CHAPTER – 1

Introduction
Fighting disease with drugs is the timeless struggle. It has begun with the existence of the mankind. Survival of the mankind on this planet has been dependent upon its success. Today the conflict is increasing continuously in the laboratory and the clinic. The scientific approach to this struggle is pharmacology. With the increasing knowledge of cell-biology and biochemistry, the field of pharmacology has also changed substantially. It has become possible through molecular analysis of receptor to design chemical compounds that act on specific cellular metabolic pathway by affecting sites directly on cell-surface receptors.

The great expansion in medicinal research in past has contributed much to the unparalleled progress of medicine. Improved and more meaningful biological test procedures of diagnosis have provided better guidance in drug discovery by pointing out suggestive observations which could be used in the design of new prophylactic and therapeutic agents. The growth of molecular biology with its chemical insight into experimental biology has contributed to more significant pharmacological theories. The elucidation of the structure of many metabolites, and that of polypeptides, enzymes, polynucleotides and other biopolymers helped in more rational study of the chemical mode of action of such compounds, and their interaction with drugs. Medicinal chemistry has taken advantage of these investigations and refined the pertinent chemical theories, to establish itself firmly as an interdisciplinary science. Medicinal chemistry has become the acknowledged meeting ground of modern organic, physical and biochemistry with their application of drugs, with its own literature and procedures.

Organic chemistry has its origin in the study of natural products and this still remains the most important role. Many organic compounds occur naturally and their functions are often of fundamental importance to living organisms. Today, although many compounds of carbon are still most conveniently isolated from plant and animal sources, most of them are synthesized.

The molecules of organic chemical compounds are built by a framework of carbon atoms to which hydrogen, oxygen, or other heteroatoms are attached. Carbon atoms can in particular readily join with one another to form chains of atoms. When the ends
of the chain are joined together, ring is formed, resulting into cyclic compounds; such compounds often referred as alicyclic or carbocyclic compounds.

Substitution of one or more of the ring carbon atoms in the molecule of carbocyclic compounds by a heteroatom gives a heterocyclic compound. The critical role played by heterocycles in drug design cannot be denied. Even where the natural substrate or ligand for a biological target does not contain a heterocycle, drugs, whether of natural or man-made origin, that act on that target frequently contain heterocyclic groups. These may mimic the heterocycles found in the natural ligands or substrates, or they may mimic other functional groups, such as the amides of a peptide ligand. In this later case, the heterocycle often confers stability on a ligand which would otherwise be rapidly degraded in vivo, or, equally important, enables the ligand to be absorbed from the gut and/or to penetrate the blood–brain barrier.

Today a large number of diseases can be cured or at least controlled by drug therapy. The fight against bacterial and fungal infections has been largely won and significant progress has been made. It would not be an exaggeration to claim that certain form of cancers can be cured by chemotherapy. However when coupled with other chronic conditions, it still irritates physician even today, because of the resistance offered by acting against various forms of therapy.

1.1 Medicinal chemistry: an overview

Medicinal chemistry is defined as a field which applies the principles of chemistry and biology to the knowledge which leads to the introduction of new therapeutic agents. The primary objective of medicinal chemistry is to design and discover new compounds that are suitable for use as drugs. This process involves a team of scientists from a wide range of disciplines such as chemistry, biology, biochemistry, pharmacology, mathematics, medicine and computing, amongst others. The discovery or design of a new drug not only requires a discovery or design process but also the synthesis of the drug, a method of administration, the development of tests and procedures to establish how it operates in the body and a safety assessment. Drug
discovery may also require fundamental research into the biological and chemical nature of the diseased state.

For better understanding of medicinal chemistry, it is important to understand the following terminologies.

1.1.1 Drugs

The word ‘drug’ is derived from the French word “drogue”, which means a dry herb. In general way, a drug may be defined as a substance used in the prevention, diagnosis, treatment or cure the disease in man or other animals. According to WHO, a drug may be defined as any substance or product which is used or intended to be used for modifying or exploring physiological system or pathological state for the benefit of the recipient.

For the drug to be most useful, first of all it should not be toxic, but should possess good pharmacokinetic properties. Secondly it should not be rapidly metabolized but should optimally be absorbed after oral administration. Thus, ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties of compounds must be investigated early in the drug discovery path to clarify clinical utility of new genes as targets. Very few drugs satisfy all the above conditions. However, the search for ideal drug continues.

1.1.2 Pharmacological action

In medicinal chemistry, the term pharmacological action refers to the specific biochemical interaction through which a drug substance produces its pharmacological effect. A mechanism of action usually includes specific molecular targets to which the drug binds, such as an enzyme or receptor.

For example, the mechanism of action of aspirin involves irreversible inhibition of the enzyme cyclooxygenase, therefore suppressing the production of prostaglandins and thromboxanes, thereby reducing pain and inflammation.
1.1.3 Biological target

A biological target is a biopolymer such as a protein or nucleic acid whose activity can be modified by an external stimulus (chemical substance). The external stimulus physically binds to the biological target. The interaction between the substance and the target may be noncovalent, reversible covalent (A chemical reaction occurs between the stimulus and target in which the stimulus becomes chemically bonded to the target, but the reverse reaction also readily occurs in which the bond can be broken) or irreversible covalent (The stimulus is permanently bound to the target through irreversible chemical bond formation).

Depending on the nature of the stimulus, the following can occur:

- There is no direct change in the biological target, except that the binding of the substance prevents other endogenous substances such as activating hormone to bind to the target. Depending on the nature of the target, this effect is referred as receptor antagonism, enzyme inhibition, or ion channel blockade.
- A conformational change in the target is induced by the stimulus which results in a change in target function. This change in function can mimic the effect of the endogenous substance in which case the effect is referred to as receptor agonism (or channel or enzyme activation) or be the opposite of the endogenous substance which in the case of receptors is referred to as inverse agonism.

