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Monografia orientada pelo Professor Doutor Luís Filipe Constantino, Categoria Professor Auxiliar e coorientada pelo Professor Doutor João Pedro Pais, Categoria Investigador

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Resumo

A tuberculose (TB) é a segunda maior causa de morte por infeção no mundo, com cerca de 1/3 da população infetada pelo agente causador, *Mycobacterium tuberculosis* (Mtb). A forma mais prevalente de TB é a tuberculose pulmonar, que é extremamente contagiosa e pode ser letal se não for tratada.

O surgimento de estirpes multirresistentes (MDR) e extensivamente resistentes (XDR) é uma das barreiras mais significativas para a erradicação da doença, contribuindo significativamente para a desistência pelos pacientes devido à longa duração do tratamento e efeitos colaterais de difícil manejo. Portanto, fármacos anti-tuberculares com modos de ação inovadores de alta potência e mínima toxicidade são urgentemente necessários.

O envelope da célula Mtb, que é composto por três camadas: peptidoglicano, arabinogalactano e ácidos micólicos, é essencial para a proliferação celular, virulência e resistência a antibióticos.

A arquitetura única da membrana celular micobacteriana é significativa na patogénese do Mtb. O patógenio depende de sua parede celular para o crescimento e sobrevivência. É rico em lipídios e extremamente impermeável, permitindo que a micobactéria se multiplique dentro dos macrófagos e permaneça por longos períodos de tempo no hospedeiro infetado.

A proteína de membrana micobacteriana grande 3 (MmpL3) é um transportador de membrana interna de Mtb que exporta monomicolatos de trealose (TMMs), um precursor do componente da membrana externa, dimicolato de trealose (TDM), bem como dos ácidos micólicos ligados ao arabinogalactano, que juntos constituem a micomembrana.

Quando o transportador MmpL3 é inibido, o TMM acumula-se intracelularmente e os níveis do complexo peptidoglicano micolilarabinogalactano (mAGP) e TDM diminuem, resultando em enfraquecimento e morte celular. Portanto, a MmpL3 é um alvo atraente e promíscuo para fármacos anti-tuberculares e desempenha um papel importante na biossíntese do envelope celular.

Usando abordagens genómicas e proteómicas, novos alvos de fármacos anti-tuberculosos foram identificados. Entre os fármacos que inibem MmpL3 estão derivados de etilenodiamina, derivados de amida (por exemplo, indol-2 carboxamidas, benzotiazol amidas, THPPs), pirróis, pirazoles, adamantil ureias, benzimidazóis e espirociclos.

Atualmente, não há fármacos aprovados pela FDA para terapia de tuberculose que tenha como alvo a proteína MmpL3.

Palavras-chave: Mycobacterium tuberculosis; transportador MmpL3; inibidores de pequenas moléculas; biogénese da parede celular; tuberculose resistente

Abstract

Tuberculosis (TB) is the second biggest cause of death from infection worldwide, with an estimated 1/3 of the population infected with the causative agent, *Mycobacterium tuberculosis* (Mtb). The most prevalent form of TB is pulmonary tuberculosis, which is extremely contagious and may be lethal if left untreated.

The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains is one of the most significant barriers to the disease's eradication, contributing significantly to patient dropout due to the lengthy duration of treatment and difficult-to-manage side effects. Therefore, antituberculosis drugs with innovative modes of action, high potency, chemical stability, and minimal toxicity are urgently needed.

The Mtb cell envelope, which is made up of three layers: peptidoglycan, arabinogalactan, and mycolic acids, is essential for cell proliferation, virulence, and antibiotic resistance.

The unique architecture of the mycobacterial cell membrane is significant in the pathogenesis of Mtb. The pathogen relies on its cell wall for growth and survival. It is lipid-rich and extremely impermeable, allowing the virus to multiply within macrophages and remain for long periods of time in the infected host.

Mycobacterial membrane protein large 3 (MmpL3) is Mtb inner membrane transporter that exports trehalose monomycolates (TMMs), a precursor of the mycobacterial outer membrane component trehalose dimycolate (TDM), as well as mycolic acids linked to arabinogalactan, which together constitute the mycomembrane.

When the MmpL3 transporter is inhibited, TMM accumulates intracellularly and mycolyl arabinogalactan peptidoglycan (mAGP) and TDM levels decrease, resulting in cell weakening and death. Hence MmpL3 is an attractive and promiscuous target for TB drugs that plays an important role in cell envelope biosynthesis.

Using genomic and proteomic approaches, new antituberculosis drug targets have been identified. Among the scaffolds that inhibit MmpL3 are ethylenediamine derivatives, carboxamide derivatives (e.g., indole-2 carboxamides, benzothiazole amides, THPPs), pyrroles, pyrazoles, adamantyl ureas, benzimidazoles, and spirocycles.

There is currently no FDA-approved drug for tuberculosis therapy that targets the MmpL3 protein.

Keywords: *mycobacterium tuberculosis*; MmpL3 transporter; small molecule inhibitors; cell-wall biogenesis, drug-resistant tuberculosis

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Acronyms

TB: Tuberculosis

Mtb: *Mycobacterium tuberculosis*

MmpL3: Mycobacterial membrane protein Large 3

INH: Isoniazid

RIF: Rifampicin

PZA: Pyrazinamide

EMB: Ethambutol

TDM: Trehalose dimycolate

TMM: Trehalose monomycolates

mAGP: Mycolyl arabinogalactan peptidoglycan

MAs: Mycolic acids

OL: Outer layer

IM: Inner Membrane

MOM: Mycobacterial outer membrane
AGP: Arabinogalactan/peptidoglycan

AG: Arabinogalactan
PG: Peptidoglycan

MAPc: Mycolic acid - Arabinogalactan-Peptidoglycan complex

FASs: Fatty acid synthases

ACP: Acyl carrier protein

DR: Drug-resistant

MDR: Multidrug resistance

XDR: Extensively drug resistance

RND: Rresistant modulation and cell division

PMF: Proton motive force

SAR: Structure activity relationship

NTM: Nontuberculous Mycobacteria

MWE: Mycolate ester wax

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1 Introduction

1.1 Tuberculosis - an overview

Tuberculosis (TB) is a severe and recurrent global health concern caused by infection with bacillus *Mycobacterium tuberculosis* (Mtb). The World Health Organization (WHO) anticipated 9.9 million infections and 1.3 million deaths among HIV-negative and additional 214 000 deaths among HIV-positive people in the 2021 Global TB report, making tuberculosis one of the world's leading infection (1).

TB was the largest cause of mortality from a single infectious agent until the coronavirus (COVID-19) pandemic, ranking above HIV/AIDS. The COVID-19 pandemic resulted in a significant reduction in the number of people newly diagnosed with tuberculosis across the world. This fell from 7.1 million in 2019 to 5.8 million in 2020, an 18% decline back to the level of 2012 and far short of the approximately 10 million people who developed TB in 2020 (1).

Through innovative research and education, the WHO hopes to minimize the disease's prevalence, morbidity, and mortality by improving diagnostic and treatment methods, as well as creating prevention initiatives. By 2035, the objective is to reduce TB death by 95% and the overall incidence of TB by 90% over the world (2).

TB usually affects the lungs, but it can also affect other parts of the body, such as the brain, the kidneys, or the spine. Humans are Mtb's primary host, and infection is conveyed by droplets that, spread the germs through the air when cough, sneeze, or spit, carrying live tubercular bacilli from an infected person to an uninfected one. It then travels to the lungs and terminal alveoli, where resident alveolar macrophages or tissue dendritic cells ingest the airway bacteria. Infected macrophages or dendritic cells then travel to draining lymph nodes, stimulate adaptive immunity, and then return to the site of infection, where a granuloma forms (3).

The patient's immunity, latent infection, and age all have a role in the development of active illness.

Currently, standardized regimens comprises an initial 2-months intensive therapy with a cocktail of the first-line drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), followed by a 4-months continuation phase with INH and RIF (4,5).

Despite their effectiveness in treating drug-resistant TB, these lengthy treatments, high pill count and side effects, frequently result in patient non-adherence, allowing Mtb. strains to become increasingly resistant to the few available anti-TB drugs (6).

With the advent of multidrug- and extensively drug-resistant Mtb. strains in recent decades, rising resistance to several drugs is becoming a significant problem for the treatment of TB. As a result, new goals for the design and development of new anti-TB drugs that are active against drug-resistant strains are urgently needed (7).

1.1.1 *Mycobacterium tuberculosis*: morphology and physiology

Mycobacteria's cell envelope is a waxy, hydrophobic complex coat with a high concentration of lipids (40%) that provides an efficient barrier to antibiotics and is required for cell development, drug resistance, and pathogenicity (8).

The two prominent features of the cell envelope are the presence of arabinogalactan-mycolate that is covalently linked to the cell wall peptidoglycan via a phosphodiester bond, located on the inner leaflet of the outer membrane, and a free glycolipid called trehalose dimycolate (TDM). TDM accumulates on the surface of the cell forming a thick layer of lipid on the cell's outer surface and protecting the tubercle bacillus from unpleasant chemicals and the immune system of the host. The main components of this protective layer are mycolic acids and they play other important roles as structural components of the cell wall and envelope (9,10).

This one-of-a-kind structure has distinct areas that form the bacterial cytoplasm's boundary with the extracellular environment (Figure 1). The following are the: (1) “outer layer” (OL, or capsule), (2) mycobacterial outer membrane (MOM, or mycomembrane), (3) arabinogalactan/peptidoglycan cell wall (AGP) core and (4) plasma membrane (11).

