Chapter-1
1. GENERAL INTRODUCTION TO TUBERCULOSIS

Infectious diseases are now the world's biggest killers of children and young adults. "They account for more than 13 million deaths a year - one in two deaths in developing countries" as stated by the WHO. Most deaths from infectious diseases occur in developing countries. The cause for this has been attributed to the unavailability of efficient drugs for resistant strains and if at all available, the high cost associated with those drugs. Previously, the main bottlenecks in drug discovery were the time and costs of making (or finding) and testing new chemical entities. In order to reduce costs, pharmaceutical companies have to find new technologies to replace the old "hand-crafted" synthesis and testing approaches. Since 1980, with the advent of high throughput screening and computational techniques, it has made possible to reduce the time taken as well as the cost of drug development. Development of cheap, efficient and broad spectrum of drugs for controlling even resistant bacteria is one of the major problems faced by mankind. The solution to this problem could be from drug design using computational methods.

1.1 TUBERCULOSIS (TB)

Among the various diseases that affect human beings across the globe, up to now, TB is considered the most dreadful one caused by *Mycobacterium tuberculosis* (Mtb). It has been an ancient disease held in close association with humans for millennium. This successful pathogen has managed remarkably to infect an estimated one third of the world's population (WHO fact sheet 2008). It is non motile rod shaped bacterium which is distantly related to Actinomycetes. The rods are 2-4 micrometers in length and 0.2-0.5 micrometers in width. *Mycobacterium* is an obligate aerobe always found in the upper aerated lobes of human lungs. Dr. Robert Koch discovered the tubercle bacilli on March 24, 1882 and also proposed specific staining procedure for it. For this significant event, he was awarded the noble prize in the year 1905. This bacterium is also known as "Koch's bacillus".

Different pathogenic strains of *Mycobacterium* responsible for TB are *avium*, *bovis*, *microti*, *canettii*, *africanum*, *leprae* (causative agent of leprosy). But these species don't usually infect healthy adults. *M.bovis* is the causative agent of TB in
cows and rarely in humans. Humans can also be infected by the consumption of unpasteurized milk. Both cows and humans can serve as reservoirs. *Tuberculosis meningitis* is sometimes seen in newly born children. This form of the disease is a life threatening condition. Other non TB *Mycobacterium* found in soil and water can cause disease in susceptible patients with history of cystic fibrosis, chronic lung damage, alcoholism and immuno-suppression. Since it is a typical *Mycobacterium* and can be present as colonizing organism. TB is a contagious disease that spreads through the air like common cold. Each person with active TB infects 10 to 15 people per year. The characteristic *Mycobacterium* cell envelope is the dominant feature of the biology of Mtb and other pathogens based on sugars and lipids of exceptional structure making them resistant to staining procedures. They are closer to Gram-positive organisms based on the analysis of 16s rRNA sequences (Pitulle *et al.*, 1992). Mtb is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristic of either, although the bacteria do contain peptidoglycan (murein) in its cell wall. One of the best acid staining methods for *Mycobacterium* is the Zeihl-Neelsen Stain and all bacilli appear pink in a contrasting back ground. Two media are used to grow Mtb Middlebrook's medium which is an agar-based medium and Lowenstein-Jensen medium which is an egg-based medium. Mtb colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out growing Mtb. It takes 4-6 weeks to get visual colonies on either type of media.

### 1.1.1 Transmission of the disease

The principal means of transmission of TB is by infective particles produced through coughing by patients with active TB. The release of particles in the air remains in suspension for hours without dying. When droplet of Mtb nuclei reaches nonspecifically to the alveolar macrophages initial infection begins. The host recruits macrophages to the site of infection and begins phagocytosis of the organism. However Mtb can escape phagosome-lysosome fusion, inhibit acidification or maturation of phagosomes (Armstrong and Hart, 1975) and survive even in the presence of reactive oxygen and nitrogen intermediates, therefore preventing them from being attacked by host immune mechanism. In macrophages, the bacteria continue to grow and as macrophages aggregate, Mtb
spread intercellularly to other macrophages in the aggregation. Chemokine production by infected macrophages attracts inactivated monocytes, lymphocytes and neutrophils which are not very effective in killing the bacteria but results in the formation of granulomatous focal lesions composed of macrophage derived in giant cells and lymphocytes (Vancrevel et al., 2002). These granulomas effectively limit the spread of bacteria. At this stage the individual becomes tuberculin-positive. This is due to the development of cell-mediated-immune response (CMI) (Schlesinger, 1993) by host. Secretion of gamma interferon by activated T-lymocytes (Bodnar et al., 2001) secretes lytic enzymes which results in the killing of the bacteria and formation of the caseous center of the granuloma. Bacteria are unable to multiply within these lesions due to acidic pH, low availability of oxygen and presence of toxic fatty acids but may remain dormant and viable. With effective cell-mediated immunity, these granulomas eventually heal leaving fibrous, calcified lesions which are readily visible on chest X-Ray.

Infection also transmits via blood to other parts of the body such as central nervous system, the lymphatic system, the circulatory system, the genitourinary system, joints and even the skin. The typical symptoms include weakness, chest pain, respiratory insufficiency, fever and cough and in advanced stages sputum will contain blood. TB is especially hard to treat because Mtb develops into multi-drug-resistant (MDR) and extensively drug resistant (XDR) strains. General treatment includes chemotherapy and a combination of different lines of drugs. The characteristic features of the tubercle bacillus include slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. The generation time of Mtb is very slow which takes 16-20 hours for each division compared to other bacteria. This contributes to the chronic nature of the disease, imposes lengthy treatment regimens and represents a formidable obstacle for researchers.

