

Review on Mycobacterial Metabolic Pathways as Drug Targets

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Abstract

Mycobacterium is acid fast genus of bacteria that include many pathogenic and non pathogenic species. Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. 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. The increasing emergence of drug-resistant tuberculosis along with the HIV pandemic (human) threatens disease control and highlights both the need to understand how our current drugs work and the need to develop new and more effective drugs. 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. Approximately one-fourth of the Mycobacterium tuberculosis genome contains genes that encode proteins directly involved in its metabolism. This review provides a brief historical account of tuberculosis drugs, metabolic pathways, examines the problem of current chemotherapy, discusses the targets of current tuberculosis drugs with focuses on some metabolic pathways. The identification of drug target form that unique metabolism of mycobacterium is crucial to to develop new drug for persistent and latent infection of tuberculosis.

Index terms— anti-tuberculosis agent, drug targets, metabolic pathway, mycobacterium tuberculosis.

1 Review on Mycobacterial Metabolic Pathways as Drug Targets

Summary-Mycobacterium is acid fast genus of bacteria that include many pathogenic and non pathogenic species. Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. 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. The increasing emergence of drug-resistant tuberculosis along with the HIV pandemic (human) threatens disease control and highlights both the need to understand how our current drugs work and the need to develop new and more effective drugs. 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. Approximately one-fourth of the Mycobacterium tuberculosis genome contains genes that encode proteins directly involved in its metabolism. This review provides a brief historical account of tuberculosis drugs, metabolic pathways, examines the problem of current chemotherapy, discusses the targets of current tuberculosis drugs with focuses on some metabolic pathways. The identification

of drug target form that unique metabolism of mycobacterium is crucial to develop new drug for persistent and latent infection of tuberculosis.

2 I. Introduction

Countries, either from loss of livestock, disease testing, or compensation. Worldwide, agricultural losses are estimated to be around \$3 billion a year (WHO, 2012). *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 immunised with *M. bovis* BCG, a live attenuated derivative of *M. bovis* (Brosch R et al., 2007).

The increasing emergencies of drug resistance tuberculosis and HIV infection which compromises host defense and allows latent infection to reactivate or render individual more susceptible to TB pose further challenges for effective control of the disease in human (Nachega et al., 2003).

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 (Blumberg, 2003). 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 (Blumberg, 2003). 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 (Zhang, 2004).

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 (Cole, 2002). It is widely accepted that TB is dynamic disease that result from combination of phenotypically diverse population of bacilli in continually changing host environment. Understanding host-pathogen interactions would give an important clue for developing new drugs, vaccine and diagnostic tests. The release of complete genome sequence of *M. tuberculosis* has facilitated the development of more rational and specific methods to search for new drug targets and vaccine candidates (Cole et al., 1998).

Ebsa Bushura ? & Feyera Gemedra Dima ? tuberculosis is a mycobacterium infection that affects a wide range of mammals. Human Tuberculosis is caused by *Mycobacterium tuberculosis* (human), was much more prevalent disease in the past than it is today, and it was responsible for the death of about one billion people during the last two centuries. *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 (WHO, 2003).

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 Bovine tuberculosis causes immense economic loss in many T

The recent rise in TB cases and especially the increase of drug resistant mycobacteria 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 mycobacteria have been important areas of the investigation to identify that metabolic pathway as drug target (Zhang, 2005). But among many pathogenic species of mycobacterium, many researches were done on *M. tuberculosis* metabolic pathways as drug target.

The objective of this paper is to review on mycobacterium metabolic pathways as drug targets.

4 II. 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 (Kanehisa et al., 2002). 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 *M. tuberculosis* (Tatusov et al., 2003). Among these pathways, Peptidoglycan biosynthesis and d-alanine metabolism is common to all bacterial species, and cell wall biosynthetic pathways have long been targeted for anti-microbial discovery.

5 a) Mycobactin biosynthesis

One of the key host defense mechanism is the production of siderocalins that sequester iron-laden siderophores and *M. tuberculosis* replicates poorly in the absence of these siderophores (de Voss & et al., 2000). 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 (Braun et al., 1998; ??yers and Arceneaux, 1998).

