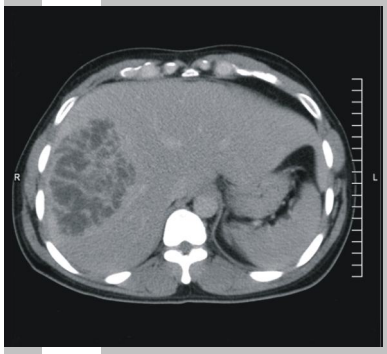
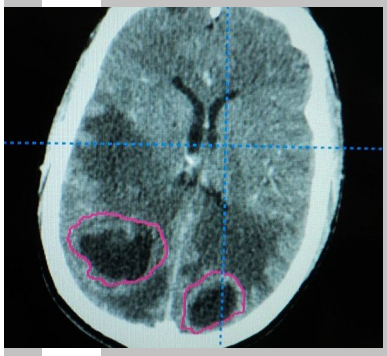
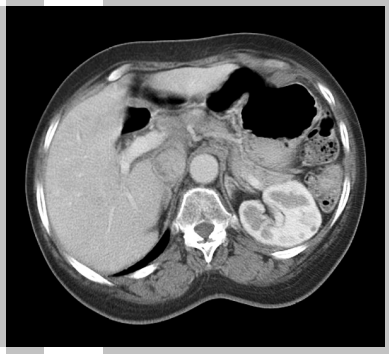
- Kurre Purna Nagasree
- Muthyala Murali Krishna Kumar

Antitubercular Drug Therapy Past, Present and Future







Antitubercular drugs, drug resistance, molecular targets , tuberculosis and *Mycobacterium tuberculosis*

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Preface

Tuberculosis is a unique, dreadful and debilitating disease. It is not just the patient or a single family but the entire society needs to be involved in the control of this relatively unyielding problem. This book is the result of our personal and professional experiences with tuberculosis. We were introduced to tuberculosis drug research by Prof. D. Sriram and Prof. P. Yogeeswari during our tenure at BITS, Pilani, Rajasthan, India and we are presently continuing it in Andhra University, Visakhapatnam, India.

This book is written in a very simple way to summarize important topics in antitubercular drug research in five chapters and can be followed by any science student. It explains the concepts of the disease pathogenesis, resistance problem and progress in anti-TB drug discovery.

The first chapter describes about the general introduction of *Mycobacterium tuberculosis*its pandemics and cell wall integrity. The second chapter deals with the history of existing antitubercular agents and their mechanisms. Third chapter gives a brief outline of clinical and preclinical agents under trials. Fourth chapter immensely emphasizes on the major problem of 'resistance' in terms of MDR and XDR and a brief discussion about the molecular basis of overcoming the resistance. The final fifth chapter focuses on the avenues for novel antitubercular drug developments.

This is the first precious book produced by us and through this writing we learnt many things. We would love to continue the task of book writing with utmost responsibility to create awareness and interest in readers through other subject themes also.

Acknowledgements

The authors are highly acknowledgeable to Prof. D. Venkata Rao and Prof. D. K. M. Lakshmi for their invaluable guidance in bringing a shape to this book and also for their consistent personal and professional support rendered during all these years. We thank Prof. V. S. Rao, Prof. D. Sriram, Prof. P. Yogeeswari and Prof. Y. Rajendra Prasad, for showering their blessings on us.

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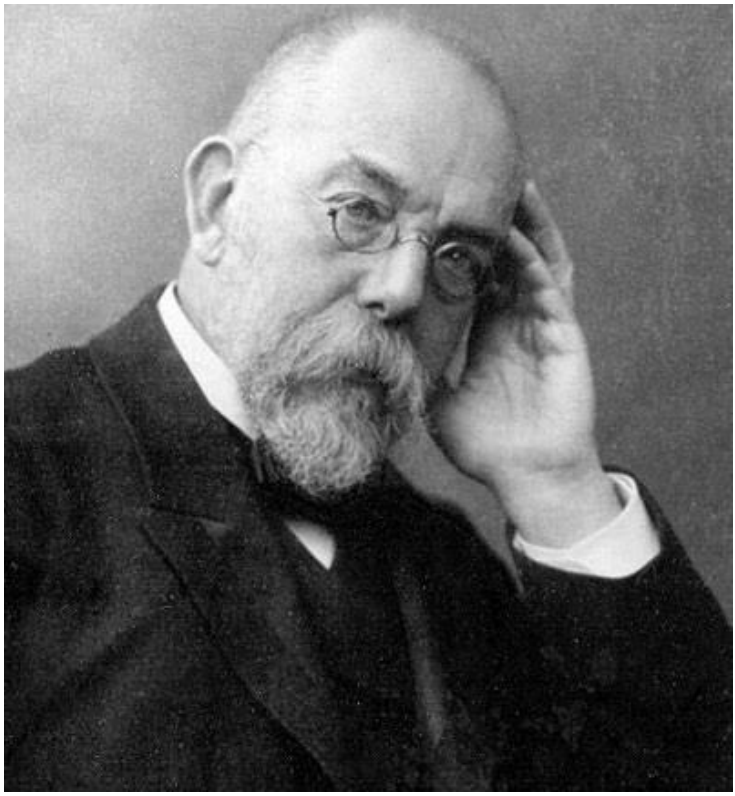

"If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings die from tuberculosis. If one only considers the productive middle-age groups, tuberculosis carries away one-third, and often more."

(Koch, 1882)

Contents

Chapter 1

Introduction

Tuberculosis (TB) is, perhaps one of the oldest known deadly diseases, caused by the bacillus *Mycobacterium tuberculosis*. Various cultures of the world gave the illness different names: *yaksma* (India), *phthisis* (Greek), *consumption* (Latin), *white plague* (Europe) and *chakyoncay* (Incan). Ancient medical literature pertaining to every civilization had references to this menace. *M. tuberculosis* is believed to kill more people annually than any other single organism on the face of the planet, and tuberculosis-related illnesses are the fourth most deadly type of infectious disease known to man. The disease attacks both man and animals, affects all ages and every organ in the body, and ranges from latent to hyperacute, killing young and old alike, the famous and the unknown. A single airborne droplet from cough or sneeze of a patient can infect others, if inhaled. Surprisingly, only 5% to 10% of these newly contacted people may ever develop symptoms. Another problem with this disease is its undefinitive manifestations which confuse even experienced physicians. Non-pulmonary forms of TB often remain undetected till it is fully blown. These unique features made TB virtually impossible to eradicate and qualified this dreadful disease to be declared a world emergency in the year 1993 by World Health Organization (WHO). As this disease becomes complicated, evasive and ubiquitous, it is important to understand what makes it so uniquely invincible.

1.1 History of Tuberculosis

Ancient Indian scriptures written in Sanskrit sometime between 1500 and 700 BC mention the first known description of tuberculous spondylitis. Conclusive evidences from the paleopathological studies on spinal columns of the Egyptian mummies have confirmed the possible presence of tuberculosis close to 5,000 years back.[1] For ages, nobody has any clue on the pathogenesis of this disease and it remained incurable till 20th century. It was considered more

like a slow death sentence to the very unfortunate patients.

Several hypotheses on pathogenesis of this disease evolved out of visionary thinking and meticulous observations. In 1720, the English doctor Benjamin Marten was the first to state that TB could be caused by "wonderfully minute living creatures." In 1839, Johann Lukas Schönlein labelled the disease "tuberculosis". In 1865, French military doctor Jean-Antoine Villemin demonstrated that TB could be passed from people to cattle and from cattle to rabbits by conducting an experiment in which tuberculous matter from human dead bodies was injected into laboratory rabbits which became infected.[2] Invention of the modern stethoscope by Rene Laennecin 1816, revolutionized diagnosis of TB.

1.1.1 Discovery of the Pathogen

Microscopic observation of pathogenic microorganisms dates back to 17th century. But the observations heavily depended on staining techniques. Mycobacterium remained unidentified till 1882, due its "stain proof" lipid-rich cell wall. The German physician and a Nobel Prize recipient, Robert Koch has identified *Mycobacterium tuberculosis* via acid-fast staining method.[3] In 1881, he began working on a tissue isolated from a deceased ape that had died of tuberculosis, isolating the rod-shaped bacteria by growing it in culture dishes separated from any other germs. He then inoculated healthy guinea pigs with the TB bacteria. The guinea pigs became sick and Koch observed the tuberculosis bacteria growing in them. He then removed some bacteria from the infected guinea pigs and grew them in yet another culture. Finally, he infected a second group of healthy animals with this cultured bacteria. When those animals contracted TB, he could be sure that the same bacteria were responsible. This involved procedure (known as *Koch's Postulates*) soon became the standard method for identification of disease-causing organisms. In 1890, Koch

discovered that a substance released from the TB bacilli caused an allergic reaction in people who have been exposed to TB. This substance, called tuberculin, is being used as a main component in diagnostic test kit for TB.

1.1.2 Tuberculosis: Global Scenario

Despite the advances in chemotherapy and the BCG (Bacillus Calmette-Guerin) vaccine, tuberculosis remains a major global threat. It is the second most prevalent infectious disease, next to HIV, in the world today than at any other time in human history. *M. tuberculosis* has an amazing ability to hide and hibernate by finding a 'safe house' inside our own tissues leading to development of latent form of the disease. But, this seemingly harmless reservoir of infected people can develop active disease at later stages of their life. They may even unknowingly transmit the disease to others, thus making it almost impossible to eradicate this scourge.

According to WHO statistics of 2012, a predicted number of 1.3 million people died with TB and around 8.6 million people newly contracted this disease (includes HIV positives also). The total resource requirements to combat TB and multi-drug-resistant TB (MDR-TB) are estimated to be US$ 4.8 billion each year over 2014-2016. The World Health Organization (WHO) and the Global Fund to Fight AIDS, TB and Malaria estimate that, 118 low and middle income countries are certainly eligible for funding from the Global fund, which totals to US$ 1.6 billion to bridge the funding gap of 2014-2016. Since 1995, 22 million lives were saved and 56 million people were successfully cured and there is a 45% decrease in the TB mortality since 1990. In spite of these achievements 3 million people are estimated to be infected every year and there is an alarming burden of MDR-TB crisis.[4] India stands alone as the highest weigh down country with 2.2 million active TB cases. In another estimate it was found that around

40% of the population contracted this germ and mostly in the latent form.[5]

1.2 Etiology and Pathophysiology

M. tuberculosis primarily invades the host through the pulmonary tract. As we know TB, the contagious microbes pass through airborne particles generated by an infected person's (especially with pulmonary or laryngeal TB) cough or sneeze or even a burst of laughter. It can also be transmitted by unpasteurized milk, as animals can be infected with the bacteria. Children nearly always contract the disease from an infected adult.

M. tuberculosis is an intracellular pathogen that establishes infection in oxygen-rich alveolar macrophages of the lung. The infection renders damaged parts of lung into a dry and cheese like tissue, which soon hardens into a scar tissue. The severity of the attack depends on whether the virulent form of bacterial infection spreads to other parts of the body or not. Tuberculosis infection in the blood, the meninges (membranes around the brain and spinal cord), and the kidneys are the most serious. Children between the ages of 6 and 24 months are the most susceptible to meningitis; it is the chief cause of tuberculosis death among children.

Once the bacteria invade the lungs, the body's immune system sends out white blood cells which build walls of fibers around the bacteria to keep them confined, forming small, hard lumps known as "tubercles." Once the body has formed tubercles to encapsulate the bacteria, the primary infection may be contained and, although the person will always test positive for the TB bacteria, the disease itself may not develop. Later in life, if the walls containing the germs are broken down, the lungs once again become infected. If the immune system is initially unsuccessful in walling off the germs, a full case of TB develops. The new bacilli

grow and multiply and the lung tissue actually dies and becomes soft. Liquid from the tissue is coughed up leaving a cavity in the lung. Cavities may have already formed before a person even notices symptoms such as a cough or fever. Eventually, however, coughing becomes painful and brings up blood with the lung tissue. By this time, the case is well advanced. If large areas of the lungs are damaged, breathing becomes difficult and the body fails to deliver necessary oxygen to tissues. The bacilli may spread to other tissues of the body causing secondary infections and complications, leading to extra-pulmonary tuberculosis. If treated with antibiotics and other drugs, the patient may recover, usually over a period of time.

1.2.1 *M. Tuberculosis* (MTB) Complex

The MTB complex consists of human and animal pathogens that are acid-alcohol fast bacilli. *M. tuberculosis* and seven very closely related mycobacterial species (*M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canetti* and *M. mungi*) together comprise MTB complex. MTB found in dogs, cats, pigs[6] and some wild animals infects human and non-human primates.[7] *Mycobacterium bovis* is an agent that causes bovine tuberculosis and infects a wide range of domestic and wild hosts. This strain is used for making BCG vaccine. Studies based on DNA homology have proved that there is a close evolutionary relationship and 95-100% DNA relatedness among the members of complex and suggests that these members belong to the same genus.[8]

M. tuberculosis is capable of affecting almost all parts of the body except hair and nail.[3] Apart from this, Mycobacterium includes more than 50 other species, often collectively referred to as non- tuberculous mycobacteria.