1.1.2 TB and HIV

TB and HIV both form lethal combination, each speeding other's progress. It has been estimated that TB accounts for around 32% of deaths in HIV infected individuals (Kochi, 1996). HIV promotes the progression of recent and latent Mtb infection to active TB disease. It also increases the rate of recurrent TB (WHO report 2009). In 2008, an estimated 1.3 million deaths, including 0.5 million deaths
among women, occurred among HIV-negative incident cases of TB. This is equivalent to 20 deaths per 100 000 population. There were an estimated 0.5 million deaths among incident TB cases who were HIV-positive. Of these HIV-positive cases, 78% were in the African region and 13% were in the South-East Asia region. The disease has brought life expectancy down to only 47 years. For HIV-positive individuals, who have compromised immune systems and other risk factors, the life time risk exceeds 30 percent. The number of TB deaths per 100000 population among HIV-negative people plus the estimated TB deaths among HIV-positive people equates to a best estimate of 28 deaths per 100000 population. People who are HIV-positive and infected with TB are 20 to 40 times more likely to develop active TB than people not infected with HIV living in the same country. Critical to saving lives is the urgent implementation of the three Is (Intensified case-finding, Isoniazid prevention therapy, and Infection control) and collaborative TB/HIV activities measures which reduce the burden of TB in people living with HIV.

1.1.3 TB the global menace

Tuberculosis has been a major health problem in developing countries, including India. In the first half of the last century tuberculosis was the main focus of attention among all respiratory diseases. India accounts for nearly 20% global tuberculosis burden even today (Dye et al., 1999). In 2008, there were an estimated 9.4 million cases of TB globally (WHO report 2008). This is an increase from the 9.3 million TB cases estimated to have occurred in 2007. Estimates of the number of cases broken down by age and sex are being prepared by an expert group as part of an update to the Global Burden of Disease study (WHO report 2009). Most of the estimated number of cases in 2008 occurred in Asia (55%) and Africa (30%), with small proportions of cases in the Eastern Mediterranean region (7%), the European region (5%) and the region of the Americas (3%). The twenty two high-burden countries (HBCs, defined as the countries that ranked first to twenty two in terms of absolute numbers of cases and which have received particular attention at the global level since 2000 account for 80% of all estimated cases worldwide. The five countries that ranked first to fifth in terms of total numbers of incident cases in 2008 are India (1.6–2.4 million), China (1.0–1.6 million), South Africa (0.38–0.57 million), Nigeria (0.37–
0.55 million) and Indonesia (0.34–0.52 million). India and China alone account for an estimated 35% of TB cases worldwide. There were an estimated 0.5 million cases of MDR-TB (Multi-drug-resistant TB) in 2009 (WHO report 2009). There are 27 countries (15 in the European region) that account for 85% of all such cases. The countries that ranked first to fifth in terms of total numbers of MDR-TB cases in 2007 were India (131,000), China (112,000), the Russian Federation (43,000), South Africa (16,000) and Bangladesh (15,000). All these statistics show that TB remains a major global health problem and has been designated as “Global Health Emergency” by WHO.

1.1.4 Status of current TB drug therapy

The current live vaccine Bacillus Calmette Gurein (BCG) attenuated strain of *M. bovis* was introduced in 1922. It does not protect all age groups as its efficacy is globally variable, and it does not provide protection in most parts of the world where TB is effectively prevalent. It is not suitable to use for immuno-compromised patients. In addition to this, BCG only reduces dissemination of Mtb to the spleen and other organs, but it does not prevent mycobacterial growth in the lungs. Therefore, an improved vaccine is required to cure aforesaid malignant ailment. Drugs available for the treatment of TB can be classified into two categories; first line drugs such as, isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STR) etc., Resistance to first line anti-tb drugs has been linked to mutations in atleast ten genes; *KatG, inhA, ahpC, kasA and ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance (Cheng et al., 2004). Second line drugs like para-aminosalicylate (PAS), kanamycin, cycloserine (CS), ethionamid e (ETA), amikacin, capreomycin, thiacetazone, fluoroquinolones etc. (Iseman 1993). These drugs are more toxic and less effective than first line drugs (WHO report 2001). Current TB therapy, also known as DOTS (Direct Observed Short Course Strategy), consists of an initial phase of treatment with four drugs, INH, RIF, PZA and EMB, for 2 months, followed by treatment with INH and RIF for another 4 months, three times a week. The targets of these drugs are varied. INH inhibits synthesis of mycolic acid, a cell wall component (Winder et al., 1970). PZA targets cell membrane where as RIF and STR interferes with the initiation of RNA and protein synthesis respectively (Garvin et al., 1974). EMB
blocks biosynthesis of arbinogalactan, a major polysaccharide present in the mycobacterial cell wall (Takayama et al., 1989) and kanamycin and capreomycin, like streptomycin inhibit protein synthesis through modification of ribosomal structure at the 16S rRNA. (Zhang et al., 2000) Cycloserine prevents the synthesis of peptidoglycan, a constituent of cell wall (Rando et al., 1975).

1.1.5 Limitations of current drug therapy
In the present scenario, due to the emergence of multi drug resistant TB (MDR-TB) and association between HIV and TB, DOTS are becoming rapidly ineffective in controlling TB. Recent reports indicate that in areas, where there is a high incidence of MDR-TB, DOTS is failing to control the disease (Kimerling et al., 1999). In such circumstances, the second line drugs are prescribed in combination with DOTS. However, these combinations of drugs are very expensive and moreover it has to be administered for a longer duration with anticipating significant side effects. One major draw back of current TB therapy is that the drugs are to be administered for at least 6 months. The length of time makes patient compliance difficult, and such patients become potent source of drug-resistant strains. The second major and serious problem of current therapy is that most of the drugs available today are ineffective against persistent bacilli, except for RIF and PZA. RIF is active against both active growing and slow metabolizing non growing bacilli, whereas PZA is active against semi-dormant non growing bacilli (Zhang et al., 2002). However, there are still persistent bacterial populations that are not killed by any of the available TB drugs. Therefore, there is a need to invent new drugs that are more active against slowly growing persistent bacilli and thus to reduce the risk of re-emerging disease. Secondly, it is important to achieve a shortened therapy to encourage patient’s compliance and to slow down the development of drug resistance in Mycobacterium.