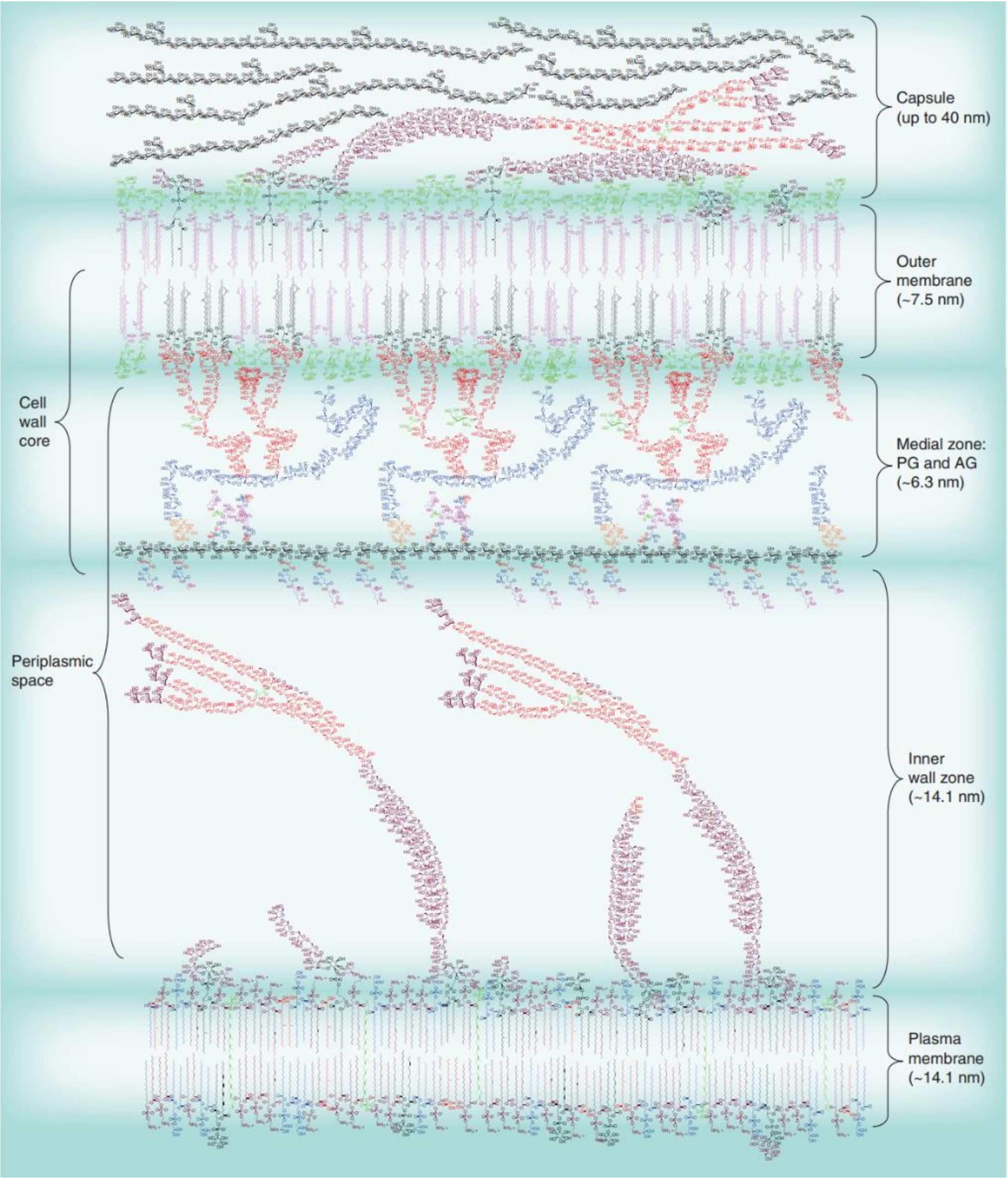


Figure 1: Representation of the mycobacteria cell envelope. The cell wall core (mycolyl AG-PG or mAGP) is made up of the PG (black with brightly colored peptides extending from it) which is connected to the mycolate layer (inner leaflet of the outer membrane) by the AG. The pink mycolic acids are attached to trehalose (TMM with a single mycolic acid and TDM with two mycolic acids) and are predicted to be present in both leaflets of the outer membrane. Source: (12)

Glycolipids extend into the periplasmic region from the inner membrane phospholipid bilayer. Three key components build up the important core cell wall structure: a cross-linked peptidoglycan polymer, a highly branched arabinogalactan polysaccharide, and long-chain mycolic acids. Solvent-extractable lipids, such as non-covalently coupled glycophospholipids and inert waxes, are intercalated into the mycolate layer to create the outer membrane. The capsule, which is mostly made up of proteins and polysaccharides, is the “outer layer” (13).

Peptidoglycan (PG) is a major component of the cell and its a polymer made up of alternating N-acetylglucosamine and N-acetylmuramic acid residues connected together by β(1 → 4) links and transpeptide bridges (14).

The role of peptidoglycan is not just to give structure and stiffness, but also to counteract turgor pressure, making it necessary for development and survival (15). Peptidoglycan is unique to bacterial cells, and it is because of this that several enzymes involved in its synthesis have been targeted by powerful antibiotics, with others representing attractive targets in the development of future antibiotics (13).

Arabinogalactan (AG) is a tree-like structure with a galactose trunk and arabinose branches, which are covalently connected to the PG through a rhamnose-GlcNAc linker unit at the trunk's base (8).

Around 30 β-D-galactofuranose residues (Galf) with alternating β(1 → 5) and β(1 → 6) connections make up the main trunk structure. Three arabinofuranose (Araf) branches are joined to the 6-linked Galf residues at the 8th, 10th, and 12th positions of the trunk, respectively (8).

The lipid- and carbohydrate-rich layers of the cell wall provide a strong physical barrier that contributes to Mtb's inherent resistance to host antimicrobial defenses and restricts anti-tuberculosis drugs entry to the bacterial cytoplasm. Investigation of the basic mechanisms that lead to the formation of the cell membrane has the potential to significantly enhance TB therapy since the cell envelope is a critical aspect of the physiology and virulence of Mtb (11).

1.1.2 Mycolic Acid Biosynthesis

Mycolic acids are long-chain fatty acids that are key factors of the cell membrane of Mtb since they are the major lipid component of the envelope and form the external mycomembrane.

Mycolic acids are either inserted into the mycomembrane as TDM and TMM, or covalently connected to the underlying arabinogalactan, which is then coupled to peptidoglycan to create the Mycolic acid - Arabinogalactan-Peptidoglycan complex

(MAPc) (16,17). Therefore, they play a significant role in cell wall architecture and impermeability, resulting in mycobacteria's inherent resistance to most antibiotics, and they are essential components in mycobacterial virulence (18).

The critical relevance of cell envelope integrity for Mtb viability has sparked research into the enzymatic mechanism for mycolic acid biosynthesis (10). Biosynthesis of mycolic acid is critical for identifying novel treatment targets for TB as well as deciphering the mechanism of action of various current antitubercular drugs (19,20).

Biosynthesis of mycolic acid precursors requires two types of fatty acid synthases (FASs), the eukaryotic-like multifunctional enzyme FAS I and the acyl carrier protein (ACP)–dependent FAS II systems, which consists of a series of discrete mono-functional proteins, each catalyzing one reaction in the pathway (18).

Unlike other bacteria's FAS II synthases, mycobacterial FAS II cannot synthesize fatty acids from acetyl-coenzyme A. Instead, it elongates medium-chain-length fatty acids already created by FAS I, resulting in meromycolic acids (18).

1.2 MDR-TB and XDR-TB- definition

Drug-resistant tuberculosis (DR-TB) is a key contributor to antimicrobial resistance globally and remains a public health problem. Approximately half a million individuals worldwide get drug-resistant tuberculosis each year (1).

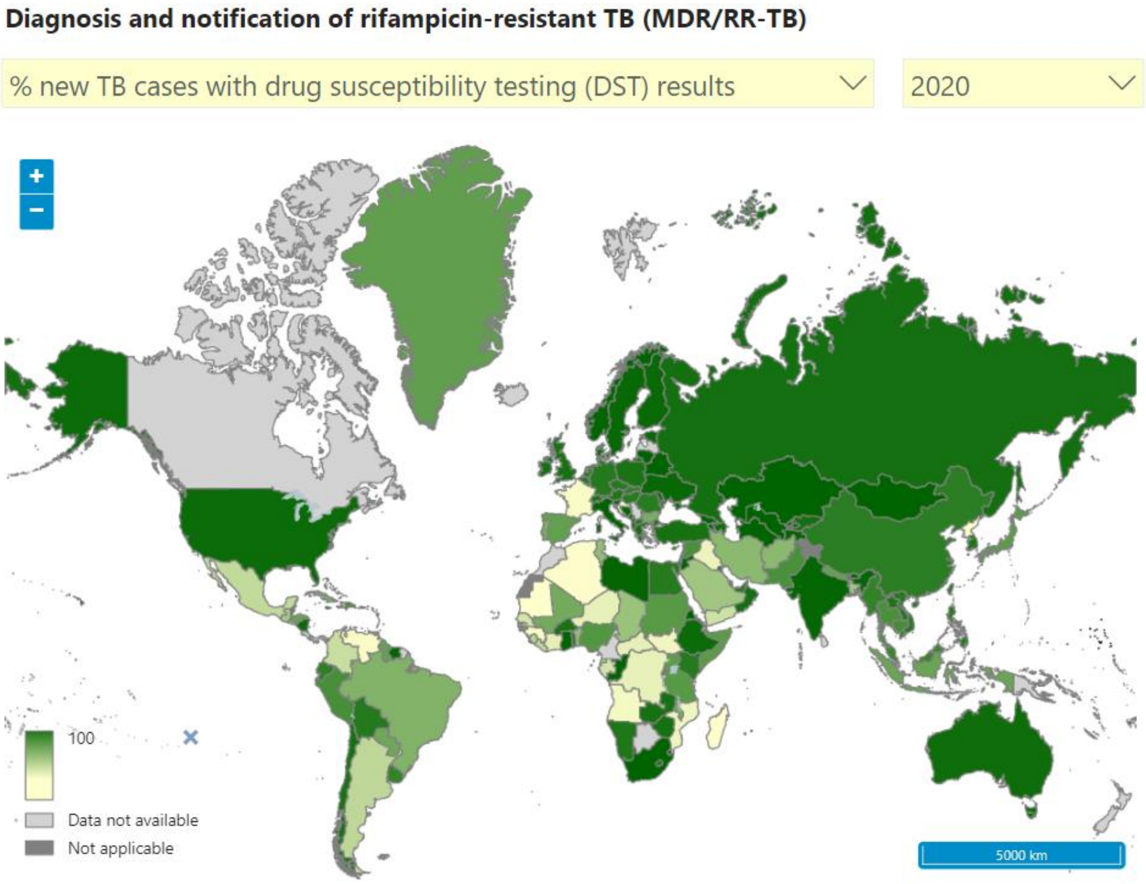
DR-TB develops when anti-TB drugs are used incorrectly, as a result of erroneous prescriptions by health care professionals, low-quality pharmaceuticals, and patients who discontinue therapy too soon (21).

There are two main types of resistance. First one is multidrug resistance (MDR), it is the strain of Mtb that are resistant to the two most effective first-line anti-TB drugs, isoniazid, and rifampicin. MDR-TB can be treated and cured using second-line drugs, however, it can take up to 2 years of treatment with second-line anti TB drugs for MDR tuberculosis to be treated (22).

