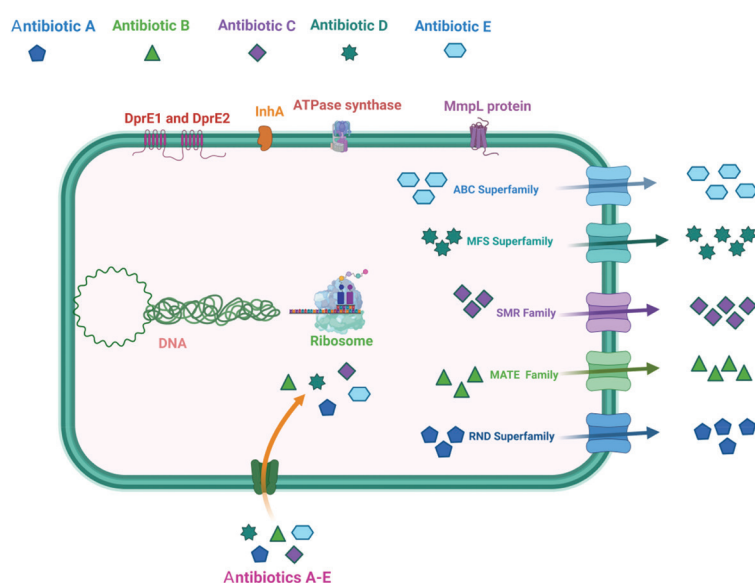


Combating Multi-drug Resistant *Mycobacterium* Species by Efflux Pump Inhibitors: Contemporary Trends

Graphical abstract



Authors

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In brief

This review highlights the critical role of efflux pumps in mediating drug resistance in *Mycobacterium tuberculosis* and non-tuberculous mycobacteria, and explores the promising potential of natural products as efflux pump inhibitors to restore antibiotic efficacy and combat multidrug resistance.

Highlights

- **Emerging Threat of Tuberculosis Drug Resistance**
A comprehensive overview of the global challenge posed by multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) and the rising incidence of drug resistance in non-tuberculous mycobacteria (NTM).
- **Efflux Pumps: A Key Mechanism Driving Resistance**
In-depth exploration of efflux pump systems as critical contributors to intrinsic and acquired drug resistance in mycobacteria, with emphasis on the roles in therapeutic failure and persistence.
- **Natural Compounds as Promising Efflux Pump Inhibitors**
Evaluation of naturally derived molecules with potential to inhibit mycobacterial efflux pumps, highlighting the role in restoring drug susceptibility and enhancing existing tuberculosis treatments.

Combating Multi-drug Resistant *Mycobacterium* Species by Efflux Pump Inhibitors: Contemporary Trends

Gautam Kumar^{1,*}, Priya Mondal², Amit Kadam², Isha Dhamija^{3,4}, Ravindra Pal Singh⁵, Deepak Nathiya⁶ and Sandeep Kumar^{5,*}

Abstract

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M.tb*) complex (MTBC), which includes *M.tb* as one of the causative bacteria. In contrast, non-tuberculous mycobacteria (NTM) refers to *Mycobacterium* spp. that do not cause TB or leprosy (MTBC spp., *M. leprae*, and *M. lepromatosis*). *Mycobacterium* spp. are responsible for the deadliest infections and remain a significant challenge in diagnosis and treatment. *Mycobacterium* spp. have developed multiple complementary mechanisms to defend against antibiotics. Specifically, the mechanisms include modifying the drug target sites, enzymatically inactivating the drugs, and lowering intracellular antibiotic concentrations by overexpressing efflux pumps. These adaptations contribute to the emergence of multi-drug resistant pathogens. This review provides an overview of antibiotic resistance in *Mycobacterium* spp. with a focus on several key factors, such as enzyme-mediated antibiotic deactivation, gene expression, biofilm formation, and the role of efflux pumps. A critical objective of this review includes *Mycobacterium* efflux pumps, the significant role in antibiotic resistance, and compounds that act against these efflux pumps.