The iron acquisition systems of many pathogenic and saprophytic bacteria mostly rely on the production of small molecules called siderophores. *M. tuberculosis* produces the mycobactin class of siderophore, which contains a salicylic acid derived moiety. Therefore, this siderophores used for mycobactin biosynthesis, d-alanine metabolism and Peptidoglycan biosynthetic pathways (De Voss et al., 2000).

Mycobactin G (Mycobactin lysine-N6hydroxylase), 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 siderophore mediated pathway (Hider & Kong, 2010). Another reason that why we are so interested in this pathway is that we can see that there is a possibility of lacing the siderophore with the drug. Since there is no siderophore mediate pathway that is important for the host it is obvious that *M. tuberculosis* inhabits one of the most hostile environments, the alveolar macrophage. Among the various defensive mechanisms expressed by the host are a potent burst of oxygen derived radical species and a dramatic restriction of available iron to support microbial growth (Kontoghiorghes and Weinberg, 1995). 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*. MbtG (Rv2378) is therefore one of the important drug targets found and is also under consideration at the TB Structural Genomics Consortium (James et al., 2000). 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. The mycobacterium can utilize Heme as an Iron source (Christopher & Niederweis, 2011).

An Mtb heme-uptake system has been defined (Tullius, M.V et al., 2011) that consists of the secreted protein Rv0203 and the transmembrane proteins MmpL3 and MmpL11; recent experiments showed that Rv0203 transfers heme to both MmpL3 and MmpL11 during Mtb heme uptake making these proteins potential targets for TB drugs (Owens et al., 2013). Hence this pathway will have to be targeted in conjugation with the main iron acquisition pathway.

6 b) Peptidoglycan biosynthesis

Mycobacterium is surrounded by a lipid-rich outer capsule that protects it from the toxic radicals and hydrolytic enzymes produced as defense by macrophages (Kolattukudy et al., 1997). The peptidoglycan layer of the cell wall serves as a base for the lipid-rich capsule. Peptidoglycan or murein 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). Isoniazid is known to inhibit mycolic acid synthesis (Zhang et al., 1992), whereas ethambutol inhibits the polymerization step of arabinan biosynthesis of Arabinogalactan (Mikusova et al., 1995).

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.

7 c) 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 with a few exceptions making 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 (alr) has been identified as a target as all the bacteria investigated contained either one or two alanine racemase genes (Strych et al., 2001). However, in mycobacteria, there is a single alanine racemase gene. One alanine racemase inhibitor, the structural d-alanine analogue d-cycloserine has been marketed clinically. Both alanine racemase (Alr) and D-Ala-D-Ala ligase are targets of D-cycloserine, a second-line anti-TB drug. These two enzymes catalyze the first and second committed steps in bacterial peptidoglycan biosynthesis. Alr 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 (LeMagueres et al., 2005). Although, this is supposed to be an excellent inhibitor of mycobacteria and other pathogenic bacteria species, serious side effects especially CNS toxicity has limited its use (Yew et al., 1993).

8 d) Polyketide Sugar Unit Biosynthesis

Arabinogalactan a heteropolysaccharide is connected via a linker disaccharide, 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 (Mills et al., 2004). Rhamnose residue and large portion of arabinogalactan polysaccharide are synthesized on GlcNAc-P-P-decaprenyl carrier lipid (Mikusova et al., 1996). 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 peptidogly can.

The rhamnose-GlcNAc disaccharide is a critical linker which connects arabinogalactan to peptidoglycan via a phosphodiester linkage. 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, 5epimerase which is involved in the arabinogalactan biosynthesis. The biosynthesis of arabinogalactan in *M. tuberculosis* begins with the transfer of Nacetylglucosamine-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 (=, Ma et al., 2001).

9 e) 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 ??GIC, 2001).

