Review Article

Indian J Med Res 117, January 2003, pp 1-9

New drug targets for *Mycobacterium tuberculosis*

Puneet Chopra, L.S. Meena & Yogendra Singh

Institute of Genomics & Integrative Biology, Delhi, India

Received January 3, 2003

In spite of the availability of effective chemotherapy and Bacille-Calmette-Guerin (BCG) vaccine, tuberculosis remains a leading infectious killer world-wide. Many factors such as, human immunodeficiency virus (HIV) co-infection, drug resistance, lack of patient compliance with chemotherapy, delay in diagnosis, variable efficacy of BCG vaccine and various other factors contribute to the mortality due to tuberculosis. In spite of the new advances in understanding the biology of *Mycobacterium tuberculosis*, and availability of functional genomic tools, such as microarray and proteomics, in combination with modern approaches, no new drug has been developed in the past 30 yr. Therefore, there is an urgent need to identify new drug targets in mycobacteria and eventually, develop new drugs. The release of the complete genome sequence of *M. tuberculosis* has facilitated a more rational, and directional approach to search for new drug targets. In general, gene products involved in mycobacterial metabolism, persistence, transcription, cell wall synthesis and virulence would be possible targets for the development of new drugs. The exploitation of host cell signaling pathways for the benefit of the pathogen is a phenomenon that deserves to be looked into with a new perspective in the current scenario to combat *M. tuberculosis*. Reversible phosphorylation and dephosphorylation, which are carried out by specific protein kinases and phosphatases have been shown to modify the host proteins and help in the establishment of disease by several pathogenic bacteria. In this review, we discuss some possible drug targets for *M. tuberculosis*.

Key words Dormancy - drugs - kinases - *Mycobacterium tuberculosis* - phosphatases

Tuberculosis continues to be a major cause of morbidity and mortality throughout the world. Five decades of tuberculosis control programmes using potentially efficacious drugs and the availability of BCG vaccine, have failed to reduce the prevalence of infection in most parts of the world. It has been reported that more than 3 billion people have been vaccinated with BCG, but still TB kills more than 50,000 people every week and approximately one-third of the world population is asymptomatically infected by *Mycobacterium tuberculosis*[1]. It has been estimated that TB accounts for around 32 per cent deaths in HIV infected individuals[2]. The situation has exacerbated because of the presence of some complicating factors like, emergence of multidrug-resistant TB[3], HIV co-infection[4], lack of patient compliance with chemotherapy, and variable efficacy of Bacille-Calmette Guerin (BCG) vaccine. A prerequisite for the effective control of tuberculosis is to understand the host-pathogen interactions and its contribution towards the development of disease. 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.

The success of mycobacteria in producing disease relies entirely on its ability to utilize macrophages for its replication and more importantly, the maintenance of viability of host macrophages that sustain mycobacteria. *M. tuberculosis* has evolved several mechanisms to circumvent the hostile environment of the macrophage, its primary host cell. In spite of extensive research, our knowledge about the virulence factor(s) of *M. tuberculosis* is inadequate. A variety of mechanisms have been suggested to contribute towards the survival of mycobacteria within macrophages. These mechanisms include (*i*) inhibition of phagosome-lysosome fusion[5]; (*ii*) inhibition of phagosome acidification[6], (*iii*) recruitment and retention of tryptophan/aspartate containing coat protein on phagosomes to prevent their delivery to lysosomes[7]; and (*iv*) host-induced expression of members of the PE-PGRS family of proteins[8].

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. It is expected that the application of functional genomic tools, such as microarray and proteomics, in combination with modern approaches, such as structure-based drug design and combinatorial chemistry will lead to the development of new drugs that are not only active against drug resistant TB but can also shorten the chemotherapy schedule.

Status of current tuberculosis drug therapy

Drugs available for the treatment of tuberculosis can be classified into two categories; first line drugs such as, isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB) *etc*., and second line drugs like para amino salicylate (PAS), kanamycin, cycloserine (CS), ethionamide (ETA), amikacin, capreomycin, thiacetazone, fluoroquinolones *etc*. 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[9]. The targets of these drugs are varied. INH, inhibits synthesis of mycolic acid, a cell well component[10]; PZA targets cell membrane whereas rifampin and streptomycin interferes with the initiation and streptomycin interferes with the initiation of RNA and protein synthesis respectively[11]. EMB blocks biosynthesis of arabinogalactan, a major polysaccharide present in the mycobacterial cell wall[12] and kanamycin and capreomycin, like streptomycin, inhibit protein synthesis through modification of ribosomal structures at the 16S rRNA[13]. Cycloserine prevents the synthesis of peptidoglycan, a constituent of cell wall[14].

