Efforts Towards the Development of New Antitubercular Agents: Potential for Thiolactomycin Based Compounds

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ABSTRACT - Development of new chemotherapeutic drugs is the need of the hour to improve tuberculosis control, particularly in the developing world. In the last fourty years no new compound has been brought to the market for the treatment of tuberculosis. However, in recent years there is an enhanced activity in the research and development of new drugs for TB. Some compounds are presently in clinical development, while others are being investigated pre-clinically in an attempt to explore new molecules for the target based treatment of TB. Simultaneously some new targets are being identified and validated for their practical usefulness. Structures based on thiolactomycin could have considerable potential in the development of target based anti-TB agents. The present review provides an overview of the drugs that are being clinically used and the compounds that are in advanced stages of clinical as well as preclinical studies. We have also attempted to highlight the efforts that are being made in the development of new molecules based on thiolactomycin as lead compound, including studies from this laboratory.

INTRODUCTION: TUBERCULOSIS AN OVERVIEW

Background

Mycobacterium tuberculosis, which causes tuberculosis (TB), is a slow growing bacterium and was evolved more than ten thousand years ago from a soil bacterium (1). Tuberculosis is a respiratory transmitted disease affecting nearly 32% of the world's population, more than any other infectious disease. Among the infected individuals, approximately eight million develop active TB and almost two million of these die from this disease per year. Of new TB cases, 95% occur in developing countries every year and approximately one million young women per year are victimized with this disease in the developing world (2-6). The occurrence of this disease is linked to dense population, poor nutrition, and poor sanitation (7). Tuberculosis, an airborne communicable disease caused by transmission of aerosolized droplets of *Mycobacterium tuberculosis* (8). The primary source of infection is viable tubercular bacilli, expelled in the environment by a patient with active TB.

Mycobacterium is a genus of bacteria, which grows slowly under aerobic conditions and is distinguished by acid-fast staining. They are Gram-positive, non-motile, rod-shaped, obligate aerobic bacteria that belong to the order actinomycetales, family *Mycobacteriaceae* (9). Several species, including *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. avium,* and *M. leprae*, are intracellular pathogens of higher vertebrates (10). The cell wall of *Mycobacterium* species in its full structural and functional integrity is essential for its growth and survival in the infected host. *M. tuberculosis* possesses a cell wall dominated by covalently linked mycolic acids, arabinogalactan and peptidoglycan (AGP), the mycolic acids of which are complimented by glycolipids such as α,α-trehalose monomycolate (TMM) (11). This mycolic acid based permeability barrier shields the organism from environmental stress and contributes to disease persistence and the refractoriness of *M. tuberculosis* to many antibiotics (12).

One of the most prominent macromolecular entities of mycobacterial cell wall is arabinan, a common constituent of both arabinogalactan (AG) and lipoarabinomannan (LAM) (13).

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In the chemical setting of mycolylarabinogalactan-peptidoglycan complex, AG forms an integral part of the cell wall proper, whereas LAM, based on a phosphatidylinositol anchor, apparently exists in a state of flux. LAM is an essential part of the cell wall core, anchored in the cell membrane and transversing the cell wall as well as appearing as an excretory product. LAM has been implicated as a key surface molecule in host-pathogen interactions. The biosynthetic pathways leading to the formation of the key mycobacterial cell wall components AG and mycolic acids are the target for the rational design of new antitubercular agents. The complete genome sequence of the best-characterized strain of *M. tuberculosis* H37Rv has been determined (14). Moreover, the TB-structural genomics consortium has undertaken an extensive study to determine and analyze the structures of over 400 proteins from *M. tuberculosis* including 40 novel folds and 200 new families of protein structures. The database of linked structural and functional information generated will have lasting impact in understanding the *M. tuberculosis* pathogenesis and for structure based drug design (15). Further, recent strategies that target various pathways related virulence, including inhibition toxin function, toxin delivery, regulation of virulence expression and bacterial adhesion could provide a number of new targets for novel antitubercular drugs (16).

Tuberculosis-HIV Combination

The current estimations reveal that one-third of the 42 million people living with HIV/AIDS worldwide are co-infected with TB (17). As per WHO reports, approximately 90% of the patients having both TB and HIV died within a few months after clinical symptoms. Therefore, WHO warned the world for ''even greater TB-HIV crisis'' and called for wide availability of free anti-TB drugs to those living with HIV. As per WHO, HIV is spreading rapidly in India with the largest number of TB cases in the world (18-21).

Drug-resistant tuberculosis (MDR-TB and XDR-TB)

Drug resistance displayed by *M. tuberculosis* is an important obstacle for the treatment and control of TB. This resistance has traditionally been attributed to the unusual multi-layer cell envelope and active multidrug efflux pumps. Recent insights into mechanisms that neutralize the toxicity of antibiotics in the cytoplasm have revealed other systems that function in synergy with the permeability barrier and efflux systems to provide natural resistance. Drugs inhibiting these intrinsic systems would enable many antibiotics, which are already available but have not been used for TB, to gain new activities against *M. tuberculosis*.

The term multidrug-resistance tuberculosis (MDRTB) refers to simultaneous resistance to at least two or more of the five first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin) (22-24). Multi-drug-resistance arises from sharing of genes between different species or genera, generally mediated by small pieces of extra-chromosomal DNA known as transposons or plasmids (25). Treatment for multidrug-resistant tuberculosis is long lasting, less effective, costly, and poorly tolerated. The most recent estimates suggest that, globally there were about 4.89 lakhs cases of MDR-TB in 2006. These cases were very unevenly spread with 86% of the cases are reported from 27 countries, of which 15 are in Eastern Europe (26).

Extensively drug resistant (XDR) tuberculosis by definition is resistance to at least isoniazid and rifampicin in addition to any quinolone and at least one injectable second-line agent (capreomycin, amikacin, kanamycin). The principles of treatment for MDR-TB and XDR-TB are the same. The main difference is that XDR-TB is associated with a much higher mortality rate than MDR-TB, because of reduced number of effective treatment options. Hence there is an urgent need for novel drugs that are active against *M. tuberculosis* in order to shorten the duration of tuberculosis therapy.

FIRST LINE ANTI-TUBERCULAR AGENTS

Chemotherapy of TB has started in the 1940's with anti-TB research resulting in the discovery of active anti-TB agents, and newer strategies have been devised in recent years to treat TB (27-30). The important first-line antitubercular drugs, which include streptomycin (SM), isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and pyrazinamide (PZA) and their mechanism of action is discussed below.

Streptomycin

Streptomycin (SM) **1** is an aminoglycoside antibiotic isolated from *Streptomyces griseus* and consists of three structural components, streptidine, streptose and *N*-methyl-L-glucosamine. Because of its poor absorbance in the gastrointestinal tract, it is administered intramuscularly and only very occasionally by intrathecal route. Streptomycin was the first really effective drug against TB, and derivatives of dihydrostreptomycin **2** also have anti-TB activities (Figure 1). Mutations in the rpsL gene of ribosomal S12 protein of mycobacteria or base substitutions in the 16S rRNA region confer resistance to streptomycin **1** (31).

Due to many toxic manifestations in the peripheral and central nervous system at higher doses and hypersensitivity reactions, it is not a drug of popular choice. Although dihydrostreptomycin **2** once was thought to be less toxic, it in fact causes severe damage to the eighth cranial nerve, inducing irreversible impairment of auditory function.

Isoniazid

Isoniazid (INH) **3** is a synthetic derivative from 4-pyridine carboxylic acid. The *M. tuberculosis* kat G gene encodes a dual function enzyme called catalase peroxidase, which confers sensitivity in *M. tuberculosis* to isoniazid. It is a prodrug that requires activation by the mycobacterial catalase peroxidase enzyme (kat G) into an active form, which then exerts a lethal effect on intracellular targets. INH is orally active and exhibits bacteriostatic action on resting bacilli and is highly active against the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*). It has very low MICs (0.02–0.06 µg/mL) against these pathogens (32). It enters the organism by diffusion and oxygen-dependent active transport, and it has been reported to have an effect on almost every aspect of mycobacterial metabolism (33). INH inhibits the mycolic acid biosynthesis in *M. tuberculosis* by affecting the enzyme mycolate synthetase, unique for *Mycobacteria* (34, 35). A mutation within the mycobacterial inhA gene has shown to confer resistance to INH in *M. smegamatis* and in *M. bovis*, suggesting that inhA is the likely target of this drug.

Rifamycins

Rifamycins RIF (rifampicin **4**, rifabutin **5**, rifapentine **6**) belong to the family of ansamycin antibiotics, so named because of their basket-like molecular architecture comprising an aromatic moiety bridged at non adjacent positions by an aliphatic chain (Latin: ansa= handle) (36-39). Rifamycins are a group of semisynthetic antibiotics of rifamycin B, isolated from *Streptomyces mediterrani* with a characteristic structure in which the chromophoric naphthaquinone group is spanned by a long aliphatic bridge which itself has very poor antimicrobial activity (40). They inhibit prokaryotic DNA-dependent RNA polymerase, an enzyme necessary for RNA synthesis (41) and acts on β-subunit of this enzyme resulting in the formation of a stable complex. However, a combination of isoniazid and rifampicin may increase a risk of hepatotoxicity. It is effective against *M. tuberculosis* with MIC ranging from 0.1 to 0.2 µg/mL.

