"RECENT ADVANCES IN ANTI-TUBERCULOSIS AGENTS"

A PROJECT SUBMITTED TO

NIRMA UNIVERSITY

In Partial fulfillment of requirements for the degree of

Bachelor of Pharmacy

By

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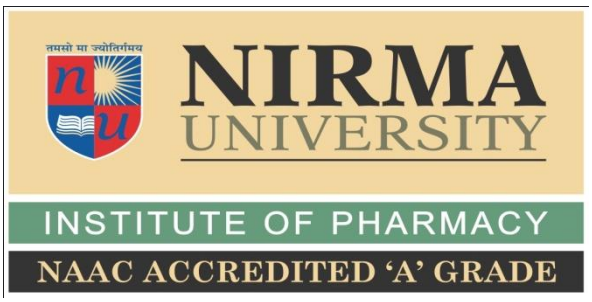
SEMESTER VII

UNDER THE GUIDANCE OF

Dr. JIGNASA SAVJANI



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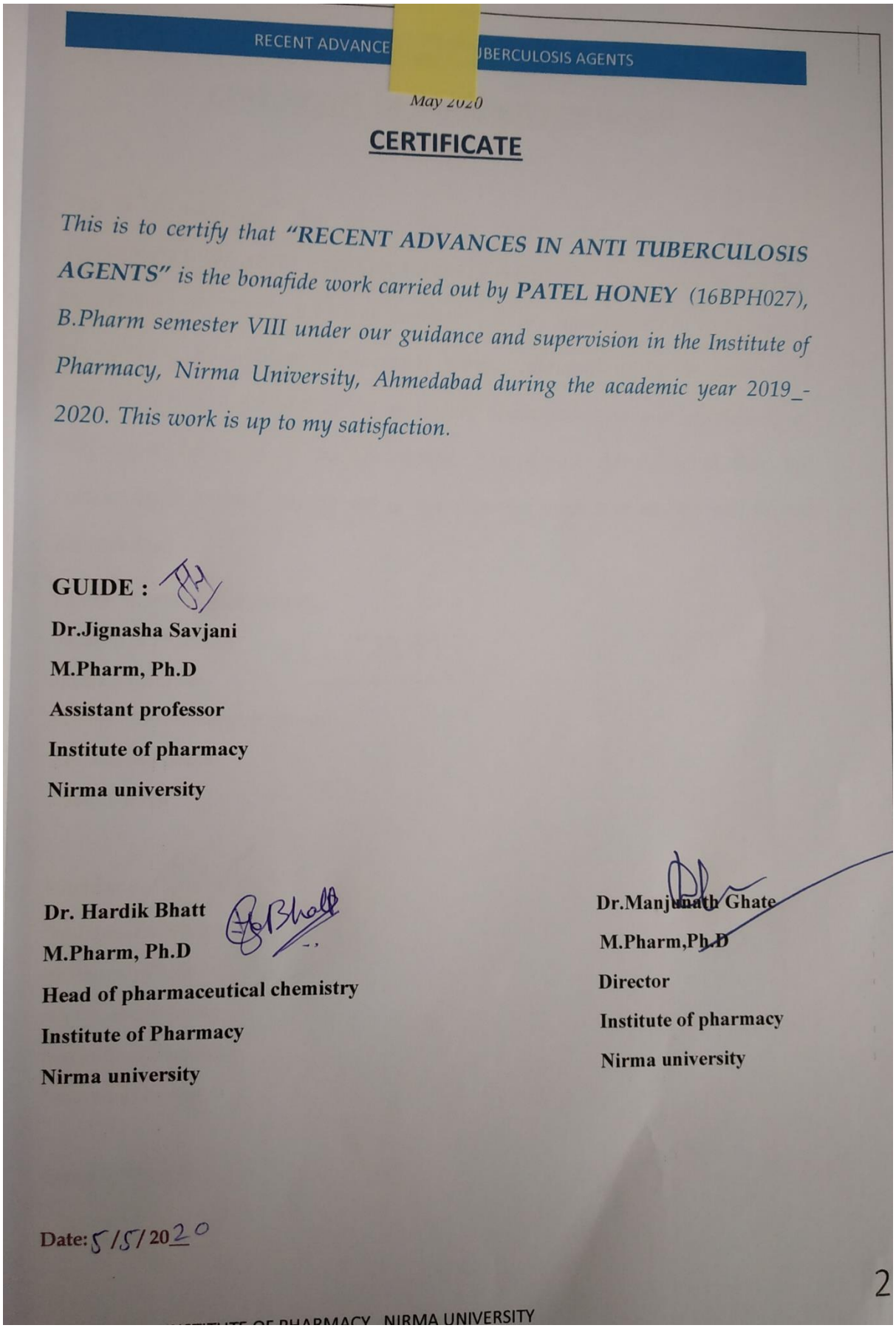

RECENT ADVANCE UBERCULOSIS AGENTS

May 2020

CERTIFICATE

This is to certify that "RECENT ADVANCES IN ANTI TUBERCULOSIS AGENTS" is the bonafide work carried out by PATEL HONEY (16BPH027), B.Pharm semester VIII under our guidance and supervision in the Institute of Pharmacy, Nirma University, Ahmedabad during the academic year 2019_-2020. This work is up to my satisfaction.

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RECENT ADVANCES IN ANTI TUBERCULOSIS AGENTS

CERTIFICATE OF SIMILARITY OF WORK

*This is to undertake that the B.Pharm. Project work entitled "**RECENT ADVANCES IN ANTI TUBERCULOSIS AGENTS**" Submitted by **PATEL HONEY** (16BPH027), B.Pharm. Semester VIII is a bonafide review work carried out by me at the Institute of Pharmacy, Nirma University under the guidance of "DR.JIGNASHA SAVJANI". I am aware about the rules and regulations of Plagiarism policy of Nirma University, Ahmedabad. According to that, the review work carried out by me is not reported anywhere as per best of my Knowledge.*

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RECENT ADVANCES IN ANTI TUBERCULOSIS AGENTS

DECLARATION

I, PATEL HONEY (16BPH027), student of VIIIth Semester of B.Pharm at Institute of Pharmacy, Nirma University, hereby declare that my project entitled "RECENT ADVANCES IN ANTI TUBERCULOSIS AGENTS" is a result of culmination of my sincere efforts. I declare that the submitted project is done solely by me and to the best of my knowledge, no such work is done by any other person for the award of degree or diploma or for any other means. I also declare that all the information was collected from various primary sources (journals, patents, etc.) has been duly acknowledged in this project report.

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1.Introduction

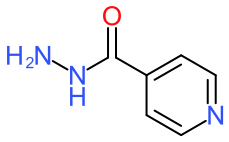
1.1 Tuberculosis

Tuberculosis (TB) is a highly infectious disease. It is caused by the pathogen Mycobacterium tuberculosis (Mtb). TB has been a scourge on humankind for centuries. The earliest predecessor of Mtb dates back approximately three million years, reportedly originating from East Africa 11. Mtb has been classed as the most dangerous microbial pathogen, killing more persons than any other microbial species . In 2015, the World Health Organization (WHO) reported a global mortality of 1.5 million people due to TB infection. In the same year 9.6 million people were diagnosed with this infection. The disease now ranks alongside Human Immunodeficiency Virus (HIV), as the main cause of death from a single infectious disease. Africa and Asia were recorded to have the highest incidence of TB estimating one-third of the TB cases globally. Africa, still remains the highest-ranking continent for patients coinfected with TB and HIV and accounts for 32% of the total reported cases of TB and HIV co-infections worldwide. In view of the impact of TB on a global scale, it is evident that this disease presents a substantial public health challenge .