1.1.6 The Stop TB Strategy
The Stop TB Strategy (WHO report 2009) is the approach recommended by WHO to reduce the burden of TB, with global targets which are setting for 2015. The six major components of the strategy are:

1. Expansion of DOTS to the all segments of the society is indispensable keeping in view of achieving effective control over TB/HIV.
2. Mere implementation of DOTS does not serve the purpose of eradication of TB/HIV, even more important thing is that the addressing TB/HIV, MDR-TB as well as other related challenges are required towards this end.

3. It is also necessary to improve health policies, human resources development, financing supplies, service delivery mechanism, authenticated information are required to be assessed, towards the context. Further more giving emphasis on infection control system in health services and other congregate settings are also a must. Up gradation of laboratory network as well as implementation to the practical approach to long health are to be taken up.

4. It is also significant to utilize the services of care providers who involve to prevent these ailments. It may also be a good idea to engage care providers from corporate, voluntary and private segments through public-private approaches (PPM). Since TB seeks at-most care from different sections of the society, taking high quality care is the most crucial factor to effectively control this ailments.

5. Further crucial factor towards subject matter is empowering the people who are suffering with TB/HIV with their respective communities through partnership persuasion of advocacy; specific communication keeping in view of eradication of TB as well as social mobilization towards the objective will help the subject purpose.

6. Community TB care projects can also show its positive role as to how people and affected communities can undertake necessary TB control tasks. Political support is also a helping aspect towards this end. Current resources are inadequate and further effort is required to mobilize additional resources from domestic as well as international sources to protect the poor and vulnerable population from TB, TB/HIV and MDR-TB. In this regard Global Drug Facility and the Green Light Committee offer countries with limited capacity the benefit of access to quality assured TB drugs at reduced prices and also facilitate access to training on drug management.

The activities such as control policies, programmes and services and social mobilization, among the public can help to build greater commitment to fighting TB. In future, the global burden of TB (per capita prevalence and death rates) will
be reduced by 50% relative to 1990 levels and incidence of active TB will be less than 1 case per million population per year.

1.1.7 Origin of *Mycobacterium tuberculosis*

The family of mycobacteria comprises pathogens and apathogenic environmental bacteria. Mycobacteria are unusual among bacteria since they have an enormously thick, hydrophobic cell wall which prevents desiccation. There are few human pathogenic mycobacteria which cause tuberculosis (*Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*) and leprosy (*Mycobacterium leprae*). Discovery of *Mtb* as an etiologic agent was reported to the "Physiologische Gesellschaft" in Berlin on March 24th, 1882 by Robert Koch. During his lecture on the "Ätiologie der Tuberkulose" Koch pointed out that it was a specific staining procedure that visualized characteristic, so far unknown bacteria in tuberculosis affected organs. It is thought that the progenitor of the *Mtb* complex, comprising *Mtb*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti*, arose from a soil bacterium and that the human bacillus may have been derived from the bovine form following the domestication of cattle. The complex lacks interstrain genetic diversity, and nucleotide changes are very rare (Sreevatsan et al., 1997). This is important in terms of immunity and vaccine development as most of the proteins will be identical in all strains and therefore antigenic drift will be restricted. On the basis of the systematic sequence analysis of loci in a large number of independent isolates it was concluded that the genome of *Mtb* is either unusually inert or that the organism is relatively young in evolutionary terms.

1.1.8 *Mycobacterium tuberculosis* H37Rv genome

Since its isolation in 1905, the H37Rv strain of *Mtb* has found extensive, worldwide application in biomedical research because it has retained full virulence in animal models of tuberculosis, unlike some clinical isolates, it is also susceptible to drugs and amenable to genetic manipulation. An integrated map of the 4.4 megabase (Mb) circular chromosome of this slow-growing pathogen had been established and ordered libraries of cosmids and bacterial artificial chromosomes (BACs) were available (Cole et al., 1998). The complete genome sequence analysis of *Mtb* H37 Rv unraveled the genetic information of the strain at molecular level which indicated the genome comprises 44,11,529 base pairs, with around 4,000 genes.
(Fig. 1.1) and has a very high guanine + cytosine content (65.6%) which is reflected in the biased amino-acid content of the proteins (Cole et al., 1998). This represents one of the largest bacterial genome sequence currently available. The genome is rich in repetitive DNA, particularly insertion sequences in new multigene families and duplicated housekeeping genes. The G+C content is relatively constant throughout the genome indicating that horizontally transferred pathogenicity islands of atypical base composition are probably absent. The genome sequence revealed several regions showing higher than average G + C content were detected corresponding to sequences belonging to a large gene family that includes the polymorphic G + C rich sequences (PGRSs). The release of complete genome sequence of *Mtb* has facilitated the development of more rational and specific methods to search for new drug targets as well as vaccine candidates.

1.1.9 Impact of *Mtb* genome sequence on identification of new drug targets

The complete genome sequence of *Mtb* H37Rv provides an opportunity for a more focused and planned approach towards the identification of new drug targets. Genome sequence helps in compilation of all the potential gene products encoded by a particular organism, identification of functions (enzymes and pathways) that are missing or unique in a particular organism, and finally identifying the genes that are common to all (or most) prokaryotes and eukaryotes. An important advantage of this analysis is the possibility of identifying a novel target that is present in many bacteria and subsequently designing a drug that could be active against a wide range of bacteria.
In addition, availability of human genome sequence can help in eliminating the potential drug targets that have close human homologues. Thus, the possibilities of using complete genome sequences for target identification are virtually unlimited (Chitta Suresh Kumar et al., 2005).