Keywords

ABC superfamily, efflux pump, MFS superfamily, *Mycobacterium tuberculosis*, non-tuberculous mycobacteria, RND superfamily, SMR superfamily, tuberculosis.

Introduction

Tuberculosis (TB) remains the deadliest infection from single infectious organisms and continues to hold centre stage among bacterial infections and remains a significant challenge in terms of diagnosis and treatment [1, 2]. The WHO Report 2023 stated that 7.5 million individuals were diagnosed with TB in 2022 and approximately 1.6 million people died from TB, including 187,000 co-infected with HIV and TB [3]. Macrophages phagocytose most internalized *Mycobacterium tuberculosis* (*M.tb*) bacilli that reach the physiologic system and eliminate significant bacilli populations. Some bacteria can escape the phagosome compartment and remain inactive, allowing patients to remain asymptomatic [4, 5]. The *M.tb* bacilli subpopulation is known as persisters, which are phenotypically tolerant to antibiotics. Also, persisters remains metabolically inactive and do not grow like resistant mutant *M.tb* bacilli [6]. A schematic representation depicting genetic and phenotypic resistance is shown in **Figure 1**.

Non-tuberculous mycobacteria (NTM) are species that belong to the same genus (*Mycobacterium*) but refers to bacteria other than the *M.tb* complex (MTBC) that do not cause leprosy or TB. NTM are ubiquitous and can survive in a wide range of environmental conditions. Most likely, drinking water or other materials are sources of NTM. Moreover, NTM can survive chlorine treatment and enter tap water [7–9]. NTM species are classified according to the Runyon classification based on growth rates in solid culture medium and pigment formation. Notably, Runyon types I, II, and III NTM are slow-growing *Mycobacterium* spp. and are characterised based on pigment formation or colour development. Runyon type IV *Mycobacterium* spp. are rapidly growing bacteria that can grow in <7 d. Pigments are categorized as Runyon type I and are considered photochromogens when developed after exposure to light. Mycobacteria classified as Runyon type II develop pigments, even in the dark; these bacteria are known as scotochromogens. Mycobacteria are categorized as Runyon type III if the pigments are not intense and