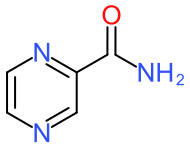
TB is an airborne disease that is identified by simple culture. The mechanism of transmissibility and identification are the evolutionary contributions of researchers JeanAntoine Villemin and Robert Koch, respectively. Transmission of the disease occurs predominantly through the inhalation of infected droplets. Although the primary site of infection is the lung (pulmonary), infection can also present at other sites (extra-pulmonary TB). The extent of the infection depends primarily on the status of the host's immune system, thereby resulting in cure, latent/dormant or active TB. Active pulmonary TB often presents as a chronic productive cough, weight loss, anorexia, fever, night sweats and haemoptysis. It is estimated that one-third of the world's population is infected with latent tuberculosis (LTB). Patients with latent tuberculosis infection (LTBI) are classed as high risk, as there is always a possibility of LTB progressing into active TB at a later stage. LTB patients also become reservoirs for transmission, thus it is essential to treat these patients effectively so as not to progress into active TB.

streptomycin was the first anti-tuberculosis agent to be discovered. The first large scale clinical trial of the drug was performed in 1948. This stimulated a 'rolling stone' effect with the subsequent release of thiacetazone and para-aminosalicylic acid (PAS), onto the market . The combined use of both drugs reported high curability rates and reduced propensity for antibiotic resistance. However, the vast appearance of drug-resistance to streptomycin perpetuated the design, discovery and development of new anti-TB drugs . These include: isonicotinic acid (isoniazid-INH) – discovered in 1951, pyrazinamide (PZA) and cycloserine – 1952, ethionamide - 1956, rifampicin (RIF)1957, and ethambutol – 1962.

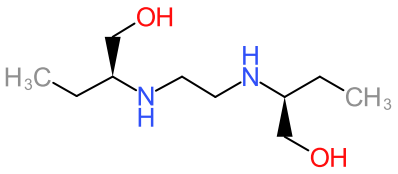
Current anti-TB therapy protocols comprise a six-month combination course of four drugs: INH, RIF, PZA and ethambutol. (Figure 1, Table 1). The first two months are known as the intensive phase. During this time all four drugs are taken. In the last four months (the continuation phase) only RIF and INH are taken. The length of treatment is crucial for the effective and complete eradication of the different sub-populations of the TB bacilli. Side effects and adverse drug reactions (ADRs) of current anti-TB drugs coupled with combination drug regimens and lengthy treatment durations often complicate the therapy. Drug-resistance to the available anti-TB drugs has also become a major public health problem. Drug-resistance is a complex issue, and can range from resistance to one drug toresistance toward many drugs, i.e. multi-drug resistant (MDR) TB; extensively drug-resistant (XDR) TB; or totally drug-resistant (TDR) TB.



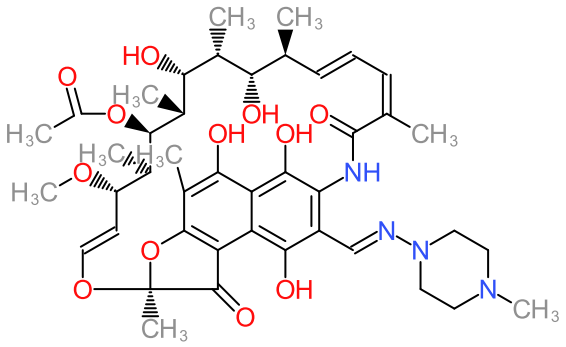
Isoniazid



pyrazinamide



ethambutol



rifampicin

Figure 1. First-line anti-tubercular drugs used in clinical therapy.

MDR-TB has been defined as an infection that expresses resistance to INH and RIF, two of the main frontline anti-tuberculosis agents. Thus the drug regimen used to treat MDR-TB requires a longer duration (6 – 9 months) with a combination of drugs (Figure 2, Table 1). XDR-TB is defined as MDR-TB with an additional resistance to fluoroquinolones and to any one of the following second-line injectable agents: amikacin, capreomycin or kanamycin. Recently, there have been reports of few cases in which patients have been resistant to all available anti-TB drugs, i.e TDR-TB. Cases

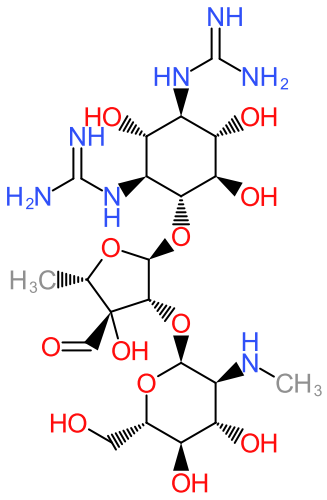
of TDR-TB resistance have become prevalent in China, India, Africa and Eastern Europe. The U.S Food and Drug Administration (FDA) have commissioned the use of drug delamanid, as a form of compassionate care for individuals diagnosed with XDR-TB and TDR-TB.

1.2 Current drug therapies

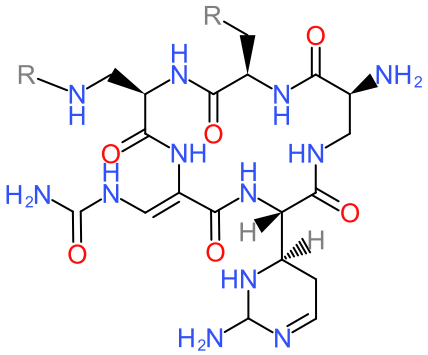
First-line drugs (Figure 1, Table 1) used in the treatment of TB are INH, RIF, PZA and ethambutol. Second-line drugs (Figure 2, Table 1) include streptomycin, ofloxacin, kanamycin, capreomycin, amikacin, ethionamide, PAS and cycloserine. Linezolid (PNU-100480) (Figure 2, Table 1) a broad spectrum oxazolidine antibiotic 30, has been approved for third-line treatment in South Africa. The WHO recommends a further classification of anti-TB drugs into five groups (Table 2). The grouping system is constructed based on data for safety, efficacy, administration and drug class.

INH (Figure 1, Table 1 & 2) has been the staple complement of the Mtb treatment. Despite INH having a simple chemical profiles (pyridine ring and a hydrazine group), the mechanism of action of INH is complex. It is a prodrug, which requires catalytic activation by catalase/peroxidase, an enzyme encoded by the gene katG. The activated drug interferes with the synthesis of essential mycolic acids (MA) by inhibiting the NADH-dependent enoyl acyl carrier protein reductase, which is encoded by inhA. Thus two molecular mechanisms of INH-resistance have been identified due to mutations in katG and mutations in inhA.

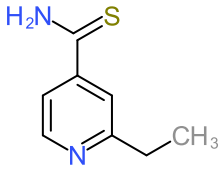
RIF (Figure 1, Table 1 & 2) is another powerful first-line anti-TB drug. It has been classed as a lipophilic ansamycin, targeting the β-subunit of RNA polymerase, where it binds and inhibits the transcription of messenger RNA (mRNA). An exceptional characteristic of RIF lies in its ability to exert its effect upon both actively growing and slowly metabolising bacilli. Resistance to RIF has been attributed to mutations in the rpoB gene, encoding the βsubunit of RNA polymerase. It has been found that almost all RIF-resistant TB strains have demonstrated resistance to other drugs, particularly to INH, making RIF-resistance detection as a proposed alternate molecular marker for MDR.