Ethambutol

Ethambutol (EMB) is a synthetic aminoalcohol **7** (ethylene diamino-di-*l*-butanol), an orally effective bacteriostatic agent that is active against most strains of *Mycobacterium* (42-44). The proposed site of action of this first-line drug ranges from trehalose dimycolate, mycolate and glucose metabolism to spermidine biosynthesis. However, recent studies provided evidence that the primary site of action is arabinan biosynthesis both in arabinogalactan and LAM. Activity of EMB is stereospecific and the dextro isomer exhibited maximum anti-tubercular activity (*S,S* form is 600 times more active than *R,R*) (45).

Pyrazinamide

Pyrazinamide (PZA) **8**, a structural analogue of nicotinamide, is another first-line drug for short course TB therapy. It is also active against semidormant bacilli not affected by any other drug, has strong synergy with INH and RMP, and shortens the therapy period to six months (46-47). The drug has no significant bactericidal effect and is thought to act *via* a sterilizing effect. The activity of PZA depends on the presence of bacterial amidase, which converts PZA to pyrazinoic acid,

the active anti-TB molecule. Mutation in the pncA gene responsible for the production of pyrazinamidase has been shown to be the reason for resistance against this drug (48-49).

SECOND LINE ANTI-TB DRUGS

According to WHO there are six classes of second-line drugs that are used in the treatment of tuberculosis (50). A drug may be classified as a second-line because of one of two possible reasons: 1) it may be less effective than the first-line drugs or it may have toxic side-effects or 2) it may be unavailable in many developing countries. These comprise of different classes namely, aminoglycosides: (amikacin, kanamycin), polypeptides: (capreomycin, viomycin),

fluoroquinolones: (ciprofloxacin, moxifloxacin), thioamides: (ethionamides, prothioamide), cycloserine and *p*-aminosalicylic acid

Amikacin and kanamycin

Amikacin **9** is a aminoglycoside antibiotic used for the treatment of different types of bacterial infections including *M. tuberculosis* (51). It acts by binding to the bacterial 30S ribosomal subunit, causing misreading of mRNA and thus leaving the bacterium unable to synthesize proteins vital to its growth (52). It is most often used for treating severe, infections with multidrug resistant bacteria. However, amikacin causes kidney damage as well as hearing loss. Kanamycin **10** also belongs this class and has similar properties (Figure 2) (53).
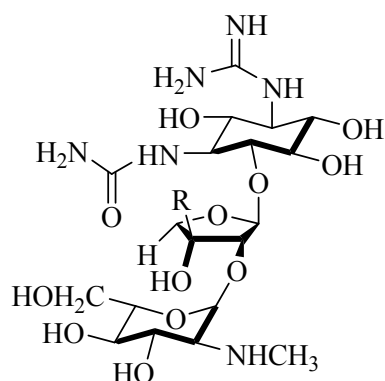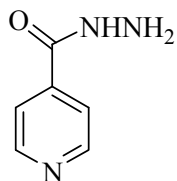

R = CHO   Streptomycin **1**

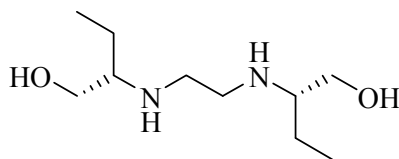R = CH$_2$OH dihydrostreptomycin **2**
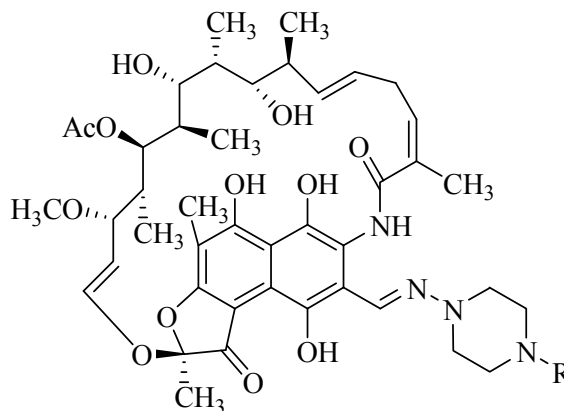
R = CH$_3$ Rifampicin **4**

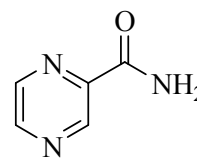R = CH$_2$CH(CH$_3$)$_2$ Rifabutin **5**

R =   Rifapentine **6**

Isoniazid **3**   Ethambutol **7**   Pyrazinamide **8**

Figure 1.

Amikacin **9**

Kanamycin **10**
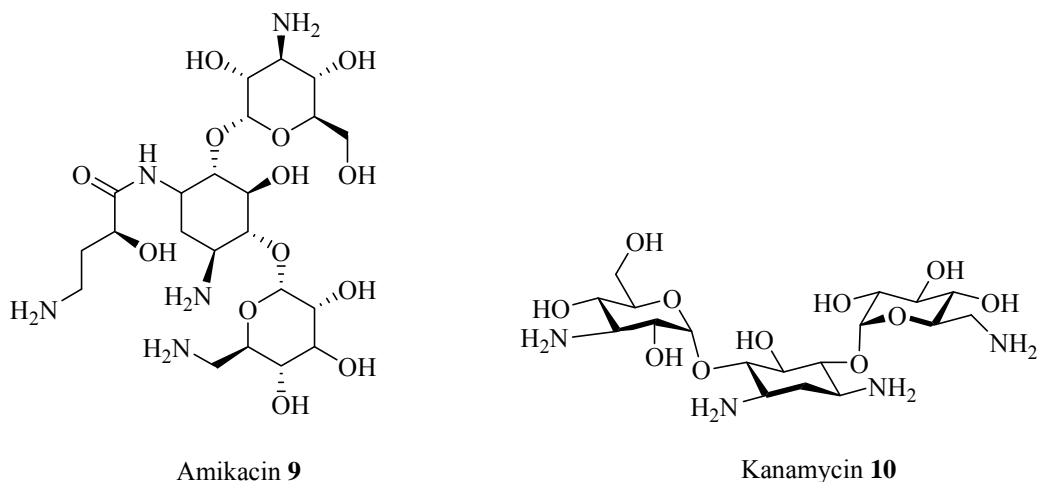
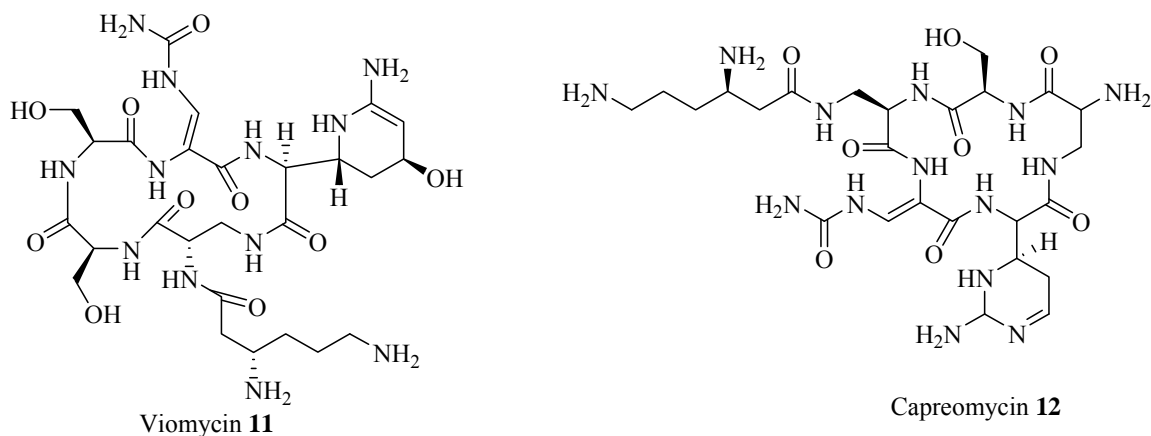**Figure 2.**

Viomycin **11**

Capreomycin **12**

Figure 3.

Viomycin and capreomycin

Viomycin **11** is an polypeptide antibiotic used for the treatment of tuberculosis. It is produced by the actinomycete *Streptomycespuniceus* that binds to RNA and inhibits prokaryotic protein synthesis and certain forms of RNA splicing (54). Capreomycin **12** also belongs to this class of antibiotic, which is given in combination with other antibiotics for treating tuberculosis (Figure 3). Unfortunately, it exhibits adverse effects including nephrotoxicity and eighth cranial nerve toxicity (55).

Fluoroquinolones

Fluoroquinolones (7-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid) are an important class of fluorine containing compounds (Figure 4). This class possesses potent antibacterial activity with a broad spectrum of activities against gram-positive, gram-negative and mycobacterial organisms as well as anaerobes with great therapeutic potential, particularly those resistant to other classes of antibacterial drugs (56). A large number of fluoroquinolones have been synthesized with further improvement, such as the solubility,

antimicrobial activity, prolonged serum half-life, lesser adverse side-effects and both oral and parenteral routes of administration. Some notable examples of this class are ciprofloxacin **13**, moxifloxacin **14,** ofloxacin **15** (levofloxacin **15a**, the chiral form of ofloxacin is more effective), gatifloxacin **16** (*S*-form is more effective), trovafloxacin **17**, enofloxacin **18** and sparfloxacin **19** etc. (56, 57).