A significant proportion of the *M. tuberculosis* genome is devoted to lipid metabolism. It possesses more than 250 enzymes involved in lipid metabolism, which includes enzymes for lipid biosynthesis as well as degradation. Degradation of host cell lipids is essential for the intracellular life of the organism. Host cell membranes provide precursors for many metabolic processes. They are also potential precursors of mycobacterial cell wall constituents through the action of beta oxidative enzymes encoded in multiple copies in the genome. Among these secreted proteins of *M. tuberculosis* which could act as virulence factors are a series of phospholipases C, lipases and esterases which might attack cellular or vacuolar membranes (The Genome International Consortium, 2001). Notable amongst these are phospholipases plcA (Rv2351c), plcB (Rv2350c), plcC (Rv2349c) and serine esterase (Rv2301) (GIC, 2001). The targets cutinases Rv3451 and Rv3452 are news ??Anishetty et al., 2005) The targets aceARv0467, aceAaRv1915, aceAbRv1916, glcBRv1837, Rv2205 identified, are related to mycobacterial persistence. They are enzymes from the glyoxylate by pass, which is important for mycobacterial persistence (e.g., Isocitrate Lyase and Malate Dehydrogenase). It has been proposed by Waynes and Lin (1982) 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 (Waynes and Lin, 1982).

10 f) In silico comparative metabolic pathways analysis

In some recent researches five unique pathways, C5-branched dibasic acid metabolism, carbon fixation pathways in prokaryotes, methane metabolism, lipopolysaccharide biosynthesis, and peptidoglycan biosynthesis with 60 new nonhomologous targets, were identified through in silico comparative metabolic pathway analysis of *Homo sapiens* and *M. tuberculosis* H37Rv using KEGG database. Pathways which are not present in the *Homo sapiens* but present in the *Mycobacterium* are designated as unique pathways. Design and targeting inhibitors against these nonhomologous sequences could be the better approach for generation of new drugs. Thus total 5 unique metabolic pathways have been taken in *M. tuberculosis* (Asad Amir et al., 2014) i.

11 Identification of unique pathways and potential drug

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. Therefore, computational approach for drug targets identification, specifically for *M. tuberculosis*, can produce a list of reliable targets very rapidly. These methods have the advantage of speed and low cost and, even more importantly, provide a systems view of the whole microbe at a time. Since it is generally believed that the genomes of bacteria contain genes both with and without homologues to the host (WHO, 2005).

ii. 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 ??Lamichhane et al., 2003).

iii. 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 (Asad Amir et al, 2014). Further, the functional analysis using Uniprot showed involvement of all the unique enzymes in the different cellular components (Asad Amir et al., 2014)

12 III. 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 (sunny. j et al., 2013). In recent years, a number of new genes and their products in *M. tuberculosis* have been identified, which can be possible drug targets for tuberculosis. 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 (Chopra et al., 2002).

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 (Chopra I, 2002).

New drugs that inhibit novel targets are needed. In choosing targets for drug development, it is important that they be involved in vital aspects of bacterial growth, metabolism, and viability. 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 (Zhang et al., 2005).

13 a) Targeting Mycobacterial persistence

Mycobacterial persistence refers to the ability of tubercle bacillus to survive in the face of chemotherapy and/or immunity (McDermott, 2000). The nature of the persistent bacteria is unclear but might consist of stationary phase bacteria, post-chemotherapy residual survivors and/or dormant bacteria that do not form colonies upon plating (Zhang, 2004). 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 (GAFTDD, 2001).