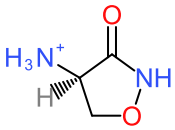
Streptomycin



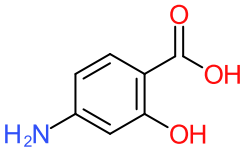
capreomycin



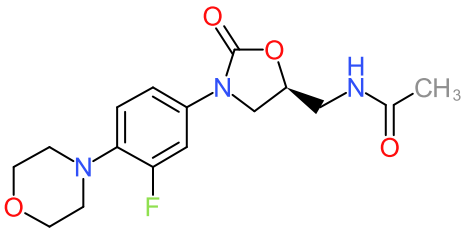
Ethionamide



cycloserine



p amino salicyclic acid



linezolid

Figure 2. Second- and third-line anti-tubercular drugs used in clinical therapy.

First line drugs used for TB: Table : 1(a)

Drug	target	Mechanism of action
Isoniazid	Having multiple targets. Acts on enoyl acyl carrier reductase	Inhibits mycolic acid synthesis. (affects DNA, lipids, carbohydrates)
Rifampicin	Acts on b-subunit of RNA polymerase	Acts by Inhibiting RNA synthesis
Pyrazinamide	S1 component of the 30S ribosomal subunit Membrane energy potential and membrane transport.	Inhibits protein translation inhibits Coenzyme A synthesis Inhibits PDIM synthesis
Ethambutol	Arabinose transferases	Inhibits cell-wall arabinogalactan synthesis

Drugs used in second line treatment of TB Table : 1(b)

Streptomycin	S12 protein and 16S rRNA components of 30S ribosomal subunit	Inhibits protein synthesis
Kanamycin, Amikacin	30S ribosomal subunit	Inhibits protein synthesis
PAS	Dihydropteroate synthase	Inhibits folate biosynthesis

Linezolid	50s ribosomal subunit	Inhibits protein synthesis
Cycloserine	D-alanine eiracemase and ligase	Inhibits peptidoglycan synthesis
Ethionamide	NADH-dependent enoyl acylcarrier protein reductase (InhA)	inhibits mycolic acid synthesis
Ofloxacin	DNA gyrase and DNA topoisomerase	Inhibits DNA supercoiling
Capreomycin	Interridge B2a between 30S and 50S ribosomal subunits	Inhibits protein synthesis

PZA (Figure 1, Table 1 & 2) has been an essential first-line drug since the 1950's. It is a prodrug that requires hydrolysis to reveal the bioactive form, pyrazinoic acid (POA). PZA has an in vivo accelerated lesion sterilisation effect, by interfering with the synthesis of a virulence factor, highlighting its potent antibacterial activity. Gopal et al. suggested that PZA/POA has two mechanisms of action and resistance. The first mechanism involves the depletion of co-enzyme A (CoA), whereby resistance is perpetuated by mutations in the aspartate decarboxylate panD, which prevents the depletion of CoA. The second mechanism implicates the phenolthiocerol dimycocerosate (PDIM) synthesis. Resistance is conferred by the loss of functional mutations in the synthesis of PDIM genes mas (mycocerosic acid synthase) and ppsA-E (phenolthiocerol synthesis type-I polyketide synthases-Pks).

The first implementation of ethambutol (Figure 1, Table 1 & 2) against TB was in 1966. The drug is active against multiplying bacilli by inhibiting the biosynthesis of arabinogalactan (AG) in the cell wall. It has been postulated that possible resistance toward ethambutol may be conferred in some cases through mutations in the embCAB gene. The embCAB gene encodes for the enzyme mycobacterial arabinosyl transferase. The centers for disease control and prevention (CDC) have developed a molecular screen for detecting drugresistance to ethambutol. The resistance mechanism has been attributed to structural changes in the E-binding site of arabinosyl transferase imposed

by mutations in the embB gene. Architectural alterations disrupt the drug-protein interactions conferring genotypic and phenotypic resistance.

WHO grouping of anti-TB drug: (Table 2)

Group 1. First-line oral agents	Isoniazid Rifampicin Ethambutol Pyrazinamide Rifabutin Rifapentin
Injectable drugs	Streptomycin Kanamycin Amikacin Capreomycin
Fluoroquinolones	Levofloxacin Moxifloxacin Gemifloxacin
Oral bacteriostatic second-line drugs	Ethionamide Prothionamide Cycloserine Terizidone para-amino salicylic acid
Group 5. Drugs with limited data on efficacy and long-term safety	Bedaquiline Delamanid Linezolid Clofazimine Amoxicillin clavulanate Imipenem/cilastatin Thioacetazone Clarithromycin

Streptomycin (the first antibiotic used in the treatment of TB) (Figure 2, Table 1 & 2) is a second- line anti-TB drug. It is an aminocyclitol glycoside based antibiotic which was isolated from the soil bacterium Streptomyces griseus. The drug's inhibitory activity prevents the initiation of protein translation by binding to the 16S rRNA. The resistance of Mtb to the inhibitor lies in mutations in the rrs, gidB (encoding a conserved 7-methylguanosine methyltransferase) or rpsL genes. The presence of mutations in these regions alter the binding site of streptomycin. The most common mutation is the K43R which occurs in rpsL.

Fluoroquinolones have been classed as second-line anti-TB drugs. The first analogue was found by chance as a by-product in the purification of the antimalarial, chloroquine, in 1965. Since then synthetic derivatives nalidixic acid, ciprofloxacin and ofloxacin (Figure 2, Table 1 & 2) have been discovered. Fluoroquinolones specifically target the type II topoisomerase (Deoxyribonucleic acid-DNA gyrase) in Mtb (Figure 2, Table 1 & 2). Reported resistance to this drug class has been associated with mutations in the binding region of gyrA or gyrB.

Kanamycin and amikacin (Figure 2, Table 1 & 2) are aminoglycosides which act on the 30S ribosomal subunit inhibiting protein synthesis. Capreomycin and viomycin are cyclic peptide antibiotics. These are second-line drugs used the treatment of MDR-TB. Aminoglycosides and cyclic peptides exert their inhibitory effect at the level of protein translation. A common mechanism of drug-resistance has been attributed to mutation, A1401G, in the rrs gene. Due to the structural similarity, cross-resistance has already occurred between capreomycin and viomycin. Mutations in the gene tlyA, have been implicated in the emergence of resistance.

Ethionamide (Figure 2, Table 1 & 2) is a pro-drug requiring activation by ethA-encoded mono-oxygenase. The activated drug forms an adduct with NAD thus inhibiting NADHdependent enoyl acyl carrier protein reductase. The emergence of resistance toward this drug can be related to mutations in the ethA and inhA genes.

p-Amino salicyclic acid (PAS) (Figure 2, Table 1 & 2) was the first drug to be used in a combinatorial drug regimen and is still effective against TB. It has been suggested that PAS is a competitive inhibitor of dihydropteroate synthase, an enzyme which is essential for folate biosynthesis. Mutations in the thyA gene, encoding thymidylate synthase A, have shown distinct resistance toward the drug. Macrolides have also been used to treat Mycobacterium infections. Resistance to macrolides has been associated with low cell-wall permeability and expression of the erm37 gene. This gene encodes the enzyme that methylates a binding site in the 23S rRNA, thus preventing activity of the drug.