Ciprofloxacin **13** and moxifloxacin **14** are broad spectrum antibiotics, active against Gram-positive and Gram-negative bacteria. They function by inhibiting DNA gyrase, a type II topoisomerase, which is an enzyme necessary to separate replicated DNA, thereby inhibiting cell division (58). Fluoroquinolones are increasingly contraindicated

for patients due to growing prevalence of antibiotic resistance.

Thioamides

The two main drugs from the thioamide (or thionamide) family that can be used for treatment of TB are ethionamide and prothionamide are structural analogues of isoniazid (Figure 5). Ethionamide (ETA: 2-ethyl thioisonicotinamide) **19** and prothionamide (PTA: 2-propyl thioisonicotinamide) **20**, are important drugs of second-line therapy for MDR-TB (59). ETA and PTA have almost identical inhibitory effects on mycolic acid biosynthesis as INH.
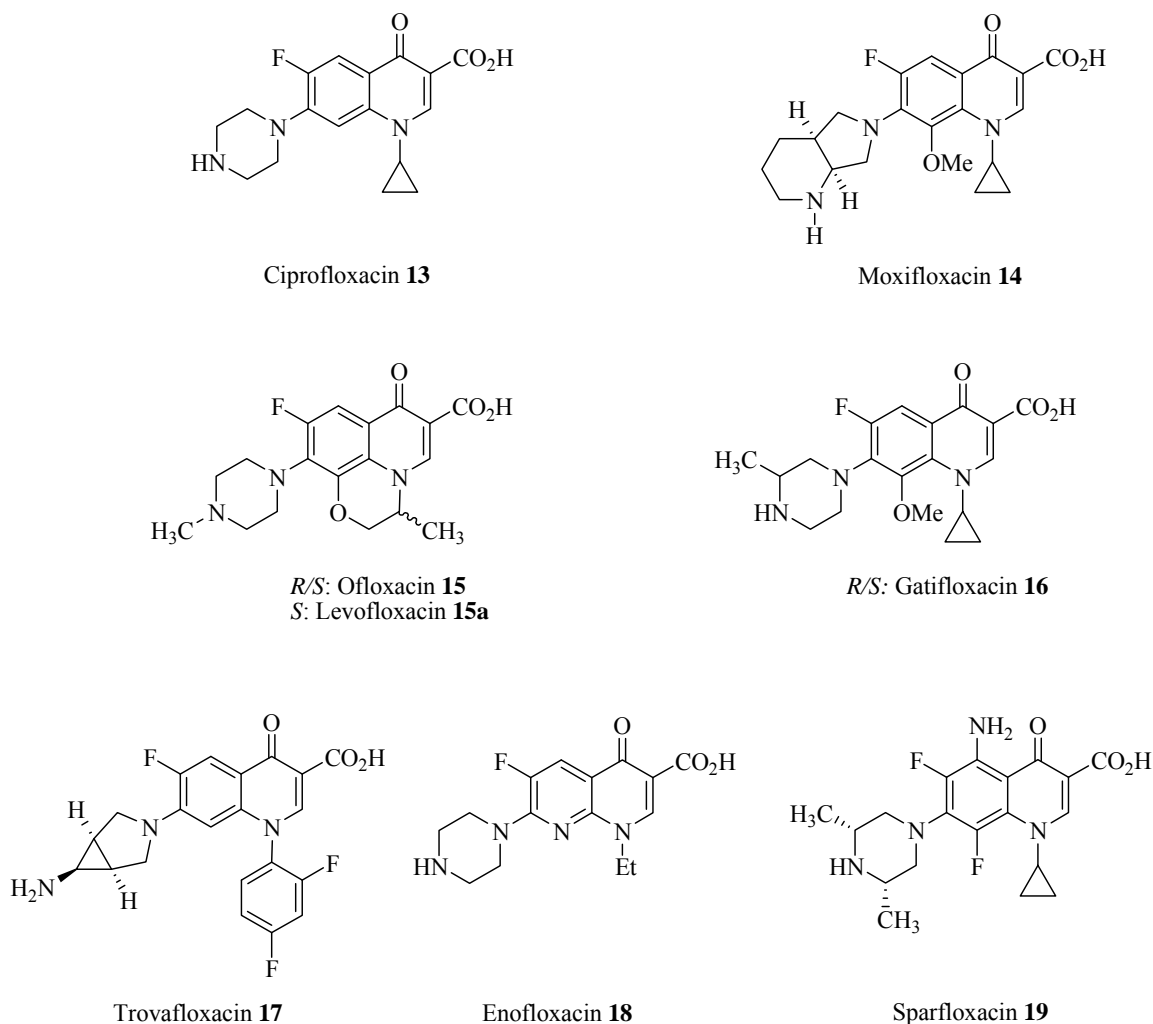

Ciprofloxacin **13**

Moxifloxacin **14**

R/S: Ofloxacin **15**
S: Levofloxacin **15a**

R/S: Gatifloxacin **16**

Trovafloxacin **17**

Enofloxacin **18**

Sparfloxacin **19**

Figure 4.

ETH is active against *M. tuberculosis, M. leprae, M. kansasii*, and some strains of the *M. avium complex* (60). Adverse effects associated with ETH and PTA are gastrointestinal disorders (such as anorexia, salivation, nausea, abdominal pain, and diarrhea), diverse mental disturbances (such as depression, anxiety psychosis, dizziness, drowsiness, and headache) and hypersensitivity skin reactions, side effects which restrict their common usage (61).

Cycloserine

D-Cycloserine (Cs) **21** a structural analogue of the amino acid D-alanine, possess an oral broad spectrum antibiotic effective against *M. tuberculosis*, by inhibition of cell wall synthesis at an early stage of peptidoglycan synthesis (61). The side effects of this drug are mainly CNS manifestations such as headache, irritability, depression, convulsions (62).

p-Aminosalicylic acid

p-Aminosalicylic acid (PAS) **22** is an antimycobacterial agent and is used in combination with isoniazid and streptomycin (63). PAS exerts a bacteriostatic effect on *M. tuberculosis* by competitively blocking the conversion of *p*-aminobenzoic acid into folic acid but is less effective.
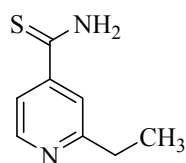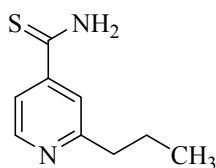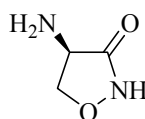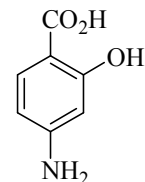
DRUGS FOR HIV/TB

Clarithromycin

Clarithromycin **23** is a macrolide antibiotic used in HIV infected TB patients to treat the *Mycobacterium avium complex* (MAC) (64). It prevents bacteria from growing by interfering with their protein synthesis (Figure 6). It binds to the subunit 50S of the bacterial ribosome, and thus inhibits the translation of peptides. It has a similar antimicrobial spectrum as erythromycin, but is more effective against certain Gram-negative bacteria, especially *Legionella pneumophila* (65).

Thioacetazone and thioridazine

Thioacetazone **24** is useful in preventing resistance to more powerful drugs like isoniazid and rifampicin. It is never used on its own to treat tuberculosis. The use of thioacetazone is declining since it can cause severe skin reactions in HIV positive patients (66). Thioridazine **25** is also known to kill multidrug-resistant *M. tuberculosis*. It is no longer recommended for treatment due its side effects like dry mouth, urination-difficulties, obstipationglaucoma and postural hypotension (67).

Ethionamide **19**   Prothionamide **20**   Cylcoserine **21**   *p*-Aminosalicylic acid **22**
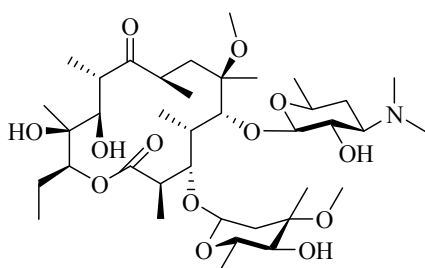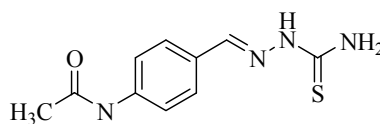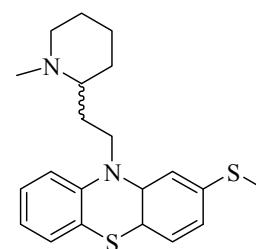
Figure 5.

Clarithromycin **23**   Thioacetazone **24**   Thioridazine **25**

Figure 6.

Now, the situation is further complicated by the emergence of multidrug-resistant TB and by infections with lethal synergy with HIV/AIDS. Patients with MDR-TB are being treated with a combination containing second line drugs that are less effective, more expensive and higher toxicity. TB's lethal synergy with HIV/AIDS puts HIV-positive individuals with latent tubercle bacilli infection (LTBI) at a greater risk of developing active TB, making TB as the number one killer among patients with AIDS.