1.2.2 Structural Complexity of *M. Tuberculosis* Cell Wall

M. tuberculosis is a bacillus shielded by a unique, thick lipid-rich cell wall which differs from that of most other bacteria (Fig. 1.1). This forms a diffusion barrier, 100–1000 fold less permeable to hydrophilic molecules than that of *Escherichia coli*.[9] The cellular envelope is composed of a core of three macromolecules covalently linked to each other. They are peptidoglycan (PG), arabinogalactan (AG), and mycolic acids (long fatty acids i.e. C60-C90) and a lipopolysaccharide, lipoarabinomannan (LAM), which is thought to be anchored to the plasma membrane. The cross linked network of peptidoglycan constitutes the arabinogalactan, where few of the muramic acid residues are substituted with complex polysaccharide and in addition it is acylated at its distal end with mycolic acids to the peptidoglycan. The entire complex is abbreviated as the mAGP (mycolylarabinogalactan–peptidoglycan) and is essential for the viability in *M. tuberculosis* and other mycobacteria.[10]

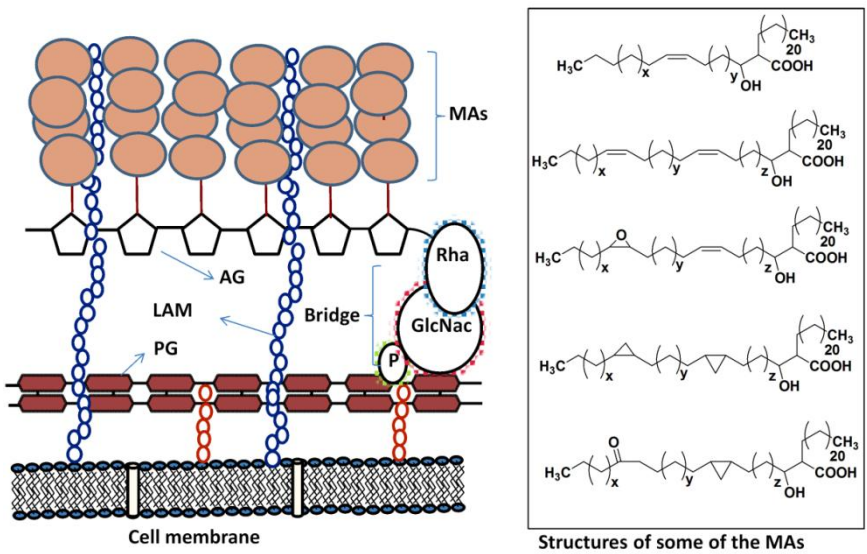

Fig. 1.1 *Cell envelope of M. tuberculosis with structural components.*

Mycolic acids are high molecular weight fatty acids carrying an ά-alkyl-β-hydroxy functionalities and bound typically as bundles of arabinogalactan. They appear primarily as tetramycolylpentaarbinosyl clusters, but also in extractable lipids mainly as trehalose 6, 6'- dimycolate (cord factor). The main part of the branched chain is called ''meromycolic acid'' and the other part ά-branch.[11] The characteristic features of these mycolic acids are that they are of the largest (C_{60}-C_{90}) and they have the largest ά -branch (C_{20}-C_{25}). The long chains are embedded with cyclopropane ring and double bond functionalities, which help maintain its constituency by producing 'kinks' in the molecules. In addition to β -hydroxyl group, it is assumed that the above groups might also contain oxygen functionality and the main carbon backbone may have methyl branches.[12]

The unusually complex mycobacterial cell wall contains many other macromolecules including LAM[13, 14], many extractable lipids, including glycolipids (glycopeptidolipids, GPL[15, 16]; lipooligosachharides, LOS[17, 18]; phenolic glycolipids, PGL [19-22]) and other classes of free lipids (sulpholipids, SL; phthioceroldimycocerosate, PDM).[23-26] LAM and arabinomannan exhibits a wide spectrum of immune regulatory functions including immune suppression, suppression of T-cell activation, inhibition of murine macrophages activation mediated through γ-interferon; cytotoxic oxygen free radical scavenging and protein kinase C activity inhibition. The glycopeptidolipids or phenolic glycolipids which are located either on the cell surface or outside the cell wall also prone to be involved in the generation of pathogenesis.[26] Cell walls containing carbohydrate layers also are indicted in virulence by averting non specific phagocytosis.

Mycolic acids are biosynthesized by Claisen type condensation and reduction of C_{16} fatty acids.[27] The four distinct steps involved in the biosynthesis include -

synthesis of C_{24}-C_{26} straight chain saturated fatty acids to provide C-1 and C-2 of the ά-alkyl chain; synthesis of the backbone of meromycolic acids of C_{40}-C_{60}; modification of meromycolic acids to introduce functional groups other than β-hydroxy and the final condensation step to provide mycolic acids. The role of several enzymes in the biosynthetic processes of mycolic acids were thoroughly studied at the gene level and can be used for development of effective antimycobacterial agents.[28-31]

The outer membrane of the mycobacterial cell wall is an important target for anti-mycobacterial agents, in particular the biosynthesis of cell wall components. The mycobacterial cell wall is very hydrophobic, resulting in an efficient barrier to a range of antimycobacterial agents.[32] Uptake of any drugs through the outer membrane requires the drugs to be lipophilic in nature although there is evidence of the presence of porin channels in the mycobacterial cell envelope through which both nutrients and drugs could diffuse.[33, 34] Currently TB is treated with agents that target mycolic acid biosynthesis including isoniazid (INH), inhibitors of nucleic acid biosynthesis such as rifampicin (RIF) which binds and inhibits mycobacterial DNA-dependent RNA polymerase, and the aminoglycoside antibiotic streptomycin (SM) which targets protein synthesis.[35]

The winning strategy of this humble microbe may now be attributed to its highly evolved chemical armory including unusually waxy cell wall, which could conceal most of the bacterial proteins from getting exposed to the host immune system and also release chemicals to modulate immune responses. The microbe also has developed several suave adaptation tactics to survive in varied environmental conditions including hypoxia, nutrient deprivation, exogenous stress conditions and intraphagosomal environment.[36] Though this lends a very complicated picture to comprehend, fortunately, unrelenting efforts by innumerable people around the world fructified and helped mankind overpower

this menace. The following chapters succinctly present all these invaluable accounts.

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Chapter 2

Chemotherapeutic Agents Used for Tuberculosis

2.1 History

Discovery of broad spectrum antimicrobials is perhaps one of the greatest achievements of scientific community in the 20th century. It is very difficult to imagine today's "relatively" healthy society with an average life expectancy of more than 65 years without these wonder drugs. Tuberculosis appeared invincible throughout human history. Attempts made by several generations of scientists, doctors and spiritual heads and their success or failure stories not only labelled TB as unique, but also helped evolve several aspects of antimicrobial chemotherapy from diagnosis to drug design&discovery.

Pre-Antibiotic ERA

It is evident from the history and primordial manuscripts that, consumptive disorders co-evolved with human race. One of the oldest ancient medical scripture "Ayurveda" written in Sanskrit (~2000 BC), mentioned about "Yakshma", a consumptive disorder with typical manifestations comparable to tuberculosis. There, concoctions of various herbal extracts containing Aswagandha, Asparagus, Pepper, Opium and Allium were suggested for the disease management. The therapy included using highly nutritious food, large quantities of milk, various types of meat and relaxation. Taking care of the patient to be in good humour is also necessary as depression was found to be an aggravating factor for TB.[1]

The role of rest in recuperating the tubercular lungs is well established. By 1930's, several sanatoriums were established throughout the world in serene environments to offer healthy air. The patients have also received sun bath therapy as sun rays were believed to cure this ailment, especially the skin lesions.[2] Cod liver oil rich in vitamin D, once sold as "liquid sun" was also used

in this treatment. In the early 20th century, usage of metals like gold, arsenic were suggested for treating infectious diseases. Surgical interventions to modulate lung function or removal of the severely damaged part proved successful in improving the health condition of many patients and are still in practice as an adjuvant therapy, especially in chronic MDR-TB cases.[3]

Throughout the history, several illustrated medical men including Susruta, Hippocrates and Galen pronounced that TB is incurable, infectious patients are to be isolated. But, 20th century saw the birth of "Chemotherapy" out of prudent thinking coupled with serendipity and luck to bring phenomenal changes in the management of TB.

2.2 Post Antibiotic ERA

Early observations of antimicrobial activity in chemical dyes by Paul Ehrlich, laid foundations for the development of synthetic antibacterials in the modern era. Domagk synthesized first ever antibacterial "prontosil". Further optimization of sulfonamide chemistry accidentally gave isonicotinic acid hydrazide, the most effective first line antitubercular agent ever discovered.[4] Later, structure activity relationship studies on nicotinamide derivatives resulted in another successful anti TB drug, pyrazinamide.[5] Careful observation of rate of oxygen uptake in tubercle bacillus under the influence of benzoates and salycilates by Berheim paved way for the discovery of another drug p-aminosalycilic acid (PAS). Lehmann of Sweden screened several salycilic acid derivatives and found PAS as a very effective anti-TB drug against "actively replicating" mycobacteria. This drug is nontoxic and soon became a very popular clinical agent.[6]

Accidental discovery of the antibiotic Penicillin from a fungus ascertained the idea of effective "chemical communication" by the microbes for protection of

their own turf from the invaders. This has led many academicians and pharmaceutical companies to explore the potential of microbes as a resource for bioactive secondary metabolites and thus began the golden era of antibiotics.[7] Mycobacterium, unfortunately is not sensitive to penicillin and many other natural or synthetic antibiotics. Selman Walksman of Rutgers University took microbial screening to a whole new level and obtained first ever antitubercular antibiotic Streptomycin from a soil sample.[8] Development of resistance to monotherapy of most of these drugs by early 1950's necessitated search for more effective anti-TB agents and optimized multi drug regimens. Though, many antibiotics possessed feeble anti-TB activity, none were found to be clinically useful. It is during 1960's, another remarkable anti tubercular antibiotic rifampicin entered into clinics. Discovery of important class of synthetic antibiotics, fluoroquinolones, in the early 1980's sealed the fate of TB forever. The uniqueness of challenges offered by anti-TB drug discovery is clearly evident from availability of a sparse 20 odd drugs for clinical use.

Chemotherapeutic agents for TB may be divided into two main classes, first line agents (isoniazid, rifampicin, streptomycin, pyrazinamide and ethambutol) and second line agents (ethionamide, *p*-aminosalicylic acid, cycloserine, rifapentine, clarithromycin, kanamycin, amikacin, ofloxacin, ciprofloxacin, viomycin and capreomycin). Current TB drugs and their targets were given in (Table 2.1).

Table 2.1 *Current Anti Tb Drugs and Their Targets.*

Drug (year of discovery)	Target	Effect
Group 1 TB drugs: First Line Oral Agents		
Isoniazid (1952)	Multiple targets including Acyl carrier protein reductase (InhA) and β-ketoacyl synthase (KasA)	Inhibits mycolic acid synthesis
Pyrazinamide (1954)	Disruption of membrane function and energy metabolism, Inhibition of fatty acid synthesis	Disruption of member function and energy metabolism, Inhibition of fatty acid synthesis, acidifies cytoplasm
Ethambutol (1961)	Arabinosyltransferases	Inhibits arabinogalactan biosynthesis
Rifampicin (1963)		
Rifabutin (1975), Rifapentin 1965)	RNA polymerase, beta subunit	Inhibits transcription
Group 2 TB drugs: Injectable Agents		
Streptomycin (1944)	S12 and 16S rRNA componentsof 30S ribosomal subunit	Inhibits protein synthesis
Kanamycin (1957)	30S ribosomal subunit	Inhibits protein synthesis
Capreomycin (1963)	Interbridge B2a between 30Sand 50S ribosomal subunits	Inhibits protein synthesis
Amikacin (1972)	30S ribosomal subunit	Inhibits protein synthesis
Group 3 TB drugs: Fluoroquinolones		
Ofloxacin (1980), levofloxacin (1983), moxifloxacin (1999)	DNA gyrase and DNA topoisomerase	Inhibits DNA supercoiling
Group 4 TB drugs: Oral Bacteriostatic Second Line Agents		
Para–aminosalicylic acid (1946)	Dihydropteroate synthase	Inhibits folate biosynthesis
Cycloserine (1952)	D-alanine racemase and ligase	Inhibits peptidoglycan synthesis
Terizidone (1952)	L-alanine racemase and D-alanine ligase	Inhibits peptidoglycan synthesis
Ethionamide (1956)	Enoyl-[acyl-carrier-protein] reductase	Inhibits mycolic acid biosynthesis
Protionamide (1956)		
Group 5 TB drugs: Agents with an unclear role in the treatment of drug resistant TB		
Clofazimine (1952)	Multiple mechanisms. Membrane destabilization, redox cycling	Membrane disruption, DNA damage
Linezolid (2000)	P site of the 50S ribosomal subunit	Inhibits protein synthesis
Amoxicillin/Clavulanate	Penicillin binding protein/β-lactamase	Inhibits cell wall synthesis
Thioacetazone (1952)	Cyclopropanemycolic acid synthases	Inhibits mycolic acid synthesis
Imipenem/cilastatin	Penicillin binding proteins/ renal dihydropeptidase.	Blocks cell wall synthesis/Cilastatin blocks imipenem metabolism
High dose isoniazid	Same as INH	Same as INH
Clarithromycin (1991)	50S ribosomal subunit, inhibits transfer of peptidyl t-RNA from A site to P site.	Inhibits protein synthesis

2.2.1 Streptomycin (SM) and Other Aminoglycoside Antibiotics

The first clinically used antitubercular antibiotic, streptomycin (Fig. 2.1), was isolated from the microbe *Streptomyces griseus*, by Albert Schatz and Selman Walksman in the year 1944, in their intense search for an antibiotic against gram –ve and other clinically important microbes. The general structure of the aminoglycosides is characterized by an aminocyclitol ring connected to one or more amino sugars by a glycosidic connection. This drug inhibits the translation of mRNA *via* interaction with the 30S ribosomal subunit. Mutations on the genes coding for the 16S rRNA and ribosomal protein S_{12} confers streptomycin resistance.[9, 10]

Resistance to streptomycin has become less common due to the wider use of ethambutol as the fourth drug in WHO standard treatment schedule. One of its derivatives, dihydrostreptomycin also showed anti-TB activities. It has an MIC value of 1 μg/mL with 50–60% plasma protein bound and a half-life of 5–7 hr. It penetrates the inner membrane of *M. tuberculosis* and binds to the 30S subunit of the ribosome.[11] Variety of synthetic derivatives of streptomycin have been synthesized and evaluated against *M. tuberculosis*.[12] Due to unwarranted toxic effects streptomycin and its derivativesare now largely replaced by other aminoglycoside antibiotics like kanamycin and amikacin used in anti-TB therapy as second-line agents.[13, 14]

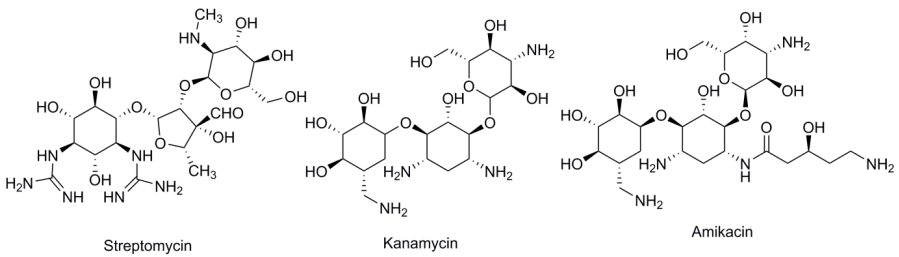

Fig. 2.1 *Clinically important aminoglycoside antibiotics.*

2.2.2 Isoxyl (Thiocarlide) & Thiacetazone

A number of diacylthioureas have shown significant anti Tb activity in experimental models. One such agent, 4, 4´-diisoamyloxydiphenylthiourea (4, 4´-diisoamyloxy diphenylthiocarbanitide, isoxyl, thiocarlide) [15-17] has proved to be clinically useful. In a study involving exposure of *M. bovis* to this drug revealed the mode of action of to be the inhibition of mycolic acid biosynthesis. Thiacetazone was discovered to have antitubercular activity in 1940s and was used as an antitubercular agent despite its toxic side effects.[18, 19]