The term biological target is frequently used in pharmaceutical research to describe the native protein in the body whose activity is modified by a drug resulting in a desirable therapeutic effect. In this context, the biological target is often referred to as a drug target. The most common drug targets of currently marketed drugs include proteins, G protein-coupled receptors, enzymes, ion channels, ligand-gated ion channels, voltage-gated ion channels, nuclear hormone receptors, structural proteins such as tubulin, membrane transport proteins and nucleic acids.
1.1.4 Pharmacophore and toxicophore

A pharmacophore, according to IUPAC definition, is "an ensemble of steric and electronic features that is necessary to ensure the optimal intermolecular interactions with a specific biological target and to trigger (or block) its biological response". When a pharmacophore is introduced in biologically inactive compound, the introduction of it makes the compound biologically more active than the parent compound. Some examples of pharmacophores are alkyl, hydroxy, alkoxy, carbonyl, carboxylic acid, amine, nitro, nitrile, unsaturated compound, halogens and unsaturated lipids.

Drugs and chemicals can be metabolized in living organisms in an effort to remove them from the body. During this excretion process, certain chemicals can be transformed into unstable, highly reactive species. This can result in the overwhelming of cellular defense, allowing the reactive metabolite to bind to cellular macromolecules, and leading to abnormal cellular function. It is widely accepted that this process can lead to clinically observed toxicities in the form of adverse drug reactions. This toxic properties of compounds can be related to chemical structures, and more specifically, to particular substructures, called toxicophores. A toxicophore is a feature or group within a chemical structure that is thought to be responsible for the toxic properties, either directly or via metabolic activation. Reliability and accuracy of mutagenicity, hepatotoxicity, or cardiotoxicity predictions may be achieved by identifying toxicophores. These predictions can guide the design of chemical libraries for hit and lead optimization. As such, a thorough molecular knowledge in drug-induced toxicity is required to aid the development of new therapeutic agents and prevent the release of potentially toxic drugs into the market.

1.1.5 Chemotherapy

The treatment of infectious disease by using a chemical agent is called chemotherapy. The substance so employed is referred to as chemotherapeutic agent. These agents are designed in such a way that they kill or destroy the disease-producing organisms without any harmful effect on the cells in which organisms are
present. Paul Ehrlich (1854-1915) did outstanding and pioneering work in medicinal chemistry and therefore is called ‘Father of Chemotherapy’. He gave original ideas about the models of action of drugs. According to him, there are some cellular constituents in mammalian cells, which were named as receptors later by Langley (1878). Ehrlich defined chemotherapy as the use of drugs to injure an invading organism without causing injury to the host.

1.1.6 Chemotherapeutic drugs

According to Ehrlich chemotherapeutic agents are chemical substances with high parasitotropism and low or no organotropism. In other words, they are selectively toxic, being harmful to the invading organism but innocuous to the host.

The infectious diseases are caused by certain species of metazoa, protozoa, fungi, bacteria, rickettisa and viruses. Drugs active on these pathogenic agents are classified according to a types of pathogens on which they act. Some representative examples are: antimalarial, antiprotozoal, antifungal, antibacterial, antiseptic, antituberculosis and antilepral, antibiotics, antineoplastic, antiviral agents, etc.

1.2 Outline of drug development process

Following is an outline which briefs the stages through which a drug discovery/development project proceeds from inception to marketing and beyond. Observing this outline, one can find the complexity of the task undertaken for finding new therapeutic candidates.

- **Stage-I:** Identification of target disease, establishment of a multidisciplinary research team, selection of a promising approach, framing a sufficient budget, procurement of research funds.
- **Stage-II:** Initiation of chemistry (normally involves the synthesis based on available chemicals or collection of natural product sources (extracts)). Pharmacological studies also include suitable screening methods and choice of receptor binding/ enzymatic assays.
• **Stage-III:** Confirmation of potential utility of initial classes of compounds in animals, focusing on potency, selectivity and apparent toxicity. Initial Hit identifications, applying ligand based (2D or 3D-QSAR), structure based design strategies for the generation of potential analogues of Hit candidates. Exploring solubility, membrane permeability, blood brain barrier parameters, hepatotoxicity, cytochrome P450 (CYP450) analogues inhibition and selectivity indexes of lead compounds.

• **Stage-IV:** Analogue synthesis of the most active lead compounds (lead optimization stage), planned after careful examination of experimental results, literature, patents and using medicinal intellect. Study of more elaborated pharmacology in order to elucidate mode of action, efficacy, acute/chronic toxicity, and genotoxicity as well as ADME (adsorption, distribution, metabolism, excretion) properties. Planning of large scale synthesis and initiation of formulation studies. Application for patent protection.

The above four stages, which typically last 4–5 years, are followed by extensive time-and resource demanding clinical, regulatory, and marketing phases, which normally last about 10-15 years. Clinical trials involving new drugs are commonly classified into four phases. Each phase of the drug approval process is treated as a separate clinical trial. The drug-development process will normally proceed through all four phases over many years. If the drug successfully passes through Phases 0, 1, 2, and 3, it will usually be approved by the national regulatory authority for use in the general population.

• **Phase 0:** Pharmacodynamics and Pharmacokinetics
  The Phase 0 trials are the first-in-human trials. Single subtherapeutic doses of the study drug are given to a small number of subjects (10 to 15) to gather preliminary data on the agent's pharmacodynamics (what the drug does to the body) and pharmacokinetics (what the body does to the drugs).
• **Phase 1**: Screening for safety
  In this trial, researchers test an experimental drug or treatment in a small group of people (20-80) for the first time to evaluate its safety, determine a safe dosage range, and identify side effects.

• **Phase 2**: Establishing the testing protocol
  In this, the experimental treatment is given to a larger group of people (100-300) to see if it is effective and to further evaluate its safety.

• **Phase 3**: Final testing
  In Phase 3 trial, the treatment is given to large groups of people (1,000-3,000) to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow it to be used safely.

• **Phase 4**: Post approval studies
  The Phase 4 trials are the post marketing studies which delineate additional information, including the treatment's risks, benefits, and optimal use.

After these project stages from initiation to successful therapeutic application after approval, the patent protection expires, normally after 17–25 years, and generic competition becomes a reality.

This outline of a drug development process illustrates that, at best, it takes many years to introduce a new therapeutic agent, and it must be kept in mind that most projects are terminated before marketing, even at advanced stages of clinical studies.