The pharmaceutical industry however, has generally shown little interest in developing new, more effective drugs to address these needs, and as a result, no new anti-TB agent with a novel mechanism of action has been launched since the introduction of RFP in 1966. Consequently, global health and philanthropic organizations are now pleading for new chemotherapy interventions that can shorten the total duration of therapy, provide improved efficacy against MDR-TB, safely treat patients co-infected with HIV/AIDS, and target LTBI.

RECENTLY DISCOVERED ANTI-TUBERCULAR AGENTS

The recommended five first-line drugs for standard treatment regimen are highly effective and the rate of severe adverse reactions is low. However, unpleasant side effects and a relatively long duration of treatment are drawbacks, which also increase the rate of non-compliance to treatment regimen. Such non-adherence with the course of treatment leads to treatment failure and the development of drug resistance. The second line drugs used for MDRTB are more expensive, less effective and more toxic than the five drug standard regimen.

The goal now is to develop bactericidal drugs in a cost-effective manner, which efficaciously treats infectious MDR strains of *M. tuberculosis* and latent infections with shortened treatment periods as well as reduced frequency of dosage. Some of recently discovered anti-Tb agents are discussed below.

Tryptanthrin

Tryptanthrin **26** is a potent structurally novel indoloquinazolinone alkaloid, active against MDR

strains of *M. tuberculosis* and the MIC value of it is 0.5–1.0 μg/mL. After the determination of the toxicity data, this structural prototype can possibly be utilized in MDR TB therapy (68).

Nitroimidazopyrans and nitroimidaoxazoles

A series of bicyclic nitroimidazopyrans (NAP) have recently been reported to possess anti-tubercular activity (Figure 7). One of the compounds, PA-824 **(27)** has emerged as a lead molecule as it is effective against both replicating and latent *M. tuberculosis* cells with a MIC ranging from 0.015 to 0.25 μg/mL. Poly and multi-drug resistant strains are susceptible to PA-824, indicating that there is no cross-resistance with current drugs (69). The mode of action of this class of compound is by a mechanism dependent on *M. tuberculosis* F420 cofactor, inhibition of protein biosynthesis and cell wall lipids (69). Another orally active analog PA 1343 **(28)** has been developed and is in preclinical studies with MIC of 0.015 μg/mL (70). The compound OPC-67683 **(29)**, a nitro-dihydro-imidazooxazole derivative is found to possess higly potent activity against TB, including MDR-TB at a concentration (MIC) range of 0.006-0.024 μg/mL.

Oxazolidinones

Oxazolidinones are totally synthetic, orally active anti-bacterial agents developed by DuPont (71, 72). They are bacterial protein synthesis inhibitors, with inhibition uniquely in the initiation phase of protein synthesis. Some of the morpholine and thiomorpholine analogues of oxazolidinones like linezolid **30** and U-100480 **(31)** have shown potent *in vitro* activity against *M. tuberculosis* whereas the other oxazolidinone derivatives **(32**, **33)** displays lethal toxicity in the rat models (73) as shown in Figure 8.

Clofazimine Analogues

The tetramethyl piperidine substituted phenazines B4169 **(34)** and B4128 **(35)** (TMP phenazines) have been found to possess significantly more activity against *M. tuberculosis*, including MDR clinical strains than clofazimines **36** (74).
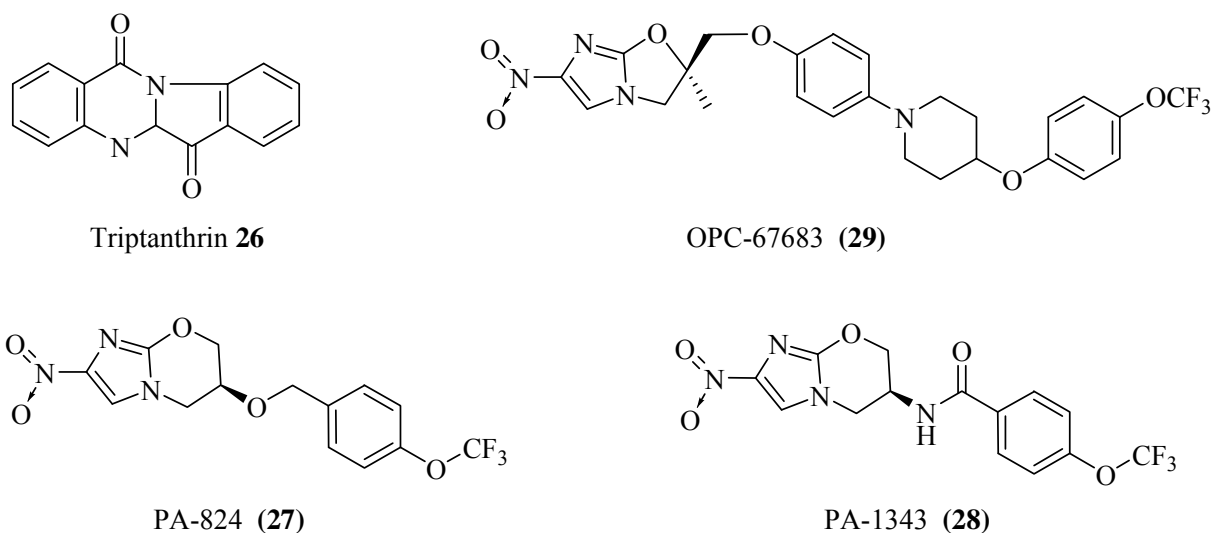
Triptanthrin **26**

OPC-67683 **(29)**

PA-824 **(27)**

PA-1343 **(28)**

Figure 7.

Linezolid **(30)**

U-100480 **(31)**

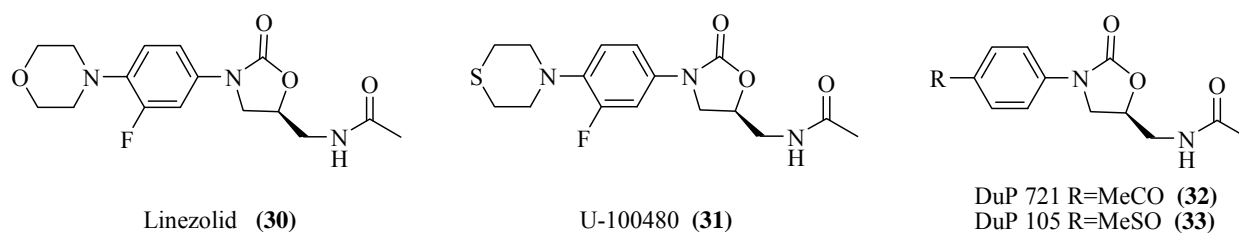DuP 721 R=MeCO **(32)**
DuP 105 R=MeSO **(33)**

Figure 8.

The most important virtues of tetramethyl phenazines are intracellular accumulation in mononuclear phagocytic cells, anti-inflammatory activity, a low incidence of drug resistance and slow metabolic elimination rate, which make them attractive candidates for the treatment of mycobacterial infections (Figure 9).

Recently, in this laboratory new conjugates of phenazine with phthalimido and naphthalimido moieties **37** have been designed and synthesized as antitubercular compounds (75). Some of the compounds in this new class of phenazine hybrids have shown promising results in the inhibition of *M. tuberculosis* ATCC 27294 as well as their clinical isolates (sensitive and resistant). This study revealed that there is a potential to design such type of phenazine hybrids for the development of new antitubercular agents.

Nitrofuranyl amides

Lee and co-workers (76) have prepared a series of nitrofuran derivatives which were tested for MIC activity against *M. tuberculosis* H37Rv. One of the compound **38** in this series has shown excellent MIC_{90} value 0.025 μg/mL, which is comparable to that of the frontline anti-tubercular agents like isoniazid and ethambutol. Structure-activity relationship studies have shown that the nitro group is necessary for biological activity.