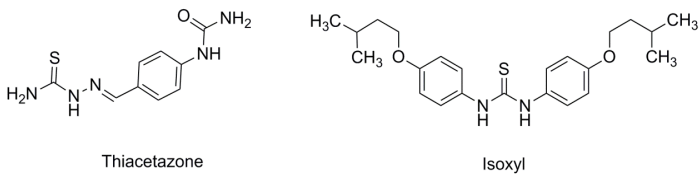

Fig. 2.2 *Thiourea containing Antitubercular drugs.*

Thiacetazone, similar to the thioisonicotinamides, is activated by EthA resulting in a reactive intermediate that inhibits mycolic acid oxygenation as well as cyclopropanation.[20, 21] Thiacetazone causes gastrointestinal disturbances and particularly in HIV-infected patients, can cause severe life-threatening skin reactions known as Stevens–Johnson syndrome.[22]

2.2.3 Isoniazid (INH)

Antitubercular activity of isoniazid (isonicotinicacidhydrazide, INH) was discovered accidentally in the year 1952, while screening synthetic products obtained from SAR studies of an anti-TB agent, thiacetazone (Fig. 2.3). INH revolutionized the TB treatment after its entry in the year 1952 and is still considered the best synthetic anti-TB drug ever discovered. This agent is orally effective, inexpensive, free from toxicity and highly active in both acidic and

basic conditions. INH is a specific antimycobacterial agent as it lacks inhibitory activity on other microbes. It acts by selectively inhibiting the synthesis of mycolic acids essential for mycobacterial cell wall. INH is a prodrug and gets activated by mycobacterial catalase-peroxidase (*katG*), which transforms the drug into a nucleophilic radical. Reaction of this radical, with the cofactor NAD^+, yields a potent inhibitor of enoyl- ACP (acyl carrying protein) reductase. This particular enzyme is essential for the mycolic acid synthesis of bacterial cell wall. The enormous class of mutations in INH-resistant *M. tuberculosis* map to a gene which encodes the catalase-peroxidase.[23] INH is orally active and shows bacteriostatic action on resting bacilli and is highly active against the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. microti* and *M. africanum*,). It has very low MICs (0.02–0.06 μg/mL) against these pathogens.[24] INH enters the organism by diffusion and oxygen-dependent active transport, and this diffusion and active transport was reported to have an effect on almost every aspect of mycobacterial metabolism.[25] A large number of compounds related to INH have been synthesized and evaluated against *M. tuberculosis* $H_{37}Rv$. Anti-TB drugs like Ethionamide and PZA are the result of these research works. This drug has a few limitations including its ineffectiveness against dormant bacteria and some unwanted effects like peripheral neuritis and liver problems.

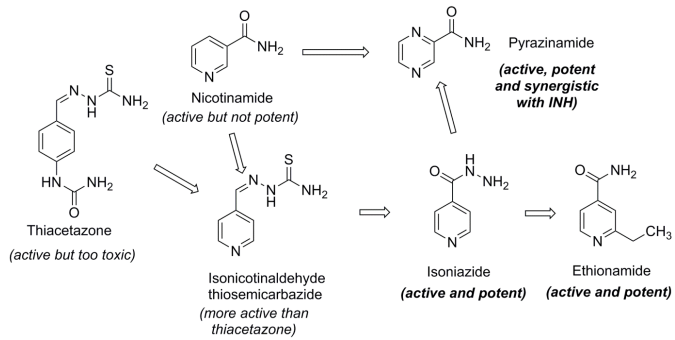

Fig. 2.3 *Development of INH and pyrazinamide.*

2.2.4 Pyrazinamide (PZA)

This, a structural analogue of nicotinamide, is a first-line drug of short course TB therapy. It is also active against semi-dormant bacilli not affected by any other drug. It has strong synergy with INH and RMP and shortens the therapy period to 6 months.[26, 27] PZA likely kills MTB by intracellular acidification following hydrolysis by Mtb-nicotinamidase/pyrazinamidase,[28] although inhibition of fatty acid synthase has also been proposed as a mechanism.[29-31] The activity of PZA depends on the presence of bacterial amidase that converts PZA to pyrazinoic acid, which is an active anti-TB molecule; this pyrazinoic acid conversion is highly prcise to *M. tuberculosis*. Mutation in the *pncA* gene is responsible for the production of pyrazinamidase and is shown to be the reason for resistance against this drug.[26, 32, 33] Interestingly, some pyrazinoic esters were also reported to possess good antitubercular activities.[34] Extensive SAR studies were conducted on this drug to ultimately find that the structural features like pyrazine ring, amide group, position of amide group are essential for bioactivity. Any change in these may either significantly reduce or nullify bioactivity.[35-38]

2.2.5 *P*-Aminosalicylic Acid (PAS)

The anti-mycobacterial activity of PAS was first reported in 1946, although it was synthesized long before.[39] It is a highly specific and effective inhibitor of *M. tuberculosis*.[40] Follow up of DOTS (Directly Observed Treatment, Shortcourse), is hardly ever used today. However, it is seldom used in the regimens for the treatment of TB caused by MDR-TB. The mechanism of action of this drug is still unclear, but it was suggested that it interferes with the salicylate-dependent biosynthesis of the iron chelating mycobactins involved in iron assimilation.[41]

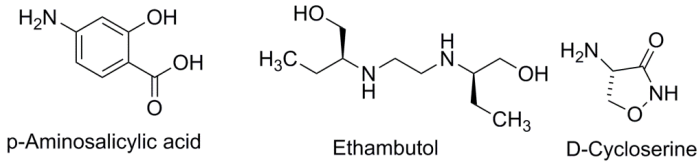

Fig. 2.4 *Structures of PAS, EMB and cycloserine.*

2.2.6 Ethambutol (EMB)

Significant anti TB activity was first observed in diisopropylethylenediamine, while screening an assortment of chemical compounds for antimicrobial activity at Lederle Laboratories in the early 1950s. Structure modification studies of this compound resulted in EMB as a potential antitubercular agent in the year 1961. It is a synthetic amino alcohol (ethylene diamino-di-1-butanol), orally effective bacteriostatic agent that is active against most strains of mycobacterium.[42-44] Structural requirements for antitubercular activity of EMB are very rigid. Several QSAR studies conducted on EMB confirmed that the ethylene-diamine unit is the minimum pharmacophore required for antitubercular activity. Any alterations in the linker region of the molecule including lengthening, incorporation of heteroatoms, or branching of the ethylene linker led to reduced activity.[45] The nitrogens must be replaced very carefully, as any change in the basicity of either amino group led to decreased antimycobacterial activity.[46, 47]

The proposed site of action of EMB is ranged from trehalosedimycolate, mycolate and glucose metabolism to spermidine biosynthesis. The critical target for EMB lies in the pathway for the biosynthesis of cell wall arabinogalactan. It inhibits arabinosyltransferase, responsible for the polymerisation of arabinose into the arabinan of arabinogalactan. Disturbing the biosynthesis of arabinogalactan would destroy the macromolecular assembly of the mycolyl-arabinogalactan-peptidoglycan complex ofthe cell wall, permitting

drugs with intracellular targets (such as rifampicin) to enter the cell without any hardships. EMB resistant *M. tuberculosis* strains carry mutations in one certain part of the gene encoding for arabinosyltransferase. In the case of MDR-TB, if there is still a consistent susceptibility, EMB might be a valuable drug for preventing the emergence of resistance with other active drugs.[48, 49]

2.2.7 Cycloserine

It is a structural analogue of the amino acid D-alanine, produced by *Streptomyces* sp. Cycloserine possesses activity against a wide range of bacteria [50] and inhibits *M. tuberculosis*at concentrations of 5–20 μg/mL. It obstructs peptidoglycan biosynthesis by inhibiting the enzymes D-alanine racemase by forming an irreversible isoxazole-pyridoxal adduct.[51] It also inhibits D-alaninyl alanine synthetase involved in synthesis of the terminal D-alanine–D-alanine of the peptidoglycan UDP-N-acetylmuramyl-pentapeptide.[52] When cyloserine was used in treating microorganisms, they accumulate a muramic-uridine -nucleotide-peptide, which differs from that produced by mycobacteria in the absence of terminal D-alanine dipeptide.[54, 54] As the bioactivity is highly conserved in the chemistry and stereochemistry of cycloserine, no novel synthetic derivative could be found with better activity.[55, 56] Cycloserine produces side effects in the central nervous system that can also generate psychotic states with suicidal tendencies and epileptic convulsions and hence used mostly in lower concentration as a second line anti-TB drug.

2.2.8 Rifampicin (RMP)

Rifamycins comprise of a complex mixture of novel antibiotics isolated from the microbe *Amycolatopsis mediterranei*. These compounds possess a highly unusual ansamycin skeleton containing a hydroxyl naphthalene core and a

19 atom polyketide ring (Fig. 2.5). Extensive QSAR studies were performed on rifampicin and found that the position and substitution of the aliphatic bridge is very critical in stabilizing the overall conformation of the molecule and positioning the phenolic –OH at C-1 and C-8 and the aliphatic –OH at C-21 and C-23 for optimal inhibition of their bacterial target, RNA polymerase.[57] It binds to a pocket of RNA polymerase within the DNA/RNA channel, but greater than 12 A˚ away from the active site. It acts by directly blocking the path of elongating RNA when the transcript becomes 2 to 3 nucleotides in length. RMP is effective against *M. tuberculosis* with MIC ranging from 0.1-0.2 μg/mL. As it diffuses freely into tissues, living cells, and bacteria, it is extremely effective against intracellular pathogens like *M. tuberculosis*. However, bacteria develop resistance to rifampicin with high frequency. Mutations conferring rifampicin resistance map almost exclusively to the *rpo B* gene that encodes the RNA polymerase *β*- subunit.[58] Resistance may also occur through ADP-ribosylation of the alcohol at position C(21).[59] However, a combination of INH and RMP may increase a risk of hepatotoxicity.

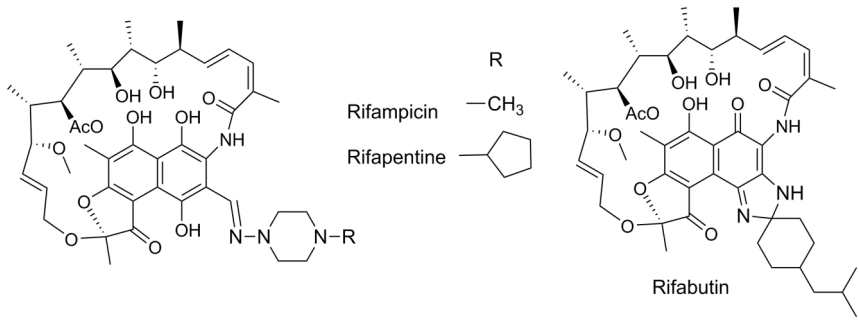

Fig. 2.5 *Rifampicin and its analogues used in anti TB therapy.*

Rifapentine was obtained from rifampicin by replacing the methyl on piperazine by a cyclopentyl unit. This is a first line anti-TB agent recommended by WHO and approved by FDA for pulmonary TB treatment in 1998. Rifapentine is a long-acting derivative of rifampicin and the drug is taken just once or twice

weekly by patients. Adding up, clinical studies also demonstrated that rifapentine could potentially shorten the current six-month treatment regimen for latent TB.[60] In contrast, once-weekly rifapentine and isoniazid treatment administered under direct observation showed a comparable effectiveness and a higher treatment completion rate compared with a 9-month isoniazid therapy regimen for the treatment of latent TB infection.[61] However, a major drawback of rifamycins is that they induce cytochrome P450 enzymes in the liver, which lead to drug–drug interactions with antiHIV agents (particularly protease inhibitors) and other TB drug candidates such as bedaquiline.[62] Hence, there is a significant interest in developing rifamycin-free regimens.[63, 64]

2.2.9 Fluoroquinolones

Serendipitous discovery of antibacterial activity in nalidixic acid, an impurity obtained during synthesis of chloroquine analogues in the year 1962, revolutionized antibacterial chemotherapy.[65, 66] Fluoroquinolones (Fig. 2.6), synthetic derivatives of nalidixic acid, display broad-spectrum antimycobacterial activity.[67-69] Ciprofloxacin, Ofloxacin and Moxifloxacin are used as second line anti- TB agents in the treatment of MDR TB patients. Structural modifications of fluoroquinolones (FQs) to optimize antimycobacterial activity have been extensively carried out to produce candidates that are more efficacious than earlier FQs. Their bactericidal effects involve an interaction of the drugs with DNA-gyrase and DNA-topoisomerase IV leading to altered DNA topology and cell death.[70] But the genome sequence of Mtb was found to be devoid of topoisomerase IV [71] thus making DNA gyrase as the primary target for its antitubercular activity. Excellent oral availability and diffusion of drug into all critical body compartments including CNS (Central Nervous System) made this a valuable drug in treating different forms of tuberculosis. Though these drugs are well tolerated, causing mild side effects that tend to be self-limiting and rarely

require discontinuation or regimen changes.[72] The most frequent adverse events reported include: gastro-intestinal upset, disturbances of the CNS and skin reactions.[73, 74] Some of the serious side effects including tendonitis and tendon rupture due to collagen damage, have been reported for FQs. Though rare, hepatotoxicity, kidney and liver dysfunction, and dysglycemia were also reported.[73] A few combinations with Gatifloxacin and Moxifloxacin with other anti-TB drugs are undergoing clinical trials as an attempt to optimize therapeutic regimen for shortening therapy to less than 4 months.[75]

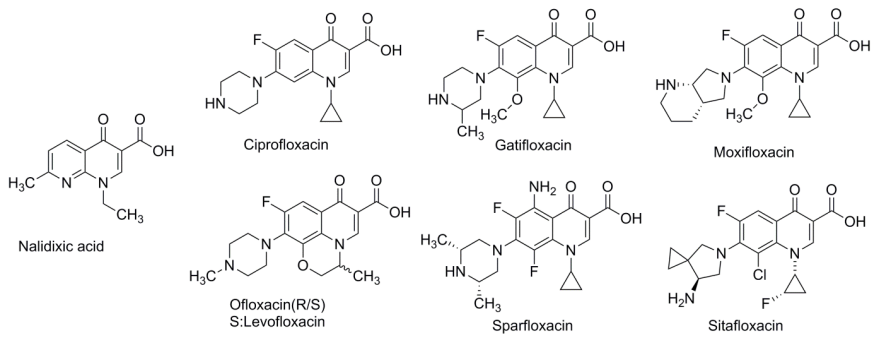

Fig. 2.6 *Clinically useful fluoroquinolones.*

2.3 Conclusions

With its unusually lipid rich cell wall and very quick adaptability makes Mycobacterium a very tough target to aim and hit. Last century saw immense progress in our understanding of this disease, biochemistry of the pathogen and its countless tricks. Though it appeared to be limited by the end of 1980's, but TB resurfaced in the early 1990's due to increase in HIV infection and much graver MDRTB form. Though very strict FDA norms and costlier clinical trials posed a complex picture, sustained efforts by the international community resulted in a few remarkable success stories, which are briefly discussed in the next chapter.

