Attack on the Scourge of Tuberculosis: Patented Drug Targets

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Abstract: Tuberculosis is one of the most devastating bacterial diseases, with increasing rates of morbidity and mortality, despite the presence of effective chemotherapy and Bacillus-Calmette-Guerin (BCG) vaccine. The success of *Mycobacterium tuberculosis* lies in its ability to spread by aerosol droplets, evade the host immune system and to persist in pulmonary granulomas. The advancement in the field of molecular and cellular microbiology and the availability of transcriptome and proteome data of *M. tuberculosis* have aided in understanding the pathogenesis of this organism for developing more effective drugs. The current strategy of drug design is to identify gene products, which are essential for survival and virulence. To date, several gene products of mycobacteria, ranging from proteins involved in cell wall synthesis to energy generation and from entry into host to persistence, have been shown to be essential for the survival or virulence of *M. tuberculosis*. These proteins and their associated pathways are considered as promising drug targets against *M. tuberculosis* and several of these have been patent protected. Herein, we enlist drug targets against *M. tuberculosis* for which patents have been filed and issued during the last ten years. The significance of these drug targets in the development of drug is also discussed. This review presents a comprehensive account of the pivotal information for drug discovery and drug design to all researchers involved in tuberculosis research.

Keywords: *Mycobacterium tuberculosis*, tuberculosis, drugs, cell envelope, MabA, sigma factor, RNA polymerase, signal transduction, serine/threonine kinases, metabolism, virulence, isocitrate lyase, dormancy.

INTRODUCTION

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the biggest killers amongst the infectious diseases despite the availability of effective drugs and an attenuated Bacillus-Calmette Guerin (BCG) vaccine. Streptomycin was the first drug introduced in 1944 for the treatment of tuberculosis but almost immediately after its introduction many patients started showing resistance to this antibiotic [1-3]. Para-aminosalicylate (PAS) was introduced in 1946 that largely overcame the emergence of resistant strains [4]. A few years later, isoniazide (INH) was developed and initial treatment with both INH and streptomycin was even more effective. To date, many drugs are available, which are classified into two categories. First line therapy includes five medications: isoniazide (isonicotinic acid hydrazide), pyrazinamide (analog of nicotinamide), ethambutol [(S,S´)-2,2´(ethylenediimino)di-1-butanol], rifampicin (lipophilic ansamycine) and streptomycin (aminocyclitol glycoside) [5, 6]. Second line therapy, which is used exceptionally in the cases of drug resistance, includes cycloserine, capreomycin, fluoroquinolones, ethionamide, PAS, thioacetazone, rifabutin, clofazimine and some macrolides [7].

The major setback in controlling tuberculosis was the emergence of multidrug resistant tuberculosis (MDR-TB) during 1990-92. Currently, at least 50 million people are estimated to be affected with MDR-TB. A few MDR strains of *M. tuberculosis* were found to be resistant to many first line agents as well as some of the second line drugs [8]. Moreover, the high rate of coinfection with human immunodeficiency virus (HIV) presented a challenge to the existing chemotherapies. One of the limitations of currently available drugs is their inability to act on latent bacilli. People carrying latent infection are at a risk of reactivation and this is one of the major barriers in controlling tuberculosis. Therefore, there is an urgent need to develop novel drugs that can act against both actively growing and dormant bacteria. The efforts for drug development are being coordinated by Global Alliance for TB Drug Development (www.tballiance.org), an organization that has been involved in developing public-private partnerships to bring out new, faster-acting and affordable drugs against tuberculosis.

Rational development of a new antitubercular agent requires the exploration of new means to understand the genetics and physiology of *M. tuberculosis*. In this regard, availability of the genome sequence of *M. tuberculosis* [9] and powerful genetic tools for manipulating mycobacteria have provided valuable information about the potential targets. Also, the information available from X-ray crystallographic studies of many of these targets has helped in designing novel chemotherapeutic agents. This review exclusively focuses on mycobacterial targets that have been patent protected from all over the world in the last ten years, Fig. (**1**). Herein, we present the information vis-à-vis proteins that are important for the survival of *M. tuberculosis* during infection and persistence in the host environment. The potential drug targets compiled in this review are likely to lead to new medication that should facilitate in controlling the spread of tuberculosis.

PATENT PROTECTED DRUG TARGETS

A patent is a set of exclusive territorial rights granted by the government of a country to the assignee for their creative ideas and expressions of the human mind that have