Cycloserine (Figure 2, Table 1 & 2) is an analogue of D-alanine. The drug inhibits the synthesis of peptidoglycan (PG) by interfering with the action of D-alanine: D-alanine ligase (Ddl). It also inhibits D-alanine racemase (Alr) which is an integral component in the interconversion of L-alanine and D-alanine i.e. a substrate of Ddl. Overexpression of alrA conveys resistance toward cycloserine .

Linezolid (Figure 2, Table 1 & 2) belongs to the oxazolidinone chemical class of third-line anti-TB drugs. This chemotherapeutic agent inhibits the early stage of protein synthesis by binding to the ribosomal 50S subunit. Although, Mtb resistance to linezolid is rare, resistance has been reported. The mutations G2061T and G2576T in the 23 rRNA gene have been implicated in the mechanism of resistance.

1.3 Overview

The introduction of effective first-line treatments in the first half of the 20th century, together with improved sanitation led to a dramatic decline in the number of deaths from TB infection worldwide. This occurred especially in developed countries which led to a halt in new antiTB drug research. For a long time, there was little incentive for investment in anti-TB drug research. Globalisation has been a major contributor to the spread of infectious diseases. This together with the HIV epidemic further fuelled the spread of TB. Since the 1990's, there has been a resurgence of interest in new anti-TB drug development, as TB once again became an internationally significant public health risk. The rapid appearance of resistance to the available drugs, bacterial persistence, latency, as well as long-treatment durations resulting in poor adherence, further emphasized the urgency of new drug development in this arena.
It has also become increasingly apparent that the development of new drugs, necessitates new target identification. The new regimen should ideally have: a) a shorter treatment duration, b) a good bactericidal and sterilizing activity against all TB bacterial sub-populations, c) a better safety and tolerability profile than existing anti-TB drugs, and d) compatibility with other drugs used in TB chemotherapy and for those patients co-infected with HIV.Our work provides an overview of the recent progress in anti-TB drug discovery. By reviewing the currently available research and literature, we wish to systematically outline the investigative methods for the discovery of anti-TB chemotherapies. The predominant focus is on drugs that are currently in the pipeline. Some of the basic aspects for precision drug discovery in TB include: 1) Identifying and understanding current drug therapies and their resistance mechanisms; 2) The use of genomics, structural genomics and crystallography in target identification and function; 3) The use of computational techniques for drug design and rapid in silico screening of newly designed inhibitors or repurposed drugs; and 4) Whole-cell and target based screening of potential drugs. The intention of this work is to promote and streamline future efforts in anti-TB drug design and discovery, by highlighting some of the advances already embarked.

2. Identification of anti-tubercular targets

2.1. Genomics

The complete sequencing of the Mtb genome was accomplished in 1998. This was followed by re-annotation in 2002. In the current post-genomic era, gene sequencing and knowledge of the functions of the various proteins have given rise to a number of potential drug targets. Favourable drug targets are ones that are essential for the survival of the microorganism, but which, have little or no sequence homology to their human counterparts. This is to minimize the toxicity to the host.

In the 1990's Camacho and co-workers utilised signature-tagged mutagenesis for the identification of important genes required for virulence. One of the main studies in the quest to find genes that were essential to the organism was conducted by Sassetti et al. A approximately 600 essential genes were identified using transposon site hybridization (TraSH). Other methods for the identification of important Mtb genes include gene knockout, conditional mutagenesis, transcriptional profiling and proteomic analyses. Brown et al., proposed whole-genome sequencing of Mtb. This provided a method for simultaneous identification of all known resistance mutations and markers. The quality of the sequence data accurately linked mutations associated with resistance to first- and secondline anti-TB drugs. The method presents a unique opportunity to investigate the biological insights into the evolution of Mtb.

2.1.1 Structural genomics

Structural genomics is the generation of high-throughput 3-D structures from gene products 2,. 3-D structures provide essential information on protein folding, function and are an important element in the anti-TB drug discovery process 60. A large reservoir of these structures can be found in the protein data bank (PDB) (Figure 4). The TB Structural Genomics Consortium, is an international collaboration of researchers who are dedicated to the comprehensive determination of the 3-D protein structures of Mtb. The Consortium employs state-of-the-art technologies for gene cloning, protein expression, crystallization and X-ray data collection. At present it contains a deposit of crystal structures from Mtb. Knowledge of the 3-D structures is imperative for binding site identification and gaining insight into interactions that are crucial for ligand-receptor binding. The availability of structures of various Mtb proteins can also be used for molecular docking and in silico screening

2.1.2.Comparative genomics

Comparative genomics is a science that compares genomes within and between species. The method can be used to identify those genes that have been lost as well as those that have been inherited. If genes are conserved between species, then they are most likely to be essential for the organism. A study looking at genomes of clinical and laboratory strains of M. tuberculosis, M. leprae and other mycobacteria showed that 219 genes were conserved across species.

The ability to prioritize targets is also important in drug discovery. Hasan et al. from the Novartis institute for tropical diseases developed a software programme called AssessDrugTarget. This programme essentially ranks potential drug targets according to essentiality, drugability, epidemiology, and distinct Mtb metabolic signatures. Sequence and structural similarity to other mycobacteria and humans is also taken into account.

2.1.3. Selective TB targets

As mentioned before, favourable drug targets are ones that are essential to the survival of the microorganism but have little or no sequence homology to humans. Details about the mechanistic features of some of these targets are described in the text to follow. These also include the anti-TB targets with available structural data, which are summarized in.

The cell wall is crucial to the survival of Mtb. It provides protection and by being impermeable to the number of drugs, it also confers inherent resistance. The bacterial cell wall is comprised of two parts, 1). The outer compartment consisting of lipids and proteins 2) the inner compartment which is composed of PG, AG, and MA. The substances in the outer compartment are unique to the Mtb microorganism. Enzymes involved in cell wall synthesis are absent in humans, and thus present primary targets for anti-tuberculosis drugs.

PG gives the cell wall its rigidity and is made up of N-acetylglucosamine and Nacetylmuramic acid. Translocase I (TL1) is an enzyme which plays a role in PG synthesis. Alanine racemase is an enzyme involved in the racemisation of L-alanine to D-alanine, which is also a key component of PG. L,D-transpeptidase (LdtMt2) is responsible for the 3–3 transpeptide linkages in the PG layer.

AG is also an essential component of the cell wall. Lipoarabinomannan (LAM) is a glycolipid located within the cell wall. LAM has immunomodulating activity and possibly plays a role in the prevention of macrophage activation. It therefore presents a major virulence factor. The enzymes ribosyltransferase, UDP-galactopyranose mutase, galactofuranosyl transferase, dTDP-6-deoxy-L-lyxo-4-hexulose reductase, Rm1B and Rm1C in the AG and LAM biosynthetic pathways are possible drug targets. Decaprenylphosphoryl-β-D-ribose 2'-epiramase (DprE1) forms a heterodimer with DpeE2 to form the active enzyme which is responsible for the conversion of decaprenylphosphoryl ribose to decaprenylphosphoryl arabinose, a component of the Mtb cell wall. DprE1 is a validated target for a number of inhibitors (BTZ-043, PBTZ-169, TBA-7371), currently in the GLP toxicity phase.