1.3 Antitubercular chemotherapy

1.3.1 Overview of *Mycobacterium tuberculosis*

The designation of tuberculosis (TB) as a global public health crisis by the World Health Organization in the mid-1990s has underscored the severe challenges facing the research community globally. It has been estimated by the World Health Organization (WHO) that almost one-third of the world’s population, around 2 billion people, is infected with the disease. Every year, more than 8 million people develop an active form of the disease, which subsequently claims the lives of nearly 2 million. In 2002, the WHO estimated that if the worldwide spread of tuberculosis
was left unchecked, then the disease would be responsible for approximately 36 million more deaths by the year 2020.

In 2011, *Mycobacterium tuberculosis* (MTB) remained the second cause of death from infectious disease worldwide. Most importantly, one third of the world population is infected with latent infection and 10% of those infected people will develop active TB in their life. The agent responsible for this burden is *Mycobacterium tuberculosis*. Bacteria are spread through the air by active TB patients and most commonly affect the lungs. The major obstacles to the global control of this infectious disease include the difficulties to detect and cure a sufficient number of cases to interrupt transmission.

Specific and highly effective antitubercular drugs are still not found and traditional antibiotics are currently being used for TB curing but the effectiveness of such treatment is rather controversial. The footprint of the global TB epidemic is the footprint of poverty. As such, there is little incentive for pharmaceutical companies to adequately invest in improving the current standard TB treatment regimen, which is nearly half a century old and relatively inexpensive, but unfortunately inadequate to control the epidemic. Although many compounds are in clinical trials, there have been no new drugs registered to treat TB in the past four decades. This reflects the inherent difficulties in discovery and clinical testing of new agents and the lack of pharmaceutical industry research in treatment of tuberculosis.

**1.3.2 Types of MTB and stages of dormancy**

The pathogenesis of MTB infection in human has been depicted in Figure 1.1. Humans become infected by inhaling aerosol droplets carrying *M. tuberculosis* when a host with active tuberculous cavity in the lungs coughs or sneezes. The bacilli are ingested initially by alveolar macrophages. Two distinct events follow at this stage depending on the immune status of the macrophages. Activated macrophages kill the bacilli by the lethal antimicrobial action of phagolysosomes. In native macrophages, phagosomes containing bacilli do not mature and leave the bacilli unharmed to establish a primary infection. Again, two distinct events can follow at this stage in
the native macrophages: *M. tuberculosis* either multiplies intracellularly or maintains a dormant infection. These two fundamental events translate into two clinically different presentations of the disease. The active multiplication of *M. tuberculosis* results in TB disease. This represents only 3–10% of healthy individuals that become infected and are unable to contain primary infection. Those individuals that are able to limit *M. tuberculosis* proliferation develop a clinically asymptomatic latent infection. However, the latent infection can reactivate into active TB if dormant bacilli divide. It is estimated that one-third of the human population is latently infected with *M. tuberculosis*. The risk of reactivation is approximately 10% over the lifetime of a latently infected individual who otherwise is free of other immune-compromising conditions. Although the percentage that can reactivate seems small, it translates to a potential epidemic of 100–200 million people with active TB over the lifetime given the large reservoir of latent infection. *M. tuberculosis* mainly is of two types. One is Latent MTB and the other is non-latent MTB. A latent infection can be defined as one which is ‘subclinical’ – that is, an infection without noticeable symptoms. However, for the prognosis of *M. tuberculosis* infection, it is important to distinguish between a recent infection, where symptoms have not yet developed, a long-term latent infection, where the host successfully contains the pathogen, and an advanced stage of infection (here defined as incipient disease) that leads to clinical disease.

It is important to understand which component(s) of the immune response can be used to diagnose the different stages of TB infection and disease. MTB is an intracellular pathogen that resides mainly within macrophages and is able to survive for many years in an intracellular habitat in a slowly-replicating or non-replicating state that is induced by host immune responses and fibrotic encapsulation. Recently, there has been a breakthrough in the understanding of the adaptation of MTB to the hostile intracellular environment of the immune host. MTB responds to the host immune system with dynamic transcriptional changes of a subset of its 4000 genes. Mimicking growth conditions *in vivo* by O₂ depletion, nutrient starvation or nitric oxide (NO) addition has led to the identification of several MTB genes, the
expression of which is rapidly altered to enable intracellular survival [e.g. the dormancy (DosR) regulon, which consists of 48 genes].

Figure 1.1 Pathogenesis of MTB infection

1.3.3 Treatment of TB and drug resistance

The main objective of anti TB drug therapy is to kill all actively metabolizing bacilli in the lungs and eliminate less actively replicating and near-dormant bacilli that may otherwise cause a relapse of the disease. The WHO-recommended DOTS (directly observed treatment, short course) anti TB therapy involves the administration of four drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol
(EMB) or streptomycin (SM), whose structures are shown in Figure 1.2. Treatment with these first-line drugs is carried out initially over two months, leading to the destruction of bacteria in all growth stages, after which treatment continues with RIF and INH alone for four months, where any residual dormant bacilli are eliminated by RIF and any remaining RIF-resistant mutants are killed by INH.\textsuperscript{14,15} Furthermore, serious threats to control and treatment of TB have emerged in the form resistant (MDR-TB) strains. These strains are resistant to the drugs normally used to treat the disease. Drug-resistant TB is transmitted in the same way as drug-susceptible TB, and is no more infectious than drug-susceptible TB. However, delay in the recognition of drug resistance or prolonged periods of infectiousness may facilitate increased transmission and further development of drug resistance.

\textbf{Figure 1.2} Chemical structures of the first-line anti TB drugs

In this present era, the main drawback of anti TB drugs is resistance. This criteria further divides the TB in two groups. One is MDR (multi drug resistance)-TB stains which do not respond to the first-line TB drugs\textsuperscript{16,17} and the other is XRD (extremely drug resistance)-TB strains which are resistant to isoniazid and rifampin, and any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). Because XDR-TB is resistant to first-line and second-
line drugs, patients are left with treatment options that are more toxic, more expensive, and much less effective. The fatal point is the cases of resistant MTB bacterial infection which is continuously growing worldwide (Figure 1.3).