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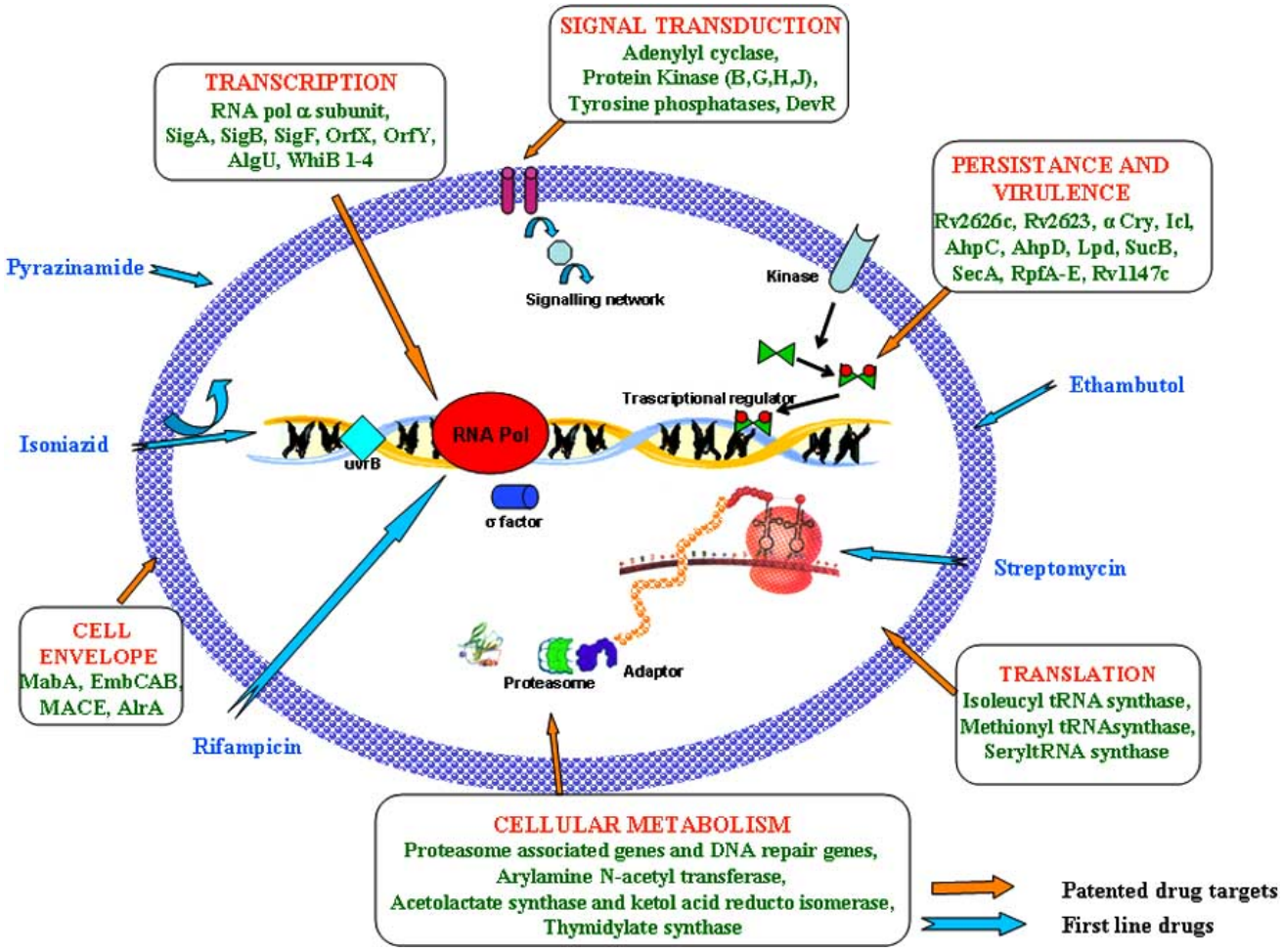

Fig. (1). An outline of Patent Protected drug targets of *Mycobacterium tuberculosis*.
The patented targets against which antitubercular drugs can be developed are shown in boxes. These targets are known to control various cellular processes required for survival or virulence of *M. tuberculosis*. The site of action of first line drugs (Rifampicin, Isoniazid, Pyrazinamide, Ethambutol and Streptomycin) are shown by arrows.

commercial value. At present, several gene products, which are part of many important pathways such as cellular metabolism, transcription, translation, cell envelope synthesis, signal transduction, persistence and virulence have been patented owing to their importance as promising drug targets (Table **1**).

PROTEINS INVOLVED IN CELL ENVELOPE METABOLISM

Mycobacteria belong to a suprageneric taxon, the mycolata, which also includes Corynebacteria, Nocardiae and Rhodococci, by virtue of similarity in their cell wall architecture [10]. The mycobacterial cell envelope is a complex structure comprising mycolic acids covalently bound to peptidoglycan via arabinogalactan [11]. The cell wall of *M. tuberculosis* confers resistance against many antibacterial agents known to inhibit cell wall synthesis such as isoniazide, ethionamide, isoxyl, and the thiosemicarbazones. An enriched knowledge of the biosynthesis and assembly of the cell envelope components has provided vital clues for the development of novel antimycobacterial agents.

After phagocytosis by macrophages, *M. tuberculosis* overcomes several environmental stresses, such as those caused by toxic reactive oxygen and nitrogen species. The detrimental effect of the reactive radicals is circumvented by modifications such as cyclopropanation of mycolic acids, which renders mycobacterium resistant to lipid peroxidation [11]. Mycolic acid cyclopropanating enzyme (MACE, US5573915) [12], also known as cyclopropane mycolic acid synthase (*cma*) is responsible for cyclopropanation at distal position of the mero chain in the α-mycolic acid series [13]. Non-pathogenic mycobacteria, *M. smegmatis* do not cyclopropanate their mycolic acids. Heterologous expression of *cma* in *M. smegmatis* not only resulted in cyclopropana-tion of α-mycolates but also significantly protected recombinant *M. smegmatis* from oxidative stress [13]. A homolog of *cma* is also present in *M. leprae*, suggesting that cyclopropanation of mycolic acids is specific to pathogenic forms of mycobacteria [14]. Therefore, therapeutic agents, which affect the activity of MACE, hold the key to successful elimination of pathogenic mycobacteria. PCT patent numbers WO9638581 and US5573915 issued in

Table 1. Patent Protected Drug Targets of *M. tuberculosis*

S. No.	DRUG TARGET	CLASSIFICATION	PATENT NUMBER[a]	PDB ID
1	Arylamine N-acetyl tranferases	Cellular Metabolism	EP1082441, EP2002516109T, WO9961625, GB9811407	1GX3
2	Proteasome associated genes and DNA repair genes	Cellular Metabolism	US2004213776	–
3	Acetolactate synthase and ketol acid reducto isomerase	Cellular Metabolism	AU2451397, US5998420, WO9737660	–
4	Thymidylate synthase	Cellular Metabolism	WO02072805, GB0105763D	–
5	SigA and SigB	Transcription	AU706367, AU4550896, AU5018496, CA2171600, CN1201465, EP0832116, GB2298862, IE960132, JP2000503525, NZ286081, WO9638478, SE9501976, SE9502596, SE9503246,	–
6	SigF, OrfX, and OrfY	Transcription	AU732858, AU2580297, CA2249208, EP0910403, JP2000508525T, US5700925, US5824546, US6004764, WO9735611	–
7	AlgU	Transcription	CA2278314, EP0970192, JP200150903, US6355469, WO9831789	–
8	RNA Polymerase alpha-subunit	Transcription	AU7625298, CA2274098, EP0956347, EP2001505438, US6355464, WO9824891	–
9	WhiB1, WhiB2, WhiB3 and WhiB4	Transcription	EP1245683, US6645505, US2002164573	–
10	Adenylyl cyclase	Signal Transduction	CA2447110, EP1258528, EP1390487, US2005048603, WO02092805	Crystallization done, No PDB ID available[b]
11	Protein kinase B, G, H and J	Signal Transduction	AU2003206594, WO03074728	1MRU
12	Tyrosine phosphatases	Signal Transduction	WO2005005639	1U2Q
13	DevR	Signal Transduction	EP1472339, GB2398302, WO03066838	–
14	Isoleucyl tRNA synthase	Translation	US5756327	_
15	Methionyl tRNA synthase	Translation	US5798240	_
16	Seryl tRNA synthase	Translation	US5656470	–
17	MabA (FabG1)	Cell Envelope	AU2003258723, EP1490491, FR2837836, WO03082911	1UZN
18	EmbC, EmbA, and EmbB	Cell Envelope	AU6467598, US6015890, WO9841533	_
19	MACE	Cell Envelope	AU5881496, US5573915, WO9638581	1L1E
20	D-alanine racemase	Cell Envelope	US2003133952	–