MA's are synthesized in the fatty acid synthesis (FAS) pathway, which consists of the FAS I and FAS II pathways. The FAS I pathway is catalysed by a single enzyme that is similar to eukaryotes. The FAS II pathway, however, is absent in mammalian systems. Discrete enzymes catalyse each step in the pathway. The enzymes - Pks13, Acyl-AMP ligase, FadD32, AccD4, FabH, MabA and InhA thus present as valuable

drug targets. MmpL3, is an enzyme involved in the transport of MAs, and is the target for SQ-109 which is currently in Phase II trials.

The Shikimate pathway is responsible for the synthesis of chorismate, which forms the backbone of aromatic amino acids. This pathway is absent in mammalian systems, therefore enzymes (AroB, AroC, AroE, AroG, AroK and AroQ) within this pathway present useful targets. The enzyme governing the Shikimate pathway, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAH7PS), has shown promise as a potential anti-TB target. DAH7PS condenses phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAH7P). The role of DAH7PS is isolated to the synthesis of aromatic amino acids in the bacterium, making it a vulnerable target.

Micronutrients such as vitamin B2 (riboflavin) and B5 (pantothenate) are essential for the survival of the organism. Riboflavin is critical to certain metabolic reactions, such as the final metabolic conversion of monosaccharides to ATP. Pantothenate synthetase (PS) is responsible for the catalysis of pantoate to pantothenate (vitamin B5). Vitamin B5 is required for CoA and acyl carrier protein synthesis, which are required for energy and fatty acid metabolism. Unlike humans who obtain vitamin B2 and B5 from their diet, bacteria depend on the endogenous synthesis of these vitamins, hence the enzymes involved in panthothenate biosynthesis (Pan B-E) and lumazine synthase (LS) are prospective targets.

2.1.4 DNA metabolism

Two classes of ribonucleotide reductases (RNRs) exist and are essential to Mtb. These enzymes catalyse the reduction of ribonucleotides to deoxyribonucleotides . Class I are oxygen dependent and are further subdivided into Class Ia (NrdA or NrdE) and Class Ib (NrdB or NrdF). Class II are oxygen independent.

DNA ligases are enzymes responsible for linking double-stranded DNA. They are either classed as NAD+ dependent (LigA) or ATP dependent (LigB). LigA is only found in some bacteria and viruses and hence present good drug targets. The thymidine kinase (TK) or phosphotransferase enzyme also has an important role in DNA synthesis and cell division.

Recently, the DNA polymerase III β-sliding clamp (DnaN) was identified as a potential antiTB drug target. It has been validated using the streptomyces derived Griselimycin and its derivatives. In vitro and in vivo studies have proved that Griselimycin is effective in killing mycobacteria .

Mtb leucyl t-RNA synthase (leuRS) belongs to the class I aminoacyl-tRNA synthetase, which share a representative Rossmann fold in the synthetic domain. The enzyme is

characterised by two additional domains: 1) the connective peptide (CP)-1 and 2) the anticodon-binding domain. LeuRS is responsible for the catalytic formation of aminoacyl-tRNAs by providing material for protein synthesis. LeuRS is the target of GSK-070, a drug candidate which is currently in pre-clinical development.

In prokaryotes the start codon for the translation of mRNA contains formylated methionine. Following mRNA translation, deformylation of methionine by peptide deformylase occurs. Methionine is then hydrolysed by methionine aminopeptidase. The absence of methionine aminopeptidase in mammalian hosts makes it a useful enzyme to target.

2.1.5 The glyoxylate pathway

Fatty acids are a source of energy and carbon. The glyoxylate pathway uses Acetyl-CoA from fatty acid metabolism, and is responsible for the assembly of carbon units involving the synthesis of C4 dicarboxylic acid from C2 units. Enzymes in the glyoxylate shunt pathway also present as good targets, as they are absent in vertebrates. Persistence of Mtb in macrophages and mice requires the glyoxylate shunt enzyme, isocitrate lyase (ICL). The first step in the glyoxylate pathway is catalyzed by ICL. In Mtb there are two ICLs (ICL1 and ICL2).

2.1.6 Regulatory proteins

ArgP, GlnD, GlnE and IdeR are regulatory proteins responsible for the regulation of other proteins and co-factors. Arg P is a transcription factor involved in the regulation of arginine transport. IdeR controls the transcription of genes responsible for iron procurement and storage, as well as those genes required for the survival of macrophages. GlnE is an adenyl transferase enzyme. GlnD, a uridylyl transferase, plays a role in the control of GlnE activity. Other distinctive TB targets

ATP synthase is essential for ATP biosynthesis, therefore disruption of this enzyme leads to ATP depletion and also affects pH homeostasis 63. Bedaquiline is a newly registered anti-TB drug which displays inhibitory activity against ATP synthase.

The respiratory cytochrome b subunit (QcrB) of the cytochrome bc1 complex is a validated target for Q203, a drug which is currently in phase 1 clinical trials. The enzyme complex is an essential component of the respiratory electron transport pathway in the synthesis of ATP. Q203 depletes intra-cellular ATP, without inhibiting P450 isoforms and without disrupting the P-gP efflux pumps. It also interferes with the electron transport of the iron-sulphur protein.

The enzymes within the Menaquinone (vitamin K2) pathway are attractive targets because they do not have human homologues, as humans obtain vitamin K2 from their diet.

Mycothiol is a low molecular weight thiol that serves as a protectant against antibiotics and toxic oxidants. The biosynthesis of mycothiol requires the four-enzymes that are encoded by genes (MshA, MshB, MshC, MshD). MshC encodes for mycothiol ligase which is essential, as disruption of this gene reduces mycothiol production. The condensation of cysteine and glucosamine inositol to cysteine glucosamine inositol, in the biosynthesis of mycothiol, is catalysed by the enzyme mycothiol ligase.

Maltosetransferase GlgE plays a key role in the conversion of trehalose to glucan. GlgE utilises maltose-1-phosphate in the lengthening of 1,4-glucan chains. The inhibition of GlgE leads to a build-up of maltose-1-phosphate, resulting in self-poisoning of the organism. An accumulation of maltose-1-phosphate has an adverse effect on the respiratory electron transport system.

Recently, the discovery of a potential anti-TB drug target biliverdin reductase Rv2074 was reported. This enzyme is responsible for the reduction of biliverdin-IXα to bilirubin. The reaction serves as a safety mechanism for Mtb, protecting the bacterium from oxidative stress. Alkylhydroperoxide reductase E (AhpE), has demonstrated itself as a potential target of Mtb. Its catalytic activity is defined as the neutralisation of redox environments, which again shields Mtb from oxidative stress. Fumarate hydratase is directly involved in the tricarboxylic acid (TCA) cycle, promoting the reversible conversion of fumarate to malate in Mtb. However, the homologous nature of fumarate hydratase with the human form of the enzyme i.e. 53 % similarity, compromises the selectivity of drugs developed for this target. Acetyltransferase E has been implicated as a major co-factor for the inactivation of kanamycin, thus promoting drug-resistant TB. This could be a potential target for aminoglycoside-resistant TB.

Furthermore, crystal structures of malate synthase, lipoyl synthase, anthranilate phosphoribosyltransferase (AnPRT; trpD), enoyl-CoA hydratase, glutamate racemase, Lglutamate ligase (FbiB), 7,8-diaminopelargonic acid synthase (BioA) and Type II dehydroquinase D88N from Mtb have been deposited in the PDB and new anti-TB agents are yet to be discerned.