ISOCITRATE LYASE (ICL) Gene products involved in mycobacterial persistence (Mckinney et al., 2000). ICL catalyzes the conversion of isocitrate to glyoxylate and succinate and is an essential enzyme for fatty acid metabolism in the glyoxylate shunt pathway. Survival of *M. tuberculosis* in the adverse in vivo environment requires utilization of C2 substrates (generated by β -oxidation of fatty acids) as the carbon source (Chen B et al., 2000). ICL was induced in the Wayne "dormancy" model, inside macrophages and in the lesions of the human lung (166). 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 Pca A (PROXIMAL CYCLOPROPANATION OF ALPHA-MYCOLATES) 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 (Ying Zhang et al., 2005). 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 (Glickman et al., 2000) the stringent response induced by starvation is mediated by the signaling molecule hyperphosphorylated guanine (ppGpp) synthesized by RelA (ppGpp synthase I) RelA (ppGpp synthase) (Dahl et al., 2003). In subsequent studies aimed at characterizing mycobacterium genes that are induced in the Wayne "dormancy" model, the same two-component system was identified by microarray analysis and named Rv3133c/Rv3132c. DosR-Rv3133/DevR-DevS The two-component system DevR-DevS was initially identified as being preferentially expressed in virulent *M. tuberculosis* strain H37Rv over that in avirulent strain H37Ra in a subtractive hybridization analysis. Inactivation of DosR abolished the rapid induction of hypoxia-induced gene expression, suggesting that DosR is a key regulator in the hypoxia-induced mycobacterial "dormancy" response. The DosR mutant grew as well as the wildtype strain initially in five-day incubation, but it survived significantly less well upon extended incubation up to 40 days in the Wayne model (Boon C and Dick T. 2002). 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 (NO). DosR could be a good target for developing drugs against persisters (Ying Zhang et al., 2005). DosR (controlling a 48-gene regulon involved in mycobacterial survival under hypoxic conditions) have been identified and could be good targets for the development of drugs that target persistent bacilli (Park et al., 2003).

Rv2421c transfers phosphorous groups in nicotinate/nicotinamide salvage and de novo synthesis. 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 (Cloete et al., 2016).

14 b) 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. 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 (Sasseti et al., 2003).

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 (Lamichhane et al., 2003).

However, the function of a significant number of essential genes is unknown. Besides systematic analysis of essential genes by transposon mutagenesis, 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 (Zhang et al., 2003).

15) 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 (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*. This study implies that energy production or maintenance is important for the viability of persistent non-growing tubercle bacilli in vivo. The recent discovery of the highly effective TB drug diarylquinoline also highlights the importance of energy production pathways for mycobacteria. It is likely that energy production pathways, such as the electron transport chain, glycolytic pathways (like the Embden-Meyerhof pathway) and fermentation pathways, could be good targets for TB drug development (Zhang et al., 2003). Isocitrate lyase (ICL) is 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 propionyl CoA of the glyoxylate pathway. Inactivation of the *icl* gene leads to attenuation of both persistent and virulent strains of *M. tuberculosis*. 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 (Cloete et al., 2016).

16 d) Targeting virulence factors

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* (extracellular repeat protein), which has been shown to be essential for the multiplication of mycobacteria during the acute phase of infection in the mouse model. The most important point is that this gene has no homologues in other organisms, making it an attractive drug target. 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 (Barrett et al., 1998).

The approach of targeting virulence factors, like other approaches suffers from some serious drawbacks, like virulence factors may not be necessarily survival genes. Therefore, inhibition of virulence factors may not be lethal to the pathogen. The other very important hurdle in this approach is that drugs that target virulence factors may be of very little or of no 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 (Alksne et al., 2000).

17 e) 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 *M. tuberculosis* genes that can mastermind the intracellular survival and multiplication within macrophages as also the shutdown of mycobacteria 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 (Jaya Sivaswam et al., 2004) 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 (Hoch, 2000). 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 (Stock, A. M et al, 2000). The histidine kinases from various bacteria also present novel targets for the development of new kinase inhibitors. MtrA (Rv3246c) (magnesium transporter) and SenX3, histidine kinases 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 (Parish, T et al, 2003).

Mycobacterium tuberculosis has shown the presence of at least 12 two-component system homologues with 8 unlinked sensor kinases or response regulators. However, the exact physiological role of most of these proteins is far from being understood. 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 (Zahrt et al., 2000).

Interestingly, another two-component system, devR-devS, was found to be over expressed in a virulent strain, H37Rv (Dasgupta et al., 2000). 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 (Perez et al., 2001).

Although the inhibitors that were used in this study-for example, staurosporine were relatively nonselective, some recent researches provide the first indication that protein kinases might be important in regulating the entry and phagocytosis of mycobacteria in macrophages. Subsequently, a small-molecule kinase inhibitor-1-(5-isoquinolinesulphonyl)-2-ethylpiperazine, a sulphonyl compound belonging to the H-series was found to inhibit in vitro growth of M. Bovis BCG, and also inhibited the kinase activity of the M. tuberculosis kinase PknB (Drews, S.J et al., 2001). As PknA, PknB and PknG are required for the growth of mycobacteria in vitro; any compound that specifically blocks these kinases might be a potential candidate for a new antimycobacterial agent (Young, T. et al., 2003).