MDR-TB is becoming increasingly difficult to treat in several countries. Treatment choices are limited and costly, suggested medications are not always accessible, and patients suffer from a variety of side effects. In 2020, just around one-third of patients with drug-resistant tuberculosis received treatment (1).

In some cases, even more severe drug-resistant TB may develop, the extensively drug resistance (XDR). XDR-TB is a rare type of MDR TB that is resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). Because there are fewer alternatives available, XDR treatment is more expensive, and the success rate is lower than MDR-TB treatment (22). Inappropriate management of DR-TB can have fatal consequences, hence should be treated by or in close consultation with a TB expert.

Rifampicin resistant TB (RR-TB) is detected using genotypic or phenotypic methods with or without resistance to other first-line anti-TB drugs and requires treatment with second line drugs. MDR-TB, which is resistant to both rifampicin and isoniazid, is included in the statistics for RR-TB (23).



Diagnosis and notification of rifampicin-resistant TB (MDR/RR-TB)

% new TB cases with drug susceptibility testing (DST) results

2020

Figure 2: Map that represents % of new TB cases with drug susceptibility testing (DST) results. The map was generated with data from 2020. Source: (23)

1.3 New therapeutical targets

Tuberculosis treatment regimens are lengthy and difficult, and the medications have a life-threatening side effect, leading to patient non-compliance and the development of drug-resistant strains. Hereupon, novel approaches and new treatment strategies are required to fight global tuberculosis spread. Protein synthesis, cell wall biosynthesis, and energy metabolism can all be targets for antitubercular drugs (22).

To tackle this problem, investigators in the field have employed both target- and whole cell-based screens to identify novel anti-TB compounds. Multiple chemical scaffolds thought to kill the bacterium through inactivation of the mycolic acid transporter, mycobacterial membrane protein Large 3 (MmpL3), have been identified through whole-cell-based screens for novel small molecule inhibitors active against Mtb in culture, followed by whole-genome sequencing of spontaneous resistant mutants (24).

SQ109 was identified as a promising lead compound based on mycobactericidal activity, cytotoxicity, pharmacokinetic properties, and improved efficacy against Mtb in vivo using combinatorial chemistry designed around the active 1,2-ethylenediamine pharmacophore of EMB (25).

Surprisingly, several pharmacophores that appeared to share the same mode of action as SQ109 have been discovered in recent years. These inhibitors include: 1,5-diarylpyrrole derivative BM212 and analogs; then benzimidazole C215; tetrahydropyrazolo[1,5-a]pyrimidine-3-carboxamides (THPPs); N-benzyl-6',7'-dihydrospiro[piperidine-4,4'-thieno[3,2-c]pyrans; indolcarboxamides; and adamantyl ureas (AUs) (25).

All these inhibitors are reported to destroy the proton motive force, thus affecting the activity of MmpL3 protein, which is a proton driven antiporter efflux pump (25).

1.3.1 MmpL Proteins in Physiology and Pathogenesis of M. tuberculosis

Mycobacterium tuberculosis (Mtb) has a unique cell envelope that enhances survival and persistence in the face of host immunological responses.

The role of MmpL in the formation of the mycobacterial cell envelope is becoming recognized. MmpL's are in charge of transferring virulent envelope lipids and siderophores through the plasma membrane into the periplasm (26). Therefore, those proteins indirectly contribute to virulence since they transport substrates that have a direct impact on bacterial survival in the human host (11).

Many resistant modulation and cell division (RND) family proteins play a role in antibiotic resistance and stress response. (27) MmpL's belong to the category of RND superfamily that rely on the proton motive force (PMF) to export substrates across cell membranes. The PMF drives the activity, and the MmpL protein acts as an efflux pump, extruding numerous drugs and causing drug resistance (28).

MmpL protein-coding genes were initially identified in the M. tuberculosis H37Rv stain and this strain's genome encodes 13 putative MmpL proteins (MmpL1-13). (16,29). The majority of MmpL proteins are expected to have 12 TMD and 2 extracytoplasmic loops and vary in size from 100 kDa (MmpL3) to 122 kDa (MmpL12). MmpL6, which is shortened to 42 kDa (five TMD), and MmpL13, which consists of two contiguous open reading frames, mmpL13a and mmpL13b, are projected to encode 32 kDa (four TMD) and 50 kDa (seven TMD) proteins, respectively, are exceptions (29).

The majority of MmpL substrates found are virulence-associated envelope lipids (Figure 2). MmpL-transported lipids are integrated into both leaflets of the mycomembrane, which provides Mtb with a lot of its intrinsic resistance and immunomodulatory abilities. Other MmpL substrates are required for environmental and nutritional stress responses (11).

MmpL proteins have been implicated in mediating substrate transport across the mycobacterial membrane. MmpL3 has been implicated in both heme uptake, along with MmpL1, and TMM export (30). MmpL11 were also found to coordinate the transport of long-chain triacylglycerols (LC-TAG) and mycolate wax esters (MWE) in Mtb (31).

Mtb produces two siderophores: lipophilic mycobactin (MBT) and hydrophilic carboxymycobactin (cMBT). MmpL4 and MmpL5 share redundant functions in siderophore export and are essential for siderophore-mediated iron acquisition (32). MmpL7 mediates the transport of phthiocerol dimycocerosate

(PDIM). PDIM has numerous functions in Mtb virulence. The extremely hydrophobic structure of PDIM restricts Mtb cell membrane permeability and contributes to innate resistance to antimicrobial agents (33) whereas MmpL8 transfers the sulfolipid to the cell envelope. Sulfolipids promote Mtb virulence by blocking the activation of the host pattern recognition receptor (PRR) toll-like receptor 2, decreasing the innate immune response to Mtb. (34,35). More recently, MmpL10 was reported to participate in the translocation of 2,3-diacyltrehaloses (DAT) (36). Additionally, MmpL5 and MmpL7 have been implicated in drug efflux (37) including the recently approved drug bedaquiline (38).

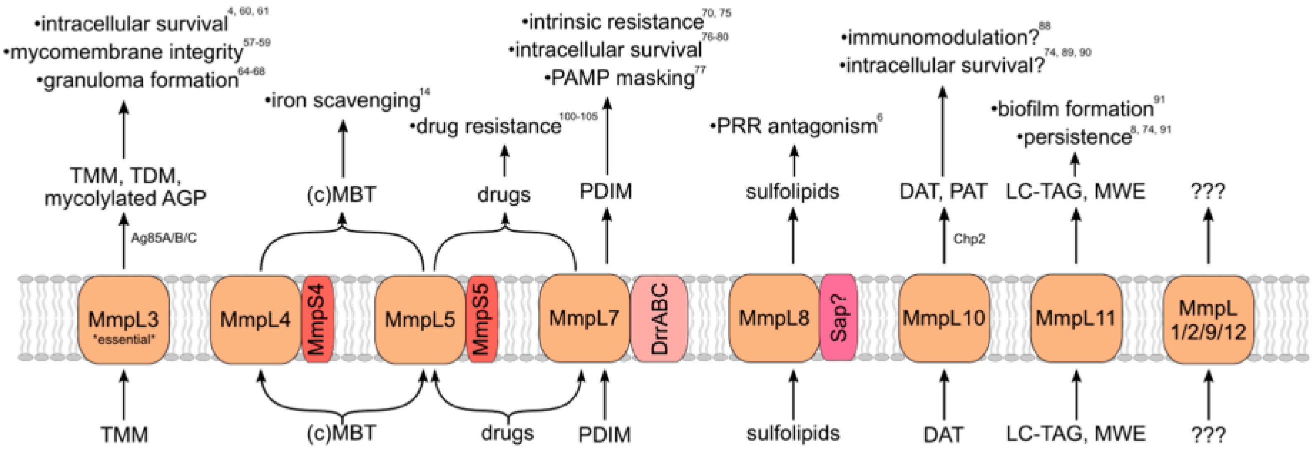


Figure 3: MmpL transporters export substrates that are important for virulence and pathogenicity. Source: (11).

Mutational analyses revealed MmpL3 is essential for Mtb viability. Understanding how MmpL substrates affect Mtb virulence is critical to grasping the MmpL protein family's importance in Mtb's pathogenic lifestyle (11).

1.3.2 The overall structure of MmpL3

The monomeric crystal structure of MmpL3 from *Mycobacterium smegmatis* was recently described and is presented in Fig. 4 (28).

The MmpL3 molecule has an approximately 2-fold pseudosymmetrical structure with 12 transmembrane α-helices (TMs 1–12) and two large periplasmic loops (loops 1 and 2), which create the periplasmic subdomains, PN (periplasmic N-terminal pore subdomain) and PC (periplasmic C-terminal pore subdomain) Fig. 4A and B (39–41).

The interaction of PN and PC resulted in the creation of a periplasmic area with a cavity at its center, which could be important for TMM transportation (39). Two additional helices (TM 1a and TM 7a) are connected to the surface of the cytoplasmic membrane (Fig. 4C).

Structural and complementation analyses identify two pairs of hydrophilic residues, Asp256-Tyr646 and Asp257-Tyr645, (Fig. 3B) connected to TM 4 and TM 10 by hydrogen bonds at the core of the transmembrane helices. These amino acids are described as essential elements for the proton relay pathway, thus inducing the PMF in MmpLs, and are essential for mycobacteria growth in cultures (24,41).