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Chapter 3

New Drugs for Treating Tuberculosis in the Clinics and Clinical Trials - An Update

3.1 Drugs in Discovery and Development Stages

Chemotherapy for tuberculosis presents a very unique set of problems to both doctors and patients. The treatment usually involves multidrug therapy with more than 2 drugs for duration of 3 to 18 months. The patient consumes over 300 pills in the course of therapy. If the patient complies, this regimen offers curative therapy. Failure to do so leads to either treatment failure and/or development of drug resistance. Though implementation of DOTS (Directly Observed Treatment Short course) increased the success rate of drug susceptible TB therapy, lack of a good treatment regimen still remained a major barrier to the scale up of access to treatment.[1]

Ideally, every anti TB drug discovery program looks for a molecule with novel mechanism of action so that the molecule can be used against bacteria resistant to existing drugs. Potency and optimal pharmacokinetics are essential for reducing the treatment duration as well as the pill burden. Compounds with selective inhibitory profile, less drug-drug interactions and increased safety window are preferred.[2]

Increase in the involvement of governments and other agencies and increased financial burden of clinical trials made many industries to wean away from this category, which apparently resulted in scarce novel clinical agents for the treatment of multidrug and extensively drug-resistant tuberculosis (MDR/XDRTB). In the year 2000, the Global Alliance for TB Drug Development (GATB) [3] was established with an objective to develop new agents that will shorten the duration of chemotherapy from the current 6–8 months to two months or less, although new drugs with activity against MDR-TB and latent TB are also needed. These efforts resulted in the discovery

of sizeable number of molecules with novel mechanism of action and clinical efficacy in the last decade. They were in various stages of clinical trials and a few potential candidates successfully made it to the market.[4, 5]

Fundamental uncertainties in many aspects of the biology of the organism have substantially hampered the ability to identify critical targets whose inhibition would correlate with sterilising activity. Sterilizing activity refers to the ability of a drug (such as pyrazinamide or rifampicin) to kill those organisms, known as 'persisters', that survive treatment with agents targeting essential processes in dividing bacteria. It is only by discovering new agents with improved sterilising activity that a shorter treatment regimen can be developed.

The past decade has seen intensive efforts to discover and develop new drugs to treat drug-susceptible-, MDR- and XDR-TB, and new combination regimens are also being devised and tested in clinical trials (Fig. 3.1; Table 3.1). New regimens will most likely employ a combination of repurposed drugs and new chemical entities (NCE) and there is a real likelihood that these regimens may contain none of the drugs previously used in TB treatment.

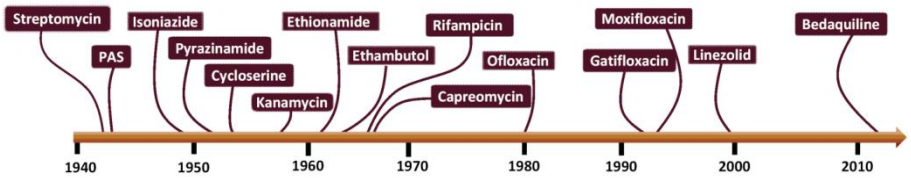


Fig. 3.1 *Timeline scale of anti TB drugs. Bedaquiline (2013) is the only novel drug approved for treating TB after rifampicin (1963).*

Table 3.1 *Global TB Drug Pipeline*.*

Lead Optimization	Early Stage Development	GLP Tox	Phase I	Phase II	Phase III
• Cyclopeptides • Diarylquinoline • DprE inhibitors • InhA inhibitor • LeuRS inhibitor • Macrolide • Mycobacterial gyrase inhibitors • Pyrazinamide analogues • Spectinamides • Translocase-1 inhibitors • Ruthenium complexes	• CPZEN-45 • DC-159a • Q203 • SQ609 • SQ641 • TBI-166	PBTZ169 TBA-354 BTZ043		• AZD5847 • Bedaquiline • Linezolid • PA-824 • Rifapentine • SQ-109 • Sutezolid **Novel regimens#** • J-M-Pa-Z • M-Pa-Z • C-J-Pa-Z • H-R-Z-E-Q-M	• Delaminid • Gatifloxacin • Moxifloxacin • Rifapentine

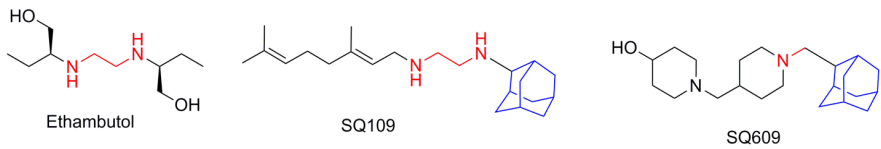
J-Bedaquiline; M-Moxifloxacin; Pa- PA-824; Z- Pyrazinamide; C-Clofazimine; H- Isoniazid; R-Rifampicin; E- Ethambutol; Q- SQ109

* Updated information can be obtained from http://www.newtbdrugs.org/pipeline.php and http://www.newtbdrugs.org/pipeline-discovery.php

3.1.1 Diamines (SQ109)

Ethambutol (EMB) is a very important first line drug for TB therapy. It has a very unusual mycobacterial specific inhibitory activity. But mycobacteria were found to develop resistance to this drug very quickly. Hence, Protopopova and his team working at Sequella Inc., in collaboration with NIH/NIAID prepared a huge combinatorial library of EMB analogues and screened for anti TB activity.[6] Out of 26 active compounds SQ109 exhibited very potent activity in broth micro-dilution assay (MIC = 0.2 mM). SQ109 is currently in Phase IIa trials. Interesting feature of SQ109 is its activity against strains resistant to isoniazid, rifampicin and ethamutol.[7] It has synergistic activity with most of the major front line antiTB agents.[8] This drug has multitarget inhibition which ultimately results

in mycobacterial cell wall damage (Fig. 3.2). In an attempt to define the target of SQ109 and its possible mechanism of action, a proteomic study of the effects of SQ109, EMB and Isoniazid was performed. The effects on ESAT-6/CFP-10 expression were more pronounced for isoniazid and ethambutol but equal for all three compounds in the case of AhpC.[9] Surprisingly SQ109 did not affect *Emb*A and *Emb*B, the target proteins for Ethambutol. The primary target of SQ109 was only recently identified as MmpL3, a transmembrane transporter of trehalosemonomycolate.[10]



Further studies on the ethylenediamine scaffold demonstrated SQ609 to be the most promising compound of this class.[11] It showed good in vitro activity against clinical isolates of *M. tuberculosis*. In a 20-day *M. tuberculosis*-induced weight-lossmouse model, SQ609 successfully restored normal health, improved survival rate and prolonged the therapeutic effect following drug withdrawal for another 10-15 days.

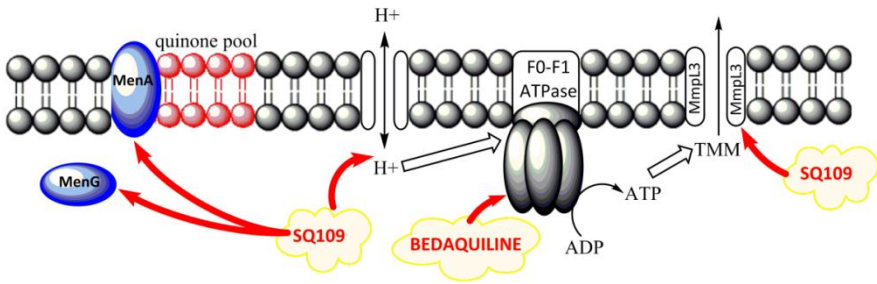
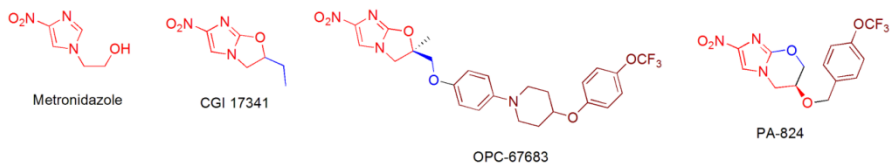


Fig. 3.2 *Mechanism of action of SQ109 and Bedaquiline.*

3.1.2 Nitroimidazofurans and Nitroimidazopyrans



The nitroheterocyclic compounds like nitroimidazoles, nitrofurans and nitrothiazoles are a very important class of antibacterial agents extremely useful to treat infections due to anaerobic bacteria and protozoa. Azomycin (2-nitroimidazole), a relatively rare antibiotic produced by *Nocardiamesenterica* [12] and *Streptomyces eurocidicus*.[13] Inspired by its novel mechanism and activity against pathogenic microbes, scientists at Rhone-Poulenc synthesized more potent and less toxic 5-nitroimidazole derivatives. One of their compounds, registered by the name metronidazole, eventually became a very important antimicrobial agent.[14, 15]

These are prodrugs and activated by metabolism and were shown to exert antibacterial activity via multiple mechanisms. In the bacteria, low-redox-potential electron transfer enzymes like Pyruvate: ferredoxin-oxidoreductase normally generates adenosine triphosphate (ATP) via oxidative decarboxylation of pyruvate (Fig. 3.3). The nitro group present in metronidazole acts as an electron trap, retaining the electrons that will be generally transferred to hydrogen ions in the cycle and produce a reactive anion; while the metronidazole stays in the cellular environment. Reduction of metronidazole generates a concentration gradient that drives uptake of more drug and encourages the formation of intermediate compounds and free radicals that are toxic to the cell. These reactive intermediates interact with nuclear material resulting in disruption of DNA and inhibition of nucleic acid/protein synthesis. These compounds doesn’t react with mitochondria containing pyruvate reductase rich cells like

aerobic microbes and human cells, hence show very good safety index.[16-18]

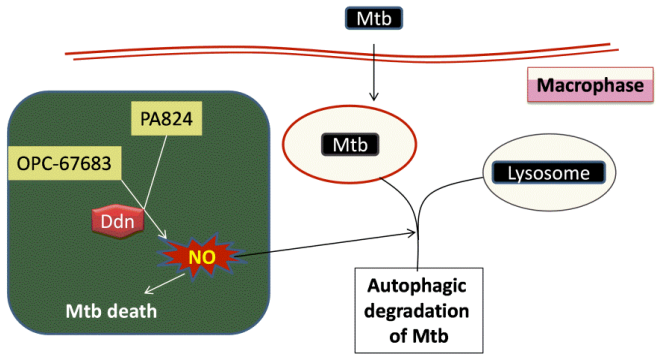


Fig. 3.3 *Mechanism of action of PA824 and Delaminid (OPC-67683).*

In pulmonary tuberculosis, oxygen concentrations are low inside granulomas and these structures are thought to contain an anaerobic environment. It is hypothesized that the Mtb residing in tubercles especially in metabolically inactive and latent forms behave in the same way as anaerobic microbes and hence the nitroimidazoles may be useful in treating tuberculosis. The promising *in vitro* anti TB results obtained for these compounds inspired many to synthesize nitroheterocycles and often succeeded in this approach.[19-21]

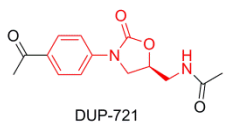
OPC-67683 (Delaminid, Deltyba®) a nitroimidazooxazole derivative recently received conditional approval in April 2014 by European Medical Agency for treating MDR-TB.[22] This drug is in its advanced phase II clinical trials in US. It has a mechanism similar to metronidazole and gets activated by the enzyme deazaflavin dependent nitroreductase (Rv3547). This results in a reactive intermediate metabolite, formed between delamanid and its desnitro-imidazooxazole derivative, which is considered to play a vital role in the inhibition of methoxymycolic acid and ketomycolic acid production. This compound showed significant selective toxicity to *Mycobacterium* and free from mutagenic problems common to nitroimidazoles. Apart from gastric disturbances,

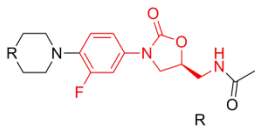
this drug suffers from cardiac side effects like prolongation of QT interval and patients need monitoring for cardiac arrhythmias.

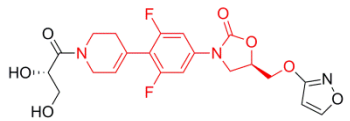
PA-824 (Pretomanid) is a nitroimidazooxazine derivative. This drug in combination with moxifloxacin and pyrazinamide (PaMZ) is advancing to Phase III clinical trials for treating MDR-TB.[22] This is a selective anti-tubercular agent with MIC ranging from 0.015 to 0.25μg/mL and has no appreciable inhibitory activity against Gram+ve or –ve bacteria. PA-824 has activity against drug susceptible, MDR/XDR strains and acts synergistically with other anti TB drugs indicating a novel mechanism for this compound.[23] Similar to delaminid, this drug is also activated by deazaflavin (F420)-dependent nitroreductase and releases reactive nitrogen species, including nitric oxide.