Limitations of current drug therapy and need for new drug targets

In the present scenario, due to the emergence of multi drug resistant tuberculosis (MDR-TB) and association between HIV and TB, DOTS is becoming rapidly ineffective in controlling tuberculosis. Recent reports indicate that, areas where there is a high incidence of MDR-TB, DOTS is failing to control the disease[15]. 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.

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[16]. 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 of developing active disease through reactivation. Secondly, it is important to achieve a shortened therapy schedule to encourage patient's compliance and to slow down the development of drug resistance in mycobacteria.

Impact of genome sequence on identification of new drug targets

The complete genome sequence of *M. tuberculosis*[17] provides an opportunity for a more focussed and planned approach towards the identification of new drug targets. 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. An important advantage of this analysis is the possibility of identifying a novel target that is present in many bacteria and subsequently designing an antibiotic that could be active against a wide range of bacteria. In addition, availability of human genome sequence can help in eliminating the potential drug targets that have close human homologues. Thus, the possibility of using complete genome sequences for target identification are virtually unlimited.

Possible drug targets

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, signal transduction 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. Some genes and their products reported in literature that can serve as drug target are discussed below.

Genes involved in dormancy or persistence

Mycobacteria has the unique property of becoming persistent or dormant for very long periods. This stage of mycobacteria poses a significant problem for effective therapy as these persistent bacilli are resistant to most of the currently available drugs for the treatment of tuberculosis. The mechanism of mycobacterial persistence or dormancy is far from being understood. Isocitrate lyase (ICL), a key enzyme of glyoxylate shunt has been shown to be involved in the persistence of *M. tuberculosis* in mice[18]. The interesting point is that ICL is not essential for the viability of tubercle bacilli in normal cultures or in hypoxic conditions, but is important for persistence of bacilli in mice. Recently, *pcaA* gene, which encodes a novel methyl transferase has also been shown to be involved in the persistence in mice. This gene is involved in the modification of mycolic acids of mycobacterial cell wall. The *pcaA* knockout strain of mycobacteria grew normally *in vitro* and replicated in mice similar to the wild type strain initially but was not able to persist[19]. Nevertheless, the role of *icl* and *pcaA* genes in the persistence of *M. tuberculosis in vivo*

and the absence of homologous genes in the host make them good drug targets, which would potentially eliminate persistent bacilli *in vivo.*

Genes involved in cell wall synthesis

Mycobacteria including *M. tuberculosis* have a unique cell wall structure. A variety of unique lipids like lipoarabinomannan (LAM), trehalose dimycolate, and phthiocerol dimycocerate which form non covalent anchorage with the cell membrane have been documented to play an important role in the virulence of *M. tuberculosis*[20]. Lipids such as cord factor have been suggested to play an important role in the virulence of *M. tuberculosis* by inducing cytokine mediated events[21]. LAM is also a major constituent of the mycobacterial cell wall and has been shown to induce TNF release from the macrophages[22] which plays a significant role in bacterial killing.

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. For example, enzymes (RmlA to RmlD) which are involved in the synthesis of dTDP-rhamnose, an essential structural component of the cell wall of *M. tuberculosis*, have been selected for an *in vitro* screening with chemical library of 8,000 compounds[23].

Thiolactomycin (TLM) targets two β-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[24]. 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[25]. 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[26]. Octanesulphonyl acetamide (OSA) has recently been identified as an inhibitor of fatty acid and mycolic acid biosynthesis in mycobacteria[27]. 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 6.25-12.5 mg/ml. Interestingly, OSA was found to be less active against fast growers such as *M. smegmatis* and *M. fortuitum*[28]. 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.

Virulence genes

On the basis of the genome sequences of pathogens, using bioinformatics approach, a few genes have been proposed to play an important role in the virulence of mycobacteria. In recent years, a number of techniques have been developed to delineate the differences between related pathogens and non-pathogens. These techniques include PCR-based subtractive hybridization that can be used to specifically amplify DNA sequences that are present in one (*e.g.*, virulent) but not in the other (*e.g.*, avirulent) strain. The other established method is signature tagged mutagenesis (STM), which provides a means for identific ation of genes which are important for bacteria to survive and proliferate *in vivo.*

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, *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[29]. 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[30], phthiocerol dimycocerosate.