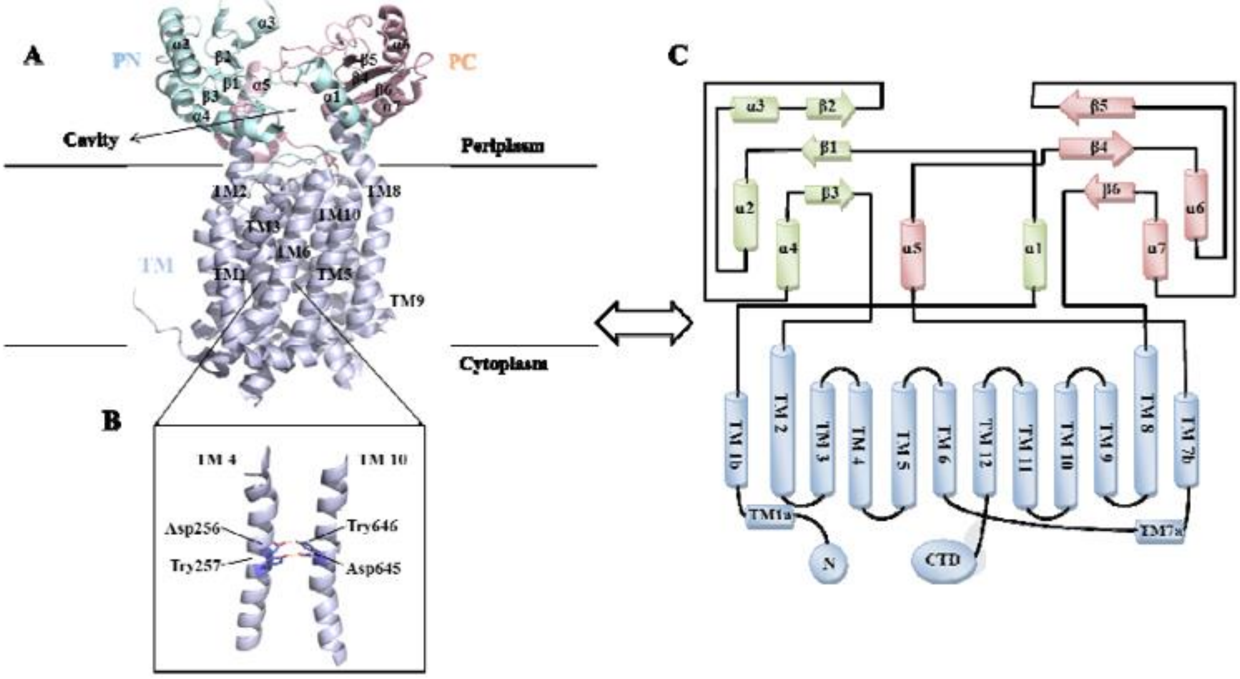


Figure 4: Overall architecture of the MmpL3. (A) Cartoon representation of the MmpL3 crystal structure. (B) Two pairs of hydrophilic residues (Asp256-Tyr646 and Asp257-Tyr645) between TM 4 and TM 10. (C) Topology diagram of MmpL3. Source: (41).

1.3.3 MmpL3 as a Promiscuous Target

Cell wall biosynthesis, iron absorption, energy generation, membrane potential, and antibiotic sensitivity are among characteristics in which MmpL3 plays a vital role (30,42,43).

MmpL3 (Rv0206c) transports TMM and is the only MmpL whose transporter activity has been established directly using a biochemical assay (44).

The outer membrane (OM) of Mtb is distinctively characterized by the presence of mycolic acids (MAs), C60–C90 long chain, α-alkyl β-hydroxy fatty acids packed together to form a bilayer with significantly decreased fluidity and permeability.

These MAs are synthesized in the bacterial cytoplasm and esterified to the disaccharide trehalose to form TMM (45). TMM is then translocated across the inner membrane (IM) by MmpL3 Fig. 4 (46–48).

Afterwards, the transport of TMM across the periplasm is presumably involved by a chaperone (44).

At the OM, the antigen 85 (Ag 85) complex (FbpA, FbpB and FbpC) transfers a mycolate chain from one TMM molecule to another to form TDM, or to the AG polysaccharides to form peptidoglycan–arabinogalactan complex of the mycobacterial cell wall, essential for Mtb growth and virulence (49–51).

The AG polysaccharides that bind the OM to the cell wall make the membrane even more stiff, rendering it impervious to a wide range of compounds, including many antibiotics (45).

MmpL3 inhibitors prevent TMM crossing the inner membrane (44). As a result, TMM accumulates in the cytoplasm, and the production of TDM and MAs transferring into peptidoglycan-arabinogalactan complex is inhibited (52–54) weakening the bacterial cell wall and lowering Mtb survival and virulence.

Other lipids, such as phosphatidylethanolamine (PE), are transported by MmpL3 to reinforce mycobacterial cell walls (44).

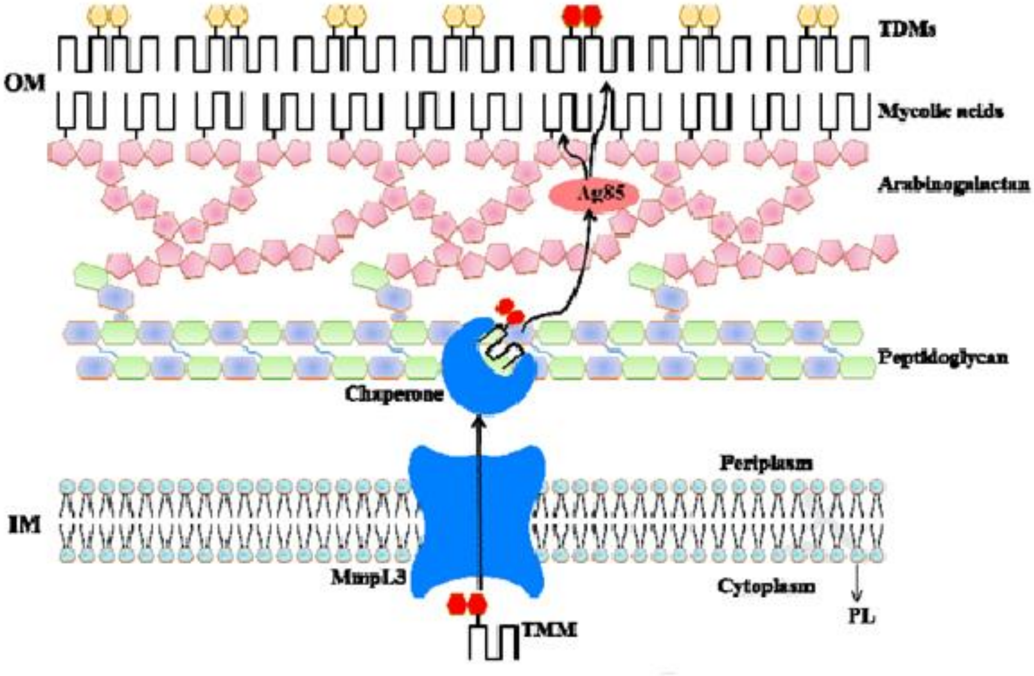


Figure 5: Steps involved in MA transport through the cell envelope. TMM transport across the IM is mediated by MmpL3. Then, with the help of a chaperone, TMM is carried through the periplasm. The Ag 85 complex at the OM transfers the MA chain from one TMM to another to produce TDM or to the peptidoglycan–arabinogalactan complex to produce wall-bound mycolates. Source: (41)

1.4 Small molecule inhibitors of MmpL3

The growing problem of MDR-TB has prompted intense research efforts from academia, nonprofit organizations, and the pharmaceutical industry, resulting in an increasing flow of novel anti-TB agents entering the drug development pipeline.

Significantly, MmpL3 has emerged as the effective anti-TB drug target in recent years. Several MmpL3 inhibitors with diverse chemical entities have been documented in several studies by several high throughput screening (HTS) programs, demonstrating MmpL3's viability as a target for anti-TB therapeutics (55).

As previously stated, the MmpL3 transporter is necessary to produce TDM and the mycolylation of the cell wall arabinogalactan, both of which are required for mycobacterial cell replication and survival.

When the MmpL3 transporter is inhibited, it results in an intracellular accumulation of TMM and a reduction in the levels of mAGP and TDM, leading to cell weakness and death (56).

Two pathways have been postulated for an inhibitor's action on MmpL3. The first is that the inhibitor prevents TMM translocation by binding to the transporter directly. The second mechanism postulated is that dissipation of the proton motive force (PMF) causes MmpL3 to be inhibited indirectly by disrupting the pH gradient and/or membrane potential which will disrupt the proton relay needed by MmpL3 to pump out TMM (56).

Several new classes of compounds including ethylenediamine derivatives, carboxamide derivatives such as indole-2-carboxamides, benzothiazole amides and tetrahydropyrazolo[1,5-*a*]pyrimidine-3-carboxamides (THPPs), pyrroles, pyrazoles, adamantyl ureas, benzimidazoles and spirocycles have been reported as MmpL3 inhibitors (41).

Even though there are numerous novel chemical entities known to target MmpL3 of Mtb, biological validation with potent in vivo activity, high oral bioavailability, and safety is often absent (28).

Because most inhibitors of MmpL3 bind to the protein's transmembrane domains, it's fascinating that they have such a wide range of chemical structures.

However, all of the compounds have one thing in common: they're all very lipophilic (clogP >4.2) and either neutral or basic (56). This feature, along with MmpL3's high sensitivity, may explain why phenotypic studies favor small hydrophobic MmpL3 inhibitors that limit enough of the transporter's function to cause growth arrest (28).

Currently, there are no FDA-approved medicine for TB treatment that targets the MmpL3 receptor. The compounds that inhibit MmpL3 do not all have the same pharmacophore and have diverse scaffold acting (28).

New compounds identified to be active against Mtb acting on MmpL3 are discussed below, as well as their physicochemical features, biological investigations, SARs, synthetic pathway, and other research (28).

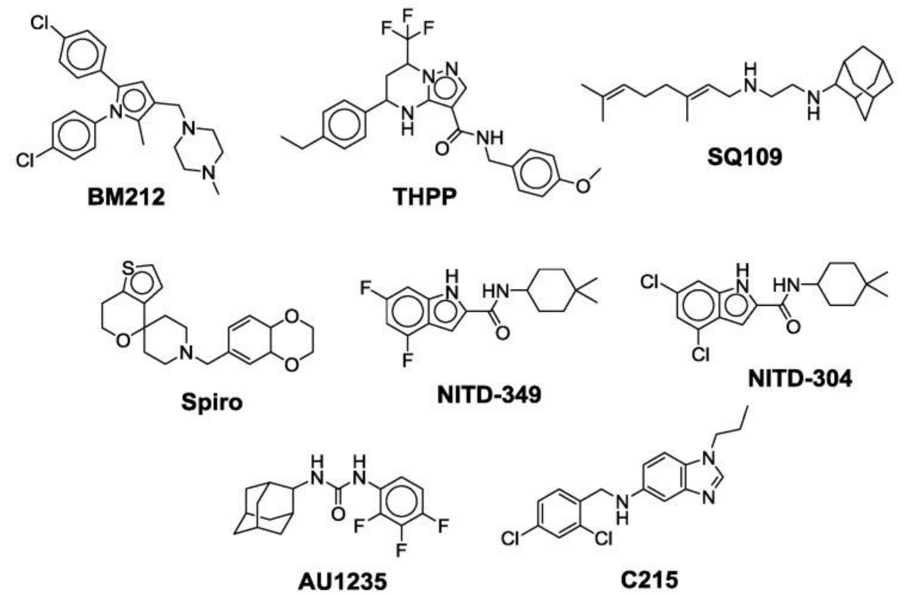


Figure 6: Structures of MmpL3 inhibitors. SQ109 (an ethylenediamine analog of ethambutol), BM212 (a 1,5-diarylpyrrole), THPP (a tetrahydropyrazolo pyrimidine), Spiro, NITD-349 and NITD-304 (an indolecarboxamide), AU1235 (an adamantyl urea), and C215 (a benzimidazole).