Nitric oxide gas is produced naturally by specific immune cells after they swallow up TB bacteria; this is one way the body fights against TB. But this immune response sometimes might not be sufficient to eliminate an infection. PA-824 mimics the body's natural immune response, but it is more precise and only releases the gas upon entering into the TB bacteria. The released nitric oxide may signify the important effectors of PA-824 killing of *M. tuberculosis* under hypoxic conditions.[25] This also affects the mycobacterial respiratory apparatus and significantly reduces the intracellular ATP levels. This drug also inhibits biosynthesis of essential cell wall lipids ketomycolates from hydroxymycolate and also inhibits protein synthesis but nucleic acid synthesis remained unaffected.[26]

3.1.3 Oxazolidinones







Oxazolidinones, exemplified by Dup - 721, are totally synthetic, orally active antibacterial agents discovered by DuPont. These compounds prevent the initiation of protein synthesis by binding to 23S RNA in the 50S ribosomal subunit of bacteria.[27-33] Linezolid, a first-generation oxazolidinoneandthe only new synthetic antibacterial agent approved after fluoroquinolones, has shown promising results to treat MDR/XDR-TB. However, the noteworthy toxicities, such as peripheral neuropathy and myelo-suppression, could limit the long-term use of this drug.[34]

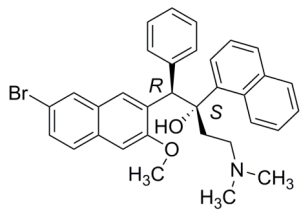
Sutezolide (PNU-100480), a thiomorpholinyl analogue of linezolid was developed recently and found to be more potent than linezolid against *M. tuberculosis* in a murine model.[35] Here, it was found that sutezolid shortens standard treatment by one month, whereas linezolid does not;[36] in the whole blood culture model, the maximal bactericidal activity of sutezolid (-0.42 log/day) is more than twice that of linezolid (-0.16 log/day, P, 0.001).[37] This drug is in later stages of Phase II clinical trials. Combination studies have been performed in whole-blood assays and these showed that sutezolid and TMC207 or SQ109 had additive effects, whereas those including PA-824 were having less than additive or antagonistic effects.[38-40]

AZD5847 is a next generation oxazolidinone developed by AstraZeneca and has recently entered Phase II clinical trials. It is a bactericidal and act synergistically with other anti TB agents.[41] This drug is safer than other

oxazolidines and effective against slow growing and intracellular mycobacteria. Oxazolidines offer promising addition to combination regimes to treat drug resistant TB.

3.1.4 Diarylquinolines (TMC207, SIRTURO^{TM})

Bedaquiline (R207910; SIRTURO^{TM}) a Janssen Pharmaceutica product, is the only novel anti TB drug approved in the last 40 years. It has received conditional approval on 20th December 2013 by European Union for use in adults with drug resistant tuberculosis and marketed by Tibotec and the TB alliance.[42, 43] This drug has extraordinary activity against both drug susceptible and drug-resistant strains of *M. tuberculosis*. It exhibits an impressive MIC value of 30–120 ng/mL, similar to or better than isoniazid and rifampicin. Interestingly, this drug rapidly kills the pathogen *in vitro* at a rate of 3 log orders of CFU (Colony Forming Units)/mL in 12 days.



Bedaquiline (TMC207; SIRTURO™)

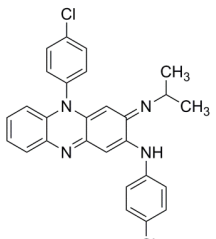
After the drug had been in development for over 6 years and a clinical trial of 47 patients showed that it is effective in the treatment of *M. tuberculosis*.[43] This drug also has shown its efficacy against *Mycobacterium leprae*, the causative agent of leprosy, in a mouse model of the disease.[44] It is found that this drug inhibits the membrane-bound F1-FoATP synthase complex results in depletion of cellular ATP levels and eventual death of the organism. Treatment of whole cells with the drug reduces ATP concentrations even in isolated vesicles.[45, 46] However,

safety concerns of this drug still remain because of an increased risk of death and QT prolongation.[47, 48]

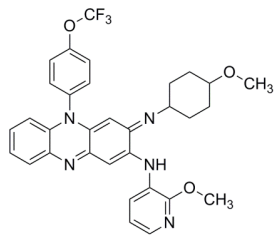
3.2 Preclinical Agents

3.2.1 Clofazimine and its Analogues

Clofazimine (CFM) is one of the oldest drugs synthesized for treating tuberculosis. It has an iminophenazine skeleton and highly lipophilic in nature. Unclear efficacy and unfavourable properties such as accumulation in fat tissues, long half-life and skin discoloration led to its discontinuation as an anti TB agent. But its activity against MDR TB,[49] the intracellular accumulation in mononuclear phagocytic cells, anti-inflammatory activity, a low frequency of drug resistance and slow metabolic elimination rate, made clofazimine an attractive lead molecule for development of newer anti TB agents.[50-52] An observation study done among 206 patients, on the effectiveness of standardized regimens for MDR-TB showed that a clofazimine-containing regimen consisting of gatifloxacin, ethambutol, and pyrazinamide achieved a relapse-free cure rate of 88%.[53]



Clofazimine

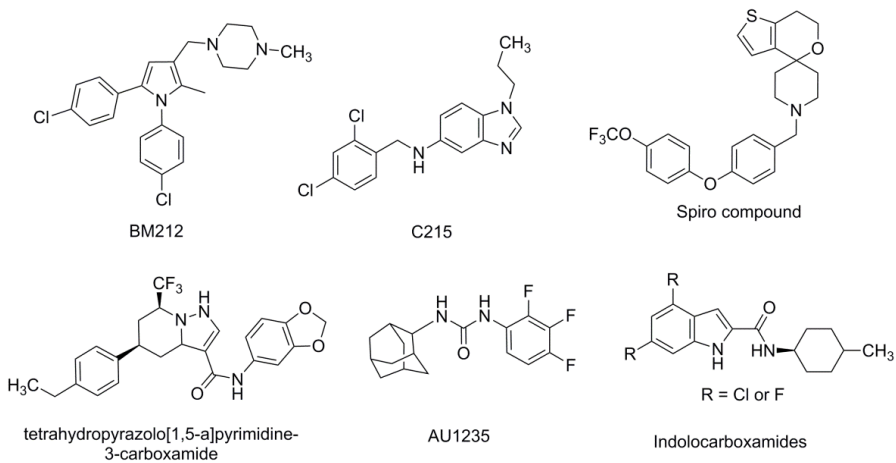


TBI-166

In a recent murine study, a CFM-containing regimen resulted in a reduced bacillary load after 2 months of treatment and negative conversion after 5 months of treatment.[54] CFM is currently used for treating MDR and XDR

patients when other choices are not available.[55] A lead optimization effort gave an important preclinical candidate TBI-166 with greater safety profile and the retained antimycobacterial activity. The mechanism of action of this compound class is still unclear but a recent study suggests that CFM is reduced by the mycobacterial enzyme NADH-quinoneoxidoreductase typeII (NDH-2) and then, spontaneous reoxydation of the reduced CFM by oxygen, is likely to produce reactive-oxygen species (ROS), probably O_2-Clofazimine reduction involves nitrogen groups on the phenazine ring together with the imino substituent and the high levels of ROS generation may provide intracellular concentrations needed for cell death.[56] It has also been suggested that CFM may act bybinding both the guanine base of DNAand stimulating phospholipase A2, which could explain its anti-inflammatory and immune-stimulating properties.

3.2.2 Diarylpyrrole Derivatives



BM212 is a novel diarylpyrrole antitubercular agent. This molecule has excellent *invitro* inhibitory profile against several clinically important mycobacteria including drug resistant and intracellular Mtb. This has an added

activity against several species of yeasts, including *Candida albicans* and *Cryptococcus neoformans.*[57] The later property is of immense use for treating immune compromised or HIV co-infected TB patients, where incidence of opportunistic infections caused by *Candida* sp. is common. Preliminary studies have confirmed that BM212 is a potent inhibitor of MmpL3.[58] MmpL3 is a putative membrane protein belonging to the RND protein family of multidrug resistance pumps that mediate the transport of a diverse array of ionic or neutral compounds as well as heavy metals and fatty acids.[59] It is surprising to notice structurally diverse group of compounds including SQ109[10], C215[65], tetrahydropyrazolo [1, 5-a] pyrimidine-3-carboxamides, [66] some indolcarboxamides [67] and AU1235 [68] showed significant anti-tubercular activity and target primarily MmpL3. These results clearly indicate that MmpL3 is a very susceptible target amenable to drug design.

3.2.3 BTZ043 and its Analogues

BTZ043 is a member of novel benzothiazinine class of antitubercular agents discovered in the year 2009.[69, 70] This compound showed an extraordinary MIC of 1ng/mL against Mtb, several folds better than existing drugs. This enzyme produces the sole source of the D-arabinose required for biosynthesis of the key cell wall components arabinogalactan and lipoarabinomannan. BTZ043 serves as a suicide substrate (Fig. 3.4) for the reduced form of decaprenyl-phosphoribose 2′-oxidase (DprE1). BTZ043 undergoes nitro reduction to yield a nitroso species that specifically attacks the thiol side chain of the active site cysteine residue Cys387 of DprE1, thereby forming semimercaptal covalent adduct and irreversibly inactivates the enzyme.[74]

Very recently, a second generation of 2-piperazino-benzothiazinones (PBTZs) were synthesized in order to improve pharmacological properties.[75] Among

several synthesized compounds, alkyl-PTBZs were found to be more active *in vitro* than BTZ043 and displayed MIC values against *M. tuberculosis* H37Rv rangingfrom 0.00019 to 0.00075 mg/mL. PTBZ169 proved to be the most active one and has many superior features thatmake it the preferred compound in BTZ series for clinical development

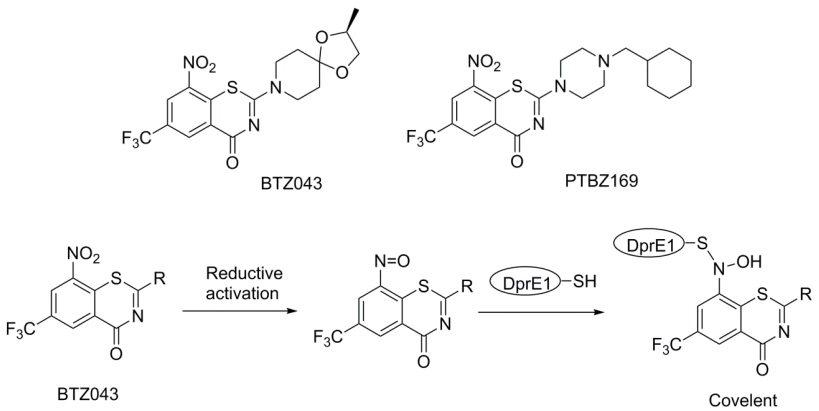
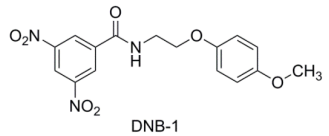
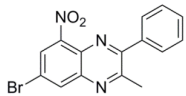


Fig. 3.4 *Mechanism of action of BTZ043.*

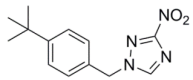
DprE1 is considered a highly vulnerable target[76] since many inhibitors with unrelated chemical structures have been reportedin literature such as dinitrobenzamides (DNB1), benzoxyquinoxalines (VI-9376),[77] and the triazole 377790.[65] To date, all of them target Cys 387 of DprE1. Very recently, series of compounds belonging to azaindoles [78] and benzothiazoles (TCA1) [79] were reported as potent DprE1 inhibitors, which showed significant activity in *in vitro* and mouse models of acute and chronic TB. These compounds have been shown to be non-covalent inhibitors as well as the generated resistors do not show missense mutation in Cys 387, suggesting that their binding mechanism is different from the covalent inhibitors.



DNB-1



VI-9376



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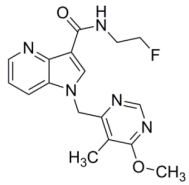
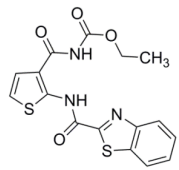


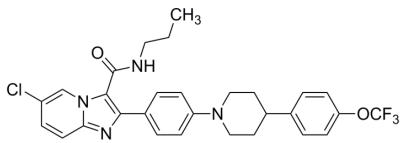
Azaindole



TCA-1

3.2.4 Imidazopyridine Amides

Imidazopyridine amides (IPAs) are a promising class of antitubercular compounds, acting by inhibition the respiratory cytochrome bc1 complex, identified by a phenotypic high-content screening of a commercial chemical libraries of 121156 compounds. A lead optimization campaign led to the optimized Q203.[80] SAR analysis around the 477 synthesized derivatives show that Q203 was active against *M. tuberculosis* H37Rv in the low nanomolar range (MIC50 0.0027 mM) as well as against MDR and XDR M. tuberculosis clinical isolates (MIC90 <0.00043 mM).

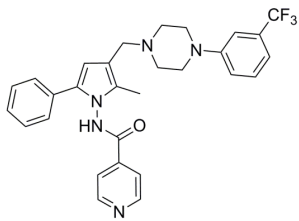


Q203

It also showed safety profile in acute model for toxicity in mice compatible with once-daily dosing. Q203 showed a bioavailability of 90% in mice and a low volume of distribution with a drug concentration in lungs 2 to 3-fold higher than in serum. In an acute mouse model of tuberculosis, it showed a reduction of more than 90% in bacterial load. In a chronic mouse model of tuberculosis

Q203 was slow acting with respect to INH, in fact the reduction of bacterial load was higher in the last 2 weeks. To date, Q203 reduced the formation of lung granulomas lesions.

3.2.5 Sudoterb (Pyrrole, LL-4858)



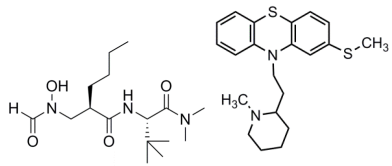
LL-3858 (Sudoterb)

Lupin Ltd., has identified a lead compound, Sudoterb (LL-4858), which has activity against sensitive and resistant strain of *M. tuberculosis*. LL-4858 was reported to have potent anti-TB activity *in vitro* and *in vivo* (mice and guinea pig) studies. LL-4858 had shown *in-vitro* bactericidal activity similar to isoniazid and was synergistic with RMP. The combination of LL-4858 with isoniazid, rifampicin and pyrazinamide led to complete sterilization of sensitive and MDR-TB strains in infected mice within 2 months. In combination with rifampicin and pyrazinamide, LL-4858 also cured TB in all animals after 3 months of treatment. LL-4858 could potentially cut the time of TB treatment to 2 or 3 months.[82, 83] The mechanism of action of this drug is not yet established.

3.2.6 Peptideformylase Inhibitor BB-3497

Bacterial peptide deformylase (PDF) is a metallo-protease that removes the N-terminal formyl group from newly synthesized proteins. Various PDF inhibitors have activity against several pathogens including *E. coli* and *S. aureus in vitro*. Six PDF inhibitors were screened against two isolates of *M. tuberculosis*

and initial testing showed that three compounds, BB-3497, BB-84518 and BB-83698 gave MICs in the range of 0.06–2 μg/mL.[84] These inhibitors were further tested against 17 isolates of *M. tuberculosis* and were found to be the most active with a median MIC of 0.25 μg/mL. Further *in vivo* evaluation is required to fully determine the potency and clinical tests must be carried out whether the drug is toxic or not in human. A recent study suggested that PDF inhibitors had no detectable effect on two different human cell lines *in vitro*.[85]



BB-3497 Thioridazine

3.2.7 Phenothiazines

Phenothiazine based antipsychotics were recently reported to possess clinically useful antitubercular activity against multi-drug/extremely drug resistant tuberculosis.[86, 87] The mechanism proposed for antimycobacterial activity of this drug includes disruption of mycobacterial respiration process most probably via type II NADH: quinone-oxidoreductase inhibition.[88] Another unusual mechanism proposed is the activation of host macrophages to effectively destroy dormant mycobacterium.[89, 90] The later mechanism draws attention of the drug designers as it does not involve biochemistry of the pathogen and hence less chances for development of resistance.[91] The only problem with this therapy is the unwanted CNS activity, neuronal and cardiac toxicity, which makes continuous therapeutic drug monitoring mandatory. Though the efficacy of thioridazine as an alternate treatment for XDR TB was ascertained in one study[92], a comprehensive clinical trial for evaluation of its safety, efficacy and development of an optimized regimen is yet to begin.