(**Table 1**) **Contd….**

S. No.	DRUG TARGET	CLASSIFICATION	PATENT NUMBER[a]	PDB ID
21	Rv3133c, Rv2623, Rv2626c	Dormancy Related Genes	AU1610002, GB0030368, GB2386420, US2004242471, WO0248391	–
22	Resuscitation Factor (RpfA-E)	Dormancy Related Genes	AU749414, AU7779798, CA2292898, EP0983361, GB9711389, GB9811221, JP2002503106, NZ501568, WO9855624, ZA9804838	1XSF
23	Rv1174c and phospholipids	Dormancy Related Genes	WO0245736	_
24	Isocitrate lyase	Dormancy Related Genes	AU3391602, US2003018166, WO0233118	1F8M, 1F8I
25	Alkyl hydroperoxide reductase (AhpC)	Virulence	WO9954479	2BMX
26	AhpD, dihydrolipoamide dehydrogenase, and dihydrolipoamide succinyltransferase	Virulence	US2003190325	1KNC
27	SecA	Virulence	AU5170896, US5885828, WO9626276	1NL3, 1NKT

[a] The first two letters in the patent number corresponds to PCT (Patent Corporation Treaty) contracting states: AU, Australia; AT, Austria; CA, Canada; CH, Switzerland; CN, China; DE, Germany; FR, France; EP, Europe; GB, Great Britain; IE, Ireland; JP, Japan; NZ, New Zealand; SE, Sweden; TW, Taiwan; US, United States of America; WO, World Intellectual Property Organization; ZA, South Africa.

[b]www.doe-mbi.ucla.edu/TB/

1996, relate to MACE and its DNA or protein compositions useful for both diagnosis and designing of therapeutics for treatment of tuberculosis and other mycobacterial infections. The patent discloses the invention of a method for determining the ability of a compound to inhibit the cyclopropanation of mycolic acids in pathogenic mycobacteria.

MabA, a NADPH-dependent β-ketoacyl ACP reductase, is involved in the biosynthesis of long chain fatty acids, the precursors of mycolic acids (WO03082911) [15]. Unlike *mabA* of *M. smegmatis*, *mabA* of *M. tuberculosis* is in an operon with *inhA*, an NADH-dependent 2-trans-enoyl ACP reductase [16]. Both genes together with KasA/B (β-ketoacyl synthase) form type II fatty acid elongation system (FAS-II) [17-19]. INH is known to inhibit InhA and MabA by formation of a covalent adduct between Mn^{III}-activated isoniazid and the MabA/InhA cofactor [20]. Crystal structure analysis revealed that MabA has specific functional and structural properties when compared to other homologous bacterial β-ketoacyl reductases, such as large hydrophobic substrate binding pocket and preference for long chain substrates [21]. These distinct properties of MabA make it a potential drug target specific for mycobacteria. Also, the mechanism of inhibition by a metabolite of INH could serve as a model for rational drug designing. WO03082911 published in 2004 relates to the recombinant native and mutant protein MabA. The invention provides crystallographic co-ordinates for designing and screening ligands inhibiting the enzymatic activity of MabA.

Ethambutol (EMB) is a frontline antituberculosis drug, which targets the mycobacterial cell wall. Resistance to EMB was used as a tool to identify genes participating in cell wall biosynthesis, which led to the identification of mycobacterial *embCAB* gene cluster (US6015890) comprising *embC*, *embA* and *embB* genes [22-24]. EmbA and EmbB are arabinosyl transferases, which utilize arabinofuranosyl phosphodecaprenol for arabinosylation of cell wall arabinogalactan, the major polysaccharide of the mycobacterial cell wall [25-27]. Over expression of Emb proteins [23] and mutations of conserved residue Met306 are associated with resistance to EMB in mycobacteria [22, 23, 28]. Identification and characterization of resistance to EMB has provided information for development of new chemotherapeutic agents against these mutated Emb proteins. WO9841533 published in 1998 and US6015890 issued in 2000 relate to the identification, cloning, sequencing and characterization of the *embCAB* operon. This patent also provides one or more single-stranded nucleic acid probes, which specifically hybridize to the wild type *embCAB* operon or the mutated *embCAB* operon that may be used in the diagnosis of drug-resistant mycobacterial strains.

D-Alanine is an essential component of dipeptide D-alanyl-D-alanine, involved in the cross-linking of peptidoglycan strands in bacteria [29]. D-alanine racemase (alrA) catalyzes the conversion of L-alanine into D-alanine [30], and D-alanine-D-alanine ligase catalyzes the subsequent dimerization of D-alanine into the key dipeptide, D-alanyl-D-alanine [31]. Inactivation of D-alanine racemase was shown to affect the survival of *M. smegmatis* in phagocytic cells (US2003133952) [32]. Thus, inhibitors can be designed against D-alanine racemase as novel antituberculosis drugs targeting peptidoglycan biosynthesis in mycobacteria. US2003133952 published in 2003 relates to methods of making live-attenuated vaccines against pathogenic mycobacteria using *alrA* mutants. Furthermore, *alrA* mutants

can be used for screening antimycobacterial agents that are synergistic with peptidoglycan inhibitors.

