhancements of existing drugs.
- The existing (currently in use) drugs should be modified because of continuous development of drug resistance.
- TB drugs should be tested and combined with different drugs to shorten therapy, to reduce toxicity and to enhance its activity.
- More research should be conducted on molecular targets of *Mycobacterium.*
- Researcher should actively participate in finding better and more effective drugs that reduce time of treatment and less toxic

Abbreviations: AG: Arabinogalactan; CB: Constraint Based; DOTS: Directly Observed Treatment, Short course Erp: Extracellular repeat protein; ICL: Isocitrate lysase; KEGG: Kyoto Encyclopedia of Gene and Genome; LAM: Lipoarabinomannan; Mbt: Mycobactin; MDR-TB: Multi Drug Resistant Tuberculosis; MIC: Minimum Inhibitory Concentration; NTM: Non Tuberculosis Mycobacteria; OMPs: Outer Membrane Proteins; PAS: Para-aminosalicylic Acid; TCS: Tow Component System; TLM: Thiolactomycin; TNF: Tumor Necrosis Factor; GSMN-TB: *M. tuberculosis* genome scale metabolic network ; PcaA: proximal cyclopropanation of alpha-mycolates

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