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[31].

Genes of signal transduction

The exploitation of host cell signaling pathways for the benefit of the pathogen is a phenomenon that deserves to be looked into with a new perspective in the pathogenesis and drug target identification of *M. tuberculosis*. Reversible phosphorylation and dephosphorylation is the key mechanism by which extracellular signals are translated into cellular responses. These processes are carried out by specific protein kinases and phosphatases[32]. It has been shown that, upon infection, the phosphatases and kinases of several pathogenic bacteria modify the host proteins that helps in the establishment of disease. Phosphorylation generally takes places at histidine, serine, threonine or tyrosine residues. Lipoarabinan (LAM) from the virulent species of *M. tuberculosis* has been shown to modulate host signaling pathways linked to bacterial survival by phosphorylation of an apoptotic protein (Bad) in phosphatidylinositol 3-kinase (PI-3K)-dependent pathway in THP- 1 cells[33]. Earlier, it had been shown that a major anti-phosphotyrosine reactive protein was present only in mycobacteria belonging to the *M. tuberculosis* complex[34].

Serine/threonine protein kinases: Serine and threonine kinases have been found to coordinate stress responses, developmental processes and pathogenicity in several microorganisms[32]. Serine/ threonine kinase (YpkA) of *Yersinia pseudotuberculosis* helps in the virulence of pathogen by disrupting and reprogramming the host signaling network[35]. These kinases have also been suggested and control the late stages of development, sporulation or secondary metabolite production in bacteria[32]. Unlike *Yersinia*, *Listeria monocytogenes* invades mammalian cells and alters the host signaling by directly stimulating mitogen-activated protein (MAP) kinase upon attachment to epithelial cells[36]. Another example of an active interaction between invasive bacteria and the host is provided by the human gastric epithelial pathogen *Helicobacter pylori* which induces cytoskeletal rearrangements following attachment to gastric cells, as well as inducing phosphorylation of two host protein[37].

The genome sequence of *M. tuberculosis* suggested the presence of eleven putative serine/threonine protein kinases[17]. Presence of these kinases, in such a large number in *M. tuberculosis* indicates a likely role for these proteins in the specific signal transduction events with host ligands. It has been suggested that protein kinase G and F may change the phosphorylation pattern of host proteins upon infection, thereby, promoting the bacterial survival[38]. Recently PknA has been shown to have a role in cell growth, division, and elongation[39]. Moreover, kinase inhibitors, genistein, staurosporin and K252a have been shown to inhibit the development of few bacteria like, *Myxococcus xanthus*[40]. Recently, it has been shown that an isoquinoline inhibitor reduces the growth of two mycobacterial species, *M. smegmatis*

mc2 155 and *M. bovis* Bacille Calmette Guerin (BCG). This inhibitor also blocked the activity of PknB, a serine threonine kinase of *M. tuberculosis* in a dose dependent manner. It has been speculated that this inhibitor may be active against other members of this family of kinases as well[41].

Tyrosine phosphatase : Pathogenic bacteria *Yersinia pseudotuberculosis,* secretes YopH, a tyrosine phosphatase in the host[42] in order to down regulate the cell signaling pathways of macrophages that are involved in phagocytosis[43] and generation of the respiratory burst[44]. Similarly, another important intracellular pathogen, *Salmonella typhimurium* also secretes tyrosine phosphatase, SptP in the host through type III secretion system. Secreted SptP binds and activates the intrinsic GTPase activity of GTP binding proteins Rac and Cdc42 leading to the disruption of host cytoskeletal network required for bacterial internalization[45].

The molecular basis of the pathogenicity of *M. tuberculosis* is far from being understood. However, both entry and subsequent survival of *M. tuberculosis* in the host cell appears to involve a specific cross talk between the host and pathogen. This is validated by the fact that the uptake of *M. tuberculosis* by the macrophage is associated with a number of early signaling events such as recruitment and activation of Src family protein tyrosine kinases. Recruitment of these proteins results in increased tyrosine phosphorylation of multiple macrophage proteins, and activation of phospholipase D(PLD)[46]. Similarly, activation of protein tyrosine kinases appears to enhance stimulation of PLD activity and the associated increase in phosphatidic acid (PA). It has been shown that increased phosphorylation may trigger a number of down stream processes necessary for membrane remodeling during phagocytosis and intracellular survival of *M. smegmatis* in the host cells[47]. It is known that *M. tuberculosis* has two functional secretary tyrosine phosphatases. Moreover, of these two tyrosine phosphatases, one phosphatase MptpB is present exclusively in the members of *M. tuberculosis* complex suggesting that MptpB might play a role in the survival of mycobacteria. These phosphatases may dephosphorylate some of the host proteins which may be helpful in the invasion and establishment of disease[48].