1.4.1 Ethylenediamine derivatives

One strategy to identify new anti-Mb agents has been to review well-known medications like EMB, RIF, or INH, with the goal of finding analogs with better physicochemical features, potency, and toxicity (25).

Based on a [1,2]-ethylenediamine pharmacophore of EMB, one of the four drugs indicated in the current therapy regimen for active pulmonary TB, a 67 238 compound library was generated via combinatorial chemistry (57).

With strong effectiveness against live M. tuberculosis, minimal cytotoxicity in grown mammalian cells, and drug-like pharmacokinetic and pharmacodynamic characteristics, SQ109 was chosen as the best in class of this library and the lead drug candidate (57,58). It presented a high antitubercular activity (MIC of 0.7-1.56 μM) against both replicating and non-replicating Mtb. (23,55) and also a low toxicity (25,58).

An analysis of SQ109 as a replacement for EMB in the standard four-drug treatment regimen shows it has the potential to decrease treatment time since it enhances RIF accumulation in Mtb (59) and interacts with RIF, INH, and bedaquiline in a synergistic manner (57,60).

In phase I and early phase II clinical studies, SQ109 was proven to be safe and well tolerated, and it is presently being tested in patients with pulmonary tuberculosis (61). SQ109 is broadly distributed and accumulates in the lungs, which may contribute to its higher effectiveness than EMB (62).

Despite being designed as an analog of EMB and hence an inhibitor of arabinogalactan production, SQ109 does not elicit the characteristic depletion of cell wall arabinose or the transcriptional profile seen with EMB therapy (63). SQ109 is a multitarget drug that targets MmpL3 as well as the MenA and MenG menaquinone production enzymes (54,64).

Additionally, it has been reported to function as an uncoupler, leading to the dissipation of the PMF (65) . Because of the loss of PMF, SQ109 also has significant efficacy against non-replicating hypoxic Mtb (66) and non-MmpL3-containing pathogens such *Helicobacter pylori*, *Candida albicans*, and *Plasmodium falciparum* due to the absence of PMF (64,67).

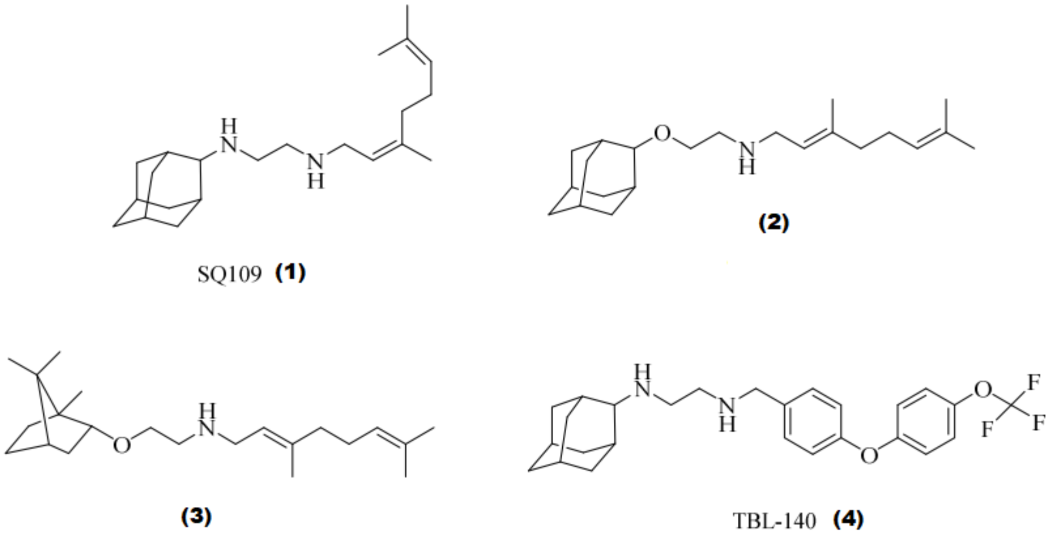
SQ109 might help reduce TB treatment periods when used in conjunction with conventional TB treatments, according to the studies. SQ109 is now in Phase 2b-3 clinical trials (41).

Other SQ109 analogues studied as MmpL3 inhibitors are shown in Fig. **7**. N-geranyl ethanolamines (compound **1** and **2**, Fig. **7**) with more effective antitubercular action than SQ109 were developed by varying the adamantane headgroup and the ethylenediamine linker (64).

The nitrogen center (compound **1**) was replaced with oxygen, which lowered its activity, suggesting that the nitrogen core is required for powerful action.

TBL-140 (compound **4,** Fig. **7**) inhibits the replication of Mtb (MIC99 = 2.5 μM) and decreases the survivability of nonreplicating Mtb (68) and also works against Gram-negative and Gram-positive bacteria showing the same broad-spectrum impact as SQ109 (41).

TBL-140 has better metabolic stability than SQ109, however further study is needed to evaluate its antituberculosis potential.



SQ109 **(1)**

(2)

(3)

TBL-140 **(4)**

Figure 7: Structures of SQ109 and its analogues 2-4. Source: (41)

1.4.2 Carboxamide derivatives

1.4.2.1 Indole-2-carboxamides

Indole-2-carboxamide derivatives are one of the most well-studied MmpL3 inhibitor class. This family are a valuable class of compounds with a low molecular weight, a straightforward synthesis and has shown to be effective against Mtb and Nontuberculous Mycobacteria (NTM) species (54).

An indole-2-carboxamide scaffold was identified from a library of 6,800 compounds (69). Compound **5** (Fig. **8**) shows significant anti-Mtb activity (MIC = 0.93 μM) and low toxicity, however, revealed poor pharmacokinetic characteristics. To overcome this problem further optimization led to the NITD-304 (compound **6**, Fig.**8**) and NITD-349 (compound **7,** Fig. **8**) (41).

NITD-304 and NITD-349 show significant anti-MDR-TB activity with MICs ranging from < 0.04 to 0.08 μM and are now in pre-clinical testing (70).

Despite their poor water solubility and high lipophilicity, these compounds have excellent pharmacokinetic characteristics following oral treatment. In both acute and established infection NITD-304 and NITD-349 were effective. After two weeks of drug treatment, the effectiveness of NITD304 and NITD-349 was equivalent to that of the first-line TB RIF and better than that of EMB in the established infection model (70).

The antitubercular activity of analogues was dramatically reduced when the cyclohexane ring was replaced with an unsubstituted phenyl ring or a nitrogen-containing heterocyclic ring, according to preliminary SAR data. It has also been postulated that the insertion of a tiny ring resulted in a complete loss of activity, whereas bulkier substituents resulted in molecules with high activity. SAR analyses revealed that the two NH groups on the indole and amide moieties, as well as the indole-2-carboxamide core, are required for significant antitubercular action (41).

Further SAR studies of indole-2-carboxamide compounds identified two new analogues, **8** and ICA38 (compound **10**, Fig. **8).**

In this group, ICA38 exhibits the strongest anti-Mtb activity in vitro, although, this compound was discarded due to low bioavailability. Both drug-sensitive and MDR clinical Mtb isolates were found to be sensitive to NITD-304 and NITD-349. (28)(40)

Compound **9** possesses good pharmacokinetic qualities and in vivo effectiveness against drug-sensitive tuberculosis (MIC = 0.012 μM) and MDR-TB/XDR-TB (MICs = 0.006-0.023 μM). Additionally, the compounds **8**, NITD-304 and NITD-349 can act synergistically with RIF (71,72).

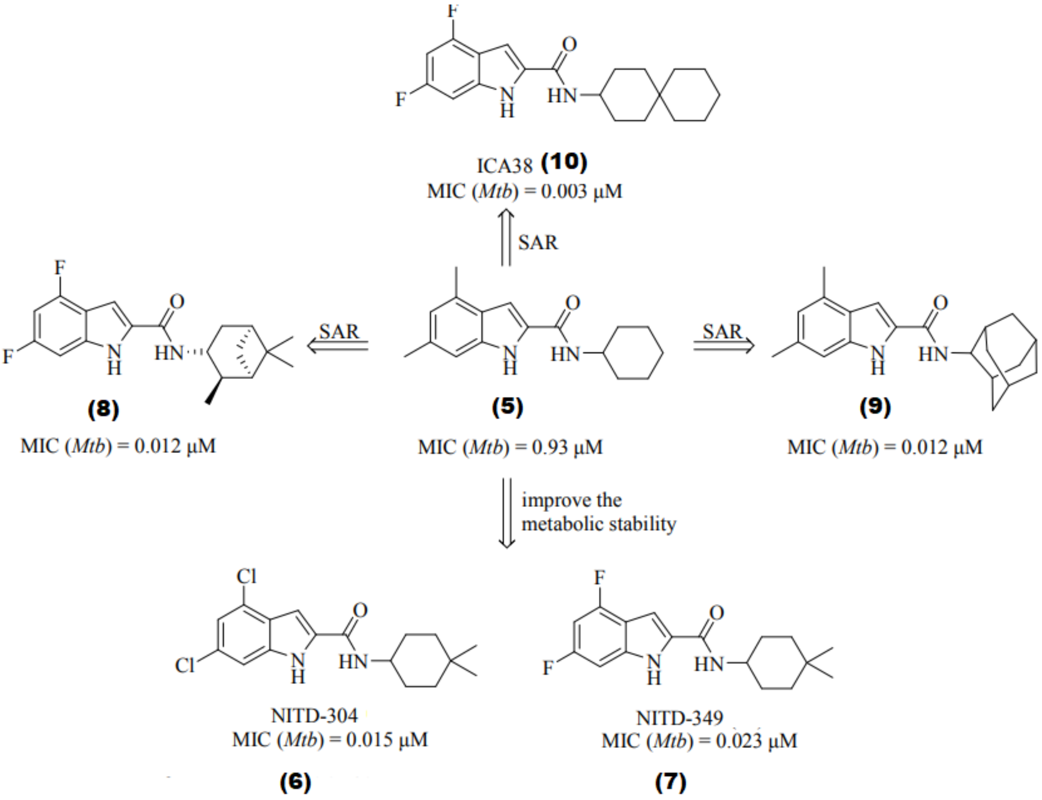


Figure 8- Structures of compounds 5-10. Source: (41)

1.4.2.2 Tetrahydropyrazolo[1,5-*a*] pyrimidine-3-carboxamides (THPPs)

The tetrahydropyrazolo[1,5-*a*]pyrimidine-3-carboxamide (THPP) compound **11** (Fig. **9**) has an MIC of 0.3 μM against Mtb and significant action against infected murine macrophages (41).