3.3 Conclusions

Uncertain biochemistry, unique cell-wall structure and survival mechanisms, lack of motivation in the pharmaceutical industry and funding agencies probably are responsible for the diminutive proportion of anti TB agents amongst the drugs in clinical trials. TB Alliance, an international organization formed in the year 2000, brought industry, academia, donors and NGOs together to intensify efforts to improve treatment options for tuberculosis. Their concerted efforts resulted in the development of novel drugs Bedaquiline and Pretomanid. These intensified efforts also filled the pipeline with more than 10 new molecules which are in various phases of clinical trials. To expand and encourage the developmental plans of new drugs for TB, government, private and public authorities need to enhance financial support for research at all levels, and adapt regulations to ease the process of evaluation, validation and approval of new drugs without altering the quality of research and life. In addition, there should be an agenda to be implemented by government, public and private agencies i.e on education and awareness, which will contribute to the prevention of TB spread and also development drug resistant MDR or XDR TB.

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Chapter 4

Drug Resistance in *Mycobacterium Tuberculosis*

Microorganisms are one of the oldest inhabitants of the earth. Immense diversity, unique survival skills made them evolve into one of the most successful diverse and prolific living organisms. Simple yet comprehensive biochemical apparatus helped them survive in heterogeneous environments, from extreme arctic temperature to the depths of ocean and to the gut of an animal. During the course of evolution, every life form strengthened the molecular mechanisms to adapt towards external environments to survive during stressful situations like lack of oxygen, nutrients, light and presence of toxic chemicals and immune attack.

Most of the life-threatening microbial infections were rendered curable with the discoveryof antibiotics. Alongside, microbes developed resistance due to

- Single drug therapy
- Inadequate dose
- Discontinued treatment
- Ingestion of wrong antibiotic due to faulty diagnosis
- Inadvertent consumption of antibiotics via food and other means

The jubilations of discovering effective anti-TB drugs like streptomycin, p-aminosalicylic acid and isoniazid evaporated after the occurrence of antibiotic resistance in *Mycobacterium tuberculosis*, almost immediately after the entry of these wonder drugs into clinics. Later, WHO recommended usage of multidrug regimen, DOTS (Directly Observed Treatment-Short course) for better management of this dreadful disease by ensuring patient compliance. This initiative was strictly implemented in many parts of the world and curtailed resistance problem significantly. Eruption of multi drug resistant (MDR) strains during 1993-1995 restored the “global health problem” status to TB. As per latest

reports, *Mycobacterium tuberculosis*, the causal agent for tuberculosis, has claimed 1.5 million lives worldwide during 2013-14, among which 22% are non HIV patients. The incidence of MDR TB increased significantly from 3,10,000 cases in 2012-13 to an estimated 4,80,000 cases in 2013-14, among which 9% belong to Extremely Drug Resistant TB (XDR TB). It is also estimated that MDR TB accounts for 14% of total deaths due to TB.[1] Though effective medicines are available for treating drug-susceptible TB infection, the chances go from bleak to null as we move from MDR to XDR or Totally Drug Resistant TB (TDR TB). Drug resistance remains a major challenge to every section involved in the health care system and is the major driving force for novel antibiotic drug discovery.

- Development of resistance due to medication is generally considered as acquired resistance. It may be due to interruption of the therapy by the patient, prescription of inadequate chemotherapy, and poor drug supply.

- The innate resistance developed in patients without prior treatment with anti-tubercular drugs is called *primary resistance*. The occurrence of primary resistance is a consequence of the level of acquired resistance in the community. The rate of primary resistance is lower than the incidence of acquired one. This resistance is more often to one drug (streptomycin or isoniazid) than to two drugs.

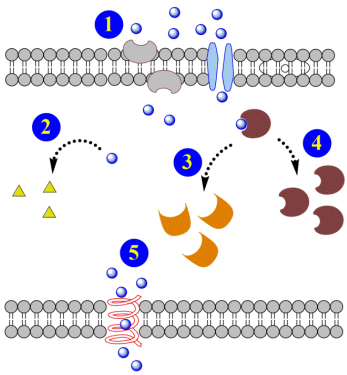
4.1 Major Mechanisms Involved in Thedevelopment of Drug Resistance in Microorganisms

Modulation of Membrane permeability–due to reduced uptake of the antimicrobial agent via modification of transporter proteins.

Degradation or Inactivation of Antibiotic–Expression of an enzyme that inactivates the antimicrobial agent by metabolic modification.

Modification of Target protein structure –

- mutation in the antimicrobial agent's target or post-transcriptional or post-translational modification which reduces the binding of the antimicrobial agent.
- overproduction of the antimicrobial agent'starget.
- expression or suppression of a gene *in vivo* in comparison to the situation *in vitro.*
- presence of an alternative enzyme instead of an enzyme that is inhibited bythe antimicrobial agent.



1. Modification of cell wall and reduced drug intake
2. Metabolic conversion of drug into inactive metabolites
3. Modification of the target protein or active site
4. Over expression of target protein
5. Drug efflux pumps

Fig. 4.1 *Drug resistance mechanisms in Mycobacterium tuberculosis.*

Increased drug clearance via Efflux pumps. These efflux pumps include the pumps of Major Facilitator Superfamily (MFS) family (lfrA, Rv1634 and Rv1258c) and ATP Binding Cassette (ABC) transporters (DrrAB, PstB and Rv2686c-2687c-2688c).[2]

4.1.1 Drug - Resistant Tuberculosis

One among three individuals in the world is infected with dormant TB germs. Only when the bacteria become active then only people become ill with TB. Bacteria become active as a consequence of anything that can decrease the person's immunity, like HIV, advancing age or some medical conditions. TB can usually be treated with a course of four first-line anti-TB drugs. In drug resistant TB, the bacteria are resistant to one or more anti-TB drugs. Essentially, drug-resistance arises in areas with poor TB control programmes.

4.1.2 Multi-Drug Resistant Tuberculosis (MDR-TB)

In MDR-TB, bacteria are resistant to several anti-TB drugs and at least to INH and RIF. It is usually found in patients after failed treatment regimens and represents a significant proportion of tuberculosis patients with acquired resistance. Only exceptionally it is observed in new cases. *Top priority is not the management but the prevention of MDR-TB*. The emergence of MDR-TB has made the scientific community throughout the world to focus on the urgent need for new anti-TB drugs. Resistance has been developed against almost every front-line drug.[3-7]

WHO recommends treatment with at least four drugs in order to reduce resistance burden further. However, unpleasant side effects and relatively long course of treatment remained major road-blocks for its success. The second line drugs used for MDR-TB are more expensive, less effective and more toxic than drugs used in the four standard regimens. It is very important to discover affordable, safer and potent bactericidal anti-TBdrugs to treat MDR-TB and latent infectionsin short treatment period with reduced frequency of doses. It is known that MDR-TB strains are sensitive to other antibiotics like

fluoroquinolones, which inhibit the topoisomerases II and IV as well as DNA gyrases, the essential enzymes to maintain the supercoils in bacterial DNA.[8] Consequently a huge effort has been made by scientists in order to discover new quinolone derivatives endowed with anti-TB activity.[9-12]

4.1.3 Extensive-Drug Resistant Tuberculosis (XDR-TB)

XDR-TB or Extensive Drug Resistant TB (also referred to as Extreme Drug Resistance) is MDR-TB that is resistant to isoniazid, rifampicin, any fluoroquinolone and at least one of the three injectable 2nd line anti-TB drugs (capreomycin, amikacin and kanamycin). XDR-TB can develop when these second-line drugs are also misused or mismanaged and therefore also become ineffective. Because XDR-TB is resistant to first and second-line drugs, treatment options are seriously limited and complicated. The emergence of XDR-TB shows that the development of novel mechanism-based anti-TB agents is necessary.[13]

4.1.4 Basic Concepts in the Development of Drug-Resistant TB

Drug-resistant TB is not a recent phenomenon. *M. tuberculosis* strains that were resistant to streptomycin (SM) appeared soon after the introduction of drug for TB treatment in 1944. Genetic resistance to an anti-TB drug due to spontaneous chromosomal mutations appears at a frequency of 10^{-6} to 10^{-8} mycobacterial replications. The probability of developing bacillary resistance to three drugs used simultaneously becomes 10^{-18} to 10^{-20}. In theory, the chance of drug resistance is thus virtually non-existent when three effective drugs are used in combination for TB treatment. Interestingly, plasmids and transposons mediated resistance is absent in *M. tuberculosis.* Because such mutations resulting in drug resistance are unlinked. Hence development of drug resistance

is largely due to human error including poor patient compliance, ‘monotherapy’ due to irregular drug supply and inappropriate doctor prescription.[14]

Subsequent transmission of resistant *M. tuberculosis* strains from the carrier to others further aggravates the problem. The MDR/XDR phenotype is caused by sequential accumulation of mutations in different genes involved in individual drug resistance. Although the definitions of ‘acquired’ and ‘primary’ drug resistance are conceptually relatively clear, in reality they are often subject to misclassification when previous treatment cannot be readily ascertained. The term ‘initial’ drug resistance is thus often preferred to ‘primary’ drug resistance to include ‘unknown’ or ‘undisclosed’ acquired drug resistance. The matter is currently further simplified by categorizing drug resistance in new cases and previously treated cases of TB.[15] The latter refers to cases with treatment lasting for at least one month.

4.2 Molecular Basis of Drug Action and Resistance

A great deal of progress has been made in our understanding of the molecular basis of drug action and resistance in *M. tuberculosis.* An update on this topic is provided below.

4.2.1 Isoniazid (INH)

INH is the most widely used first-line anti-TB drug. Since its discovery in 1952, INH has been the cornerstone of all effective regimens for treatment of TB, including the latent form. *M. tuberculosis* is highly susceptible to INH (MIC 0.02–0.2 μg/mL) but is virtually not active against non-replicating bacilli or under anaerobic conditions. INH is a prodrug that is activated by the catalase peroxidase enzyme (*Kat*G) encoded by the *kat*G gene [16] to generate a range of

highly reactive species which then attack multiple targets in *M. tuberculosis.*[17] The reactive species generated by *Kat*G-mediated INH activation include both reactive oxygen species such as superoxide, peroxide and hydroxyl radical,[18] nitric oxide [19] and reactive organic species such as isonicotinic-acyl radical or anion [21, 22] and certain electrophilic species.[23] InhA enzyme (enoyl-acyl carrier protein reductase) which is involved in the elongation of fatty acids in mycolic acid synthesis is one of the prime targets of INH.[23] The active species (isonicotinic-acyl radical or anion) derived from KatG-mediated INH activation reacts with NAD(H) (nicotinamide adenine dinucleotide) to form an INH-NAD adduct, and then attacks InhA.[20, 21] A recent study showed that INH-NAD(P) adducts react with other protein targets besides InhA, such as *Dfr*A (an NADPH-dependent dihydrofolatereductase involved in DNA synthesis).[24] Resistance to INH occurs more frequently than for most anti-TB drugs, at a frequency of 1 in 105 bacilli *in vitro*.[25] It is also found that catalase and peroxidase enzymes were absent in the INH-resistant clinical isolates of *M. tuberculosis* [26] encoded by *kat*G, especially in high level resistant strains (MIC > 5 μg/mL).[32] Low level resistant strains (MIC < 1 μg/mL) often still possess catalase activity.[25] Mutation in *kat*G is the main mechanism of INH resistance and KatG S315T mutation is the most common mutation in INH resistant strains, accounting for 50–95% of INH-resistant clinical isolates.[16, 17, 27] Over expression of InhA via mutations in the promoter region of *mab*A/*inh*A operon, or lowering the InhA affinity by mutations at the InhA active site, are also observed in resistant strains.[20, 23] Mutations in *inh*A or its promoter region are usually associated with low-level resistance (MICs = 0.2−1 μg/mL) and are less frequent than *kat*G mutations.[17, 27] Additional mutations in the *katG* conferred higher levels of INH resistance.[28] Mutations in *inh*A was also linked to cross-resistance to the structurally related drug, ethionamide (ETH).[23] About 10–25% of low-level INH-resistant strains does not have mutations in *kat*G or

*inh*A[27], and may be due to new mechanism(s) of resistance. Recently, mutations in another important enzyme *msh*A, encoding an enzyme involved in mycothiol biosynthesis, have been shown to confer INH and ETH resistance in *M. tuberculosis* strains *in vitro*,[29] but its role in clinical resistance remains to be demonstrated.

4.2.2 Rifampicin (RMP)

RMP is an important first-line drug for the treatment of TB. RMP is bactericidal for *M. tuberculosis*, with MICs ranging from 0.05 to 1 μg/mL on solid or liquid media, but the MIC is higher in egg media (MIC = 2.5–10 μg/mL). Strains with MICs < 1 μg/mL in liquid or agar medium or MICs < 40 μg/mL in Löwenstein- Jensen (LJ) medium are considered RMP-susceptible. RMP is active against both growing and latent phase bacilli. The latter activity is related to its high sterilizing activity *in vivo*, correlating with its ability to shorten the 12–18 months TB treatment to 9 months.[30] RMP interferes with RNA synthesis by binding to the β subunit of the RNA polymerase. The RMP-binding site is located upstream of the catalytic centre and physically blocks the elongation of the RNA chain. In *M. tuberculosis*, resistance to RMP occurs at a frequency of 10^{-7} to 10^{-8}. As in other bacteria, mutations in a defined region of the 81 base pair region of the *rpo*B are found in about 96% of RMP-resistant *M. tuberculosis* isolates.[31] RMP-resistant strains are often found to carry mutations at positions 531, 526 and 516. Mutations in *rpo*B generally result in highlevel resistance (MIC > 32 μg/mL) and cross-resistance to all rifamycins. However, specific mutations in codons 511, 516, 518 and 522 are associated with lower level resistance to RMP and rifapentine, but retain susceptibility to rifabutin and rifalazil.[32, 33] The circumstances under which the RMP-dependent strains arise remain unclear, but they often occur as MDR-TB and seem to develop upon repeated treatment with rifamycins in patients with repetitive treatments.