Genes of two-component systems: 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[49].

It has been reported that disruption of a multitude of TCS in *Streptococcus pneumoniae* greatly reduced the ability of the pathogen to cause disease[49]. In addition, TCS have also been shown to be involved in the regulation of bacterial virulence in a number of organisms. The genome sequence of *M. tuberculosis* has shown the presence of at least 12 two-component system homologues with 8 unlinked sensor kinases or response regulators[17]. 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*[50]. Interestingly, another two-component system, devR-devS, was found to be over expressed in a virulent strain, H37Rv[51]. Disruption of the *phoP* component of the PhoP/PhoR (TCS that controls transcription of virulence genes in a number of intracellular bacterial pathogens such as *Salmonella, Shigella* and *Yersinia*) 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[52], 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.

Transcription factors

Gene products that are involved in transcription regulation have long been used as target for drugs in a number of pathogens. For example rifampin, a well-known drug for tuberculosis, targets RNA polymerase. The sigma factors have been shown to regulate gene expression in response to numerous environmental conditions in a number of bacterial species. Genome sequence of *M. tuberculosis* revealed the presence of 13 sigma factors. Sigma factors of mycobacteria like other bacteria also perform a multitude of functions. Sigma factor RpoV (same as SigA) has been shown to be a virulence factor in *M. bovis*, as point mutation (arginine 522 to histidine) in RpoV has been shown to cause attenuation of virulence in a guinea pig model[53]. It has been speculated that attenuation of virulence caused by the RpoV mutation might be due to the inability of the mutant RpoV to switch on certain virulence genes. Besides the virulence genes, RpoV also controls many other housekeeping genes. SigB has been shown to be induced during stationary phase[54]. Similarly, SigE is involved in heat stress, oxidative stress, stress due to exposure to SDS and survival in macrophages[55]. Expression of both SigB and SigE is under the control of SigH[56]. Both SigE and SigH play a role in the resistance of *M. smegmatis* to various stress stimuli, including elevated temperature and oxidative stress. Moreover, expression of SigE and SigH of *M. tuberculosis* is markedly enhanced when the pathogen is inside the macrophages. Another sigma factor, SigF, has been shown to control the expression of Acr protein, which is induced in the macrophages and is necessary for the persistence of *M. tuberculosis*[57]. These observations clearly suggest that sigma factors especially the centrally important SigH, SigF and SigA are potential drug targets.

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 Δ-*mgtc* mutant showed *in vitro* growth defects[58]. Similarly Δ-*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[59].

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[8]. Therefore, the members of this category of genes also constitute potential drug targets.

Conclusion

Major obstacle in the cure and prevention of tuberculosis is posed by the latent or persistent *M. tuberculosis* infection. This is due to the fact that most of the currently available drugs are ineffective against latent infection. In spite of better understanding of the physiology of *M. tuberculosis*, our knowledge about the state of the bacillus during the latent period is far from being complete. Moreover, a true representative model of latent tuberculosis in the laboratory setting is not available. Establishment of such a system would certainly accelerate the efforts to

understand the physiology of mycobacteria during the latent period and eventually it will help in the identification of new drug targets that can act on the persistent mycobacteria. Recent advances in modern biology, in combination with bioinformatic tools, proteomics and microarray technology would further facilitate the search of new drug targets against tuberculosis. These exciting techniques are providing new avenues for understanding the biology of mycobacteria. The result of better understanding of the physiology of mycobacteria is manifested by the fact that the list of possible drug targets for tuberculosis is increasing day by day, the utility of these targets, however, cannot be predetermined. 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 intermediary metabolic pathways. All these targets should be explored to identify new drugs against tuberculosis that will overcome the limitations of existing drugs such as, prolonged chemotherapy, failure against persistent infection and multidrug resistance.

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Reprint requests : Dr Yogendra Singh, Institute of Genomics & Integrative Biology
Mall Road (Near Jubilee Hall), Delhi 110007, India