Because of its limited solubility compound **11** could not be studied in murine infection models. To overcome this situation, further optimization identified compound **12** with an MIC of 0.16 μM against Mtb and reduced clogP value (41).

On the other hand, the methylenedioxyphenyl in compound **12** (Fig. **9**) is sensitive to oxidation by the CYP enzyme, and the active intermediates generated are linked to in vivo toxicity (73,74).

After further adjustment, compound **13** (MIC= 0.15 μM) (Fig. **9**) was shown to be effective in mouse models of tuberculosis infection (41,75).

Replacement of the phenyl group on the left and/or right of the THPP core with polar groups showed no influence on solubility or plasma protein binding but did result in decreased activity.

Compound **14** (Fig. **9**), which had a pyrimidine group at the C5 position and a difluoromethyl group at the C7 position, had antitubercular action and improved solubility. However, compound **14**, failed to show action in vivo due to its short half-life and poor plasma distribution (41).

Later research revealed that THPP inhibits mycolic acid synthesis by targeting the enoyl-coenzyme A (CoA) hydratase EchA6 (76), indicating that THPP might be a multitarget agent.

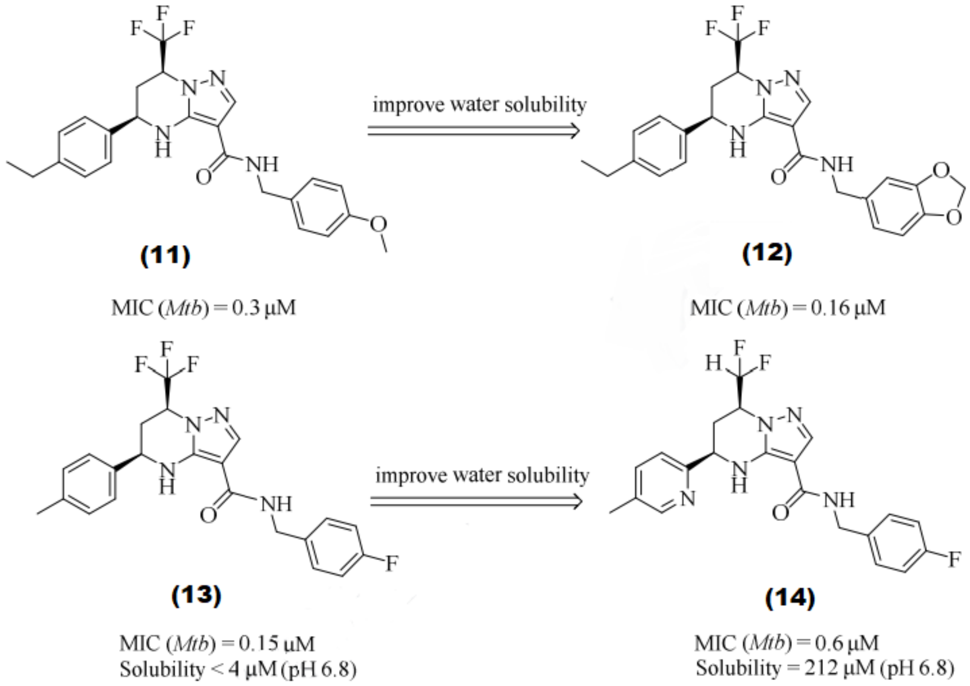


Figure 9- THPP derivatives compounds 11-14. Source: (41)

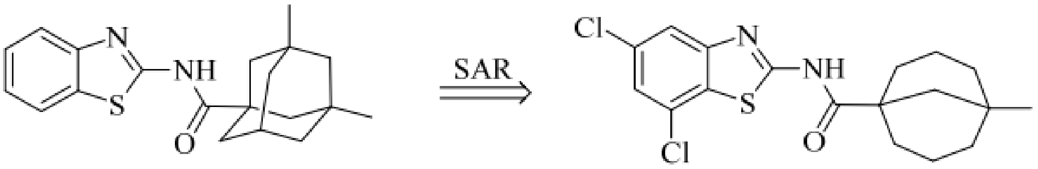
1.4.3 Benzothiazole amides

Compound **15** in Fig. **10** were identified from the high-throughput screen of 350,000 small molecule compounds (77). However, because of its high lipophilicity and propensity for nonspecific binding, the adamantyl group in compound **15** might be a liability. As a result, first technique for this series was to trim the adamantyl group to find the smallest amount of lipophilic structure that still retained activity (77).

The position and degree of substitution around the cyclohexane ring influenced anti-mycobacterial action. Generally, the MICs were elevated as the number of carbons was trimmed resulting in loss of antimycobacterial activity.

CRS400393 (Fig. **10**), an advanced lead chemical with good potency and a mycobacteria-specific spectrum of action, was developed by replacing the adamantyl group with cyclohexyl derivatives and subsequently developing this series (41,77).

To identify the mechanism of action, researchers used whole-genome sequencing on a drug-resistant Mtb strain to reveal a mutation in the MmpL3 gene. The parental strain showed overexpression of the mutant MmpL3 gene. Mtb culture was treated with synthetic derivatives and radiolabelled lipids, and quantitative TLC analysis was performed. TDM production was inhibited, and the quantity of mycolated arabinogalactan was reduced, indicating that these derivatives act on the MmpL3 membrane transporter (78).



(15)

MIC (*Mtb*) = 4 μg/mL
MIC (*M. abs*) = 1 μg/mL
MIC (*M. avium*) = 2 μg/mL
MIC (*M. intracellulare*) = 2 μg/mL

CRS400393, (16)

MIC (*Mtb*) ≤0.12 μg/mL
MIC (*M. abs*) = 0.03 μg/mL
MIC (*M. avium*) = 2 μg/mL
MIC (*M. intracellulare*) = 2 μg/mL

Figure 10: Benzothiazole amide derivatives 15-16. Source: (41)

1.4.4 Adamantyl ureas

Anti-tubercular action of adamantyl ureas and their derivatives has previously been investigated and MICs of less than 0.1g/mL have been reported to be effective (53). **AU1235** (compound **17**, Fig **11**) was identified in the LeadScreen (Tripos), a pre-formatted set of high-throughput screening compounds, library of 12,000 (53).

Adamantyl ureas and their derivatives were reported to inhibit Mtb epoxide hydrolase but also human soluble epoxide hydrolase (sHE), resulting in a reduction in therapeutic applications. MmpL3, a protein involved in the secretion of TMM, has also been demonstrated to be targeted by these compounds (79).

AU1235 was further optimized to improve its solubility, metabolic stability, and selectivity without altering sEH. SAR studies of **AU1235** involving aryl ring, alkyl ring, or urea group yielded to 42 variants (80).

Compounds **18** and **19 (**Fig. **11)** have shown the strongest activity, MIC values of 0.02 and 0.01 g/mL, but demonstrate considerable inhibition against sEH (41).

Although a series of amantadine sulfonamides enhanced solubility, they lost antitubercular efficacy. Furthermore, pyridine, pyrimidine, thiazole, and triazine derivatives had weak to moderate antitubercular action, whereas isoxazole derivatives shown the highest activity (41).

The isoxazole series SAR revealed that hydrophobic groups at the C5 position of the isoxazole ring boosted antitubercular activity. When compared to the tertiary 1-position, the insertion of an isoxazole group at the secondary 2-position of the adamantyl group considerably increased antitubercular action (**20 versus 21,** Fig. **11)**.

An oxadiazole derivative **22 (**Fig. **11)** demonstrated comparable SAR to isoxazoles while exhibited lower human sEH activity (41).

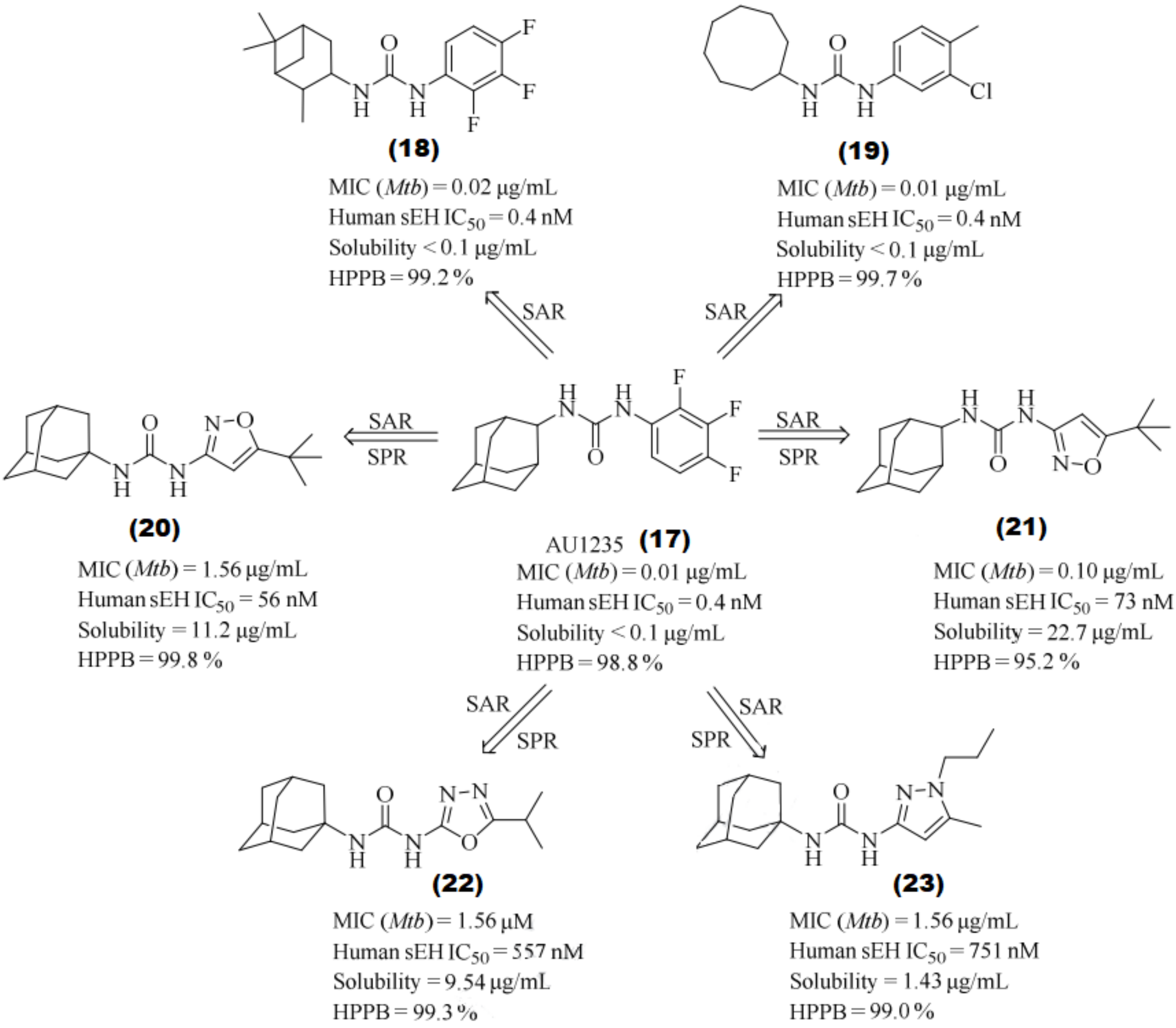


Figure 11: Structures of AU1235 and its analogues 18-23. Source: (41)

1.4.5 Pyrroles and pyrazoles

With the rise of MDR, the demand for a novel anti-Mtb molecule is expanding. BM212, commonly known as 1,5-diphenyl pyrrole derivative, is one of the potential pyrrole-ringed compounds. This molecule was identified in a library of azole compounds and proved to be active against Mtb replication (MIC = 5 µM) (52,81).