2.2. Exploiting physiology that is unique to Mycobacterium tuberculosis

There are certain weaknesses/deficiencies in the TB bacterium that could be exploited for the design of new drugs. One such weakness is the deficiency of the bacterium in its efflux ability of POA, hence it is susceptible to PZA. PZA is particularly effective in an acidic medium where its accumulation is highest. It is thought that due to a slow metabolism, Mtb displays defectiveness in its ability to maintain membrane potential and pH gradient, and is henceforth highly susceptible to weakly acidic drugs.

Studies on the mechanism of action of INH have shown that Mtb has a deficiency in its defence against endogenously produced oxygen radicals, which are generated by KatG, on the activation of the prodrug to its active form. This seems to be due to a defective OxyR gene and poor removal mechanism of the oxygen species and organic radicals. Mtb also appears to be susceptible to reactive nitrogen species, which is why drugs such as niclosamide, nitroimidazopyran (PA-824) and nitrofurans whose activation leads to the generation of reactive nitrogen – are particularly active against non-replicating bacteria.

2.3. Drug screens

To overcome the problem of drug-resistance in TB, the National Institutes of Health (NIH), GlaxoSmithKline (GSK), NIAID, Otsuka Pharmaceutical and the Global Alliance for antiTB drug development have sponsored many drug discovery programs. Each of the program involve both whole-cell and target-based screening. Drug screening was successful in identifying many of the marketed anti-tubercular agents that are in clinical use. The few compounds with anti-tuberculosis activity are currently in the pipeline of anti-TB. Those have also been discovered through high-throughput screening of whole-cells or targeted enzymes.

Whole-cell screening has been more successful in yielding plausible hits, as target based screening does not take into account the poor penetration and efflux problems. It is recommended that whole-cell screening be initially performed to scout for potential hits followed by target-based screening to distinguish the mechanism of action of a specific drug candidate. One successful candidate discovered through this method was TMC-207.

The anti-tuberculosis activity of actinomycetes and adamantanoids, drug candidates that are currently in the hit-to-lead stage, were discovered through whole-cell screening. A number of hit compounds of malate synthase inhibitors 87 were identified through a target-based screen. These compounds were further modified through structure-based drug design and chemical modification. A target-based screen of isoprenoid biosynthetic enzymes filtered out certain compounds that possessed anti-tuberculosis activity. Biological target-based screening of Mtb RNA polymerase, ATP synthase and Ndh-2 identified a number of compounds with activity against their respective targets.

Drugs: Capuramycins (TL1 inhibitors), Azaindoles (DprE Inhibitors), SPR-113, ureas and Ruthenium (II) phosphine/diimine/picolinate complexes – which are currently in the lead optimization stage – were discovered through screening for anti-tubercular activity,

using whole-cell based assays. GSK's InhA inhibitors (thiadiazoles) and Anacor Pharmaceuticals LeuRS inhibitors (oxaboroles) were discovered through biochemical target-based screening against Mtb.

Although the method of whole-cell based drug screening of molecules works well against growing bacilli, it is not useful for screening non-replicating persisters. In persisting bacteria there is also no correlation between low minimum inhibitory concentration (MIC) and sterilising activity. One example of this is INH, which is highly active against growing bacteria but inactive against persisters. In contrast, PZA is poorly active against growing bacteria but is more effective against non-replicating bacteria. PZA, which has excellent sterilising ability against non-replicating bacteria, would have been missed if the screening process had been conducted based on MICs. Owing to the different sub-populations and in vivo growth environments present in a TB infection, it is imperative that screens are performed against both replicating and non-replicating bacteria and under different culture conditions. Standardized aerobic culture conditions are partial to actively replicating bacteria. Recently, screens to cover different culture conditions were developed. This is thought to be a better representative of the in vivo environment and is more likely to determine the inhibitors with activity against persisters. Screening against Mtb that is present in macrophages has been successful and has recently yielded the compound Q203 which is currently in phase I clinical trials.

High molecular weight, lipophilic drugs are more likely to partition and accumulate in the cell membrane and have poor penetrability into intra-cellular compartments. Large molecular weight, highly lipophilic drugs also seem to have a greater potential for hepatotoxicity, as well as affect mitochondrial function. These properties can also present a problem in downstream pharmaceutical formulation optimization to improve the solubility. This should be taken into account when designing the whole-cell screen. It is therefore better to prioritize the compounds with suitable physiochemical properties over those compounds with higher potency but poor penetration capabilities. However, it should be noted that bedaquiline (TMC-207) was successfully developed despite its high clogP value of Methods that have been recently reported to determine intra-cellular compound levels will certainly have value in correlating intra-cellular drug levels with bacterial killing potential. The use of matrix-assisted laser desorption/ionization (MALDI) spectrometry imaging is an invaluable method to visualize the unlabelled drugs in different tissue sections, essentially mapping where these anti-TB drugs concentrate.

3. Computational approaches in anti-tubercular drug design/discovery and to understand the mechanism of action of anti-TB agents

To provide a more cost-effective and streamlined approach, CADD can be used as a complementary tool to aid experimental work. CADD can significantly expedite the drug discovery process by aiding the discovery and refinement of potent novel drugs. Virtual screening (VS) is an effective tool for in silico screening of large compound libraries for obtaining potential leads. The ligand-based approach is founded on the properties of known actives, whereas structure-based drug design uses the 3-D structure of the protein. The incorporation of filters can significantly narrow the size of the library to be selected. The CADD approach is guided by the amount of structural and other information available. Assays are then used to confirm the actives.

GSK employed computational approaches for the identification of potential anti-TB targets. Their protocol integrated the chemical properties of the ligand together with structural similarity and bioassays. This study was based on a chemogenomics space search (CHEM), a structural space search (STR) and a historical assay space search (HIST). The approach correlated the structural similarity against validated targets. It has been suggested that this type of approach could be useful for the development of future novel anti-TB drugs.

A TB drugome approach was used to empirically reposition an established drug that is effective in another disease against Mtb. Anti-TB targets with similar ligand-binding sites to other organisms were analysed. These targets are then screened using established drugs. The study tested twenty-three reported drug candidates. Known anti-cancer agents, tamoxifen and 4-hydroxytamoxifen, were found to suppress Mtb. These drugs also enhanced the antimicrobial effect of rifampicin, INH, and ethambutol.

Docking algorithms are inexpensive VS tools. Molecular dynamics (MD) simulations can be used after VS for fine-tuning of docked complexes and to obtain binding affinity information. There is a range of commercially available software for the visualisation of ligandprotein complexes that can provide important information on the binding mode and active conformation of the ligand.

Malate synthase inhibitors work in the glyoxylate pathway. Krieger et al. employed a structure-based drug design where a library of 35 small molecules having a common phenyldiketo acid (PDKA) scaffold was assayed against GlcB. Some of these compounds were crystallised in complex with the receptor. The consequent derivation of structural information and ligand-protein interactions allowed them to further optimise these compounds from the lead chemical structure.

Linezolid, an oxazolidinone derivative, was recently evaluated for activity against MDR-TB. Structure-based bioisosteric replacement of the parent scaffold resulted in the development of linezolid analogues Radezolid, Torelozid and PNU-100480 (Sutezolid). These drugs are currently in different stages of clinical trials for use as anti-TB agents.