1.1.10 HISTORY OF ANTITUBERCULOSIS DRUGS

The TB drugs in use today reflect their origins in two sources of antimicrobial agents, i.e., chemical origin and antibiotic origin. Albert Schatz and Selman Waksman discovered the first effective TB drug streptomycin (Fig.1.2) from *Streptomyces griseus* in 1944 (Schatz et al., 1944), a discovery that marked the beginning of modern TB chemotherapy. The modern chemotherapeutic treatment of TB also had its beginning in sulfa drugs developed by Domagk for
the treatment of gram-positive bacterial infections (Domagk, 1935). In 1938, Rich and Follis from Johns Hopkins University found that sulfanilamide at high doses significantly inhibited the disease pathology in experimental TB infection in guinea pigs (Rich and Follis, 1938) but without significant effect in treatment of human TB in tolerable doses. This finding stimulated further effort to refine sulfa drugs for the treatment of TB and subsequently led to synthesis of thiosemicarbazones such as Conteben (also called amithiazone), which were more active than sulfanilamide and had definite clinical value but were not as effective as streptomycin (Hinshaw and Dermott, 1950). In 1946, two years after the discovery of streptomycin, Lehmann from Sweden discovered para-aminosalicylic acid (PAS) (Fig. 1.2) as an effective TB drug (Lehmann, 1946), a discovery based on a curious observation made by Bernheim that salicylate and benzoate stimulated the oxygen consumption of tubercle bacillus (Bernheim, 1940).

Fig. 1.2: Chemical Structure of Effective TB Drugs
**INTRODUCTION**

Fig. 1.3 Special bacterial populations and TB chemotherapy

<table>
<thead>
<tr>
<th>Drug (year of discovery)</th>
<th>MIC (^{\text{a}}) (µg/ml)</th>
<th>Effect on bacterial cell</th>
<th>Mechanisms of action</th>
<th>Targets</th>
<th>Genes involved in resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (1952)</td>
<td>0.01-0.2</td>
<td>Bactericidal</td>
<td>Inhibition of cell wall mycolic acid synthesis and other multiple effects on DNA, lipids, carbohydrates, and NAD metabolism</td>
<td>Multiple targets including acyl carrier protein reductase (InhA)</td>
<td>inhA, katG</td>
</tr>
<tr>
<td>Rifampin (1966)</td>
<td>0.05-0.5</td>
<td>Bactericidal</td>
<td>Inhibition of RNA synthesis</td>
<td>RNA polymerase (\beta) subunit</td>
<td>rpoB</td>
</tr>
<tr>
<td>Pynuzinamide (1952)</td>
<td>20-100</td>
<td>Bacteriostatic/bactericidal</td>
<td>Inhibition of proteus synthesis and energy depletions</td>
<td>Membrane energy metabolism</td>
<td>pks, pknA</td>
</tr>
<tr>
<td>Ethambutol (1961)</td>
<td>0.1-5</td>
<td>Bacteriostatic</td>
<td>Inhibition of cell wall arabinogalactan synthesis</td>
<td>Arabinosyl transferase</td>
<td>catB</td>
</tr>
<tr>
<td>Streptomycin (1944)</td>
<td>2-8</td>
<td>Bacteriostatic</td>
<td>Inhibition of protein synthesis</td>
<td>Ribosomal 312 protein and 16S rRNA</td>
<td>rpm, rps</td>
</tr>
<tr>
<td>Kanamycin (1957)</td>
<td>1-8</td>
<td>Bacteriostatic</td>
<td>Inhibition of protein synthesis</td>
<td>16S rRNA</td>
<td>rks</td>
</tr>
<tr>
<td>Quinolone (1963)</td>
<td>0.2-4</td>
<td>Bactericidal</td>
<td>Inhibition of DNA synthesis</td>
<td>DNA gyrase</td>
<td>gyrB, gyr</td>
</tr>
<tr>
<td>Ethionamide (1956)</td>
<td>0.6-2.5</td>
<td>Bacteriostatic</td>
<td>Inhibition of mycolic acid synthesis</td>
<td>Acyl carrier protein reductase</td>
<td>inhA, inhB</td>
</tr>
<tr>
<td>PAS (1956)</td>
<td>1-8</td>
<td>Bacteriostatic</td>
<td>Inhibition of folic acid and iron metabolism?</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cycloserine (1952)</td>
<td>5-20</td>
<td>Bacteriostatic</td>
<td>Inhibition of peptidoglycan synthesis</td>
<td>(\beta)-alanine racemase</td>
<td>apa, Dap</td>
</tr>
</tbody>
</table>

*MIC is based on isolated & sulfonamides (15).
*KatG, PknA, and RpoB/RpoC are enzymes involved in the production of proteus (PAS, PZA, and INH, respectively).
*In last growing of inactivity.

Table 1.1: List of TB Drugs and its mechanism of action of their specific targets

### 1.1.13 Possible drug targets *Mtb*

In recent years, a number of new genes and their products in *Mtb* have been identified, which can be possible drug targets for designing anti-tuberculosis drugs (Table 1.1). The gene products that control vital aspects of mycobacterial metabolism are:
physiology like, metabolism, persistence, virulence, signal transduction and cell wall synthesis would be attractive targets for new drugs. A large number of gene products are being studied in the search for new drug targets using various computational approaches. The approach of targeting virulence factors, like other approaches suffers from some serious drawbacks, like virulence factors may not be necessarily survival genes. Therefore, inhibition of virulence factors may not be lethal to the pathogen. The other very important hurdle in this approach is that drugs that target virulence factors may be of very little or of no use if the disease has already been established. However, inhibitors of these virulence gene products may be used in combination with existing drugs to improve the regime of chemotherapy (Alksne and Projan, 2000).