18 f) Targeting cell wall synthesis

The mycobacterial cell wall is a complex structure that is required for cell growth, resistance to antibiotics and virulence (Bansal-Mutalik, R. & Nikaido, H, 2014) It is composed of three distinct macromolecules; peptidoglycan, arabinogalactan and mycolic acids -which are surrounded by a noncovalently linked outer capsule of proteins and polysaccharides (Sani, M. et al., 2010) The outermost, the mycolic acids (Brennan et al., 1995) are 70 to 90 carbon-containing, branched fatty acids which form an outer lipid layer in some way similar to the classical outer membrane of gram-negative bacteria (Brennan et al., 1968). Mycolic acids are strong hydrophobic molecules oriented perpendicular to the plane of the membrane and provide a special lipid barrier responsible for many physiological and disease inducing aspects of Mtb.

They are thought to be significant determinant of virulence in Mtb. Probably they prevent attack of mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. The mycolic acids are esterified to the middle component, arabinogalactan (AG), a polymer composed primarily of Dgalactofuranosyl and D-arabinofuranosyl residues. AG is connected via a linker disaccharide, α-L-rhamnosyl-(1-3)-α-D-A/-acetylglucosaminosyl-1-phosphate, to the sixth position of a muramic acid residue of peptidoglycan (PG) (Me Neil and Brennan 1990) which is the innermost of the three cell wall core macromolecules. A complex consisting of mycolic acids, arabinogalactan and peptidoglycan constitutes "the core" of the cell wall (Crick et al., 2001) which is often referred as "mycolyl-arabinogalactan-peptidoglycan" (MGP) complex. This covalently linked structure is intercalated with numerous glycolipids such as lipoarabinomannan (LAM), the phosphatidyl inositol containing mannosides (PIMs), trehalose dimycolate (TDM; so-called cord factor), trehalose-monomycolate (TMM), which play an important role in virulence of Mtb (Glickman and Jacob 2001). The dominating heteropolysaccharide LAM is noncovalently attached to cell wall and may be anchored to the cytoplasmic membrane via phosphatidyl-myoinositol (pi) unit.

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 (Karen J. Kieser & Eric J. Rubin, 2014) Mycobacteria including M. tuberculosis have a unique cell wall structure. A variety of unique lipids like lipoarabinomannan (LAM), trehalose dimycolate, and phthiocerol dimycerate which form non covalent anchorage with the cell membrane have been documented to play an important role in the virulence of M. tuberculosis (Glickman et al., 2001).

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 mycobacterial cell wall and has been shown to induce TNF release from the macrophages which plays a significant role in bacterial killing (Puneet Chopra, 2003).

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. Enzymes involved in this pathway have always been preferred targets in drug development efforts (SchullerLevis et al., 1994).

Thiolactomycin (TLM) targets two β -ketoacyl-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, (Puneet Chopra, 2003). TLM has also been shown to be active against MDR-TB clinical isolate. Several TLM derivatives have been found to be more potent in vitro against fatty acid and mycolic acid biosynthesis.

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 (Parrish et al., 1999). 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 (Jones et al., 2000).

19 g) 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 β -*mgtc* mutant showed in vitro growth defects (Buchmeier et al., 2000).

Similarly β -*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. Members of PE-PGRS family of proteins that are highly expressed within tissue granulomas have been shown to be essential for the virulence of mycobacteria. Therefore, the members of this category of genes also constitute potential drug targets (De Lencastre et al., 2002).

20 h) TB genomics and drug targets

The first bacterial genome was sequenced by Fleischmann and colleagues at The Institute for Genomic Research (TIGR) in 1995 (Fleischmann et al., 1995). 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 genomicsbased approaches (Dougherty et al., 2002).