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Received: February 6 2025

Revised: April 1 2025

Accepted: May 3 2025

Published Online: June 2 2025

Available at:
<https://bio-integration.org/>

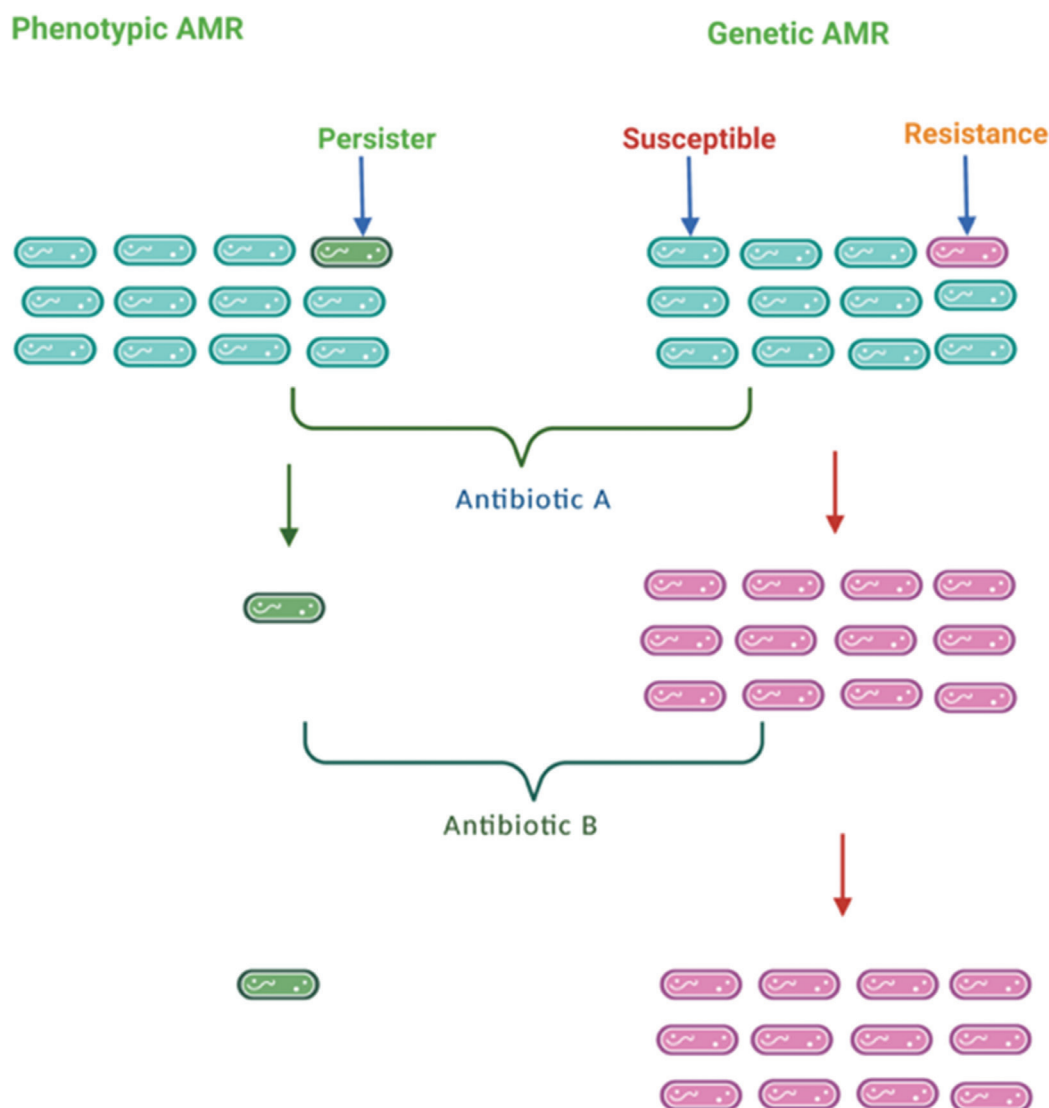


Figure 1 Schematic representation depicting antimicrobial resistance (phenotypic and genetic) is displayed above. *Mycobacterium* persisters do not have the effects of antibiotics A and B. The genetically mutated *Mycobacterium* survives and multiplies in the proximity of antibiotics A and B.

these mycobacteria are known as non-photochromogens [10]. Runyon classification of NTM and their species are given in Table 1.

NTM can infect humans and are responsible for severe infections in immune-compromised and -competent individuals [11, 12]. NTM species, including *M. kansasii*, *M. simiae*, *M. goodii*, *M. szulgai*, *M. scrofulaceum*, *M. avium* complex (MAC), and *M. malmoense*, are responsible for the majority of pulmonary infections [13–23]. NTM species, such as *M. marinum*, *M. ulcerans*, *M. chelonae*, *M. fortuitum*, and *M. abscessus*, are responsible for skin infections. In addition, *M. chimaera* and *M. abscessus* are the most common NTM species responsible for soft tissue infections [24–26].

Because *M.tb* and NTM species react favourably to the standard smear acid-fast staining process, clinical detection of *M.tb* and NTM species poses a considerable problem and frequently results in incorrect interpretations. As a result, the frequency of NTM has been underestimated due to the prevalence of TB in several countries [27].

Treatment of TB includes a cocktail of first-line anti-TB drugs, including isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA) [28]. Second-line anti-TB drugs (groups 2–4) are prescribed to treat drug-resistant TB. Group 2 drugs include injectable aminoglycosides (streptomycin [STR], kanamycin, and amikacin) and polypeptides (capreomycin and viomycin). Group 3 drugs include oral and injectable fluoroquinolones [FQs] (gatifloxacin, moxifloxacin, and levofloxacin). Group 4 drugs include the following oral medications: *p*-aminosalicylic acid; cycloserine; teridazone; ethionamide; prothionamide; thioacetazone; and linezolid [LZD] [28]. Third-line anti-TB drugs (group 5) have unclear efficacy or an undefined role. Group 5 drugs include clofazimine, linezolid, amoxicillin plus clavulanate, imipenem plus cilastatin, and clarithromycin [29]. In recent years, several compounds have been identified as potential anti-TB agents and are summarised in Table 2 [30–32].