1.1.13.1 Mycobacterial cell wall

The cell wall of Mtb and its related genera is unique among prokaryotes. The wall is necessary for viability (Anuradha et al., 2010) and several known drugs such as isoniazid (Winder, 1982) and ethambutol (Deng et al., 1995) inhibit cell wall synthesis. The mycobacterial cell wall core consists of three interconnected macromolecules (Fig 1.3). The outermost, the mycolic acids (Brennan et al., 1995) are 70 to 90 carbon-containing, branched fatty acids which form an outer lipid layer in some way similar to the classical outer membrane of gram-negative bacteria (Brennan et al., 1968). Mycolic acids are strong hydrophobic molecules oriented perpendicular to the plane of the membrane and provide a special lipid barrier responsible for many physiological and disease inducing aspects of Mtb. They are thought to be significant determinant of virulence in Mtb. Probably they prevent attack of mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. The mycolic acids are esterified to the middle component, arabinogalactan (AG), a polymer composed primarily of D-galactofuranosyl and D-arabinofuranosyl residues. AG is connected via a linker disaccharide, α-L-rhamnosyl-(1-3)-α-D-N-acetyl-glucosaminosyl-1-phosphate, to the sixth position of a muramic acid residue of peptidoglycan (PG) (McNeil and Brennan 1990) which is the innermost of the three cell wall core macromolecules. A complex consisting of mycolic acids, arabinogalactan and peptidoglycan constitutes "the core" of the cell wall (Crick et
which is often referred as "mycolyl-arabinogalactan-peptidoglycan" (MGP) complex. This covalently linked structure is intercalated with numerous glycolipids such as lipoarabinomannan (LAM), the phosphatidyl inositol containing mannosides (PIMs), trehalose dimycolate (TDM; so-called cord factor), trehalose-monomycolate (TMM), which play an important role in virulence of Mtb (Glickman and Jacob 2001). The dominating heteropolysacharide LAM is noncovalently attached to cell wall and may be anchored to the cytoplasmic membrane via phosphatidyl-myco-inositol (pi) unit.

LAM is a potent inducer of chemoattractant cytokines and also induces a potent proinflammatory response in macrophages and dendritic cell and in the inhibition of phagosome maturation, apoptosis and cytokine secretion, thus may contribute to circumvent the activation of a pulmonary granulocytic response, which may be important in the early immune response to mycobacterial infection and intracellular survival (Schuller-Levis et al., 1994). Lipids such as cord factor have been suggested to play an important role in the virulence of Mtb by inducing cytokine-mediated events (Devergne et al., 1992).

Possible drug targets in Mtb Cell wall

In recent years several genes products and enzymes involved in the cell wall synthesis as well as fatty acid, mycolic acid synthesis are being studied in the search for new drug targets using various approaches. Progress made in this regard is reviewed here. Current available TB drugs such as INH, ETA, and EMB target mycobacterial cell wall synthesis. Thiolactomycin (TLM) targets two alpha-
ketoacyl-acyl-carrier protein syntheses, KasA and KasB enzymes that belong to the fatty acid synthase type II system involved in the fatty acid and mycolic acid biosynthesis (Slayden et al., 1996). TLM has also been shown to be active against MDR-TB clinical isolate. Several TLM derivatives have been found to be more potent invitro against fatty acid and mycolic acid biosynthesis (Zhang and Amzel, 2002). Cerulenin, an inhibitor of fatty acid synthesis, has also been shown to inhibit mycobacterial lipid synthesis and is active against Mtb in-vitro with a minimum inhibitory concentration (MIC) of 1.5-12.5 mg/ml (Parrish et al., 1999). Octanesulphonyl acetamide (OSA) has recently been identified as inhibitor of fatty acid and myolic acid biosynthesis in mycobacteria (Jones et al., 2000). The inhibitor was found to be active against both slow growers such as Mtb and also MDR-TB strains with a MIC of about 6.25-12.5 mg/ml. Interestingly, OSA was found to be less active against fast growers such as M. smegmatis and M. fortuitum (Parrish et al., 2001). In recent years enzymes involved in the cell wall pathway have been attracting as targets in drug development. For example, enzymes (RmlA to RmlD) which are involved in the synthesis of dTDP-rhamnose, the precursor of L-rhamnose, an essential structural component of the cell wall of Mtb, have been selected for an in vitro screening with chemical library of 8,000 compounds (Ma et al., 2001). Determination of the pathway for rhamnose biosynthesis in mycobacteria- cloning, sequencing and expression of the Mtb gene encoding alpha-D-glucose-1-phosphate thymidyl transferase (Ma et al., 1997). The purification, crystallization and structural elucidation of dTDP-D-glucose 4,6-dehydratase (RmlB), the second enzyme in the dTDP-L-rhamnose synthesis pathway from Salmonella enterica serovar typhimurium (Allard et al., 2000). High resolution X-Ray structure of dTDP-glucose 4, 6-dehydratase (RmlB), the second enzyme in the dTDP-Lrhamnose synthesis pathway from Streptomyces venezuelae (Allard et al., 2004). Crystal structure of dTDP 4-Keto-6-deoxy-D-hexulose 3, 5 epimerase (RmIC) from Methanobacterium thermoautotrophicum complexed with dTDP (Christendat et al., 2000). Identification of a terminal rhamnopyranosyltransferase (RptA) involved in Corynebacterium glutamicum cell wall biosynthesis (Helen et al., 2009). Despite of intensive research on potential drugs targets of Mtb till date there are only few reports available to procure comprehensive information pertaining to the rhamnose
enzymes of *Mycobacterium* as of today. These enzymes are envisaged as good targets for the development of inhibitors against resistant strains.