PROTEINS EMPLOYED IN TRANSCRIPTION AND TRANSLATION

Genes required for survival following uptake by macrophages can provide insight into mycobacterial pathogenesis and provide novel targets for developing antibacterial agents. The ability to adapt to intracellular stress requires regulation of complex gene expression mediated mainly by sigma factors. Sigma factors are involved in transcription initiation by interacting with RNA polymerase. DNA-dependent RNA polymerase is a basic unit of the bacterial transcription apparatus. The holoenzyme is a complex consisting of five protein subunits: two copies of α subunit and one copy each of β, β' and sigma subunit. The β and β' subunits are invariant in a given bacterial species and together with α subunit form core RNA polymerase. The α subunit is involved in protein-protein interactions with transcription activators and protein-DNA interactions [33-35]. The amino terminal domain of α subunit is also required for the assembly of core RNA polymerase. RNA polymerase is a well-documented drug target and rifampicin is a highly specific inhibitor of mycobacterium RNA polymerase [36]. Due to emergence of resistant strains, development of new compounds that interfere with the enzymatic activity of RNA polymerase or disrupt interaction of a subunit with the core enzyme is urgently required. Healy *et al.*, have patented the method for high-throughput screening of RNA polymerase inhibitors and identified anti-tuberculosis compounds that specifically inhibit mycobacterial transcription (EP0956347) [37]. Patent applications WO9824891 published in 1998, EP0956347 and US6355464 issued in 2002 relate to novel nucleic acids encoding the RNA polymerase alpha subunit from *M. tuberculosis*. The invention also provides vectors comprising the nucleic acids, cells comprising the vectors, and methods for producing *M. tuberculosis* alpha subunit.

Sigma factors are interchangeable RNA polymerase subunits that are responsible for promoter recognition. Prokaryotes usually have a constitutively expressed principal sigma factor, which is responsible for the transcription of essential housekeeping genes, and a number of alternative sigma factors that are transcriptionally and/or post-translationally activated in response to specific environmental signals [38]. Mycobacterial genome encodes at least 14 sigma factors [9]. Sigma factor A (*sigA*; *rpoV*), (WO9638478) [39], a principal sigma factor, is an essential gene and has been shown to be involved in virulence of *M. tuberculosis* [40]. A mutation in *sigA* from arginine to histidine at amino acid residue 522 resulted in the loss of virulence of *M. bovis* and complementation of the attenuated *M. bovis* mutant with the *M. tuberculosis* wild type *sigA* restored virulence [40]. The transcription of *sigB* (WO9638478) [39] is regulated differentially from that of *sigA*. Transcription of the *sigB* gene increases significantly when *M. tuberculosis* enters the stationary phase at 10 days of microaerophilic incubation and under various stress conditions [41]. These findings suggest that SigB may be an alternative or secondary sigma factor, which controls a large stationary-phase regulon.

WO9638478 published in 1996 and GB2298862 issued in 1999 relate to the provision of novel nucleic acid molecules coding for SigA and SigB subunits of *M. tuberculosis* RNA polymerase. The invention further provides screening assays for compounds, which inhibit the interaction between a sigma subunit and a core RNA polymerase.

sigH, (AlgU, US6355469) [42] was shown to be induced after heat shock and after exposure to the thiol-specific oxidizing agent, diamide [43]. *M. tuberculosis* mutants lacking *sigH* showed reduced immunopathology in infected animals [44]. *M. tuberculosis sigF* mutant strain was shown to be attenuated in immunocompetent mice [45, 46] and moreover, the expression level of *sigF* is upregulated in stationary phase and in stress conditions [47]. SigF is regulated by *M. tuberculosis* OrfX (anti-sigma factor) and OrfY (anti-anti-sigma factor) proteins. The *M. tuberculosis* SigF, OrfX and OrfY (WO9735611) [48] can be used to screen for dormancy activators, which trigger growth arrest during active tuberculosis infection and can also be used to screen for antagonists that induce reactivation in patients with latent tuberculosis. Reactivation will render antimycobacterial drugs more effective, because the available drugs are typically more potent toward actively growing bacilli. Thus, sigma factors are logical targets for the development of the transcriptional inhibitors. US6355469 patent issued in 2002 and WO9831789 published in 1998 relate to DNA encoding *M. tuberculosis* RNA polymerase AlgU sigma subunit protein and methods of detecting inhibitors of the RNA polymerase. The invention also encompasses sequence-conservative and function-conservative variants of this sequence. US5700925 patent issued in 1997 is directed to a gene involved in latency and a diagnostic method for detecting latent *M. tuberculosis*. The invention also relates to *M. tuberculosis* vaccines expressing mutant *sigF* genes. Patent application WO9735611 published in 1997 and AU732858 patent issued in 2001 disclose a method for identifying a gene or a protein, which is regulated by a sigma factor of *M. tuberculosis*.

WhiB proteins were originally described in *Streptomyces coelicolor* and were shown to be involved in sporulation and cell septation [49]. WhiB family proteins are also present in *M. tuberculosis* and *M. bovis* BCG. WhiB proteins (WhiB1, WhiB2, WhiB3 and WhiB4, US6645505) [50] of *M. tuberculosis* have been patented and disruption of these genes in *M. bovis* BCG revealed that *whiB1* is an essential gene and *whiB2*, *whiB3* and *whiB4* are involved in growth and septum formation. Furthermore, WhiB proteins were characterized by the yeast two-hybrid system that demonstrated DNA binding and transcriptional activation properties of these proteins [50]. WhiB3 has also been shown to be involved in virulence of *M. tuberculosis* as gene inactivation led to reduction in the survival in immunocompetent mice [51]. Patent number US6645505 issued in 2003 discloses an *in vivo* drug screening method taking advantage of the yeast two-hybrid and provides a method of using *whiB* genes. The drugs against WhiB2 and the WhiB4 of *M. tuberculosis* H_{37}Rv and *M. bovis* BCG will be particularly useful where drug resistance has developed against the WhiB1 and WhiB3 or where the anti-WhiB1 and anti-WhiB3 drugs are allergic or toxic.