The complete genome sequence of *M. tuberculosis* provides an opportunity for a more focused and planned approach towards the identification of new drug targets (Puneet Chopra et al., 2003). Genome sequence helps in compilation of all the potential gene products encoded by a particular organism, identification of functions (enzymes and pathways) that are missing or unique in a particular organism, and finally identifying the genes that are common to all or most prokaryotes and eukaryotes (S Gerdes, 2011).

The common targets can then be over expressed for biochemical assays in drug screens or structure determination, to be used in the drug design. So far, however, no company has been successful in developing a drug using a genomics approach. The availability of the *M. tuberculosis* genome sequence (Cole et al., 1998) opens up a new opportunity to understand the biology of the organism and provides a range of potential drug targets (Cole, 2002).

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 (Wilson et al., 1999), and by INH, TLM and triclosan (Betts et al., 2003). Microarray was also used to identify genes that are switched on in the Wayne "dormancy" model under hypoxic and nitric oxide stress conditions (Voskuil et al., 2003), a discovery that led to the identification of a 48-gene "dormancy regulon" controlled by DosR (Voskuil et al., 2003).

A proteomic approach was used to identify potential proteins that are induced in starvation as an in vitro model of persistence (Betts et al., 2002). Two unique *M. tuberculosis* proteins with homology to each other were identified: Rv2557 and Rv2558. Rv2557 were also induced in side granulomatous lesions in the lung. Genes identified by microarray analysis or proteins identified by a proteomic approach should be further validated as potential drug targets by gene knockout and in vivo testing in mice before they are selected as targets for drug development (Fenhalls et al., 2002). As described recently the chemotherapy of tuberculosis has much evolved along the years since it started with the introduction of streptomycin 1946. By 1955, the combination of streptomycin, paraaminosalicylic acid and isoniazid was adopted as a standard treatment by the western world (Datta et al., 1993). More recent clinical data suggest using at least five regimens of adequate anti-tuberculosis drugs (Mirsaiedi et al., 2005). The choice of these drugs will be driven by the actual or presumed (in view of past failed treatment) resistance characteristic of the strains of *M. tuberculosis* considered. In order of preference they can be chosen from the following. amoxicillin and the penicillinase inhibitor clavulanic acid has an anti-mycobacterial effect in vitro. The same is true for clarithromycin although its clinical efficacy remains to be established (Forget et al., 2006).

21 a) Status of current tuberculosis drug therapy

The current live vaccine *Bacillus Calmette Gurein* (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 (Andrea M. and Shabaana A, 2009) .

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 ??WHO,2000). The targets of these drugs are varied. INH inhibits synthesis of mycolic acid, a cell wall component (PZA targets cell membrane whereas 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 (Zhang et al., 2000). Cycloserine prevents the synthesis of peptidoglycan, a constituent of cell wall (Puneet Chopra, 2003).

22 b) Limitation of current tuberculosis therapy

In the present scenario, due to the emergence of multi drug resistant tuberculosis (MDR-TB) and association between immune compromising disease and TB, DOTS is becoming rapidly ineffective in (Maus et al., 2005) ? In any case, the first line agents still active on the patient: isoniazid, rifampin, pyrazinamide and ethambutol. ? This is followed by the group of injectable drugs: streptomycin, kanamycin, amikacin, capreomycin or viomycin/tuberactinomycin B and the related tuberactinomycins A, N and O. ? One of the many related antibacterial fluoroquinolones such as ciprofloxacin, ofloxacin, levofloxacin or the more recent sparfloxacin, gatifloxacin, moxifloxacin and sitafloxacin should be included in the regimen. This class of antibiotics has now been proven as indispensable treatment for MDR tuberculosis (Chan et al., 2004). ? Second line bacteriostatics, with established clinical efficacy, usually have more important side effects (Newton et al., 1975). They are para-aminosalicylic acid, ethionamide (the propyl analogue prothionamide is also used) and cycloserine. ? Other drugs are also considered. Their use is the subject of debate and only time and proper observations will provide the necessary data. Clofazimine is among these compounds and is also used against *M. leprae*. The combination of Table ??:

controlling tuberculosis in human. 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 (Kimerling et al., 1999). 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 nongrowing 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

23 V. Conclusion 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 ¹

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