Enzymes involved in the D-alanine metabolism are Alanine racemase (EC:5.1.1.1), D-alanine-D-alanine ligase (EC: 6.3.2.4) and D-alanine-D-alanyl carrier protein ligase (EC: 6.1.1.13). Alanine metabolism in murein synthesis is particularly interesting since the pentapeptide possesses three alanyl residues, two of these being of the dextro configuration. D-Alanine is thus a key residue in the peptidoglycan assembly and cross-linking. The enzymes mainly involve to synthesize the D-alanine branch in peptidoglycan are: Pyridoxal phosphate-dependent alanine racemase and ATP-dependent D-Ala-D-Ala ligase and these two key enzymes implicated in the elaboration of the peptidoglycan precursor (Fig 1.4). The other enzyme in D-alanine metabolic pathway is ATP dependent D-alanine-D-alanyl carrier protein ligase, involved addition of D-alanine to teichoic acids reduces the negative charge of the cell envelope, thereby influencing the binding and interaction of various compounds. In recent years these enzymes have been extensively investigated in terms of molecular biology, protein purification, and catalytic mechanisms. A particular emphasis has been on the mode of action of selective inhibitors and analyses of opportunities for design of new antibacterial agents based on enzyme mechanistic rationales.
Fig. 1.4: Alanine racemase (EC: 5.1.1.1.), D-alanine-D-alanine ligase (EC: 6.3.2.4) and D-alanine-D-alanyl carrier protein ligase (EC: 6.1.1.13) are involved in D-alanine metabolism and are linked to peptidoglycan synthesis.

Obtaining the biological function of a protein is essential for determining its potential as a therapeutic target and its utility as part of structure-based drug design effort. Furthermore, understanding the biological function for a protein provides the basis for exploring its cellular activity. Till to date only few reports are available on key enzymes $\text{Ar}$ and $\text{DltA}$ in $\text{Mtb}$, among purification and preliminary crystallography studies in alanine racemase (Strych et al., 2007) and a functional $\text{dlt}$ Operon, encoding Proteins ($\text{DltA}$) required for incorporation of D-Alanine in teichoic Acids in Gram-Positive Bacteria, Confers Resistance to Cationic Antimicrobial Peptides, but no reports were observed on $\text{Ddl}$ either molecular level or crystallographic studies. In addition to this, few reports are available in detailed study of $\text{Mtb}$ strains resistant to beta-lactam antibiotics and role of neuraminidase, autolysin and superoxide dismutase in respiratory tract infections. Hence, in difficulties on determination of protein structures by NMR or
X-ray crystallography on these enzymes, it is necessity to predict the three dimensional structure (3D) structure from a homologous protein of known structure and design drug like compounds on targeted proteins of Mtb through High-throughput docking (HTD) method. HTD is one of the most important approaches among structure-based virtual screening methods for hit/lead discovery in both pharmaceutical industry and academia.

1.1.13.3 Inhibitors of CellWall Synthesis

\textbf{INH} INH is a prodrug that requires activation by \textit{Mtb} catalase-peroxidase (KatG) (Zhang et al., 1992) to generate a range of reactive oxygen species and reactive organic radicals, which then attack multiple targets in the tubercle bacillus. The primary target of inhibition is the cell wall mycolic acid synthesis pathway (Winder and Collins, 1970), where enoyl ACP reductase (InhA) was identified as the target of INH inhibition (Benarjee et al., 1994). The active species for InhA inhibition has been found to be isonicotinic acyl radical, which reacts with NAD to form INH-NAD adduct and then inhibits the InhA enzyme (Rozwarski et al., 1998, Broussy et al., 2003). The reactive species produced during INH activation could also cause damage to DNA, carbohydrates, and lipids (Winder et al., 1982) and inhibit NAD metabolism (Winder and Collins, 1968, Sri prakesh and Ramakrishnan, 1969). Changes in the NADH/NAD ratios caused by mutations in NAD dehydrogenase II (\textit{ndh}) could cause resistance to INH (Miesel et al., 1998, Lee et al., 2001). The cidal activity of INH is very likely to be due to its effect on multiple targets in tubercle bacillus (Zhang et al., 2004). Mutations in KatG involved in INH activation (Zhang et al., 1992), in the INH target InhA (Banerjee et al., 1994), and Ndh II (NADH dehydrogenase II) (Lee et al., 2001) could all cause INH resistance. KatG mutation is the major mechanism of INH resistance (Wade and Zhang, 2004, Zhang et al., 2004).

\textbf{ETH}/\textbf{PTH} ETH, structurally related to INH (Figure 1), is also a prodrug that is activated by the enzyme EtaA (a monoxygenase, also called EthA) (Debarber et al., 2000, Baulard et al., 2000) and inhibits the same target InhA as INH (Banerjee et al., 1994) of the mycolic acid synthesis pathway. PTH (prothionamide) shares almost identical structure and activity as ETH, where the R group in ETH is C2H5 and the R group in PTH is C3H7 (Figure 1). EtaA is an 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 sulfinic acid intermediate (Vennelli et al., 2002). EtaA also activates thiacetazone, thiobenzamide, and perhaps other thioamide drugs (Vennelli et al., 2002). Mutations in the drug-activating enzyme EtaA/EthA and the target InhA cause resistance to ETA (Moricock et al., 2003).

**EMB** EMB [(S,S)-2,2-(ethylenediimino)di-1-butanol] (EMB) interferes with the biosynthesis of arabinogalactan, a major polysaccharide of mycobacterial cellwall (Takayama and Kilberg, 1989). It inhibits the polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (Mikusov et al., 1995) and induces accumulation of D-arabinofuranosyl-Pdecaprenol, an intermediate in arabinan biosynthesis (Woluka et al., 1994). Arabinosyl transferase, encoded by *embB*, an enzyme involved in synthesis of arabinogalactan, has been proposed as the target of EMB in *Mtb* (Telenti et al., 1997) and *M. avium* (Belanger et al., 1996). In *Mtb*, *embB* is organized into an operon with *embC* and *embA* in the order *embCAB*. *embC*, *embB*, and *embA* share more than 65% amino acid identity with each other and are predicted to encode transmembrane proteins with 12 transmembrane-spanning domains (Telenti et al., 1997). Mutations in *embCAB* operon are responsible for resistance to EMB and are found in approximately 65% of clinical isolates of *Mtb* resistant to EMB (Sreevatsan et al., 1997).