The components of the translational apparatus are prominent targets for antibiotics. Aminoacyl-tRNA synthetases catalyze the transfer of specific amino acid to its corresponding tRNA to form aminoacyl-tRNAs, which are used during protein synthesis. Eukaryotic-like isoleucyl-tRNA synthetase (IleRS, US5756327) of *M. tuberculosis* [52, 53] has been found to be resistant to the prokaryotic IleRS-targeted antibiotic, pseudomonic acid [54, 55]. Moreover, *M. tuberculosis* methionyl-tRNA synthetase (MetRS, US5798240) [56] has characteristic class I signature sequences (HVGH and KMSKS) but lacks the Zn^{2+} binding motif and the C-terminal dimerization appendix [57]. Because the amino acid sequences of the tRNA synthetases have diverged with time, significant differences exist between the structures of the enzymes from mammals and mammalian pathogens [58]. These differences can be exploited by identifying inhibitors, which have specific activity against these mycobacterial tRNA synthetases. Moreover, the seryl-tRNA synthetase (US5656470) [59] of *M. tuberculosis* has also been characterized by biochemical assays and antisense strategy. US5756327 patent issued in 1998 relates to IleRS [52]. Recombinant DNA constructs encoding IleRS can be used for the construction of tester strains to identify inhibitors of the essential tRNA synthetase. US5798240 patent issued in 1998 relates to the isolated and/or recombinant nucleic acids, which encode MetRS [56]. The invention also provides antisense nucleic acid that can hybridize to the mRNA of MetRS of mycobacteria. US5656470 patent issued in 1997 relates to isolated and/or recombinant nucleic acids, which encode seryl-tRNA synthetases of mycobacterial origin [59].

PROTEINS OF SIGNAL TRANSDUCTION PATHWAYS

After entering macrophages, cross-talk modalities exist between the mammalian host and the pathogen, with the result that the host's defense system is bypassed and the pathogen survives and proliferates. *M. tuberculosis* genome encodes 15 putative adenylyl cyclases (ACs), indicating the presence of signal transduction pathways mediated through cAMP as a second messenger. It has also been reported that cAMP levels increase in macrophages infected with mycobacteria leading to an inhibition of phagosome-lysosome fusion [60]. Amongst the 15 putative ACs, Rv2435c and Rv1625c are grouped with mammalian ACs based on their sequence homology (WO02092805 and US2005048603) [61, 62]. As the closest progenitor of mammalian AC, Rv1625c gene product comprises a protein with six transmembrane helices and a single cytosolic catalytic domain, which dimerizes to form a 12-transmembrane, homodimeric enzyme, with two substrate-binding sites at the dimer interface in contrast to the heterodimeric mammalian enzyme [63]. Structural and biochemical analysis revealed that despite the high (60% similarity to guanylyl cyclases) sequence similarity to the mammalian enzymes, Rv1625c has a unique substrate binding pocket that has not been reported in any other cyclase so far [63, 64]. These differences could in principle be exploited in designing novel drugs. US2005048603 published in 2005 and WO02092805 published in 2002 relate to a method for expression of membrane proteins wherein a portion of a nucleotide sequence coding for the membrane protein is fused to a portion of a nucleotide sequence coding for an adenylyl cyclase of *M. tuberculosis* [61, 62]. The main advantage of this inventive method is that the recombinant membrane protein can be effectively expressed in prokaryotic as well as in eukaryotic systems. The invention further provides a kit for expression of membrane proteins.

Protein kinases and phosphatases are essential for virulence in a number of bacterial species that modulate the host-signaling network. *M. tuberculosis* has two functional tyrosine phosphatases, MptpA and MptpB (WO2005005639) [65], which are secreted into the culture filtrate by actively growing mycobacterial cells [66]. *mptpA* and *mptpB* knock out strains of *M. tuberculosis* were attenuated in the lungs and spleen of infected animals. The ability of mutant strains to survive in macrophages activated with IFN-γ was highly impaired [67]. This suggests that tyrosine phosphatases of *M. tuberculosis* help in its survival in the host cells by dephosphorylating proteins that are involved in IFN-γ mediated signaling pathways. WO2005005639 published in 2005 relates to the role of *mptpA* and *mptpB* in the virulence and pathogenesis of mycobacteria. The invention provides mutant mycobacterium strains harboring a modified tyrosine phosphatase gene (*mptpA* or *mptpB*) wherein the mutant mycobacterium strain is incapable of expressing the active tyrosine phosphatase.

Eukaryotic type serine/threonine kinases of *M. tuberculosis* [68] are also attractive therapeutic targets. Because of their significance in signal transduction and their role in circumventing the hostile environment, many of them have been patent protected. Mutant strains of protein kinase G (PknG) [69, 70] and protein kinase H (PknH) were unable to survive under *in vivo* conditions (WO03074728) [71]. PknG was shown to be secreted in host cells, causing phagosomal maturation block [70]. The inhibition of phagosome-lysosome fusion may be mediated by phosphorylation of host proteins by PknG. PknH can phosphorylate EmbR, a protein hypothesized to modulate the levels of arabinosyltransferases involved in arabinan biosynthesis of arabinogalactan, a key molecule of the mycobacterial cell wall [72, 73]. *pknB* (protein kinase B, WO03074728) [71], one of the four *M. tuberculosis* kinases, conserved in the downsized genome of *Mycobacterium leprae*, is presumed to play an important role in the processes that regulate the complex life cycle of mycobacteria. Transposon-mutagenesis has shown that PknB and PknG are required for growth of mycobacterium under *in vitro* conditions [74]. PknB inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (WO03074728) [71, 75] was found to inhibit the growth of *M. bovis* (BCG) and *M. smegmatis*. WO03074728 published in 2003 relates to a method for identifying compounds capable of affecting the activity of serine/threonine kinases (PknB, PknG, PknH and PknJ) of *M. tuberculosis*. Knockout mutants of PknG and PknH display slower growth and viability both *in vitro* and *in vivo*. The compounds of this invention can be incorporated into a variety of formulations for therapeutic administration.