Treatment regimens for fast-growing NTM include macrolides, aminoglycosides, fluoroquinolones, oxazolidinones,

Table 1 Non-Tuberculous Mycobacteria (NTM) Runyon Classification and the *Mycobacterium* spp

Runyon Classification	NTM Species	Ref.
Runyon type I (photochromogens)	<i>M. kansasii</i>	[13, 14]
	<i>M. simiae</i>	[15]
	<i>M. marinum</i>	[16, 17]
Runyon type II (scotochromogens)	<i>M. goodii</i>	[18, 19]
	<i>M. szulgai</i>	[20]
	<i>M. scrofulaceum</i>	[21]
Runyon type III (non-photochromogens)	<i>M. avium</i> complex (<i>M. avium</i> and <i>M. intracellulare</i>)	[22]
	<i>M. malmoense</i>	[23]
	<i>M. xenopi</i>	[23]
	<i>M. terrae</i> complex	[214]
	<i>M. genavense</i>	[23]
	<i>M. haemophilum</i>	[23]
	<i>M. ulcerans</i>	[23]
	<i>M. abscessus</i>	[23, 24]
	<i>M. chelonae</i>	[23, 215, 216]
	<i>M. smegmatis</i>	[217]
Rapid-growing Runyon type IV	<i>M. fortuitum</i>	[25]

tigecycline, carbapenems, and cephalosporins. In contrast, RIF, macrolides, EMB, and amikacin are front-line agents for slow-growing bacilli NTM species [12, 33]. The antibacterial activity of macrolides, such as azithromycin and clarithromycin, inhibits the 50S ribosomal subunit protein synthesis. It possesses activity against MAC, *M. kansasii*, *M. malmoense*, and *M. abscessus* [34, 35]. The antibacterial activity of aminoglycosides, including streptomycin and amikacin, inhibits protein synthesis by acting on the 30S ribosomal subunit. Aminoglycosides efficiently cure patients with cavitary MAC lung disease [36, 37]. The antibacterial activity of oxazolidinone class antibiotics inhibits binding of 23S rRNA to the catalytic site of the 50S ribosome, which in turn prevents the formation of a functional 70S initiation complex. Delpazolid has antibacterial activity against *in vitro* and *in vivo* models of macrophage and murine *M. abscessus* infections [38]. Tedizolid exhibits antibacterial activity against *M. fortuitum*, *M. chelonae*, and *M. abscessus* subsp. *massiliense*, *M. marinum*, *M. kansasii*, MAC, and *M. simiae* [38, 39]. Tetracycline, doxycycline, and tigecycline effectively treat NTM infections. The tetracycline analog, omadacycline, has inhibitory activity against *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, *M. chelonae*, *M. immunogenium*, *M. fortuitum*, and *M. mucogenicum*, including resistant strains [40]. The fluoroquinolones, such as ciprofloxacin and moxifloxacin, are active against MAC and *M. intracellulare* [41, 42]. SPR719 is a gyrase B inhibitor that effectively reduces the mycobacterial burden in lungs of infected mice [43]. SPR719 is active against multiple clinical strains of NTM, including MAC and *M. abscessus* [44]. SPR720 is a phosphate prodrug of SPR719 that effectively treats human NTM and pulmonary TB infections [45]. The antibacterial activity inhibits DNA-directed RNA polymerase (RNAP). Rifabutin is an RIF derivative active against *M. abscessus* subsp. *abscessus*. Rifabutin also inhibits other NTM, including MAC, *M. kansasii*, *M. malmoense*, and *M. xenopi* [46, 47]. The anti-mycobacterial activity of