**CS** inhibits the synthesis of cell wall peptidoglycan by blocking the action of D-alanine racemase (Alr) and D-alanine:D-alanine ligase (Ddl) (David et al., 1969, Strych et al., 2001). Alr is involved in conversion of L-alanine to D-alanine, which then serves as a substrate for Ddl. The D-alanine racemase encoded by *alrA* from *M. smegmatis* was cloned and its overexpression in *M. smegmatis* and *M. bovis* BCG caused resistance to cycloserine (Cacersetal et al., 1997). Inactivation of *alrA* (Chacon et al., 2002) or *ddl* (Belanger et al., 2000) in *M. smegmatis* caused increased sensitivity to CS. Overexpression of *Alr* conferred higher resistance to CS than Ddl overexpression in *M. smegmatis*, suggesting Alr might be the primary target of CS (Feng and Barletta, 2003). Consistent with this finding, CS also preferentially inhibited Alr over Ddl in *M. smegmatis* (Feng and Barletta, 2003). However, the mechanism of resistance of CS in *Mtb* remains to be identified.
1.1.13.4 DNA Gyrase:

DNA gyrase is a type IIA topoisomerase, which introduces negative supercoils into closed-circular DNA (Gellert et al., 1976). DNA gyrases are found in all prokaryotes and in eukaryotes such as *Plasmodium falciparum*, *Plasmodium vivax* and *Arabidopsis thaliana*. *E. coli* DNA gyrase was the first type II topoisomerase to be discovered (Gellert et al., 1976). The enzyme maintains the homeostatic balance of chromosomal superhelical density by catalysing the introduction of negative supercoiling into DNA, a prerequisite for replication and transcription, and relaxes positive supercoils generated ahead of the transcription complex. The DNA supercoiling mechanism involves transient breakage and resealing of both strands of DNA. It also catalyses catenation and decatenation of DNA rings, and knotting and unknotting of duplex DNA, but this activity is weaker in comparison to topo IV. *E. coli* DNA gyrase is composed of two subunits of GyrA and two subunits of GyrB. The molecular weights of *E. coli* GyrA and GyrB are 97-kDa and 90-kDa respectively (Wang, 1985). The *E. coli* DNA gyrase domain boundaries have been well defined and characterised. GyrA is divided into a 64-kDa N-terminal domain (breakage-reunion domain) that contains the QRDR (Quinolone-resistance determining region), and a 33-kDa C-terminal domain around which the DNA is wrapped (Reece & Maxwell, 1989). The N-terminal domain forms a DNA-binding saddle known as the DNA breakage-reunion domain. It also forms a large 30-Å cavity that can accommodate a DNA duplex. The GyrA C-terminus adopts a β-propeller fold and is largely basic around the outside suggesting the involvement of the domain in DNA wrapping (Corbett & Berger, 2004, Liu & Wang, 1978a). GyrB is divided into a 43-kDa N-terminal ATPase domain (aminocoumarin interaction site) (Wigley et al., 1991) and a 47-kDa C-terminal domain (Brown et al., 1979) that interacts with GyrA and DNA. *E. coli* DNA gyrase has a 170 amino acid insertion within the C-terminal domain, in comparison to other bacterial DNA gyrase (*Mtb*) and type IIA topoisomerases. This insertion is important for the DNA binding capabilities of *E. coli* DNA gyrase. Recently has been established that the insertion is important for DNA binding and communication between different functional domains (Schoeffler et al., 2010). The crystal structures for different domains of *E. coli* gyrase subunits are available as shown in the Figure 1.15. *Mtb* DNA gyrase is a validated target for anti-tubercular
drug discovery, because its inhibition results in mycobactericidal activity (Mdiluli & Ma, 2007). Inhibitors of this enzyme are also active against non-replicating, persistent mycobacterium, which may play a role in shortening the duration of TB therapy. The \textit{Mtb} DNA gyrase genes have been cloned in \textit{E. coli} (Madhusudan \textit{et al.}, 1994, Takiff \textit{et al.}, 1994). Based on the sequencing data and genome analysis, \textit{Mtb} genes encoding DNA gyrase were identified as a \textit{gyrB-gyrA} contig in which \textit{gyrA} and \textit{gyrB} encode the A and B subunits, respectively; they are organized as a bicistronic unit. The \textit{gyrA} gene is located 34 nucleotides downstream of \textit{gyrB}. Both the genes are transcribed from the promoter elements located upstream of \textit{gyrB} coding sequence.

Like \textit{E. coli} DNA gyrase, \textit{Mtb} DNA gyrase is an A$\alpha$B$\beta$ tetramer. \textit{GyrA} is $\sim$97-kDa and 838 amino acids, while \textit{GyrB} is $\sim$72-kDa and 714 amino acids. The N-terminal domain of \textit{GyrB} harbours the ATPase activity; the C-terminal domain of \textit{GyrB} is the domain that interacts with \textit{GyrA} and forms a complex capable of catalyzing DNA breakage and reunion. The N-terminal domain of \textit{GyrA} is the catalytic centre of gyrase, which harbours the DNA cleavage–reunion activity, and the C-terminal domain of \textit{GyrA} is involved in binding and wrapping the DNA. Recent research shows that Y577, R691 and R745 are among the key DNA-binding residues in the C-terminal domain of \textit{Mtb} \textit{GyrA}, and that the third blade of the \textit{GyrA} C-terminal domain is the main DNA-binding region (Huang \textit{et al.}, 2006). The pairwise comparisons of the gyrase proteins from \textit{Mtb} and \textit{M. smegmatis} showed that \textit{GyrA} of \textit{M. smegmatis} shares 88.5\% identity and 93.9\% similarity with that of \textit{Mtb} at the amino acid level. \textit{M. smegmatis} \textit{GyrB} is also highly homologous (86\% amino acid sequence identity) to \textit{Mtb} \textit{GyrB} protein. There are two structural differences between \textit{E. coli} and \textit{Mtb} DNA gyrase. There is an insertion of 170 amino acids within the Toprim fold of \textit{E. coli} \textit{GyrB} that is not present in \textit{Mtb}.