![Countries that had notified at least one case of XDR-TB by the end of 2011](image)

**Figure 1.3** Cases of XDR-TB worldwide according to WHO 2011 report

Drug-resistant TB disease can develop in two different ways, called primary and secondary resistance. Primary resistance occurs in persons who are initially infected with resistant organisms. Secondary resistance or acquired resistance, develops during TB therapy, either because the patient was treated with an inadequate regimen, did not take the prescribed regimen appropriately, or because of other conditions such as drug malabsorption or drug-drug interactions that led to low serum levels.

1.3.4 **Drugs under clinical trials for the treatment of MTB infections**

A number of new potential antituberculosis drug candidates with novel modes of action have entered clinical trials in recent years. These agents are most likely to be effective against resistant strains. Figure 1.4 represents the current portfolio of anti TB drug development project undertaken by various pharmaceutical giants.
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worldwide. Figure 1.5 represents various new chemical entities in several preclinical and clinical stages of development.

![Figure 1.4](image1.png)

**Figure 1.4** Drug discovery portfolio worldwide (Information from www.newtbdrug.org/world portfolio, updated November 2012)

![Figure 1.5](image2.png)

**Figure 1.5** New chemical candidates in various preclinical and clinical stages of development (Information from www.newtbdrug.org/pipeline-discovery, updated November 2012)
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Figure 1.6 represents the structures of drug candidates which are under development in Phase-I and Phase-II trials.  

1.3.5 Identified targets for the treatment of *Mycobacterium tuberculosis*

Since the determination of the *Mycobacterium tuberculosis* genome sequence, various groups have used the genomic information to identify and validate targets as the basis for the development of new antituberculosis agents. Validation might
include many components: demonstration of the biochemical activity of the enzyme, determination of its crystal structure in complex with an inhibitor or a substrate, confirmation of essentiality, and the identification of potent growth inhibitors either \textit{in vitro} or in an infection model.

Next few sections summarize the identified targets in \textit{M. tuberculosis} that have been validated beyond initial genetic identification. Advancing these defined targets for the development of inhibitors has the potential to produce new drugs with novel mechanisms of actions in treating TB.

1.3.5.1 Targeting the mycobacterial cell wall biosynthesis

The mycobacterial cell wall is mainly comprised of three components, peptidoglycan, arabinogalactan and mycolic acids, covalently linked to each other. Because the enzymes involved in biosynthesis of cell wall do not have homologues in mammalian system, cell wall biosynthesis is considered as good source of molecular target. Currently used anti TB drugs include inhibitors of mycolic acids (isoniazid and ethionamide), arabinogalactan (ethambutol) and peptidoglycan (cycloserine).

1.3.5.1.1 Peptidoglycan biosynthesis

Both alanine racemase (Alr)\textsuperscript{20} [PDB code: 1XFC] and D-Ala-D-Ala ligase\textsuperscript{21} [PDB code: 3LWB] are targets of D-cycloserine, a second-line anti TB drug. These two enzymes catalyze the first and second committed steps in bacterial peptidoglycan biosynthesis. Alr is a pyridoxal 5-phosphate (PLP) containing enzyme that catalyzes the racemization of L-alanine into D-alanine, while D-Ala-D-Ala ligase catalyzes the ATP-driven ligation of two d-alanine (d-Ala) molecules to form the d-alanyl:d-alanine dipeptide. These molecules are key building blocks in the biosynthesis of peptidoglycan layer in bacterial cell walls.

1.3.5.1.2 Fatty acid biosynthesis

Long chain fatty acids are part of the keys for the mycobacterial ability to withstand chemical injury such as the one produced by macrophage phagolysosome.\textsuperscript{22}
Moreover, the acid-fast staining, characteristic of mycobacteria, is actually caused by the mycolic acids. There are two principle classes of fatty acid synthase pathways (FAS I and FAS II) in *M. tuberculosis* for the synthesis of these long chain fatty acid. FAS I system produces C16-C26 fatty acids and co-operates to FAS II system which extends these fatty acids up to C56 to make precursor of mycolic acid. The FAS I system utilizes a single large multi-functional polypeptide and is common in both mammals and mycobacteria. While, FAS II system is characterized by the use of discrete mono functional enzymes for fatty acid synthesis. Thus, inhibitors of this pathway are being investigated as possible antibiotics.

The enzymes involved in FAS II pathways are (I) MabA [PDB code: 1UZM] (also called FabG) and InhA [PDB code: 2H7I]. These are NaDH dependant enolyl carrier protein reductase which catalyses reduction steps of fatty acid synthesis (respectively, the β–carbonyl reduction & α,β–unsaturation reduction). (II) FabH [PDB code: 1U6E], KasA and KasB are three different keto-acyl carrier protein synthases, which catalyse the condensation steps of fatty acid synthesis.

S-adenosylmethionine dependant methyl transferases (SAM-MT’s) which catalyse the introduction of key chemical modification in defined position of mycolic acids, are another important targets for the development of new anti TB drugs. One of the eight genes that encode putative mycolic acid SAM-MT’s in MTB (*mma2* or *hma*) has been characterized. Except CmaA1 [PDB code: 1KPG] which has no discernable role in mycolic acid modification, all mycolic acid SAM-Mt’s three dimensional structures known so far are those of the cyclopropane synthases CmaA2 [PDB code: 1KPI] and MmaA2 [PDB code: 1TPY]. Hma, also known as MmaA4 [PDB code: 2FK8], is unique in that it is the enzyme responsible for the production of precursor for all oxygenated mycolates in MTB.

Pks13 is type 1 polyketide synthase that catalyses the final condensation step in mycolic acid biosynthesis. The enzyme contains four catalytic domain required for the condensation reaction. Recently, high resolution crystal structures were determined for the apo, palmitoylated and carboxy palmitoylated forms of “acyl transferase” domain of this enzyme [PDB codes: 3TZW, 3TZX, 3TZY,
Moreover, acyl-AMP ligase FadD32 which catalyses the final steps in mycolic acid bio-synthesis is shown to be essential for the bacterial growth. Multimethyl branched fatty acids are found uniquely in the cell envelope of pathogenic mycobacteria. These unusually long fatty acids are essential for the survival, virulence, and antibiotic resistance of MTB. Therefore, inhibitors aimed at the biosyntheses of these multimethyl-branched fatty acids have high potential to become new antituberculosis therapeutics. Acyl-CoA carboxylases (ACCases) commit acyl-CoAs to the biosynthesis of these unique fatty acids. Unlike other organisms such as *Escherichia coli* or humans that have only one or two ACCases, *M. tuberculosis* contains six ACCase carboxyltransferase domains, AccD1–6, whose specific roles in the pathogen are not well defined. Previous studies indicate that AccD4, AccD5, and AccD6 are important for cell envelope lipid biosynthesis and that its disruption leads to pathogen death. Lin *et al.* showed that AccD5 [PDB code: 2A7S], which produces methylmalonyl-CoA (the substrate for the biosyntheses of multimethyl-branched fatty acids such as mycocerosic, phthioceranic, hydroxyphthioceranic, mycosanoic, and mycolipenic acids), is an effective target for future anti TB drug development.