The two-component system plays a central role in the adaptation of pathogenic bacteria to the environmental signals prevailing within host tissues. The genes encoding

the response regulator DevR (Rv3133c/DosR, EP1472339) [76] and cytoplasmic region of the histidine kinase DevS (Rv3132c/DosS), are part of a well characterized two-component system of *M. tuberculosis* [77]. Expression of Rv3134c-devR-devS operon has been shown to be induced in hypoxic cultures of *M. tuberculosis* [78]. Guinea pigs infected with the mutant strain of DevR showed a significant decrease in gross lesions in lung, liver and spleen compared to guinea pigs infected with the parental strain [79]. The mutant strain also showed loss in viability in oxygen deprived cultures [76]. These observations suggest that DevR-DevS system is involved in the virulence of *M. tuberculosis* and acts as a key regulatory link between oxygen limitation and the initiation and maintenance of adaptive response to hypoxia. Therefore, this genetic system could serve as a vital target for the development of new drugs for elimination of dormant bacilli. WO03066838 published in 2003 relates to a process for identifying a novel target for the development of therapeutic modalities and drugs effective against tuberculosis. The patent relates to testing of *M. tuberculosis devR* mutant strain for virulence in guinea pigs.

ENZYMES INVOLVED IN CELLULAR METABOLISM

The proteins involved in metabolic pathways are also promising targets as they are essential for survival of the pathogen. Arylamine *N*-acetyltransferases (NATs, WO9961625) [80] belong to a unique family of proteins found in a wide range of organisms, catalyzing the acetyl-CoA dependent *N*-acetylation of the arylamines, N and O acetylation of arylhydroxy amines and acetylation of aromatic hydrazines. Human NAT2 acetylates and inactivates the antituberculosis drug, isoniazid (INH) and is polymorphic. A homologue of human NAT2 in *M. tuberculosis* has also been shown to acetylate and inactivate INH *in vitro* [81]. Introduction of a substrate or an inhibitor, which may diminish the activity of NAT against INH can act as an anti-mycobacterial drug. WO9961625 published in 1999 relates to arylamine *N*-acetyltransferase proteins from *M. tuberculosis* and *M. smegmatis* together with antibodies raised against these proteins, and methods of detecting mycobacteria using such antibodies. WO9961625 has also described the methods for screening compounds that are ligands of arylamine *N*-acetyltransferase. Such ligands, designed using three-dimensional structures of NATs can be used for treatment of mycobacterial infections.

Microbes synthesize branched-chain amino acids such as isoleucine, valine and leucine. The three reactions of the isoleucine and valine biosynthesis catalyzed by enzymes are common to both pathways. One of the intermediates of the valine pathway is used for the synthesis of leucine. Therefore, inhibition of the isoleucine-valine pathway enzymes, acetolactate synthase (ALS) and ketol acid reductoisomerase (KARI) will result in elimination of all the three branched-chain amino acids (US5998420) [82]. Herbicides that inhibit plant branched-chain amino acid biosynthetic enzymes were tested for inhibition of *M. tuberculosis* growth *in vitro* [83, 84]. Sulphometuron methyl and metsulphuron methyl, inhibitors of ALS, were indeed able to affect the growth of *M. tuberculosis*. Furthermore, inhibitors of both ALS and KARI were effective against drug-resistant clinical isolates. Animal studies showed that sulphometuron methyl significantly prevented the growth of *M. tuberculosis* in lungs [85]. As mammals do not have branched-chain amino acid biosynthetic enzymes, treatment with these compounds should be specific to pathogenic organism. US patent no. 5998420 issued in 1999, patent applications AU2451397 and WO9737660 published in 1997 relate to a method for treating tuberculosis by administering therapeutically effective amount of a compound that inhibits an enzyme of the branched chain amino acid biosynthetic pathway in *M. tuberculosis* [82, 86, 87].

Thymidylate synthase is a ubiquitous enzyme, which catalyzes the essential methylation of dUMP to dTMP, one of the four bases required for DNA synthesis. The reaction requires N^5, N^{10}-methylene H_4 folate as a cofactor. Thymidylate synthase activity is strongly linked to the activity of the two enzymes responsible for replenishing the cellular folate pool: Dihydrofolate reductase and Serine transhydroxymethylase. Thymidylate synthase of *M. tuberculosis* (Rv2492) has been cloned and characterized (WO02072805) [88]. The thymidylate synthase reaction is a crucial part of the pyrimidine biosynthesis pathway, which generates dCTP and dTTP for incorporation into DNA. Inhibition of dTMP synthesis leads to a loss of DNA production, an arrest of the cell cycle and eventually a 'thymine-less' cell death. The emergence of multi-drug resistant bacteria in recent years has prompted research into the use of mycobacterial thymidylate synthase inhibitors as antibiotics. WO02072805 published in 2002 relates to the identification of a novel protein, thymidylate synthase and its use in the diagnosis, prevention and treatment of disease. Moreover, thymidylate synthase has been implicated in the pathogenicity of the *M. tuberculosis* and the ligands of this protein are likely to be effective in controlling disease.

Macrophages produce nitric oxide and other reactive nitrogen intermediates (RNI) to control infection by *M. tuberculosis* [89]. Despite the protective effect of RNI, mycobacteria persist and multiply in macrophages. Genes required for resistance against host RNI were identified using transposon mutagenesis and the mutants were screened for hyper susceptibility to acidified nitrite [90]. The study yielded seven genes with transposon insertions, including the genes required for DNA repair (*uvrB*) and synthesis of a flavin cofactor (*fbiC*) (US2004213776) [91]. Five mutants had insertions in two proteasome-associated genes that encode a proteasome-associated adenosine triphosphatase (ATPase) called Mpa (Rv2115c) and a proteasome-associated factor called Paf (Rv2097c) [90, 91]. Unlike wild type *M. tuberculosis*, mutants (Rv2115c and Rv2097c) failed to grow in resting primary macrophages isolated from wild type or $iNOS^{-/-}$ mice. An inhibitor of the human proteasomal protease, *N*-[4-morpholine]carbonyl-β-[1-naphthyl]-L-alanine-L-leucine boronic acid blocked proteosomal protease activity in *M. tuberculosis* and suppressed the growth of *M. tuberculosis* in culture conditions [90]. A specific inhibitor of bacterial proteasome might be useful to sensitize *M. tuberculosis* to the immune system if they are combined with chemotherapeutic agents that target enzymes involved in RNI resistance. US2004213776 published in 2004 provides methods for screening compounds that inhibit proteasomal

and protease activity, DNA repair enzyme activity, or flavin-like co-factor synthesis enzyme activity, where the inhibitory compounds have an ability to sensitize bacteria to the antibacterial effects of oxidative/nitrosative stress.