After screening genomic libraries and employing whole genome sequencing mutations in the mmpL3 gene have been found to be responsible for resistance to **BM212.** It has also been showed that drug resistance caused by mutations in the mmpL3 gene is not attributable to increased drug efflux, suggesting that the mmpL3 gene product is the cellular target for BM212 action (52).

The identification of **BM212** as a hit within this compound class generated impetus for the development of new structures with less potential toxicity and improved activity.

Various substituents with different substitution patterns were added to the pyrrole nucleus at N1 and C5 positions to see how they affected Mtb activity. It has been shown that replacing the piperazine moiety with a morpholine or thiomorpholine led to compounds with better in vitro activity and lower toxicity (82).

BM533 (compound **24**, Fig. **12**), resulted from the thiomorpholine substitution, possesses strong anti-Mtb activity, however is cleared at a faster rate in mouse microsomal fractions (41).

BM635 (compound **25**, Fig. **12**), resulted from the substitution of thiomorpholine by morpholine, has improved anti-Mtb activity and microsomal stability. It has been demonstrated by in vivo studies that BM635 has comparable activity to RIF, INH, and moxifloxacin (41).

Despite their significant in vivo efficacy, pyrrole derivatives have poor solubility and metabolic profiles, as well as high plasma binding and hERG channel blockage. A large number of compounds (>300) were developed to increase potency and physicochemical qualities while reducing cytotoxicity, which is caused by caused by hERG channel inhibition (56).

With the aim of improving water solubility, optimization of **BM635** where performed involving modification of three main substitutions: the N1 phenyl ring (N1Ph), the C-5 phenyl ring (C5Ph), and the C3 morpholine moiety (C3M) (83).

The modification of C3M resulted in a considerable reduction of activity, according to SAR analysis. The addition of trifluoromethyl at the para position of the C5Ph, as well as alkyl, cycloalkyl, pyridine, or tetrahydropyrane ring substituents at N1, lowered hydrophobicity while retaining activity (56).

Compound 27 (CLND solubility = 199 μM) was found to be more soluble than **BM635** (CLND solubility = 1 μM) and to possess substantial antitubercular action **(**Fig. **12).** Despite a poor mean bioavailability, further in vivo experiments revealed that compound **27** decreased bacterial numbers in the lungs compared to untreated mice (41).

By replacing the pyrrole core of **BM635** with a pyrazole ring SAR synthesized a series of 1,3,5-trisubstituted pyrazole derivatives. In SAR investigations compounds containing 4-isopropylphenyl or 4-trifluoromethylphenyl at the C5-position of pyrazole were well tolerated (41).

Among these pyrazole derivatives, **compound 28 (**Fig. **12)** outperformed **BM635** in terms of water solubility (solubility = 152 μM).

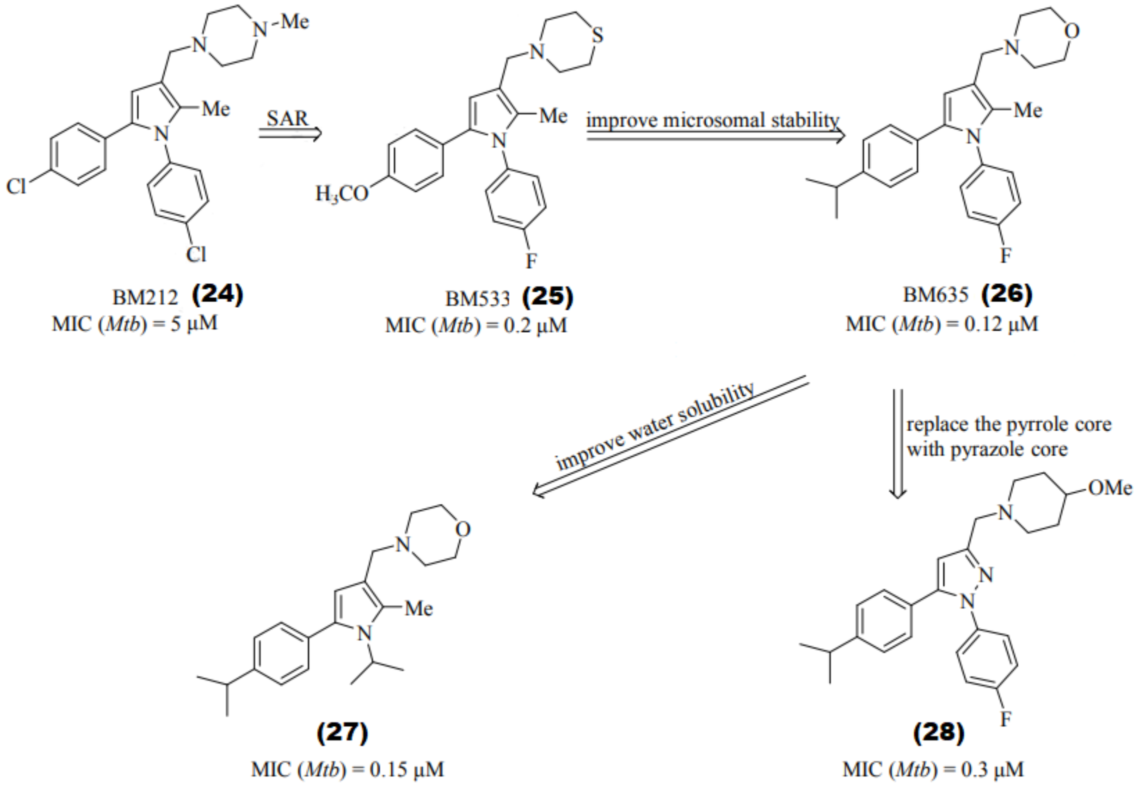


Figure 12: Pyrrole and pyrazole derivatives 24-28. Source: (41)

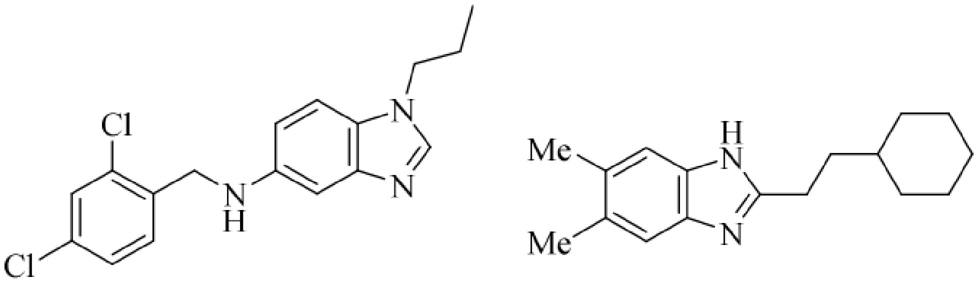
1.4.6 Benzimidazole Derivatives

C215 (compound **29,** Fig. **13**) is an inhibitor with action against Mtb with an IC90 of 16 µM, limited nonspecific toxicity against mammalian cells and effectiveness against Mtb growing in macrophages that was identified in a whole-cell screening assay (84).

SAR of 1H-*benzo[d]imidazole* derivatives revealed that compounds having cyclohexyl ethyl at C2 and one or two halogen atoms or two methyl groups on the benzimidazole seemed to have the strongest antitubercular action. On the other hand, bulky substituents (such as methylsulfonyl or phenylsulfonyl) at the N1 position lowered antitubercular action (78,85).

EJMCh-6, (Compound **30**, Fig **13**) with minimal cytotoxicity, has significant inhibitory actions against Mtb (MIC = 0.75 µg/mL) and *M. abscessus* (MIC = 0.125 µg/mL) (86).

MmpL3 in Mtb was shown to be affected by benzimidazoles derivates, since it inhibit TDM production and arabinogalactan mycolylation (78).



C215 (29)

IC_{90} = 16 µM

EJMCh-6 (30)

MIC (*Mtb*) = 0.75 µg/mL
MIC (*M. abs*)= 0.125 µg/mL)

Figure 13: Benzimidazole derivatives 29-30. Source: (41)

1.4.7 Spiropiperidines

SPIRO is an active member of the spirocycles family and belongs to a novel class of spiropiperidines inhibitors. Based on high-throughput phenotypic screening, it was identified novel spiropiperidine agent (compound **31**, Fig **14**) with a MIC of 0.3 µM but high clearance values in mouse microsomes (87).

Following further optimization, compound **32** was found to have powerful action (MIC = 0.06 μM) and lower clearance values in mouse microsomes (1.2 mL/min/g) than compound **31** (> 30 mL/min/g) (41).

Other spiropiperidines, such as spiroindenes, were also identified as antituberculosis drugs (88).

Further SAR research yielded compound **33** with a MIC of 0.3 μM. Because **33** is extremely lipophilic (clogP > 4), has low water solubility (1 μM), and plasma free fractions (<1% free), additional optimization by adding polar side- at the R1 or R2 locations was a good way to improve it. However, modifications towards the R1 position resulted in no improvement (41). Furthermore, compounds **34** and **35** demonstrated efficacy against a range of clinical isolates and resistant Mtb bacteria (MICs = 0.39–3.12 μM). To increase the lipophilicity and metabolism of these molecules, further optimization is required (41).