Bioisosterism was also used as a tool to modify the ligand scaffold of ethambutol, which led to the design of SQ-109. SQ-109 is currently in Phase II clinical trials for the treatment of TB.

In PA-824 (Pretomanid) development, binding affinities obtained from molecular docking were evaluated against Mtb bactericidal activity. Docking was also used to gain insight into key binding interactions within the active site. PA-824, has recently entered Phase III clinical trials to evaluate the efficacy, safety, tolerability and pharmacokinetics.

Molecular docking studies were also employed to discern the influence of the double-bond arrangement, and size of the isoprenoid unit of thiolactomycin (TLM) on the binding affinity. TLM acts on KasA and KasB in Mtb. Due to difficulty in crystallization of Mtb; FabB, from E. coli was used. The study demonstrated that large conformational changes of FabB occurred upon TLM binding. Active site residues His298 and Phe392 altered its position to accommodate the inhibitor. The use of homology modelling for the β-ketoacyl-ACP synthases (KasA and KasB) was also employed to identify differences at the active sites of these Mtb enzymes.

Benzothiazinone (BTZ-043), which binds to the DprE1, was found to have selective activity against Mtb. Tiwari et al. used computational molecular docking studies to gain insight into the binding potential of BTZ-043 oxidative derivatives [1,3-benzothiazinone sulfoxide (BTZ-SO) and 1,3-benzothiazinone sulfone (BTZ-SO2)]. Molecular docking suggested similar binding patterns occurred for BTZ-SO and BTZ-SO2. Biological assays confirmed that BTZ-SO was also highly potent in pathogenic and non-pathogenic mycobacterial strains.

A novel series of spectinomycin analogues were generated using a structure-based drug design approach. The study involved the building of a homology model of the Mtb 16S helix spectinomycin-binding site using the 30S E. coli spectinomycin structure as a template, followed by molecular docking and dynamics to optimise the analogues.

4. Anti-TB drugs currently in the pipeline

In recent years, considerable efforts have been employed in the discovery and development of new anti-TB drugs. Currently there are a number of drug candidates in different phases of the discovery, pre-clinical and clinical development There are also a number of ongoing trials using repurposed drugs, where different combinations and doses of drugs that are currently on the market, are being tested with a view of optimizing therapies.

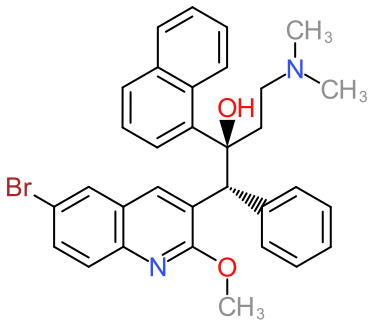
4.1. Pre-clinical development (early stage)

CPZEN-45 is a nucleoside antibiotic, which works through the inhibition of decaprenyl-phosphate–GlcNAc-1-phosphate transferase. It has in vitro activity against both replicating and non-replicating bacteria and has demonstrated efficacy against both drug- sensitive and XDR-TB in murine models.

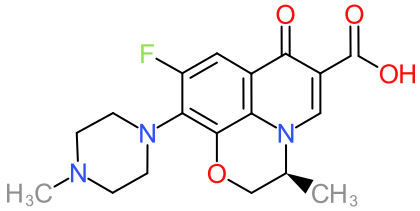
Lead compound SQ-609, contains a dipiperidine pharmacophore, and was identified from a library of compounds. It is currently in pre-clinical trials and has in vivo activity against drug-sensitive and drug-resistant forms of TB. SQ-609 has in vitro intracellular activity against laboratory and clinical isolates of Mtb. It is an add-on drug to be taken in combination with other anti-TB drugs.

TBI-166 belongs to the riminophenazine class of drugs. This class also contains the antileprotic drug clofazimine. Clofazimine has several undesirable properties such as urine discoloration, poor solubility and an extensive half-life. TBI-166 was obtained through lead optimization, from a project designed to identify the compounds with similar efficacy to clofazimine but without its undesirable properties. TBI-166 has an improved physiochemical and pharmacokinetic profile.

The spectinamide analog 1599 was generated using structure-based drug design. It has a narrow spectrum activity and is selective for ribosomal inhibition. It was well tolerated in a number of murine models where it considerably reduced lung mycobacterial burden as well as improved the patient's survival. In vitro studies have demonstrated activity against MDR-TB.



Bedaquiline



levofloxacin

Figure 3. Drugs that are currently in pre-clinical (early stage) development.

4.2. GLP toxicity

BTZ-043 a benzothiazinone compound, is a suicide substrate for the flavoprotein subunit DprE1. BTZ-043 displayed in vitro and in vivo bactericidal activity in TB murine models. The activity was slightly lower than that of RIF and INH.

PBTZ-169 is a piperazine containing benzothiazinone also acting on DprE1. It is not stereoselective like BTZ-043, and is therefore easier to synthesise. In the zebrafish model, it displayed an improved potency, safety and efficacy profile compared with BTZ-043. It has in vivo activity in murine models, and has additive effects with a number of anti-TB therapeutic drugs and a synergistic effect if taken with bedaquiline.

TBA-7371 is currently in pre-clinical development. It has activity against DprE1, an enzyme involved in arabinan synthesis. This molecule is active against drug-resistant TB. In vivo studies have demonstrated that to date it has a good safety profile.

GSK-070 (Table 6) inhibits protein synthesis by blocking Mtb leuRS. It is selective for Mtb and displays no cross-resistance to other anti-TB drugs.

4.3. Phase I

Q203 entered phase I clinical trials at the end of 2015. It was optimised from an imidazole[1,2-α]pyridine amide (IPA) lead. Q203 works in both anaerobic and aerobic conditions, it displays inhibition against both intra-cellular and extracellular TB as well as replicating and non-replicating bacteria. It also has activity against MDR-TB and XDR-TB. Its target is the respiratory cytochrome bc1 complex where it inhibits the synthesis of adenosine triphosphate (ATP) thereby disabling energy conversion.

4.4. Phase II

Sutezolid (PNU-100480) is an analogue of linezolid. In vitro studies revealed that it was active against Mtb and M. avium. In vitro and in vivo (murine) studies have shown that sutezolid has a superior antimycobacterial activity and safety profile compared with linezolid. It is also active against drug-sensitive and drug-resistant TB. It is currently in phase IIa.

SQ-109 is an ethylenediamine derivative, derived from the ethambutol pharmacophore. Its selection was based on in vitro and in vivo results where it showed activity against wild-type Mtb as well as MDR and XDR-TB 181. SQ-109 inhibits cell wall synthesis, by inhibiting MmpL3. Since it is effective against ethambutol resistant strains it is speculated to have a mechanism of action that is different to that of ethambutol. It is currently in phase II trials.

4.5. Phase III

Pretomanid (PA-824) is a bicyclic nitroimidazofuran which is currently in phase III clinical trials as part of a drug regimen consisting of bedaquiline and linezolid (NiX TB trial) . Recently, a study to evaluate safety, efficacy and tolerablity of pretomanid in combination with bedaquiline and linezolid has been initiated. The study includes a six-month treatment of pulmonary XDR-TB, treatment of intolerant or non-responsive MDR-TB patients (https://clinicaltrials.gov). In previous study, PA-824 was shown to be active against wild-type and some drug-resistant strains. To date there has been no cross-resistance to other anti-tuberculosis drugs. It is also active against no n-replicating bacteria, making it a possible drug for the treatment of LTB. The mechanism of action occurs through cell wall lipid and protein biosynthesis inhibition Its effect on non-replicating bacteria is probably due to the production of nitric oxide that is most likely generated on conversion of the prodrug to its active form. The NC-005 trial using the drug combination: pretomanid, bedaquiline and PZA is testing the two-month efficacy of the combination in drug sensitive and MDR-TB. The PRACTECAL study conducted by the Medecins Sans Frontiers (MSF), examined the use of different combinations of pretomanid, bedaquiline, moxifloxacin, linezolid and clofazimine for a sixmonth duration against MDR-TB and has recently received ethics and regulatory approval.