ENZYMES INVOLVED IN VIRULENCE AND PERSISTENCE

M. tuberculosis virulence can be understood by finding the factors that are important for the progression of tuberculosis. In most cases, essentiality of these factors for virulence has been studied either by gene knockout, global gene inactivation by transposon mutagenesis or antisense strategy. Understanding the strategies employed by *M. tuberculosis* for persistence would allow designing of antibiotics or inhibitors that would specifically target persistent or latent bacilli, allowing shortening of time required for chemotherapy.

Hypoxia is proposed as a key signal sensed by mycobacterium to enter into the persistent state [92]. Importantly, the dormant form of the bacterium is resistant against conventional antimycobacterials [93, 94]. To identify genes induced in dormancy, Boon *et al.* subjected *M. bovis* BCG to an oxygen-limited Wayne culture system followed by proteome analysis. Their work revealed the up-regulation of response regulator Rv3133c and three other polypeptides: α-crystallin and two "conserved hypothetical" proteins, Rv2623 and Rv2626c (WO0248391) [95]. The gene encoding response regulator DevR (Rv3133c/DosR) has been shown to be involved in virulence of *M. tuberculosis* as discussed earlier. DevR, a transcriptional regulator, also regulates the expression of three other dormancy genes [96]. Thus, the dormant mycobacterium can be targeted by inhibitors of DevR, leading to down regulation of Rv2623 and Rv2626c. WO0248391 (2002) and US2004 242471 (2004) disclose a method for the identification of an anti-mycobacterial agent that modulates the activity and/or expression of a protein (Rv3133c, Rv2623 and Rv2626c) expressed by a mycobacterium in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase [95, 97].

As discussed above, the drugs available against tuberculosis are unable to eliminate dormant bacilli. The persistent bacilli can get reactivated and cause disease following immune system perturbations. The resuscitation of dormant bacilli would make the bacterium susceptible to anti-mycobacterial drugs and would lead to successful elimination of dormant bacilli. The discovery of new class of pheromones, which stimulate the resuscitation of dormant bacilli has provided opportunities for treatment of persistent mycobacterial infection. Resuscitation factor (Rpf) is a secreted growth factor, which is required for the growth of vegetative cells in minimal media at very low inoculum densities, as well as for the resuscitation of dormant cells. *M. tuberculosis* contains five genes whose predicted products resemble Rpf from *Micrococcus luteus* (WO9855624) [98]. The Rpf-like proteins of *M. tuberculosis* (RpfA-E) were cloned and were shown to stimulate bacterial growth in laboratory culture at picomolar concentrations [99]. The Rpf-like proteins of *M. tuberculosis* show cross-species activity as they were shown to stimulate the growth of the slow-growing organism, *M. bovis* BCG and two fast-growing organisms, *M. smegmatis* and *M. luteus*. Moreover, expression of Rpf in *M. smegmatis* could also stimulate its growth in minimal medium [99]. WO9855624 published in 1998 describes Rpf, their cognate receptors and inhibitors or mimetics. Pharmaceutical compositions and methods based on the Rpf and their receptors/convertases are also described in the patent.

Zhang *et al*. have also shown that dormant bacilli can be resuscitated by spent culture supernatant [100]. The components present in the culture supernatant having resuscitation activity were found to be phospholipids and peptide fragments of Rv1174c (WO0245736) [101]. Phosphatidyl-L-serine and a dioleoyl phosphatidyl-L-serine, both of which are precursors of phosphatidylethanolamine and phosphatidylcholine had significant resuscitation activity for the 6-month-old *M. tuberculosis* H37Ra cells over medium control as judged by colony forming units (CFU) assay. Phosphatidyl-L-serine allowed small inocula (10^{-7} dilution) to form visible growth and CFU on plates, whereas the control culture grew only at 10^{-5} dilution. Taken together, these results suggest that the phospholipids not only resuscitated old tubercle bacilli but also allowed small inocula to initiate growth in liquid culture. MALDI mass spectroscopic analysis of culture supernatant fractions and N-terminal amino acid sequencing revealed a peptide identical to the 29^{th} to 58^{th} aminoacids of a hypothetical protein Rv1147c of *M. tuberculosis* with unknown function [100]. WO0245736 published in 2002 provides the media for growth enhancement and resuscitation of mycobacteria. In this patent, diagnostic kits and treatment methods utilizing spent culture supernatant and cell extracts are also provided.

Alkyl hydroperoxide reductase, subunit C (AhpC) of *M. tuberculosis* (MtAhpC, WO9954479) [102] is a member of peroxidases, the peroxyredoxins found in many organisms. MtAhpC can detoxify hydroperoxides and protect against reactive nitrogen intermediates [103, 104] and specifically catalyzes the conversion of peroxynitrite ($OONO^-$) to nitrite [105]. Enhanced expression of MtAhpC is observed both in INH resistant KatG-deficient strains [106] as well as in INH-sensitive strains when challenged with the drug [107]. The crystal structure of Cys176-Ser point mutant of MtAhpC suggested a model for the peroxidase reaction that includes the generation of a large internal cavity, which encloses the reaction center [108]. The intricacies of the mechanism supported by the structural details might provide a structural framework for the design of inhibitors with potential therapeutic applications. MtAhpC together with AhpD, dihydrolipoamide dehydrogenase, and dihydrolipoamide succinyltransferase (US2003190325) comprise an NADH-dependent peroxidase and peroxynitrite reductase system, which provides support to the antioxidant defense of *M. tuberculosis* [109, 110]. The AhpD crystal structure revealed a trimer with two catalytic sulfhydryl groups; Cys-130 and Cys-133, in which each of the subunits had an identical but novel protein fold [111]. Structural details can support for designing inhibitors with potential utility as antitubercular agents. WO9954479 published in 1999 relates to the use of alkyl hydroperoxide reductase subunit C encoding gene, proteins or polypeptides to confer resistance against antimicrobial reactive nitrogen intermediates. AhpC can be used to screen drugs that inhibit the activity of AhpC and

sensitize *M. tuberculosis* to RNI produced by host cells. Alternatively, therapeutics can be developed to treat gastric infection. AhpC can be useful in vaccines to prevent infection by *M. tuberculosis*, while the antibodies raised against this protein can be employed in passive immunization of infected person. These proteins, antibodies and DNA molecules may also be utilized in diagnostic assays to detect *M. tuberculosis* in tissue or body fluids. US2003190325 published in 2003 relates to the methods of inhibiting AhpD, dihydrolipoamide dehydrogenase and dihydrolipoamide succinyltransferase in the infected person to make the pathogen susceptible to antimicrobial reactive nitrogen intermediates or reactive oxygen intermediates. Methods of producing an AhpD crystal suitable for X-ray diffraction as well as methods for designing a compound suitable for treatment or prevention of tuberculosis are also disclosed.