1.3.5.1.3 Targeting Arabinogalactam biosynthesis

A general pathway for the biosynthesis of arabinogalactam (a polysaccharide made of arabinose and galactose), which acts as the intermediate binding scaffold between many types of mycolic acids and the inner peptidoglycan, has been suggested. The cell wall mycolyl-arabinogalactan-peptidoglycan complex is essential in mycobacterial species, and is the target of several antitubercular drugs. Studies into the mechanism of action of ethambutol in *M. avium* identified a gene cluster that conferred resistance to this antibiotic when over expressed. The products of this gene cluster, EmbA and EmbB proteins were found to be involved in the formation of the proper terminal hexaarabinofuranoside motif in arabinogalactam, where mycolic acids are attached. For instance, ethambutol targets arabinogalactan biosynthesis through inhibition of the arabinofuranosyltransferases Mt-EmbA and Mt-EmbB.
Alderwick and co-workers identified two novel arabinofuranosyltransferases (AftA & AftB) involved in cell wall arabinan biosynthesis in MTB.\textsuperscript{43,44} These enzymes are not sensitive to inhibition by ethambutol but have been shown to be essential for the viability of bacteria.

5-phospho-alpha-d-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyl transferase is known to be essential for the growth of MTB. The enzyme catalyses the first committed step in the synthesis of decaprenyl-phosphoryl-D-arabinose, the lipid donor of mycobacterial D-arabinofuranosyl residues during arabinogalactan biosynthesis, by transferring 5-phosphoribosyl residue from phosphoribose diphosphate to decaprenyl phosphate to form decaprenyl-phosphoryl-5-phosphoribose.\textsuperscript{45} Two other enzymes UDP-galactopyranose mutase (Glf) and galactofuranosyl transferase, essential for the arabinogalactan biosynthesis, have been identified.\textsuperscript{40,46}

In 2009, Makarov \textit{et al.} identified DprE1/DprE2 as the target for a benzothiazinone compound that is bactericidal against MTB.\textsuperscript{47} DprE1 heterodimerizes with DprE2 to form active enzyme that converts decaprenylphosphoryl ribose into decaprenylphosphoryl arabinose for incorporation into the cell wall.\textsuperscript{48,49}

\subsection*{1.3.5.2 Targeting amino acid biosynthesis}

The absence of the leucine biosynthesis pathway in humans makes the enzymes of this pathway in pathogenic bacteria such as \textit{Mycobacterium tuberculosis} potential candidates for developing novel antibacterial drugs.\textsuperscript{50} Leucine biosynthesis occurs via the isopropylmalate (IPM) pathway, starting with the formation of \(\alpha\)-IPM from acetyl-CoA and \(\alpha\)-ketoisovalerate (\(\alpha\)-KIV), which is also the immediate precursor for valine biosynthesis. This aldol condensation-type reaction is catalyzed by \(\alpha\)-IPM synthase (\(\alpha\)-IPMS or LeuA), encoded by \textit{leuA}. The crystal structure of LeuA has been determined at 2.0 Å resolution in complex with its substrate \(\alpha\)-ketoisovalerate \textsuperscript{[PDB code: 1SR9].}\textsuperscript{51} Subsequent reactions in this pathway are catalyzed by \(\alpha\)-IPM isomerase, which is encoded by \textit{leuC/D}, and \(\beta\)-IPM dehydrogenase, which is encoded by \textit{leuB}. \(\alpha\)-IPM isomerase exists as a complex of two subunits: the large
(LeuC) and the small (LeuD) subunit. The functional LeuCD complex catalyzes the stereospecific conversion reaction of α-isopropylmalate to β-isopropylmalate. Three C-terminally truncated variants of LeuD have been analyzed by X-ray crystallography to resolutions of 2.0 Å (LeuD_1-156), 1.2 Å (LeuD_1-168), and 2.5 Å (LeuD_1-186), respectively.50

Lysine biosynthesis occurs via the diaminopimelate (DAP) pathway. The occurrence of the lysine biosynthetic pathway in microorganisms and plants, but not in mammals, suggests that specific inhibitors of this biosynthetic pathway may display novel antibacterial activity with low mammalian toxicity. Dihydrosopicolate reductase (DHPR), which catalyzes the second step in the DAP pathway, generates meso-diaminopimelate (a component of bacterial cell walls) and the amino acid L-lysine, is an important target for the development of newer anti TB drugs.52 The three-dimensional crystal structures of the MTB dihydrosopicolate reductase complexed with NADH & 2,6-PDC [PDB code: 1C3V] and NADPH & 2,6-PDC [PDB code: 1P9L] have been evaluated by Cirilli et al.53

The shikimate pathway, responsible for aromatic amino acid biosynthesis, is required for the growth of Mycobacterium tuberculosis and is a potential drug target. The pathway consists of seven enzymes (designated as AroG, AroQ, AroE, AroK, AroA, and AroC).54 The first reaction is catalyzed by 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase (DAH7PS or AroG) [PDB code: 2B7O].55 The other enzymes of these biosynthesis pathway, 3-dehydroquinate Synthase (AroB) [PDB code: 3QBE], shikimate dehydrogenase (AroE),56 5-enolpyruvylshikimate 3-phosphate (EPSP, AroA) [PDB code: 200D], type II 3-dehydroquinase (AroQ), chorismate synthase (AroC) [PDB code: 1ZTB]57 and shikimate kinase (AroK) [PDB code: 2G1J],58 have been characterized in detail. Recently many high-affinity reversible competitive inhibitors of MTB type II dehydroquinase (AroQ), were investigated and the 3 dimensional crystal structures of the protein-ligand complexes were studied.59–62

Arginine biosynthesis has been found to be essential in MTB, as an ArgF mutant strain (defective in the metabolism of L-arginine) requiring exogenous L-arginine for the growth in vitro had reduced virulence in immunodeficient SCID mice and was
Introduction

highly attenuated in immunocompetent mice. ArgA, an essential enzyme that catalyzes the initial step in L-arginine biosynthesis, namely the conversion of L-glutamate to alpha-N-acetyl-L-glutamate, has been identified.