Purines

9-Benzylpurines, with a variety of substituents on 2, 6 and/or 8 position, have been shown to possess high inhibitory activities against *M. tuberculosis* (Figure 10).
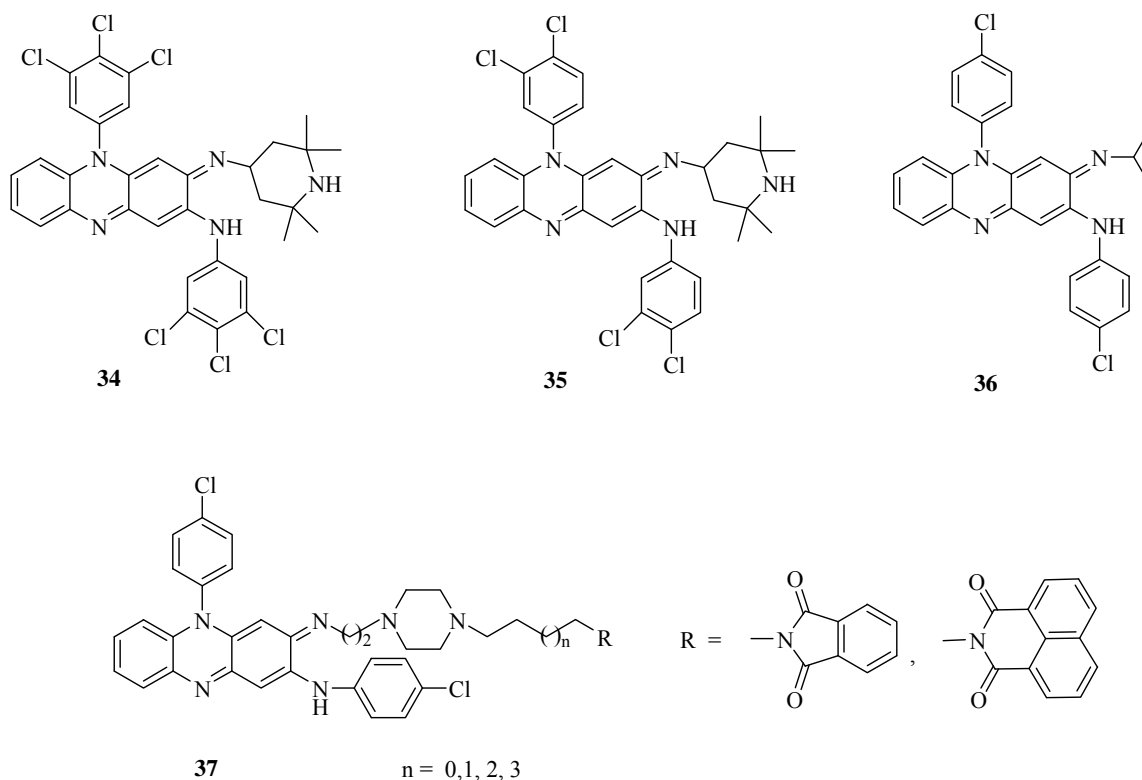
34   **35**   **36**

37   n = 0, 1, 2, 3

Figure 9.

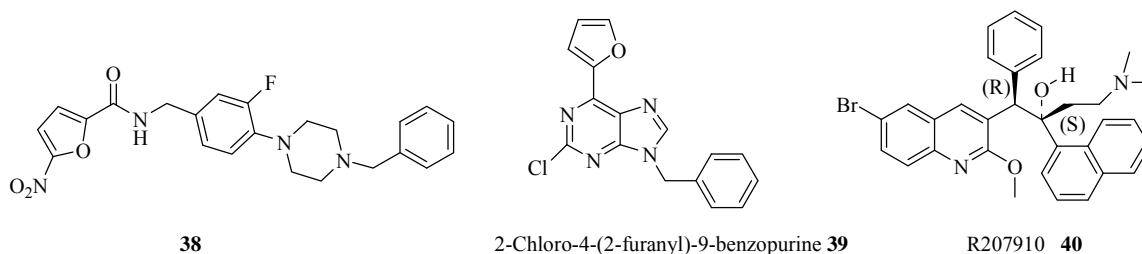**38**   2-Chloro-4-(2-furanyl)-9-benzopurine **39**   R207910 **40**

Figure 10.

One of the compounds, **39** belonging to the above class carrying trans-styryl or aryl substituents at 6 position and generally chlorine in 2 position tends to increase the activity and has MIC of 0.78 mg/mL *in vitro* (77). Antimycobacterial activity of 6-arylpurines (78) and 9-sulphonylated or sulphenylated-6-mercaptopurines are also known in the literature (79).

Diarylquinolines

Diarylquinolines (DARQs) are structurally different from both fluoroquinolones and other quinoline classes. The DARQ R207910 **(40)** is a member of a new chemical class of antimycobacterial agents and has a MIC value equal to or lower than reference compounds. It has a unique specificity towards mycobacteria including atypical species important in humans such as MAC, *M. kansai*, and the fast growers *M. fortium* and *M. abscessus* (80). This antimycobacterial specific spectrum differs from that of isoniazid, which has very poor activity against MAC. The clinical use of this will be highly targeted to the treatment of the mycobacterial infections, particularly targeting the proton pump of adenosine triphosphate (ATP) synthase (81).

1,2,4-Benzothiadiazines

Sulfonamides are well known for their antibacterial property and a large number of such compounds have been developed as antimicrobial agents (82-84). 1, 2, 4-benzothiadiazine dioxides have a close relation to sulfonamide and could be considered as cyclic sulfonamide class of molecules. These compounds are well known for a variety of biological properties, including antimicrobial activity (85, 86). Based on this finding and within a research program in this laboratory to develop new antitubercular agents - the 1, 2, 4-benzothiadiazine system was explored by incorporating other heterocyclic rings like pyridine and pyrazine moieties 41, 42 (Figure 11). Studies in this direction have afforded some molecules based on 1, 2, 4-benzothiadiazine system that exhibited interesting antitubercular activity (87, 88). Studies in this direction have afforded some molecules based on 1, 2, 4-benzothiadiazine system that exhibited interesting antitubercular activity.

Several other molecules like pyrroles **43**, (89) quinoxaline-1,4-dioxides **44** (90) and alkylsulfinyl amides **45**, (91) etc. have also been prepared and tested for their antimycobacterial activity. Recently some new targets such as signaling kinase inhibitors have been investigated. The survival of *M. tuberculosis* against the macrophage phagocytosis relies not only on a thick cell wall but also on many mycobacterial kinases and phosphatases which disrupt the host-cell defence mechanism against parasitism (92-94). Histidine kinase is the focus for the specific inhibition of two component signal transduction system in mycobacteria (95-98). Based on this signal transduction system, a series of antimycobacterial salicylanilides and related compounds have been reported (99-102). Inhibition of this type of regulation has been involved in the virulence of *M. tuberculosis* in mice (103). Eleven putative eukaryotic–like protein serine-threonine kinases (Pkn A to L) involved in signal transduction have been identified in *M. tuberculosis* H37Rv genome (11, 104-106). Based on this kinase inhibition benzothiophenes (specifically inhibits Pkn G) (107, 108) and benzoquinoxalines (inhibitors of Pkn B, Pkn G, and Pkn H) (109-112)

have been reported. Hence this intensive research on signaling kinase inhibitors could also provide target oriented lead molecules for the control of tuberculosis.

In view of the persistent drug-resistant TB problem, it is important that new drugs should address different targets, as those of currently used drugs including the shortening of TB therapy. The unique structure of the mycobacterial cell wall makes it a useful target for drug development and studies can be directed to specific sites like cell wall biosynthetic pathways (113). Thiolactomycin inhibits mycobacterial fatty acid synthase and the elongation steps of mycolic acid biosynthesis (114), with negligible toxicity and thus structures based on this lead could provide a new class of antibiotics against tuberculosis.

THIOLACTOMYCIN

Naturally occurring (5*R*)-thiolactomycin (TLM, **46**) exhibits potent *in vivo* activity against many pathogenic bacteria, including Gram-negative and Gram-positive bacteria and *M. tuberculosis* (114-116). TLM inhibits bacterial and plant type II fatty acid synthases (FAS-II) but not mammalian or yeast type I fatty acid synthases (FAS-I) (117). In *Escherichia coli*, it inhibits both β-ketoacyl-ACP synthase I to III and acetyl coenzyme A (CoA): ACP transacylase activities in *in vivo* and *in vitro* conditions (118, 119) (Figure 12).

Introduction

Thiolactomycin (TLM, **46**) is a thiolactone antibiotic isolated from a soil sample collected in Sayama city, Saitama prefecture, Japan. It is obtained from fermentation broth of *Nocardia* species, a strain of actinomycetes and has a unique chemical structure with no chemical relation to any group of known antibiotics (120, 121). This antibiotic has been detected in the fermentation broth by the use of the β-lactam antibiotic-sensitive mutant of *Pseudomonas aeruginosa* M-57740. The structure and antibiotic properties of TLM have been first reported by Oishi and coworkers in 1982 and it is the first naturally occurring thiolactone to exhibit antibiotic activity (120).
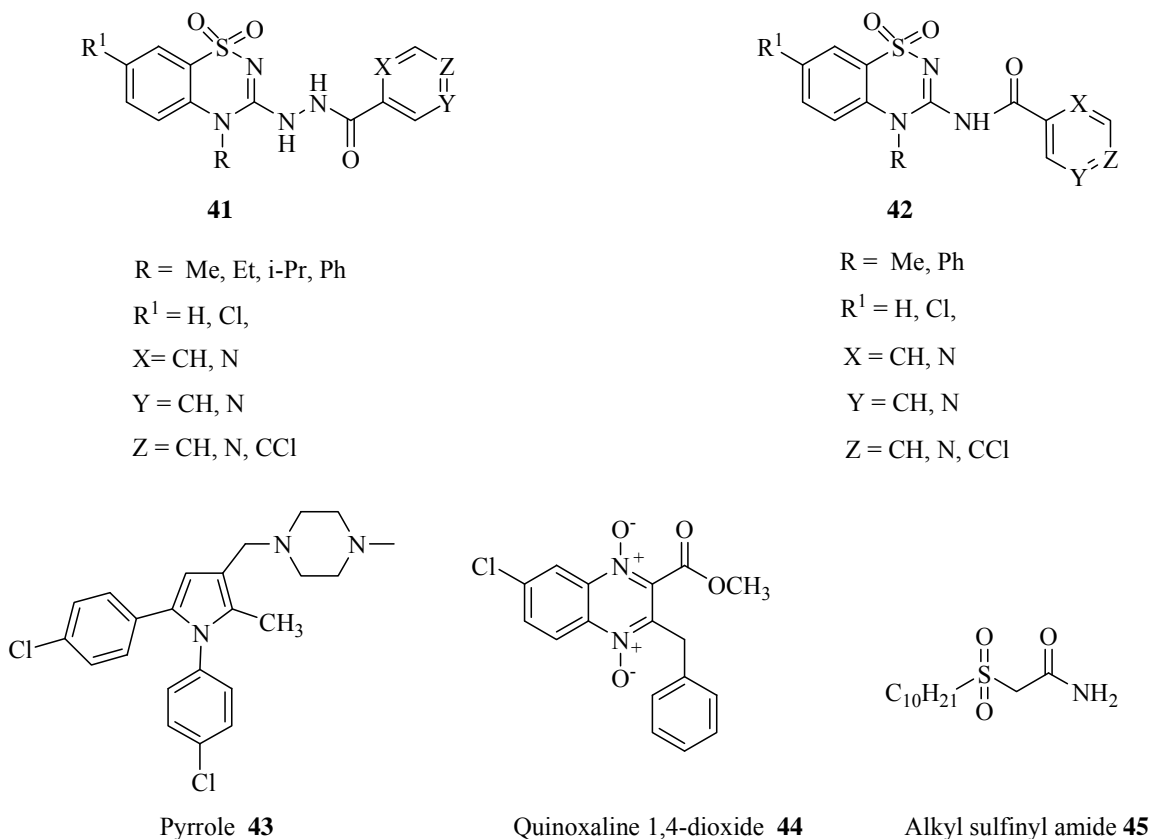
41