4.2.3 Pyrazinamide (PZA)

PZA is an important first-line drug used along with INH and RMP. PZA plays a unique role in shortening the previous 9–12 months TB treatment to 6 months because it kills a population of persistent bacilli in acidic pH environment in the lesions that are not killed by other drugs.[30] PZA is an unconventional and paradoxical anti-TB drug that has high sterilizing activity *in vivo* [34] but no activity against tubercle bacilli at normal culture conditions near neutral pH.[35] PZA is only active against *M. tuberculosis* at acid pH (e.g., 5.5).[36] Even at acid pH (5.5), PZA activity is rather reduced, with MICs in the range of 6.25–50 μg/mL. PZA activity is enhanced under low oxygen or anaerobic conditions [37] and by agents that compromise the membrane energy status, such as weak acids [38] and energy inhibitors such as DCC (dicyclohexylcarbodiimide), azide and rotenone.[39] PZA is a prodrug that requires conversion to its active form pyrazinoic acid (POA) by the pyrazinamidase/ nicotinamidase enzyme encoded by the *pnc*A gene of *M. tuberculosis*.[40] The POA produced intracellularly, reaches the cell surface through passive diffusion and a defective efflux.[41] The extracellular acid pH facilitates the formation of uncharged protonated POA, which then permeates through the membrane and causes accumulation of POA and disrupts membrane potential in *M. tuberculosis*.[39] The protonated POA brings protons into the cell and could eventually cause cytoplasmic acidification and de-energize the membrane by collapsing the proton motive force, which affects membrane transport. The target of PZA is related to membrane energy metabolism although the specific target remains to be identified. Fas-I was proposed as a target for PZA [42] but its validity is questioned.[43] PZA-resistant *M. tuberculosis* strains lose pyrazinamidase/ nicotinamidase activity.[44] Using a cloned *M. tuberculosis pnc*A, scientists have shown that defective pyrazinamidase activity due to *pnc*A mutations is the major cause of PZA resistance. Most PZA-resistant *M. tuberculosis* strains (72–97%) have mutations

in *pnc*A,[45-52] however, some resistant strains do not have *pnc*A mutations. The lower percentage of PZA-resistant strains with *pnc A*mutations (e.g., 72%) [47] reported in some studies could be caused by false resistance due to well-known problems with PZA susceptibility. PZA is active only against *M. tuberculosis* complex organisms (*M. tuberculosis*, *M. bovis* from *M. microti*), but not *M. bovis*, due to a characteristic mutation in its *pnc*A gene [40]. Strains of *M. bovis*, including BCG, are naturally resistant to PZA and lack pyrazinamidase; these features are commonly used to distinguish *M. Bovis* from *M. tuberculosis.* A single point mutation of 'C' to 'G' at nucleotide position 169 of the *pnc*A gene compared with the *M. tuberculosis pnc*A sequence, causing amino acid substitution at position 57 of the *pnc*A sequence is said to be responsible for the natural resistance to PZA in *M. bovis*. However, the correlation between pyrazinamidase activity and PZA susceptibility is not true for other naturally PZA-resistant mycobacterial species whose intrinsic PZA resistance is most likely due to their highly active POA efflux mechanism.[41]

4.2.4 Ethambutol (EMB)

EMB is an indispensable ingredient in all anti TB regimen containing other first line drugs INH, RMP and PZA. It is a bacteriostatic (MIC 0.5–2 μg/mL) and shows its activity chiefly on replicating bacilli by interfering with the biosynthesis of cell wall arabinogalactan.[53] It inhibits the polymerization of cell-wall arabinan of arabinogalactan and of lipoarabinomannan. Further, it induces the accumulation of D-arabinofuranosyl-P-decaprenol, an intermediate in arabinan biosynthesis.[53, 54] Arabinosyl-transferase (embB), a critical enzyme involved in the arabinogalactan synthesis has been proposed as the target of EMB in *M. tuberculosis* and *M. avium.* Strains resistant to EMB have MICs > 7.5 μg/mL. Mutation to EMB resistance occurs at a frequency of 10^{-5}. Resistance to EMB arise mostly due to mutations in the *emb*B geneand

occasionally *emb*C.[55] It was found that mutations leading to certain amino acid changes are indeed causing EMB resistance while other amino acid substitutions have little effect on EMB resistance.[56] However, about 35% of EMB-resistant strains (MIC < 10 μg/mL) do not have *emb*B mutations,[57] suggesting that there may be other mechanisms of EMB resistance. Further studies are needed to identify potential new mechanisms of EMB resistance.

4.2.5 Aminoglycosides (Streptomycin (SM)/Kanamycin (KM)/ Amikacin (AMK)/Capreomycin CPM)

SM is an aminoglycoside antibiotic that is active against a variety of bacterial species, including *M. tuberculosis.* SM kills actively growing tubercle bacilli with MICs of 2–4 μg/mL, inactive against non-growing or intracellular bacilli.[30] SM inhibits protein synthesis by binding to the 30S subunit of bacterial ribosome, causing misreading of the mRNA message during translation.[58] The site of action of SM is the 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA. Resistance to SM is caused by mutations in the S12 protein encoded by *rps*L gene and 16S rRNA encoded by *rrs* gene.[59] Mutations in *rps*L and *rrs* are the major mechanisms of SM resistance, accounting for respectively about 50% and 20% of SM-resistant strains.[59-61] The most common mutation in *rps*L is a substitution in codon 43 from lysine to arginine, causing high-level resistance to SM. Mutation in codon 88 is also common. However, about 20–30% of SM-resistant strains with a low level of resistance (MIC < 32 μg/mL) do not have mutations in *rps*L or *rrs*,[62] which indicates other mechanism(s) of resistance. Recently, low-level SM resistance in 33% of resistant *M. tuberculosis* isolates [63] were found to carry a mutation in *gid*B, encoding a conserved 7-methylguanosine- methyltransferase specific for 16S rRNA. In addition, some low-level SM resistance seems to be caused by increased efflux.[64] KM and its derivative AMK are also inhibitors of protein synthesis through modification of

ribosomal structures at the 16S rRNA. Mutations at 16S rRNA (*rrs*) position 1400 are associated with high-level resistance to KM and AMK.[65, 66] CPM is a polypeptide antibiotic. A gene called *tly*A encoding rRNA methyltransferase was shown to be involved in resistance to CPM.[67] The rRNA methyltransferase modifies nucleotide C1409 in helix 44 of 16S rRNA and nucleotide C1920 in helix 69 of 23S rRNA.[68] SM resistant strains are usually still susceptible to KM and AMK.

4.2.6 Fluoroquinolones (FQ)

DNA topoisomerases are a diverse set of essential enzymes responsible for maintaining chromosomes in an appropriate topological state. In the cell, topoisomerases regulate DNA supercoiling and unlink tangled nucleic acid strands to meet replicative and transcriptional needs.[69] In most bacterial species, FQs inhibit DNA gyrase (topoisomerase II) and topoisomerase IV, resulting in microbial death. DNA gyrase is a tetrameric A2B2 protein. Between the two subunits, A subunit carries the breakage-reunion active site, but the B subunit promotes adenosine triphosphate hydrolysis. *M. tuberculosis* has *gyr*A and *gyr*B correspondingly encoding the A and B subunits.[70] A conserved region, the quinolone-resistance-determining region (QRDR) of *gyr*A (320 bp) and *gyr*B (375 bp), has been found to be the most important area involved in the exhibition of FQ resistance in *M. tuberculosis*.[76] Mutations within the QRDR of *gyr*A have been found in clinical and laboratory-selected isolates of *M. tuberculosis*, basically clustered at codons [56, 68-74] with Asp94 as the pretty common one.[72, 75] For clinical isolates, *gyr*B mutations appear to be of much rarer occurrence.[73, 74, 77] Generally, two mutations in *gyr*A or concomitant mutations in *gyr*A plus *gyr*B are required for the development of higher levels of resistance.[70, 78] Recently, a new mechanism of quinolone resistance mediated by MfpA was identified.[79] MfpA is a member of the penta peptide repeat family of proteins from *M. tuberculosis*,

whose expression causes resistance to FQ drugs. MfpA binds to DNA gyrase and inhibits its activity in the form of a DNA mimicry, which explains its inhibitory effect on DNA gyrase and quinolone resistance.[79] The *M. tuberculosis Rv2686c-Rv2687c-Rv2688c* operon, encoding an ATP-binding cassette transporter, has been shown to confer resistance to ciprofloxacin and to a lesser extent norfloxacin, moxifloxacin and sparfloxacin in *M. smegmatis*.[80] The resistance level was found to decrease in the presence of efflux pump inhibitors such as reserpine and verapamil. However, it remains to be determined if clinical strains elaborate MfpA or the *Rv2686c-Rv2687c-Rv2688c* operon to develop clinical resistance to quinolones. Furthermore, it has been suggested that, regarding *M. tuberculosis* resistance to FQs, the underlying genetic mutations can show substantial disparity among different geographic regions.[74]

4.2.7 Ethionamide (ETH)/Prothionamide (PTH) and Thioamides

ETH (2-ethylisonicotinamide) is a derivative of isonicotinic acid and is bactericidal only against *M. tuberculosis, M. avium-intracellulare* and *M. leprae.* Like INH, ETH is also a prodrug that is activated by EtaA/EthA (a monooxygenase) [81, 82] and inhibits the similar target as INH, the InhA of the mycolic acid synthesis pathway. Prothionamide (PTH, 2-ethyl-4-pyridine-carbothioamide) shares structure and activity almost identical to that of ETH. EtaA or EthA is a flavin adenosine dinucleotide (FAD) containing enzyme that oxidizes ETH to the corresponding S-oxide, which is further oxidized to 2-ethyl-4-amidopyridine, presumably via the unstable oxidized sulfinicacid intermediate. EtaA also activates thiacetazone, thiocarlide, thiobenzamide and perhaps other thioamide drugs,[83] which explains the cross-resistance between ETH and thiacetazone, thiocarlide and other thioamides and thioureas.[84] Mutations in the drug-activating enzyme EtaA/EthA [81, 82] cause resistance to ETH and other thioamides. In addition, mutations in the target InhA confer

resistance to both ETH and INH.

4.2.8 Oxazolidinones

Oxazolidinones are a very important group of synthetic antibacterial agents. Linezolid, an approved drug in this category elicits bactericidal activity by binding to the ribosomal 50S subunit and blocking an early step in the protein synthesis.[85] In view of its potential anti-TB activity,[86] a new oxazolidinone PNU100480 was developed, which showed good anti-TB activity and better pharmacokinetic profile than linezolid in a murine model.[87] Further, this compound was also found to be active against drug-resistant *M. tuberculosis* isolates. When compared to other anti-TB drugs, resistance to linezolid in *M. tuberculosisis* relatively rare (1.9% among 210 MDR strains). However, in the linezolid resistant strains mutations were largely observed G2061T and G2576T mutations inthe 23S rRNA gene.[88] In another recent study mutations were also observed at T460C in rplC, encoding the 50S ribosomal L3 protein.[89] It is also possible that involvement of efflux pumps or other non-ribosomal alterations may also play important role in linezolid resistance in *M. smegmatis*.[90]

4.2.9 Cycloserine

D-cycloserine is one of the oldest anti-TB drug that inhibits the synthesis of peptidoglycan by competing with D-alanine and blocking the action of D-alanine: D-alanine ligase (Ddl). This drug was also found to inhibit alanine racemase (AlrA) which converts L-alanine to D-alanine.[91] Resistance to this drug is mainly due to reduced drug permeation and over production of AlrAenzyme.[92]

4.3 New Drugs, New Targets and New Resistance Mechanisms

Quite a lot of new drugs are being projected as candidates for the treatment of TB. They exert anti TB activity by interacting with diverse targets, which are in many cases different from the classical targets of other anti-TB drugs. Surprisingly, new mechanisms of resistance have already been identified, even before these drugs have been put into clinical use.

4.3.1 Nitroimidazoles

Bicyclic nitroimidazole derivatives, PA-824 and OPC-67683 are very important candidates for treating MDR TB.[93] They are highly potent, relatively safe and possess novel mechanism of action. Nitroimidazole derivatives are prodrugs and are activated via, deazaflavin (cofactor F420) dependent nitroreducase (Ddn) mediated bioreduction. In this the aromatic nitro group is reduced to a reactive nitro radical anion intermediate within the cell.[94] This process alsoreleases NO gas inside the bacteria, causing severe damage to its respiratory apparatus.[95] The reduction preferably takes place in anaerobic environment; hence well oxygenated host cells are spared. Resistance to bicyclic nitroimidazole compounds was found to be associated most commonly with the lack of drug specific nitroreductase enzymes or its deazaflavin cofactor. More recently, a nitroimidazo-oxazine specific protein causing minor structural changes in the drug has also been identified. The resistant strains most commonly showed mutations in the gene Rv3547, a protein with high structural specificity for these drugs.[96] The total frequency of resistance by any mechanism to PA-824 was determined by fluctuation analysis in MTB strain H37Rv to be 9.0×10^{-7}, slightly less than that of 1.3×10^{-6} for INH.