In MTB, the biosynthesis of histidine begins with the conversion of 5-phospho-a-D-ribose 1-diphosphate to phosphoribosyl-ATP by ATP phosphoribosyltransferase (HisG) and involves approximately 10 enzymatic activities to generate L-histidine. The loss of two of these enzymes, HisC and HisD, renders MTB auxotrophic to histidine, reaffirming the essentiality of histidine and its biosynthetic pathway for the growth of mycobacteria. Cho and his colleagues solved the molecular structure of HisG [PDB code: 1NH7] and used a combination of chemo-informatics approaches to identify small molecule inhibitors of this enzyme. In this study, the researchers virtually screened 500,000 compounds to select those that should bind to HisG and inhibit its function. Fifty unique compounds were selected following iterative rounds of molecular docking using multiple algorithms to minimize artifacts associated with simulating enzyme-inhibitor binding. Following the selection of the compounds that bound and inhibited HisG activity in vitro, the authors identified two nitrobenzothiazole compounds with cidal activity against M. smegmatis.

Cysteine biosynthetic genes are up-regulated in the persistent phase of Mycobacterium tuberculosis, and the corresponding enzymes are therefore of interest as potential targets for novel antibacterial agents. cysK1 is one of these genes and has been annotated as coding for an O-acetylserine sulfhydrylase (OASS). The product of this gene is a pyridoxal phosphate (PLP)-dependent enzyme which catalyzes the conversion of O-acetylserine to cysteine. The crystal structure of the enzyme was determined to 1.8 Å resolution [PDB code: 2Q3B].

Unlike mammals, bacteria also encode enzymes that synthesize branched-chain amino acids. The pyridoxal 50-phosphate-dependent transaminase performs the final biosynthetic step in these pathways, converting keto acid precursors into α-amino acids. The branched-chain amino-acid transaminase from Mycobacterium tuberculosis (MtIlvE) has been crystallized and its structure has been solved at 1.9 Å resolution [PDB code: 3HT5].
1.3.5.3 Targeting cofactor biosynthesis

Because the enzymes involved in cofactor biosynthesis pathways are not present in humans, they appear to be promising candidates for the development of therapeutic drugs.

Tetrahydrofolate and its derivatives (folates) are essential cofactors of one-carbon metabolism which are required for the biosyntheses of purines, thymidylate, serine and methionine in a wide variety of organisms; they are also required for the formylation of methionyl-tRNA in eubacteria. Whereas plants and many microorganisms obtain folate coenzymes by *de novo* synthesis, mammals depend on nutritional sources. Two enzymes of the folate biosynthesis pathway (i) 7,8-dihydropteroate synthase (DHPS) and (ii) Dihydrofolate reductase (DHFR) are the validated targets of the widely used sulfa drug trimethoprim.69

The enzyme 7,8-dihydropteroate synthase (DHPS) catalyzes the condensation of para-aminobenzoic acid (PABA) with 6-hydroxymethyl-7,8-dihydropterinpyrophosphate to form 7,8-dihydropteroate and pyrophosphate. Because humans lack, while microorganisms possess DHPS, this enzyme has been long exploited as a selective drug target. Inhibition of this enzyme's activity by sulfonamide and sulfone drugs depletes the folate pool, resulting in growth inhibition and cell death. The crystal structure with 1.7 Å resolution of the binary complex of 6-hydroxymethylpterin monophosphate (PtP) with DHPS has been established [PDB code: 1EYE].70 Dihydrofolate reductase (DHFR) [PDB code: 1DG5] catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Inhibition of the enzyme's activity leads to arrest of DNA synthesis and cell death. The three DHFR inhibitors are the anticancer drug methotrexate, the antimicrobial trimethoprim and Br-WR99210, an analogue of the antimalarial agent WR99210. Structural comparison of these complexes with human dihydrofolate reductase indicates that the overall protein folds are similar, despite only 26% sequence identity, but that the environments of both NADP and of the inhibitors contain interesting differences between the enzymes from human and MTB. These differences between the
enzymes from MTB and human provide opportunities for designing new selective inhibitors of *M. tuberculosis* DHFR.\textsuperscript{71}

Coenzyme A is an essential cofactor in lipid biosynthesis, thus CoA biosynthesis pathway proteins are potential targets for developing inhibitors against bacteria including *Mycobacterium tuberculosis*. Pantothenate kinase (PanK) is a ubiquitous and essential enzyme that catalyzes the first step of the universal coenzyme A biosynthetic pathway. In this step, pantothenate (vitamin B5) is converted to 4'-phosphopantothenate, which subsequently forms coenzyme A in four enzymatic steps. Crystal structure of pantothenate kinase from *Mycobacterium tuberculosis* (MtPanK) in complex with pantothenate [PDB code: 3AVO] has been established recently.\textsuperscript{72} Recently, two enzymes involved in this pathway: phosphopantetheine adenyltransferase (CoaD) and dephospho CoA kinase (CoaE) have been identified. Amino acids alignment and phylogenetic analysis showed CoaD to be distantly related to the human counterpart while CoaE was found to be relatively similar to the human enzyme. These make CoaD a suitable target for developing inhibitors against *M. tuberculosis*.\textsuperscript{73}