R = Me, Et, i-Pr, Ph

R^1 = H, Cl,

X= CH, N

Y = CH, N

Z = CH, N, CCl

42

R = Me, Ph

R^1 = H, Cl,

X = CH, N

Y = CH, N

Z = CH, N, CCl

Pyrrole **43**

Quinoxaline 1,4-dioxide **44**

Alkyl sulfinyl amide **45**
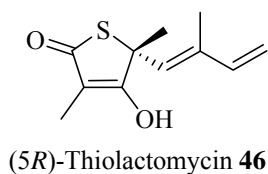
Figure 11.

(5*R*)-Thiolactomycin **46**

Figure 12.

Noto and coworkers as well as Hamada and coworkers have individually reported that TLM has moderate *in vitro* activity against a broad spectrum of pathogens, like Gram-positive and Gram-negative bacteria including *Mycobacterium tuberculosis* (120, 123). It shows complete inhibition of growth of the virulent strain *M. tuberculosis* Erdmman at 25 µg/mL (114). In rodents, thiolactomycin is well absorbed orally with an LD$_{50}$ of 1.689 g/kg (115). No reports appear to have been published on its efficacy towards *M. tuberculosis* in any animal models. Recently, it has also shown encouraging antimalarial activity, involving inhibition of type II fatty acid biosynthetic pathway in apicoplasts by the research groups of Waller and Morita (134, 140). TLM has chemotherapeutic potential, as it is non-toxic to mice and affords significant protection against urinary tract and intraperitoneal bacterial infections (116). Hayashi and coworkers have shown that TLM activity is directed towards type II fatty acid synthases (117). Jones and coworkers (127) confirmed that TLM inhibits type II dissociable fatty acid synthases by using [1-^{14}C]acetate labeling

in pea-leaf chloroplasts. By using of *in vivo* [1,2-^{14}C]acetate labeling of *Mycobacterium smegmatis* (126, 127), it was shown that it also inhibits the biosynthesis of both fatty acids and mycolic acids, which are the characteristic major 2-alkyl-branched 3-hydroxy fatty acids in *Mycobacteria* (122, 124, 125, 128, 129). Hence thiolactomycin is also a useful tool for studying and understanding the mechanisms of underlying parasitic infections and infectious diseases.

This new antibiotic has proven to be of considerable interest with its unique thiolactone moiety and because of its broad antibacterial spectrum. The favourable physical and pharmacokinetic properties of thiolactomycin, like low toxicity profile and good activity against several strains of *M. tuberculosis* which are resistant to the other drugs has made it an attractive lead molecule for the development of a drug candidate against the treatment of *M. tuberculosis*.

Mechanism of action

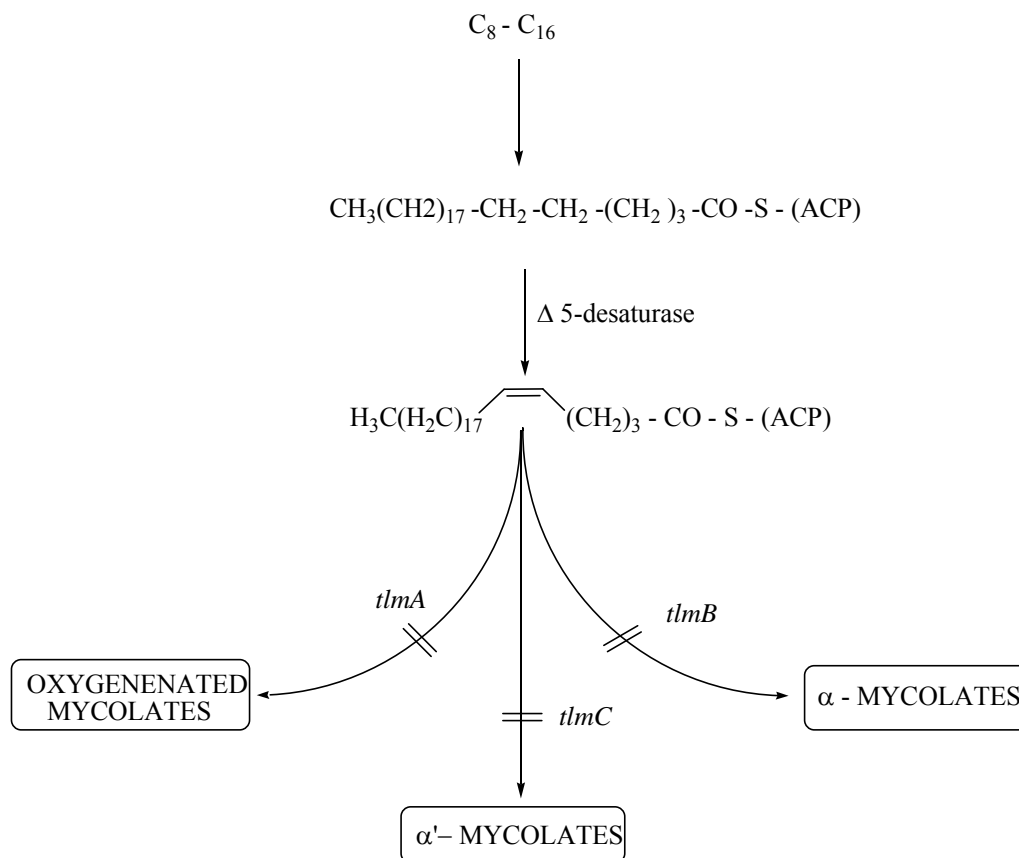**Thiolactomycin : Bacterial fatty acid synthesis inhibitor**

Fatty acid biosynthesis (FAB) in bacteria, plants and animals is carried out by the ubiquitous fatty acid synthase (FAS) system (130). FAB is an essential metabolic process for prokaryotic organisms and is required for cell viability and growth (131). Targeting this pathway is a rational approach for developing new antibacterial agents (132, 133). In the type I system of animals, including humans, FAS is a homodimer of two large polypeptides, each composed of several distinct enzyme domains and is an integral acyl carrier protein (ACP) (134). In the type II system of bacteria, plants and protozoa, the FAS components, including the ACP, exist as discrete proteins (135). The corresponding enzymes of the type I and II FAS are related in structure and function but generally lack overall sequence homology. Large multifunctional proteins termed type I FAS catalyze these essential reactions in eukaryotes (136). In contrast, the bacterial use of multiple enzymes to accomplish the same goal is referred to as type II, or dissociated FAS (137). The bacterial system and proteins bear little homology to the human system and therefore represent a set of attractive target

proteins for novel antibacterial development. Since many of today's nosochomial bacterial infections are resistant to several of the available antibiotics, compounds targeting the FAB pathway could fill a serious medical need (138).

A key enzyme responsible for the initiation of bacterial FAB has not yet received attention. FabH, a β-ketoacyl–acyl carrier protein synthase, is the bacterial condensing enzyme in Gram-positive and Gram–negative bacteria that initiates the cycle by catalyzing the first condensation step between acetyl–CoA and malonyl–ACP (139, 140). Mycolic acid, one of the structurally largest fatty acids found in nature, is a vital cell wall component of the human tuberculosis strain of *Mycobacterium tuberculosis*. The impermeability of this strain to many antibiotics has spawned much research interest into the biosynthesis of mycolic acid with a view to developing novel antibiotics (141).