4.3.2 SQ109

SQ109 is a highly potent analogue of Ethambutol, which acts synergistically with every first-line anti-TB agent including EMB. Even though the mode of action of SQ109 is not very much known, but it is understood that it affects mycobacterial cell wall synthesis in a manner unlike to that exercised by ethambutol.[97]

SQ109 inhibits biosynthesis of trehalosedimycolate (TDM) and other cell wall mycolates (methoxy, keto and alpha) chiefly by blocking transport of trehalosemonomycolate (TMM) across cell membrane via inhibiting MmpL3 transporter (Rv0206c protein).[98] Interestingly, development of resistance to SQ109 appears to be feeble as it targets a protein critical for survival of the microbe. All the resistant strains observed *in vitro*, has shown mutations in the Mmpl3 gene. In strains resistant to isoniazid, ethambutol and SQ109, it was established that there is an up-regulation of *ahp*C; which signifiesits possible role in the development of resistance to this drug.[99] Induction of the efflux pump viatranscription of the iniBAC operon required was also indicated in the SQ-109 resistance in MTB.[100]

4.3.3 Bedaquiline (TMC207, R207910, Sirturo®)

Bedaquilineis a diarylquinoline antibiotic with excellent bactericidal activity against *M. tuberculosis*. In combination with other anti-TB drugs it achieved significant sputum conversion rates in MDRTB. Several studies confirmed that bedaquiline selectively inhibits mycobacterial ATP synthase. The *in vitro* generated resistant species showed A63P and I66M mutations in the *atp*E gene which encodes Cpart in the F0 subunit of the ATP synthase.[101] The other study reveals that six distinct mutations, Asp28 → Gly, Asp28 →Ala, Leu59 →Val,

Glu61 →Asp, Ala63 →Pro, and Ile66 →Met, have been identified in the subunit forming a C ring in the ATP synthase.[102] It was also found that *atp*E gene is highly conserved in *Mycobacterium* species. One exception is *M. xenopi*, in which residue Ala63 in the *atp*E protein is replaced by Met, rendering it naturally resistant to bedaquiline. The reasons underlying exceptional specificity of this drug to mycobacterial *atp*E proteins are yet to come to light. In another recent study it was found that 55% (32 out of 58) of the *in vitro* generated resistant species showed no mutation in *atp*E gene hinting at alternate mechanism for development of resistance or even its bactericidal action.[103]

4.3.4 Benzothiazinones

Benzothiazinonesare a relatively new class of anti-TB antibiotics with excellent bactericidal activity against Mtb clinical isolates (MIC 0.75–30 ng/mL). Benzothiazinone irreversibly inhibits DprE1 subunit of the enzyme and thus inhibits epimerization of decaprenyl-phosphoryl-β-O-ribosetodecaprenyl - phosphorylarabinose, a chief component in mycobacterial cell wall assemblage. Resistance to benzothiazinones is mainly due to mutation of the dpeE1 gene, in which Cys387 codon was replaced by Ser or Gly.[104] *M. avium*, which is naturally resistant to benzothiazinones had the codon Cys387 replaced by an Ala. *M. smegmatis* is less susceptible to benzothiazinones (MIC 4ng/mL) and showed overexpression of nitroreductaseNfnB, which inactivates the critically needed nitro group to an amino group.[105] *M. tuberculosis*, however, seems to lack nitroreductases and unable to inactivate these drugs.

4.4 Conclusions

Drug resistance in Mtb is a major hurdle for the effective disease management and chemotherapy. It increases both financial and pill burden on the patient. In

MDR or XDR cases the treatment options are severely limited, available drugs are more toxic and thetreatment period often goes beyond 18 months. Successful implementation of DOTS and improving patient compliance significantly reduced the resistance problem in many parts of the world. Strict policies and legislations are to be made and implemented to avoid accidental or unnecessary exposure to antibiotics. Though drug resistant Mtb strains often carry a prominent and functional mutated gene, there are many cases of resistant strains with no trace of these predictable mutations. This intriguing complexity in the molecular mechanisms of drug resistance needed inquiry to further for knowledge which can be of immense help during development of newer drugs or biologicals.

Unlike in many diseases, TB diagnosis is a challenging task. Detection of MDR strain is even harder. Classical drug susceptibility tests take more than three weeks' time and it is probably more important to have diagnostic tools that are easy to use, inexpensive and provide rapid results of drug susceptibility or resistance of a strain.

Microbes seem to develop resistance to virtually any antibiotic. It would be wise to look for drugs to reverse drug resistance. Recently verapamil, a calcium channel blocker, increased drug susceptibility of a MDR TB strain.[106] Piperine, a natural product also showed similar activity in MRSA. Blocking p-glycoprotein mediated efflux pump was suggested as the mechanism for this activity.[107] Thioridazine, an antipsychotic drug was recently found to have excellent anti-TB activity. Activating pulmonary macrophages is one of the mechanisms ascribed to thioridazine's anti-TB activity.[108] So far no resistance was reported for thioridazine. With more and more new drugs filling the clinical trials pipeline, future appears more hopeful now than at any other time in the recent past.

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Chapter 5

Strategies for Anti-Tubercular Drug Development

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

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

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

5.1 Cell Wall Components Synthesis and Assemblage

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

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

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

5.1.1 Biosynthesis of Mycolic Acids and Other Lipids

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

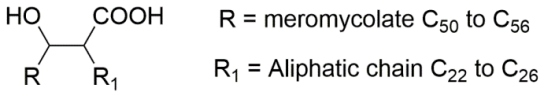

Fig. 5.1 *General structure of Mycolic acids.*

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

biosynthesis involving fatty acid synthase systems (FAS).

5.1.2 Mycobacteria Possessing FAS-I and FAS-II Enzymes

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

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

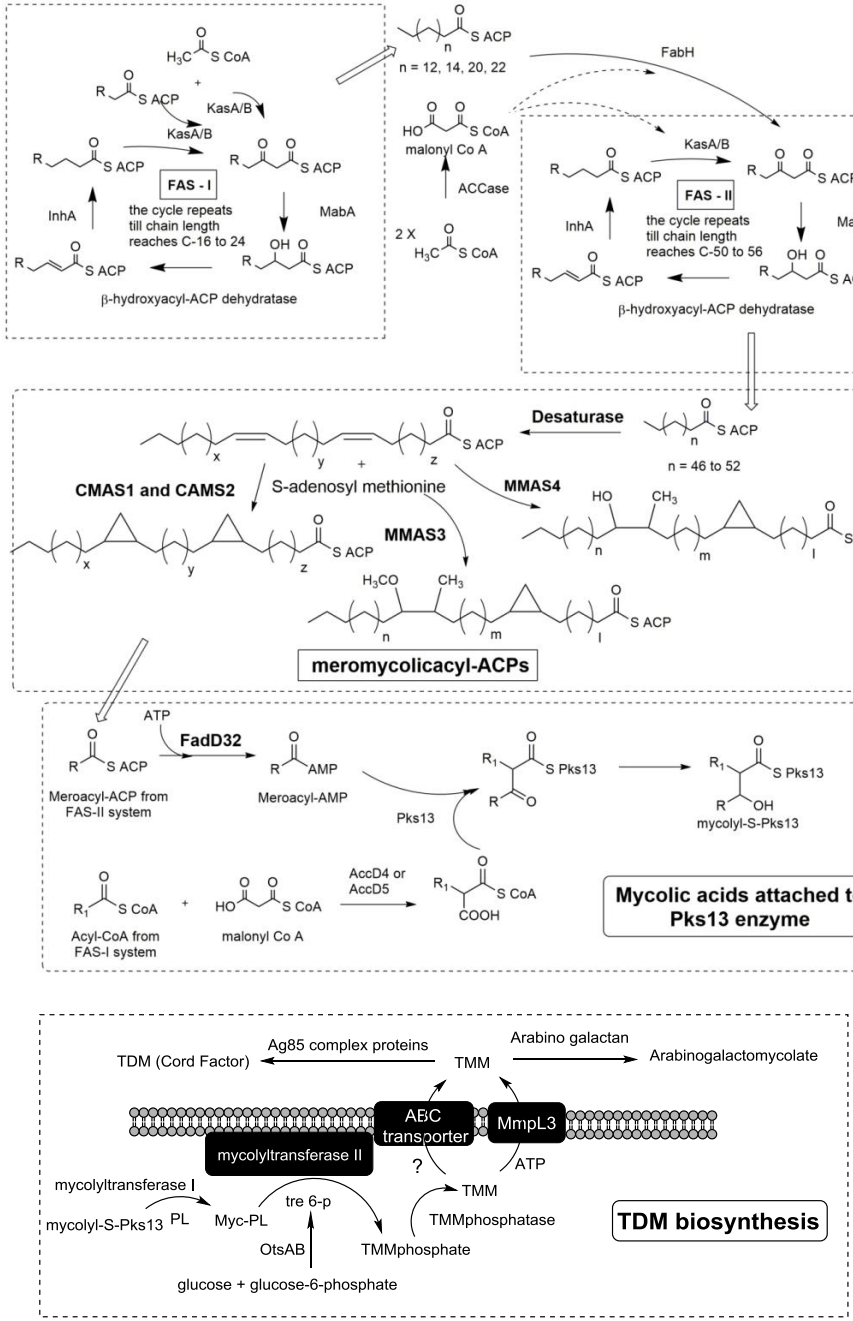

Fig. 5.2 *Biosynthesis of mycolic acids and trehalosedimycolates (TDM).*

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

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

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

5.2 Targets in Mycolic Acid Biosynthesis

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

5.2.1 INH A and Maba

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

5.2.2 Kas A and Kas B

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

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

5.2.3 B-Ketoacyl-ACP Synthaseinhibitors

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

5.2.4 FadD32 – AccD4 System

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

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

5.2.5 Methyltransferases

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

5.2.6 Polyketide Synthase System (Pks)

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

5.2.7 Mmpl3 Transporter Protein

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

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

5.2.8 Biosynthesis of Mycolyl-Arabinogalactan-Peptidoglycan Complex

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

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

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

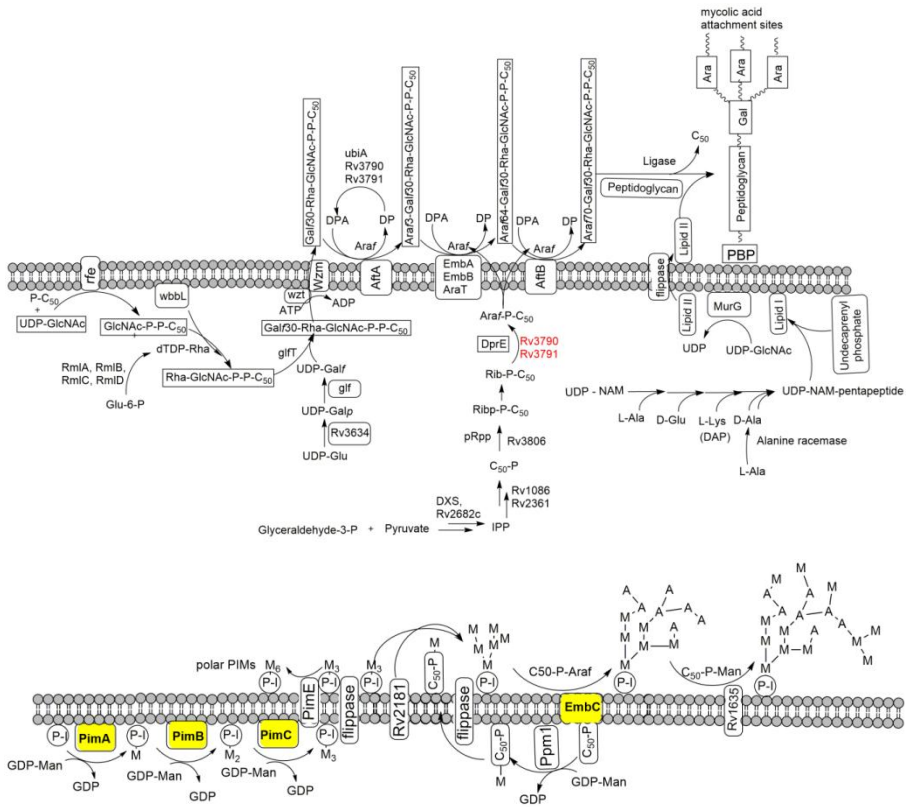

Fig. 5.3 *Mycolyl-Arabinogalactan-Peptidoglycan Complex.*[19-21]

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

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

5.3 Drug Targets for Tuberculosis

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

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

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

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

5.3.1 Peptidoglycan Biosynthesis

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

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

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

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

5.3.2 Protein Synthesis as a Target

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

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

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

5.3.3 Decaprene Biosynthesis

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

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

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

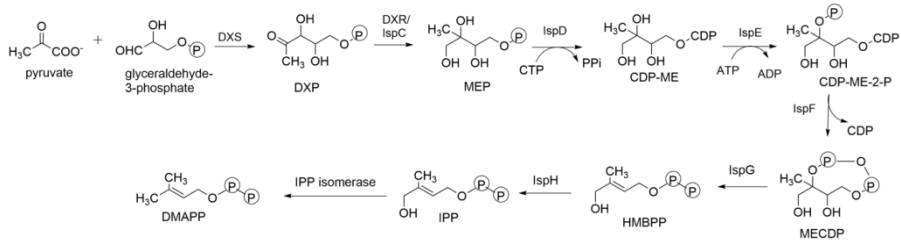

Fig. 5.4 *Biosynthesis of Decaprene.*

5.3.4 The MEP Pathway as a Drug Target

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

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

5.4 Enzymes Involved in Amino Acids or Co-Factor Biosynthesis

5.4.1 Pantothenatesynthetase

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

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

5.4.2 Quinolinatephosphoribosyltransferase (QAPRTase)

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

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

5.4.3 Shikimate Kinase (SK)

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

5.4.4 Thymidylate Kinase

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

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

5.5 Targets in DNA Biosynthesis and Metabolism

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

5.5.1 Ribonucleotide Reductases

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

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

5.5.2 DNA Ligase

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

5.5.3 DNA Topoisomerase

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

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

5.5.4 Respiratory Chain Inhibitors

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

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

5.6 Miscellaneous Targets

5.6.1 Isocitratelyase (ICL)

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

5.6.2 Mycobacterium Protein Tyrosine Phosphatase B (mPTPB)

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

5.6.3 Carbonic Anhydrase

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

5.6.4 Mycobacterial Thioredoxin Reductase (MtTrxR)

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

5.6.5 Glutamine Synthetase (GS)

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

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

5.6.6 Cysteine Biosynthetic Pathway

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

5.6.7 Acetohydroxyacid Synthase (AHAS)

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

5.7 Conclusions

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

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

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Short Introduction to the Book

Tuberculosis has several unique problems in diagnosis, disease progression and treatment. The causative organism Mycobacterium tuberculosis is distinguished by its slow growth, lipid rich membrane and unique survival abilities in-side host body. It enjoyed “incurable” status for quite a lot of period in known history. TB Alliance has brought together funding agencies, industries, philanthropists, academia and research organizations to improve the scenario. Two novel drugs entered into market in the last decade and several are in the pipe-line. Newer combinations are being formed to reduce the pill-burden of the patient and toxicity. This book helps in gaining knowledge about the history, origin of TB; drugs in use and under clinical trials; their molecular targets; emphasis was given on resistance.

Short Biography of the Author

Dr. K. Purna completed her B.Pharmacy from Siddartha College of Pharmacy, Vijayawada, Andhra Pradesh, India and Masters from BITS-Pilani, Rajasthan, India. After serving BITS, Pilani as Project Assistant and lecturer for five years, she moved to Andhra University and completed her PhD in Design, synthesis and screening of novel antitubercular agents. She is well versed with synthesis, screening, modern drug discovery tools like CADD, Pharmacophore modelling, virtual screening etc. She is a life member of several scientific societies like APTI, IPGA etc and secured IPA Domagk goldmedal. She has 15 International and National Publications in peer reviewed journals. She has presented 30 papers in various national and international conferences. Currently she is working as a DST, Women Scientist-A in AU College of Pharmaceutical Sceinces, Andhra University.

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