As the genome of *Mycobacterium leprae* has undergone a dramatic process of gene fragmentation, the fact that all riboflavin biosynthesis genes were retained in apparently functional form. This indicates that the biosynthetic pathway is of vital importance for the intracellular lifestyle of this pathogen. By extrapolation of this argument, it appears likely that the riboflavin pathway genes are also essential for *Mycobacterium tuberculosis*.\textsuperscript{74} The biosynthesis of riboflavin has been studied extensively over recent years. Two enzymes, lumazine synthase (LS) and riboflavin synthase (RS), catalyzing the penultimate and the last step of riboflavin biosynthesis, respectively, are the main targets of interest. Morgunova *et al.* synthesized five novel ribitylpurinetrione inhibitors of LS and performed calorimetric binding studies. The analysis of structures of MTB-LS in complex with these inhibitors clearly showed the ribitylpurinetrione with C4-C5 alkylphosphate chains as most promising leads for further development of therapeutic drugs against *M. tuberculosis*.\textsuperscript{75} Zhang *et al.* prepared three metabolically stable analogues of the hypothetical intermediate, proposed to arise after phosphate elimination in the LS-catalyzed reaction, and
evaluated as LS inhibitors. All three intermediate analogues were potential inhibitors of MTB-LS, thus validating LS as target for anti TB drug discovery.76

5'-Adenosinephosphosulphate reductase (APSR) catalyzes the reduction of APS to sulfite (SO3\(^2\)) (first catalytic step in sulfur assimilation pathway) and adenosine-50-monophosphate (AMP) using reduction potential supplied by the protein cofactor, thioredoxin. Senaratne et al. demonstrated that APSR is required for survival in the latent phase of TB infection.77 APSR is not found in humans and thus represents a unique target for antibacterial therapy.

1.3.5.4 Targeting Mycothiol biosynthesis

Mycothiol (MSH) is the functional equivalent of glutathione in mycobacteria78 and is associated with the protection of Mycobacterium tuberculosis from toxic oxidants and antibiotics.79 The biosynthesis of MSH is a multistep process involving four enzymatic reactions designated MshA, MshB, MshC, and MshD. This pathway is absent in humans.80

MTB \(mshC\) encodes an ATP-dependant mycothiole ligase (MshC) which catalyzes the condensation of GlcN-Ins and l-cysteine to form l-Cys-GlcN-Ins, the penultimate step in mycothiol biosynthesis. Targeted disruption of \(mshC\) yielded no colonies unless second copy of the gene was supplied which indicated the essentiality of \(mshC\) in survival of pathogen.81 MshC is essential to Mycobacterium tuberculosis and therefore represents an attractive target for chemotherapeutic intervention. A recent study validated mycothiol as a druggable target by identifying dequalinium chloride as an inhibitor of MhsC in screen of 3100 compounds.82 This study also uncovered the molecular mechanism of inhibition: dequalinium competes with ATP for binding to mycothiol ligase with high affinity. The structures for both MshB (a metal-dependant deacetylase)83 and MshD (mycothiol synthase)84 have been determined.
1.3.5.5 Targeting terpenoid biosynthesis

Mammals exclusively use the mevalonate pathway for the synthesis of terpenoids, whereas plants, many eubacteria and apicomplexan protozoa, including major human pathogens such as Plasmodium falciparum and Mycobacterium tuberculosis utilize the non-mevalonate pathway, making the pathway good source of targets for the treatment of important infectious diseases. The non-mevalonate pathway involves sequence of enzymatic reaction designated from IspC to IspH.

The natural antibiotic fosmidomycin acts via inhibition of 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR, IspC). Recently, Andaloussi et al. represented the crystal structure of MTB-DXR in complex with 3,4-dichlorophenyl substituted fosmidomycin analogues, thus providing better insights into how DXR accommodates the new inhibitors and serve as an excellent starting point for the design of more potent inhibitors. Structural and functional studies of MTB 4-diphosphocytidyl-2-C-methylerthritol synthetase (IspD), which carries out the third catalytic step, has been carried out by Björkelid and co-workers. Novel IspD inhibitor was also reported to exhibit antimycobacterial activity in vitro. Crystal structure of MTB 4-diphosphocytidyl-2-C-methyl-d-erythritol kinase (IspE) has been recently evaluated, paving the way for the design of novel MTB-IspE inhibitors. IspF (2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase) from M. smegmatis, an orthologue of MTB IspF sharing 73% amino acid sequence identity, has been evaluated at 2.2 Å resolution by Buetow and co-workers. Sequence-structure comparisons indicate that the active site and interactions with ligands are highly conserved. Recently, high through put screening of 40000 compounds as potential MTB-IspF inhibitors was resulted in an identification of some lead compounds that inhibits the enzyme at micro molar level thus validating the target for the future anti TB agents.

1.3.5.6 Targeting DNA synthesis

Thymidylate kinase (TMPK, ATP:TMP phosphotransferase) belongs to the nucleoside monophosphate kinase (NMPK) family and catalyzes the reversible
phosphorylation of dTMP to deoxothymidine 5'-diphosphate (dTDP) in the presence of adenosine triphosphate (ATP). Situated at the junction of the de novo and salvage pathways for the synthesis of deoxothymidine 5'-triphosphate (dTTP), TMPK is the last specific enzyme in these pathways, therefore being essential for cell growth and survival.91 The crystal structures of MTB-TMPK with TMP and TMP analogues as competitive inhibitors for this enzyme have been established.91–93 The MTB enzyme shows important differences from the mammalian enzyme. As opposed to the human and other TMPKs, catalysis by the MTB-TMPK necessitates the transient binding of a magnesium ion coordinating the phosphate acceptor.91 Many reports on the design and identification of lead candidates as potential inhibitors of MTB-TMPK have been presented so far.94–114

DNA ligases are important for DNA replication and repair. They facilitate the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. DNA ligases are classified as either NAD+ or ATP-dependant, depending upon their specific cofactor. Whereas ATP-dependent ligases are widely distributed in all species, NAD+ dependant ligases (LigA) are only found in some viruses and eubacteria. Genetic and pharmacological studies of the MTB ligases highlight LigA as uniquely essential for cell viability and a possible drug target for treating tuberculosis.115 Analysis of strong overproduction or depletion of LigA in some conditional mutant strains of mycobacteria by Malgorzata et al. revealed no effect on the growth or survival of mycobacteria under standard laboratory conditions. In conclusion, although NAD+-dependent DNA ligase is essential for mycobacterial viability, only low levels of protein are required for growth. These findings suggest that very efficient inhibition of enzyme activity would be required if NAD+-dependent DNA ligase is to be useful as an antibiotic target in mycobacteria. The crystal structure of MTB LigA complexed with AMP (adenosine monophosphate) has been established. The structure was utilized for identifying the novel glycosyl ureides and glycosylamine derivatives as novel inhibitors of MTB LigA using computational approaches.115-117