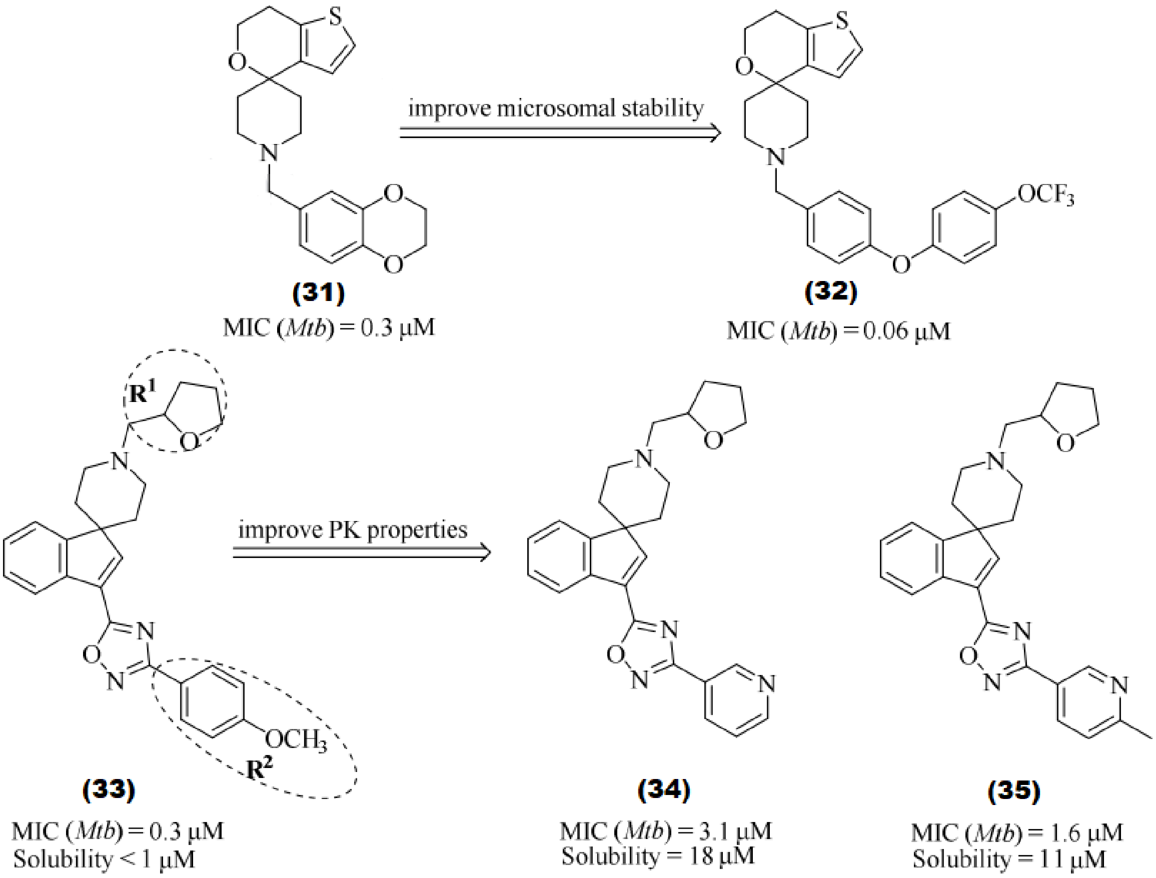


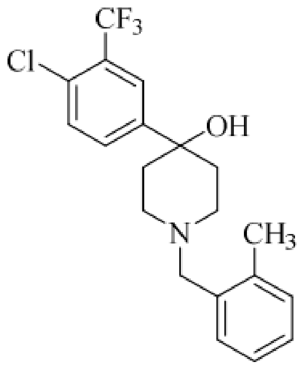
Figure 14 :Spiropiperidine derivatives 31-34. Source: (41)

1.4.8 Piperidinol

A piperidinol-containing molecule **PIPD1** (Fig. **15**), was recently found as a powerful lead agent against Mtb by high-throughput whole-cell screening of a large chemical library (89).

The results of functional experiments performed on an *M. smegmatis* strain showed that PIPD1 inhibits MmpL3-driven TMM translocation without affecting the proton motive force across the inner membrane. Hence, piperidinol anti-tubercular efficacy is achieved by direct inhibition of MmpL3 flippase activity rather than inhibition of the inner membrane proton motive force (89).

Furthermore, **PIPD1** inhibits intracellular *M. abscessus* and greatly lowers bacterial load, pathogenic symptoms, and larval mortality in zebrafish. PIPD1 is the first example of a very promising molecular scaffold for treating *M. abscessus* infections (41).



PIPD1 **(36)**

Figure 15: Structure of PIPD1. Source: (41)

1.5 Mode of action of MmpL3 targeting chemical scaffolds

As previously stated, numerous chemical scaffolds have been discovered to inhibit mycobacteria growth and target MmpL3. The abundance of potential MmpL3 inhibitors, as well as their structural diversity, has sparked heated discussion about their modes of action, including whether they act directly on MmpL3 or through indirect pathways by disrupting the pH gradient and/or membrane potential.

To resolve this debate and offer additional insight on the mechanism of action of these inhibitors there have been established in vitro and whole cell assays to find direct inhibitors of MmpL3. They showed, using fluorescence competition assays, that SQ109, BM212, AU1235, NITD304, NITD349, and THPP1 all bind directly to MmpL3. Only SQ109 and BM212 dissipated both transmembrane pH gradient (ΔpH) and membrane potential (ΔΨ), although the latter only at high concentrations (90).

Several co-crystal structures of MmpL3 with the inhibitors SQ109, AU1235, and ICA38 have also been described to better understand the mode of action (90).

Surprisingly, all three inhibitors directly bind to the same binding pocket in the TM domain's center causing conformational changes to accommodate the inhibitors by disrupting the interaction of the two Asp-Tyr pairs required in proton translocation and thereby blocking the PMF for substrate translocation (90,91).

Furthermore, the binding residues are highly conserved across mycobacteria, implying that these compounds might have a broad spectrum of effectiveness against a variety of microorganisms (64).

ICA38's binding mechanism differs from that of SQ109 and AU1235, in that its carbocyclic spiro group allows for a more extensive hydrophobic contact (91). Molecular docking experiments with six different compounds (BM212, NITD-349, GSK2200150A, C215, PIPD1 and HC2091) revealed that they all attach to the same binding pocket, indicating that the majority of MmpL3 inhibitors appear to share the same mechanism of action by inhibiting the crucial PMF pathway. This is supported by the fact that the majority of resistance mutations are found inside or adjacent to the inhibitor binding pocket (90,91).

An increasing number of MmpL3 inhibitors have been discovered to have additional target. SQ109 also inhibits MenA and MenB in Mtb, two enzymes involved in the manufacture of menaquinone, a crucial electron transport chain component. Studies have shown that THPP also targets EchA6, suggesting that MmpL3 may not be the only target. Identifying possible secondary targets of MmpL3 inhibitors should be further explored.

Several additional MmpL proteins have been demonstrated to be necessary for virulence and/or drug efflux in Mtb, despite MmpL3 being the sole essential MmpL protein. Because all inhibitors impair the proton relay route, which is a conserved property of all MmpL transporters, it opens the door to the development of compounds that target not only MmpL3 but the whole MmpL family of transporters, impacting virulence and drug resistance (90).

Both direct and indirect routes can lead to MmpL3 suppression in treated mycobacterial cells, contributing to the target's promiscuity. While not mutually exclusive, a thorough knowledge of how these two mechanisms(s) play out for each inhibitor to eventually abolish mycolic acid export would necessitate a complete examination of how each interacts with the transporter and impacts the bacterium's energy metabolism (92).

2 Conclusions

Tuberculosis (TB) is a contagious disease that is a major cause of illness and one of the top causes of mortality across the world. TB was the largest cause of mortality from a single infectious agent until the coronavirus (COVID-19) pandemic, ranking ahead of HIV/AIDS.

One of the most serious impediments to disease eradication is the growth of multidrug-resistant and extensively drug-resistant strains, which contribute considerably to patient dropout due to the extended duration of therapy and difficult-to-manage side effects. Hence, antituberculosis treatments with novel mechanisms of action, high efficacy, chemical stability, and low toxicity are urgently needed.

Inhibitors targeting MmpL3, a protein important for translocating mycolic acids from the inner membrane to the periplasm during the production of the mycobacterial cell membrane is an appealing and promising target for TB medicines since it is involved in cell envelope biosynthesis.

Several new classes of compounds have been identified as MmpL3 inhibitors, including ethylenediamine derivatives, carboxamide derivatives such as indole-2-carboxamides, benzothiazole amides, and THPPs, pyrroles, pyrazoles, adamantyl ureas, benzimidazoles.

Furthermore, some of these scaffolds interact with known antitubercular drugs, such as rifampicin, indicating that inhibiting MmpL3 might reduce TB treatment time.

These scaffolds can be further changed to increase their drug-like characteristics and pharmacokinetics/pharmacodynamics. Many lead compounds are in various phases of clinical and preclinical testing, with the potential to produce innovative drugs. Although many small compounds have been found and published in the literature, issues such as lesser water solubility, toxicity, and increased plasma protein binding must be addressed before they may be considered as a potential therapeutic candidate.

The ideal antitubercular drugs should be effective against both replicating and non-replicating Mtb. SQ109, which is an ethylenediamine derivatives, is the most promising MmpL3 inhibitor and its now in Phase 2b-3 clinical trials. Furthermore, it showed synergetic activity with isoniazid, rifampicin and bedaquiline and shortened clearance of TB in mice model.

On the other hand, indole-2-carboxamides NITD-304 and NITD-349, have entered preclinical trials. However, there is presently no FDA-approved drug for TB therapy that targets the MmpL3 receptor.

Even though several small-molecule MmpL3 inhibitors have been found, significant obstacles persist.

Over half of the MmpL3 inhibitors reported have lipophilic non-aromatic groups such as adamantyl, cycloalkyl, or halogen substituents, which may contribute to enhanced cell wall penetration and affinity for binding to MmpL3. However, they may cause problems in plasma due to their low water solubility, high protein binding rate, and high toxicity.

On the other hand, MmpL3 mutations provide resistance to these inhibitors by causing conformational changes that hinder inhibitor binding. To minimize the possible routes to resistance, next-generation MmpL3 inhibitors with more flexible conformations are necessary.

Further investigation of MmpL3's biological activity is needed since it can help on the development of improved inhibitors.

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