Biosynthesis of the fatty acyl chain involves two fatty acid synthetic systems, fatty acid synthase I (FAS I), which catalyzes *de novo* fatty acid synthesis and fatty acid synthase II (FAS II), which consists of monofunctional enzymes that elongate FAS I products into long chain mycolic acid precursors (142). Three enzymes, β-ketoacyl-acyl carrier protein synthase (Kas) A and B and the condensing enzyme FabH, have all been identified as FAS II enzymes involved in mycolic acid and fatty acid biosynthesis (142, 143). Thiolactomycin inhibits fatty acid synthesis by inhibiting FabH, KasA and KasB. TLM inhibited KasA and KasB displaying IC_{50} values of 20μM and 90μM respectively (142).

The use of *M. smegmatis* cell extracts confirmed that TLM specially inhibited the mycobacterial acyl–carrier-protein-dependent type II fatty acid synthase (FAS-II), but it did not inhibit the multifunctional type I fatty acid synthase (FAS-I). Analysis of the *in vivo* and *in vitro* data has suggested two separate sites of action for TLM, the β-ketoacyl carrier protein synthase in FAS-II and the elongation step involved in the synthesis of α-mycolates and oxygenated mycolates. In a recent study Kremer and coworkers have shown that the enzymes targeted by TLM are KasA and KasB, which are involved in fatty acid and mycolic acid biosynthesis in *M. tuberculosis* (114).

$$C_8 - C_{16}$$

$$\downarrow$$

$$CH_3(CH2)_{17} - CH_2 - CH_2 - (CH_2)_3 - CO - S - (ACP)$$

$$\downarrow \quad \Delta \text{ 5-desaturase}$$

$$H_3C(H_2C)_{17} \diagup\diagdown (CH_2)_3 - CO - S - (ACP)$$

tlmA   *tlmB*

┌─────────────────┐   ┌──────────────┐
│ OXYGENENATED   │   │ α - MYCOLATES │
│   MYCOLATES   │   └──────────────┘
└─────────────────┘

tlmC

┌──────────────┐
│ α'– MYCOLATES │
└──────────────┘

TLM and mycolic acid biogenesis. The sites of action of TLM are represented by *tlmA* and *tlmB*. The site of action, *tlmC* is involved in the synthesis of mediumchain length by the FAS-II.

α' - MYCOLATES $=$ Monosaturated mycolates of *M. smegmatis*

OXYGENENATED MYCOLATES $=$ Epoxy, keto, methoxy and wax ester mycolates typical of *M. smegmatis, M. tuberculosis and M. avium*

Figure 13.

The *in vitro* activity of thiolactomycin is substantial against a wide range of strains of *M. tuberculosis*, including those that are resistant to isoniazid, however this is at somewhat higher concentration. However, the above activity is interesting whereas it is insufficient to warrant further progression of thiolactomycin itself as an anti-TB agent (114, 143, 144). Therefore, new analogues of thiolactomycin needs to be designed and synthesized that could exhibit potential activity against *M. tuberculosis* cultures.

Structure Activity Relationship

Modifications at C-5

Minnikin and coworkers have reported various analogues of thiolactomycin at the C-5 position and evaluated their activity against whole cells of *Mycobacterium tuberculosis* H37Rv as well as mycolic acid biosynthesis in cell extracts of *Mycobacterium smegmatis* (145). The analogue with a 5-tetrahydrogeranyl substiuent showed the highest biological activity with an MIC$_{90}$ of 29 μM for *M. tuberculosis* and 92% mycolate inhibition in extracts of *M. smegmatis*, as compared to 125 μM

and 54% respectively, for TLM. Furthermore, the *trans*-geranyl analogue (**47**) was inactive against *M. tuberculosis* H37Rv, but the sequential saturation of one or two double bonds (**49 or 50**) resulted in acceptable activities (Figure 14). Increasing the length of the side chain to *trans-trans*-farnesyl (**48**) also gives enhanced activity.

Besra and coworkers have synthesized various thiolactomycin based analogues with biphenyl and acetylene side-chains at the C-5 position of the thiolactone ring (146, 147). The compounds **54** and **55**, have been derivatized with biphenyl ring, and compounds **56-58** with acetylene side chains. These compounds exhibit moderate *in vitro* inhibitory activity against the recombinant *Mycobacterium tuberculosis* β-ketoacyl-ACP synthase *mt*FabH condensing enzymes (Figure 15).

Recently, there was another report on C5 analogues of thiolactomycin (13 biphenyl analogues and two biphenyl mimics) by the same authors. These have been assessed for their *mt*FabH and whole cell *Mycobacterium bovis* BCG activity, respectively (148). Amongst them three analogues (**59-61**) exhibited a significant enhancement in the *in vitro* activity against *mt*FabH assay. The

analogue **61** (5-(4-methoxycarbonyl–biphenyl-4-ylmethyl)-4-hydroxy-3, 5-dimethyl-5*H*-thiophen-2-one) showed an IC_{50} value of 3 μM compared to 75 μM for the parent drug thiolactomycin against *mt*FabH. Finally, they reaffirm the requirement for a linear π-rich system containing hydrogen bond accepting substituents attached to the *para*-positions of the C5 biphenyl analogue to generate compounds with enhanced activity (Figure 16).

Dowd and co-workers have reported C-5 analogues of TLM and suggesting that an intact (5*R*)-isoprene unit is necessary for the activity against condensing enzymes FabH, KasA and KasB from *Mycobacetrium tuberculosis* (149). The modifications at C-5 position, like increasing the alkyl chain length **62a-f** and reduction of one or both double bond of isoprene unit **63-65,** resulted in markedly reduced activity (Figure 17). There is no significant difference in activity for the analogues **66, 67** obtained by addition and removal of methyl group. However, Besra and co-workers have synthesized C5-biphenyl TLM library with increased activity against mtFabH condensing enzyme, thus supporting C5-derivatization of TLM scaffold (148).
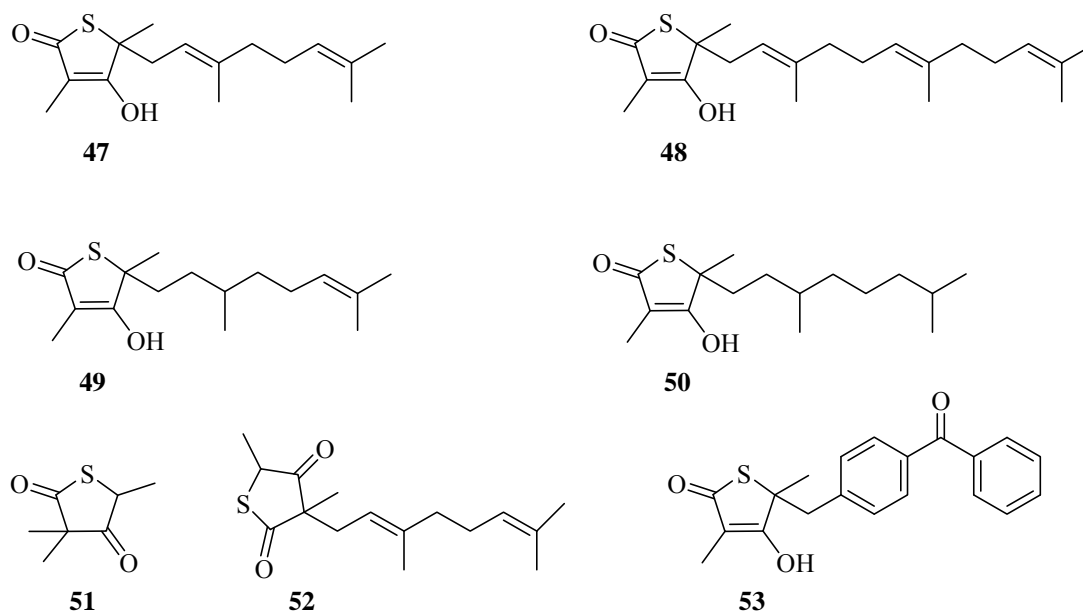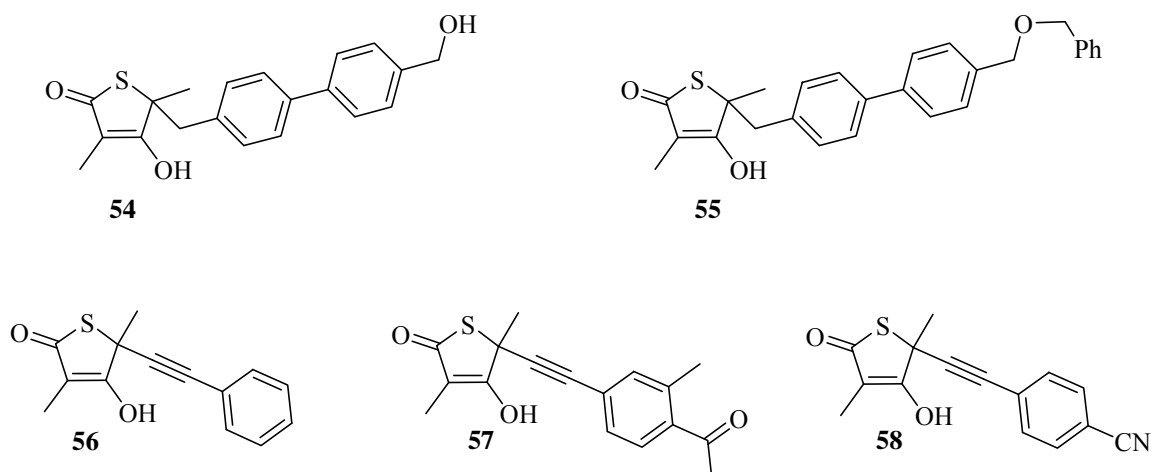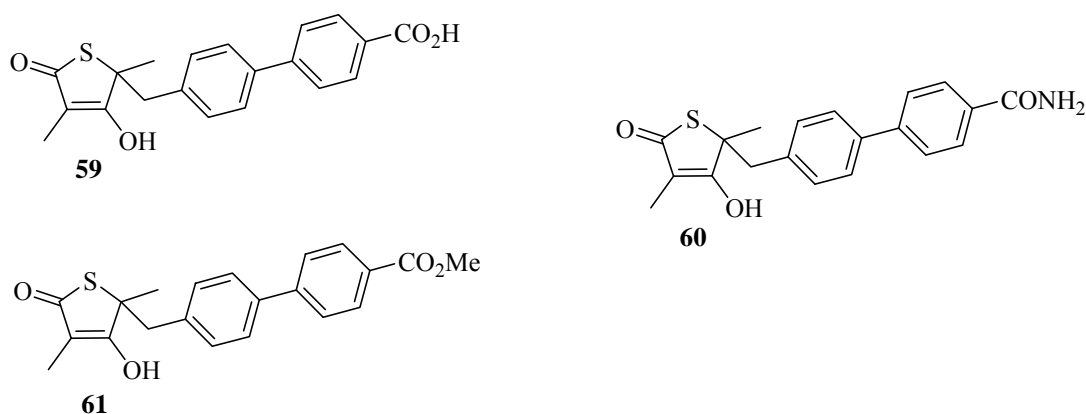