MTB usually possesses only one type II topoisomerase, DNA gyrase and is hence the sole target for quinolone action, a crucial drug active against multidrug-resistant
strains. Targeting DNA gyrase is a clinically validated therapeutic approach using fluoroquinolone antibiotics to target the gyrase subunit A (GyrA). Increasing resistance to fluoroquinolones has driven interest in targeting the gyrase subunit B (GyrB), which has not been targeted for TB. The biological activities of two potent small-molecule inhibitors of GyrB have been characterized to validate its targeting as a therapeutic strategy for treating TB. Several quinolinone derivatives as potential DNA gyrase inhibitors have been reported since last few years. Ribonucleotide reductases (RNRs) enzymes, essential for the bacterial growth, which catalyze the first committed step in DNA synthesis by reducing ribonucleotides to deoxynucleotides are important targets for the development of potential antibacterial agents. There are two classes of RNR in MTB: class Ib and class II. Class Ib RNRs consists of two subunits, the large subunit R1 and the small subunit R2; both subunits are required for catalytic activity. Several major differences between the MTB and mammalian RNR, including the observation that R1 lacks an allosteric regulatory site for dATP, have been revealed through extensive genetic, biochemical and biophysical characterizations.

1.3.5.7 Targeting the glyoxylic shunt

To establish or maintain a persistent infection, MTB appears to require the glyoxylic pathway to bypass the energy-generating tricarboxylic acid cycle (TCA). The glyoxylic pathway converts isocitrate to succinate and glyoxylic, catalysed by isocitrate lyase (ICL), followed by the addition of acetyl-CoA to glyoxylic to form malate by malate synthase. The glyoxylic pathway allows these organisms to avoid the carbon dioxide generating steps of the Krebs cycle and enabling them to use carbons from fats to carbohydrate synthesis. ICL is thus a potential target for the development of new antitubercular drugs. In MTB, glyoxylic cycle is comprised of a single gene encoding malate synthase, but two genes encoding ICL. It has been found that a double deletion of both icl1 and icl2 resulted in complete impairment of mycobacterial intracellular replication and rapid elimination of the bacteria from the lungs, validating ICL and the glyoxylic pathway as a target for compounds that
would eliminate persistent bacteria. The structure of MTB ICL has been solved in complex with the inhibitors.

1.3.5.8 Inhibiting menaquinone biosynthesis

Menaquinone (vitamin K1) is a polyisoprenylated naphthoquinone that shuttles electrons between membrane-bound protein complexes in the electron transport chain. In mammalian cells this function is performed by ubiquinone, and although menaquinone is required for blood clotting, the biosynthetic pathway for this vitamin is absent in humans. This has resulted in the proposal that enzymes involved in menaquinone biosynthesis may be promising targets for drug discovery. The menaquinone biosynthesis has been studied in detail in *E. coli* where the pathway is catalyzed by a series of enzymes including MenF, MenD, MenC, MenE, MenB, MenA and MenG. The crystal structures of the MTB MenB (1,4-dihydroxy-2-naphthoyl-CoA synthase) has been solved so far. In menaquinone biosynthesis, 2-succinyl-5-enoylpyruvyl-6-hydroxy-3-cyclohexane-1-carboxylic acid synthase (MenD), MenE (an acyl-CoA synthase), 1,4-dihydroxy-2-naphtoate prenyltrasferase (MenA) and 1,4-dihyroxy naphthoyl-CoA synthase (MenB) have recently been studied for the development of novel drug lead for gram-positive pathogens including *M. tuberculosis*.

1.3.5.9 Targeting ATP synthesis

MTB uses the proton motive force across the plasma membrane to synthesize ATP. Despite being an intuitive target, ATP synthase has not been exploited for drug development. In 2005, Andries and coworkers identified the diarylquinoline compound R207910 with activity against *M. smegmatis* in a whole cell assay. The analysis of the genome sequences of two *M. smegmatis* and one MTB isolate, all of which were resistant to R207910, revealed that the atpE gene was mutated in all three strains. Subsequent studies demonstrated that R207910 inhibited the synthesis of ATP in a dose-dependent manner. In an effort to identify the target protein bound by R207910, these researchers immobilized an analog of the compound onto a resin and passed *M. smegmatis* membrane protein extracts through it, and discovered
that the a and b subunits of ATP synthase (AtpE) copurified with the R207910 analogue. However, the researchers also observed that the R207910 analogue bound to the purified c subunit (AtpE). Based on these observations, AtpE was concluded to be the target. These studies provide direct evidence that the ATP synthase complex can be effectively inhibited to prevent the growth of MTB. A separate study showed that human mitochondrial ATP synthase was 20000 fold less sensitive to R207910 compared with its homolog in MTB, allowing selective toxicity against the pathogen. In addition, the activity of R207910 against dormant and drug-resistant clinical isolates of MTB validated ATP synthase as a novel target. R207910 (now known as TMC207) is currently being evaluated in clinical trials for its effectiveness to treat infection with drug resistant strains of MTB.

In brief, the systematic exploitation of these potential validated molecular targets can result in an identification of novel class of chemical compounds for their utilization in treatment of TB. But still, the effective inhibition of any of these targets will not guarantee the molecules tested \textit{in vitro} to become potential drug candidate. The new generation anti TB drugs must offer the solution to the difficulties to which current drug molecules fail to.

1.3.6 What should a new TB drug offer?

New-generation TB drugs must satisfy onerous criteria that:

- Should shorten the duration of chemotherapy
- Should be associated with minimal side-effects as part of a regimen that requires fewer tablets and less frequent dosing
- Should possess activity against MDR and XDR strains
- Should be compatible with drugs for chronic conditions, like HIV and diabetes
- Should be active against the organism in both intra- and extracellular host environments.

With the possible exception of drug-drug interactions, the difficulties associated with tackling TB chemotherapeutically are a direct consequence of the inherent characteristics of MTB and TB disease pathology. Therefore, efforts to improve therapeutic efficacy depend critically on an improved understanding of the physiology of the infecting organism.