### 1.1.1.13.5 DNA Gyrase inhibitors

Since DNA gyrase is the only topoisomerase that can catalyse the introduction of negative supercoils into DNA, it has been exploited as a drug target. It has been possible to develop inhibitors that inactivate gyrase without substantially affecting its human counterpart, topo II (Gatto \textit{et al.}, 1999). DNA gyrase is the target of the
fluoroquinolones (e.g., ofloxacin, ciprofloxacin, sparfloxacin, moxifloxacin, gatifloxacin), aminocoumarins (novobiocin, clorobiocin), simocyclinones (e.g., simocyclinone D8), cyclothialidines, CcdB and microcin B17 (Fig. 1.5).

Fig. 1.5: Effect of quinolone drugs on DNA Gyrase

Inhibitors of Nucleic Acid Synthesis

RIF RIF is a broad-spectrum semisynthetic rifamycin B derivative that interferes with RNA synthesis by binding to the bacterial DNA-dependent RNA polymerase β-subunit encoded by rpoB. An important feature of RIF is that it is active against both actively growing and slowly metabolizing nongrowing bacilli. Its activity against the latter is thought to be involved in shortening the TB therapy from 12–18 months to 9 months (Mitchison, 1985). Mutations in a defined 81-bp region of the rpoB are found in about 96% of RIF-resistant Mtb isolates (Telenti et al., 1993). Resistance to RIF could confer cross-resistance to other rifamycins such as rifabutin and rifapentine. Rifapentine, with a longer half-life and greater activity than RIF, is a new drug approved by the FDA in 1998 for treatment of TB (Jarvis and Lamb, 1998). Rifapentine can reduce the frequency of drug dosage required, but it is not active against RIF-resistant Mtb (Williams et al., 1998).
Fluoroquinolones

Fluoroquinolones (7-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acids) have few toxic side effects, good pharmacokinetic properties and potent activity against Gram-positive, Gram-negative and mycobacterial strains (Fig. 1.6). Norfloxacin was the first of a new generation of fluoroquinolone antibacterial. They bind reversibly to the enzyme-DNA complex and stabilize the covalent enzyme tyrosyl-DNA phosphate ester, which is normally a transient intermediate in the topoisomerase reaction. Hydrolysis of this linkage leads to the accumulation of double-stranded DNA fragments and is the bactericidal consequence of fluoroquinolone treatment. Fluoroquinolones are being used in cases of MDR-TB and have a great potential for shortening treatment duration and improving therapy of TB-HIV co-infections. Recently a low-resolution crystal structures (4 Å) of cleavage complexes formed by the S. pneumoniae 55-kD ParC breakage-reunion and 30-kD ParE Toprim domains of topoisomerase IV stabilized by moxifloxacin and clinafloxacin have provided insights into the fluoroquinolone mechanism of action (Laponogov et al., 2009). The structures reveal that the fluoroquinolones intercalate at the bent gate DNA, stacked against DNA bases and are present in the drug-stabilised cleavage complex. They intercalate in the gap between the -1' and +1 nucleotide pairs of the cleaved DNA bound to topo IV. Studies in the past have identified consensus sequences for fluoroquinolonemediated DNA cleavage by Gram-negative and Gram-positive type II topoisomerases. Wild type and mutated consensus sequences showed the following features:

1. Gn/Cn-rich sequences at and around the cleavage site are hot spots for quinolone-mediated strand breaks.
2. Symmetry of the target sequence is important for cleavage.
3. In case of S. pneumoniae, A and T at position -2 and +6 are cleavage determinants.
4. The consensus sequence adopts a heteronomous A/B conformation (Richter et al., 2007).

In the crystal structure the C-7 substituent of the quinolone interacts with DNA base pairs while the 3-carboxyl group contacts S79 (equivalent to positions 83 in E. coli GyrA) and is close to S80 (A84 of GyrA). Formation of a Mg2+ bridge is
important for drug binding due to the Mg$^{2+}$ dependence of complex formation (Sissi et al., 2001). Figure 1.25 also shows the residues in ParC (S79, D83) that are responsible for drug resistance upon mutation. Similarly, in the case of ParE, the residues (E474, E475, R456 and D435) responsible for drug-resistance are located within the Toprim domain and are close to the intercalated drug.

\begin{align*}
\text{Fig. 1.6: Chemical Structure of Fluoroquinolones Drugs} \\
\text{However, the existing DNA intercalation model has its limitations. It cannot explain the effects of certain amino acid substitutions and drug structure variations. For example the putative interaction of the C-7 substituent with position 81 as described above and the drugs substituent at position 1 and 8 that alter both the antimicrobial activity and drug-target binding constant. Thus, quinolone binding is a multi-step process that involves at least two steps before and after the DNA cleavage. A Crystal structure with a resolution of 3.35 Å of S. aureus DNA gyrase in complex with fluoroquinolone and DNA is also available (2XCT) (Bax et al., 2010). This structure provides insights into how DNA is cleaved by type IIA topoisomerases and the role of the metal-binding Toprim domains in catalysis.}
\end{align*}