Figure 14.
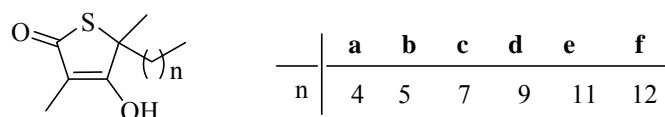
Figure 15.

Figure 16.

Townsend and co-workers have developed new structural analogues of TLM (**68-70)** at C-5 position as shown in Figure 18 and they have exhibited weak to moderate activity against Type-I fatty acid synthase (150).
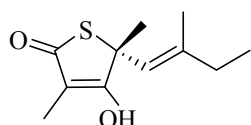
Modifications at C-4

Design and synthesis of a series of C-4 analogues **(71-74)** of thiolactomycin has been carried out in this laboratory and the molecules have been evaluated against four different *Mycobacterium* species namely *M. tuberculosis* H37Rv ATCC 27294, *M. tuberculosis* clinical isolates (sensitive and resistant), *M. avium* ATCC 49601 and *M. intracellulare* ATCC 13950 (151). The compound **72e,** having spacer of eight methylene groups and linked to methyl thioglycolate proved to be is the most active compound with an MIC value 1.0-4.0 μg/mL against drug sensitive and resistant strain of *M. tuberculosis* (Figure19).
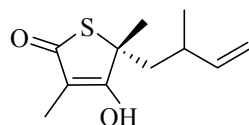
Based on these findings several new molecules have been designed and prepared in order to understand the SAR. A number of these molecules have good potential for further development and are presently undergoing detailed investigations.
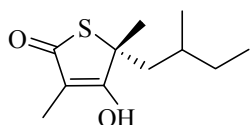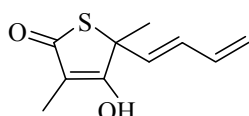
62a-f

n	a	b	c	d	e	f
n	4	5	7	9	11	12

63

64

65

66
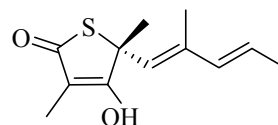
67

Figure 17.
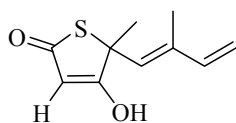
68
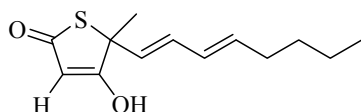
69
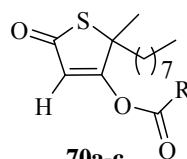
70a-c

a R = -OCH$_3$

b R = -OCH$_2$CH$_3$

c R = -OCH$_2$CH=CH$_2$

Figure 18.
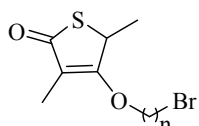
71a-f
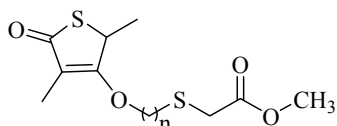
72a-f
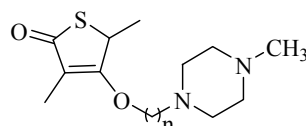
73a-f
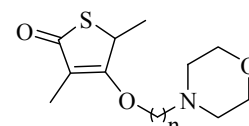
74a-f

	a	b	c	d	e	f
n	3	4	5	6	8	10

Figure 19.

Modifications at C-3 and C-5

Gilbert and coworkers have synthesized a series of analogues of naturally occurring thiolactomycin and evaluated their ability to inhibit the growth of malaria parasite *Plasmodium falciparum* (152, 153). Compounds **75**-**78** with substitutions at the C-3 and C-5 positions with long chain alkane residues show improved activity against *Plasmodium falciparum*, blood streams of *Trypanosoma brucei* and *Trypanosoma cruzi* amastigotes cultured intracelluarly (Figure 20). This shows that the analogues of thiolactomycin not only have potential in the development of new anti-TB compounds but may be useful as new antimalarial agents.

Sakaya and coworkers synthesized 3-acetyl analogues of thiolactomycin and profiled their activity against live stock pathogens (154). Compounds **79-81** have shown improved activity over thiolactomycin, against *Stapylococcus aureus* and moderate to comparable activity against *Pasteurella multocida* (Figure 21).

CONCLUSION

Inspite of the availability of the BCG vaccine and some chemotherapeutic agents, TB remains a leading infectious killer worldwide. This is mainly due to the lack of new drugs in the market, particularly for effective treatment against the spread of multi drug-resistant (MDR) and extensively drug-resistant (XDR) strains. Therefore, there is an urgent need for the development of new anti-TB drugs with lesser side-effects, improved pharmacokinetic properties to be effective against both the Gram positive and Gram negative bacteria including the resistant strains. More importantly, the newly developed drugs are required to reduce the overall duration of treatment. It is also important to note that while we pursue the development of new drugs based on inhibition of bacterial targets, we need to understand host factors such as immune mechanisms, genetic susceptibility and disease relapse. Therefore, the newer anti-TB compounds need to be developed on the understanding of the molecular mechanisms of drug action and drug resistance.

In recent years, efforts are being made to develop new molecules based on different scaffolds that act on a number of drug targets. Further, the molecular mechanisms and biosynthetic pathways are being unraveled for the already known and newly discovered lead molecules. One of the well established broad spectrum antibacterial drug targets that have been historically very effective is the inhibition of cell wall biosynthesis. Some of the anti-TB agents like isoniazid and ethambutol target different aspects of the cell wall biosynthesis.
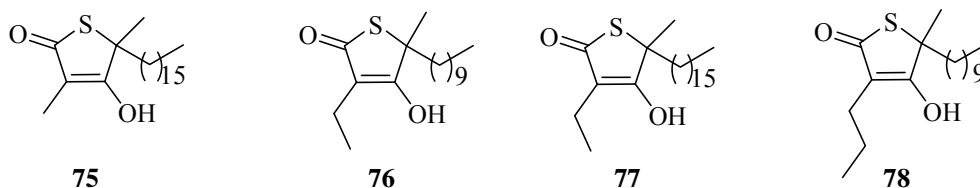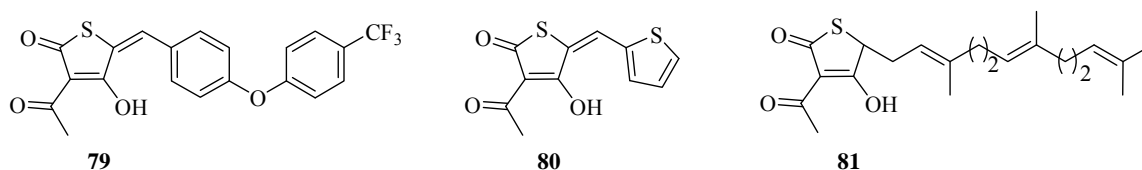

Figure 20.

Figure 21.

Hence, new molecules that are active against cell wall targets could provide valuable therapeutic options for the therapeutic application including drug resistance. Thiolactomycin, which is a natural product, inhibits bacterial as well as plant type II fatty acid synthases (FAS-II), which provide essential building blocks for the bacterial cell wall. Thiolactomycin is believed to exert its overall effect by inhibition of the β-keto acyl-ACP-synthases (Kas), key condensing enzymes involved in the chain elongation in FAS-II. Therefore, developing structurally modified compounds based on thiolactomycin structure as well as the thiolactone ring could provide novel anti-TB drugs. Based on its potential as an attractive lead molecule, efforts are being made by many research groups including our laboratory to structurally modify this natural product for the development of new anti-TB agents.

ACKNOWLEDGMENT

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