During infection, many pathogens encounter deprivation of certain essential nutrients and cofactors. Mutations in the genes encoding enzymes in the biosynthetic/degradative pathways and acquisition systems for some of these factors have helped in deciphering new drug targets. Earlier it has been reported that *M. tuberculosis* shifts from a metabolism that preferentially uses carbohydrate when growing *in vitro* to one that utilizes fatty acids when growing in the host, which is supported by the fact that over 200 genes were annotated to be involved in fatty acid metabolism [9]. One such enzyme is isocitrate lyase (Icl, WO0233118) [112] that converts isocitrate to succinate in the glyoxylate shunt and thus helps to survive on acetate or fatty acids as the sole carbon source. Icl activity increases dramatically in the stationary phase of *M. tuberculosis* growth [113] and its mRNA levels increase during macrophage infection and in the lungs of infected mice [114-116]. It has also been demonstrated that Icl is important for survival of *M. tuberculosis* in the lungs of mice during the persistent phase of infection (2-16 weeks), but is not essential during the acute phase (0-2 weeks) [117]. Given its potential as a drug target against persistent infections, its structure was solved without ligand and in complex with two inhibitors [118]. Covalent modification of an active site residue, Cys 191, by the inhibitor 3-bromopyruvate traps the enzyme in a catalytic conformation with the active site completely inaccessible to solvent. These inhibitor-bound structures not only help to establish key residues in the active site, but enable to pinpoint interactions, which are essential in forming the closed conformation of the enzyme and are likely to be a key to successful drug discovery. WO0233118 published in 2002 discloses the importance of the glyoxylate shunt in the persistent phase of various infectious agents, including mycobacteria and the identification of targets for drug development. Crystals and three-dimensional structures of *M. tuberculosis* Icl, without ligand and in complex with two inhibitors that can be used in the design of inhibitors and therapeutic agents are also disclosed.

Protein export is an important aspect of bacterial pathogenesis. Research on diverse bacterial pathogens has demonstrated that the majority of virulence factors are secretory proteins [119, 120]. In bacteria, the majority of exported proteins are translocated by the Sec system, which recognizes the signal sequence of a pre-protein and uses ATP and the proton motive force to mediate protein translocation across the cytoplasmic membrane. SecA (WO9626276) [121] is an essential protein component of this system, containing the molecular motor that facilitates translocation. There are two homologues of *secA* in mycobacteria, *secA1* and *secA2*. Using an allelic-exchange strategy in *M. smegmatis*, it was demonstrated that *secA1* is the essential housekeeping protein whereas s*ecA2* is an accessory factor for the secretion [122]. The topography of SecA of *M. tuberculosis* and its ATP binding sites are highly conserved, whereas its membrane insertion domains are species specific [123]. The crystal structure of SecA1 revealed that each subunit of the homodimer contains a motor domain and a translocation domain. The structure predicts that SecA can interact with the SecYEG pore and function as a molecular ratchet that uses ATP hydrolysis for physical movement of the preprotein [124]. A deletion of the *secA2* gene in *M. tuberculosis* led to loss of virulence because SecA2 mediates secretion of SodA, a virulence factor of *M. tuberculosis* [125]. WO9626276 published in 1996 relates to an isolated nucleic acid encoding a SecA protein of *M. tuberculosis*. The invention includes the mutant SecA protein of *M. tuberculosis* and provides methods of screening for putative virulence factors translocated by SecA**.**

CURRENT AND FUTURE DRUG DEVELOPMENTS

The availability of *M. tuberculosis* genome sequence [9] and recent advances in understanding the molecular basis of host pathogen interaction have opened new avenues for the development of novel antimycobacterial drugs. Promising new drug candidates such as PA-824 (nitroimidazopyran) have entered phase I clinical trials (www.tballiance.org). PA-824 exhibits bactericidal activity against both actively growing and static *M. tuberculosis*. The potency of PA-824 is attributed to its ability to get activated by *M. tuberculosis* F420 cofactor and inhibit the synthesis of protein and cell wall lipid [126-128]. Diarylquinoline (R207910), another molecule reported by Johnson and Johnson Pharmaceutical Research and Development, has a unique spectrum of potent and selective anti-mycobacterial activity under *in vitro* conditions. The molecule is active against both drug-sensitive and drug-resistant *M. tuberculosis* and it was suggested that the drug targets the proton pump of adenosine triphosphate (ATP) synthase [129]. Plasma levels associated with efficacy of R207910 in mice were well tolerated in healthy human volunteers. Another drug, tetrahydrobenzothiophene (AX20017) developed by Axxima Pharmaceuticals AG, Germany, was shown to inhibit the kinase activity of PknG of *M. tuberculosis.* Chemical targeting of PknG led to the localization of *M. bovis* BCG into lysosomes causing bacterial lysis within macrophages [70]. AX20017 represents a promising candidate for the development of a class of drugs that would target the intracellularly residing mycobacteria.

In the past several years, it has been realized that controlling tuberculosis needs two issues to be addressed, drug resistance and persistence. A better understanding of the biology of tubercle bacilli, development in mycobacterial genetic tools, high throughput drug screening and structure based drug designing have increased the prospect of identifying novel anti-tubercle agents to combat drug

resistant and persistent organisms. In short, investigation of *M. tuberculosis* pathogenesis has entered a new era and it is anticipated that the global challenge of tuberculosis will be surmounted in near future.

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