Bedaquiline (TMC-207) is a diarylquinoline that has activity against both drug-susceptible, MDR and XDR strains. It also displays no cross-resistance against any first-line drugs and appears to have a greater potency against drug-resistant strains. TMC-207 works by inhibition of subunit c of ATP synthase. The BedaquilinePretomanid-PZA combination is currently in phase III clinical trials for the treatment of MDR-TB. The WHO MDR-TB guidelines have incorporated bedaquiline and delamanid as additional drugs to the MDR-TB regimen to ensure the combination of five effective drugs.

Delamanid (OPC-67683) has good in vitro activity against drugsusceptible and drug-resistant bacteria. There is also no cross-resistance with any of the firstline drugs. It is a prodrug and is metabolised to its active form, which is desnitroimidazole. Delamanid's mode of action is via the inhibition of methoxy-mycolic and keto-mycolic acid synthesis. The endTB, A5343, and MDR-end trials will shed more light on delamanid's use in combination therapy. The MDR-end trial sponsored by the Korean centre for disease control contains a regimen comprising of delamanid, linezolid, levofloxacin and PZA. The A5343 trial looks at the use of linezolid and delamanid

for MDR-TB. The VTEU trial which was sponsored by NIH Division of Microbiology and Infectious Diseases, is testing the delamanid-containing injectable-free MDR-TB regimen against the standard of care. Recently, the six-month safety, efficacy and pharmacokinetic trial of delamanid in paediatric patients has been initiated against multidrug-resistant TB. This study is sponsored by Otsuka Pharmaceuticals.

4.6 Optimizing the use of approved and repurposed drugs

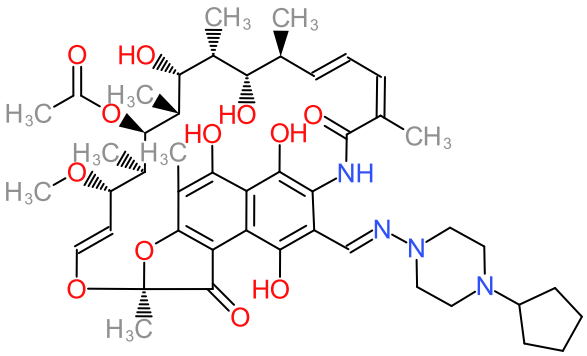
Studies are underway to optimize a higher dosage of RIF which is the first line anti-TB drug . A two-month study with higher doses of 15 or 20 mg/kg has demonstrated that the increase in dose had no corresponding increase in adverse effects. Obtaining the optimal dose may help to reduce the emergence of resistance.

Rifapentine is currently in phase II clinical trials for reducing the treatment durations of drug-susceptible TB and in phase III clinical trials for the treatment of LTB infections. Rifapentine is a semi-synthetic long acting rifamycin that requires once-weekly dosing. It acts on the β-subunit of RNA polymerase. In the Study31/A5349 trial, it is being looked at alone and in combination with moxifloxacin to reduce treatment duration times to four-months in drug-sensitive TB.

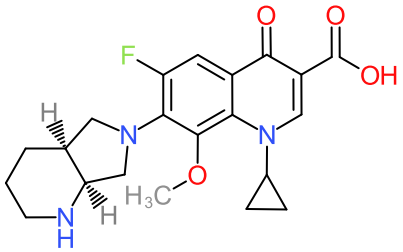
The TRUNCATE-TB study entailing four two-month regimens comparing it to the standard DS-TB treatment, has recently received protocol and ethics approval. This trial is due to commence at the end of 2016. One arm consists of a high dose of RIF, linezolid, INH, PZA and ethambutol; the second arm consists of the same regimen but substituting clofazimine instead of linezolid, the third arm uses rifapentine, levofloxacin, linezolid and PZA, and the fourth arm consists of bedaquiline, linezolid, INH, PZA and ethambutol.

Moxifloxacin (fluoroquinolone) is currently in Phase III clinical trials for its combination with PA-824 and PZA for drug-sensitive and MDR-TB . The objective of a second study was to assess the safety and efficacy profile of this drug combination to ensure whether it has an impact on reducing treatment duration times. The STREAM-1 trial uses moxifloxacin together with several of the current anti-TB drugs plus an injectable and clofazimine. In comparison to moxifloxacin, levofloxacin has lower QT prolongation effects. Currently, the Opti-Q study sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) is in the process of trying to determine the optimum dose of levofloxacin against Mtb. Clofazimine a riminophenazine analog, is a drug that has been repurposed for the treatment of TB. Originally an antileprotic drug, it is currently being used in combination with other anti-TB drugs in various clinical trials - PRACTECAL, STREAM-I, STREAM-II, and endTB studies. It has also been used off-label for the treatment of MDR-TB. Clofazimine is now undergoing evaluation for TB in randomized clinical trials (CIAM320B2202), which is currently in phase IIb/III. Linezolid is part of the NiX-TB

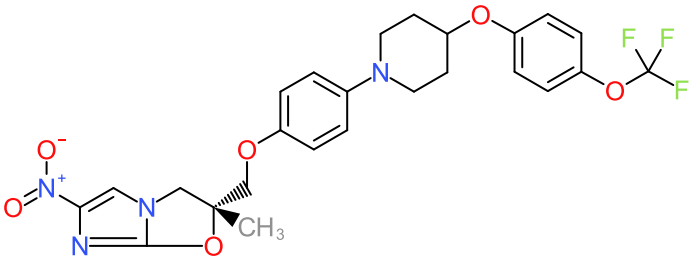
trial which is currently in phase III clinical trials. This drug has become increasingly crucial in the treatment of MDR and XDR-TB.



Rifampentine



Moxifloxacin



Delamanid

Figure 4. Drugs that are currently in clinical trials (Phase III).

5. Conclusion

The discovery and use of the four-drug regimen – INH, RIF, PZA and ethambutol – was a significant breakthrough in tackling drug-susceptible TB. However, the bacterium's resilient nature and its ability to adopt a latent state or develop resistance has entrenched TB as one of the leading causes of death from an infectious disease. Socio-economic factors and the TBHIV synergistic relationship have further contributed to increased infection rates. Combatting the TB problem is thus a complicated affair. The importance of novel drug discovery concentrating on drugs that are active against resistant, latent and dormant bacteria has been acknowledged; prompting a greater global effort in the search for novel drugs. Developments in anti-TB drug discovery show promise especially with the introduction of two new drugs delamanid and bedaquiline which are currently in Phase III clinical trials. There is also considerable interest in the investigation of the use of repurposed drugs. Recent advances and developments in the field of genomics, crystallography, CADD and highthroughput screening have proved invaluable and have certainly aided the anti-TB drug discovery process. This resulted in the appearance of a number of new drugs in the pipeline of anti-TB drug development

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