bedaquiline (BDQ) inhibits ATP synthesis and growth of MAC, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. kansasii* [48]. Clofazimine has been reported to be potent in patients with pulmonary NTM, *M. abscessus* group, and MAC infections [49]. Cell wall biosynthesis inhibitors disrupt the bacterial cells, leading to a compromise in bacteria viability [50]. β -lactams, including imipenem and ceftazidime, exhibit antibacterial activity against *M. abscessus*. β -lactamase inhibitors, including clavulanate, tazobactam, avibactam, relebactam, nacubactam, and zidebactam, in combination with other β -lactams have potential inhibitory activity against NTM species [51–55]. The chemical structures of compounds acting against NTM species are shown in Figure 2. In this review the diverse mechanisms underlying antibiotic resistance in *Mycobacterium* spp. are discussed and effective solutions are explored, including natural and synthetic compounds, to tackle the pressing challenge of antibiotic resistance in these organisms.

Antibiotic resistance in *Mycobacterium* spp.

Antibiotic resistance in pathogenic microorganisms poses a global threat. Antibiotic resistance is a condition in which conventional antibiotics cannot effectively cure a disease [56]. In most cases, when patients do not recover from TB, second-line anti-TB drugs are given in combination for 18–24 months to treat multidrug-resistant (MDR)-TB [28]. *M.tb* has demonstrated resistance to most first- and second-line anti-TB drugs. Current research has documented the development of drug-resistant TB (DR-TB), MDR-TB, extensively drug-resistant TB (XDR-TB), and totally drug-resistant TB (TDR-TB) over the past several decades [57]. *M.tb* strains exhibit various levels of drug resistance. The I-R strain is resistant to INH, while the R-R strain has

Table 2 Anti-TB Agents or Drugs under Clinical Evaluation

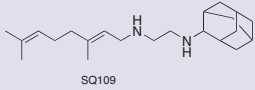
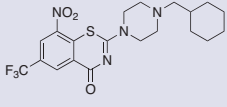
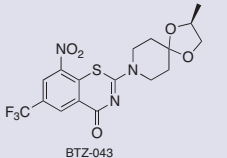
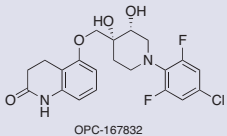
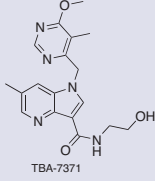
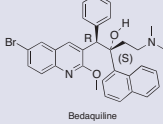
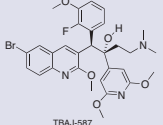
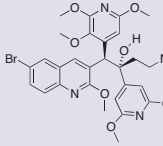
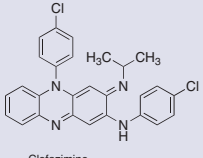
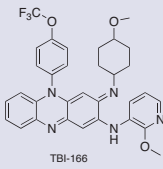
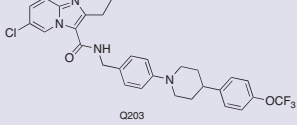
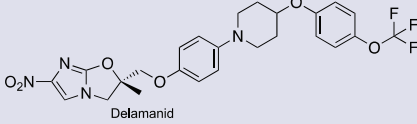
Anti-TB Agents/Drugs	Chemical Class	Mode of Action	Ref.
 SQ109	Ethylenediamine	MmpL3 transporter inhibition. Additionally, SQ109 disrupts the proton motive force of the <i>M.tb</i> membrane.	[218–221]
 Macozinone (PBTZ-169)	Benzothiazones	Inhibition of membrane DprE1 enzyme	[222]
 BTZ-043	Benzothiazones	Inhibition of membrane DprE1 enzyme	[223, 224]
 OPC-167832	Carbostyrl	Inhibition of membrane DprE1 enzyme	[225, 226]
 TBA-7371	Azaindole	Inhibition of membrane DprE1 enzyme	[227–230]
 Bedaquiline	Diarylquinoline	ATP synthesis inhibition	[231, 232]
 TBAJ-587	Diarylquinoline	ATP synthesis inhibition	[233, 234]
 TBAJ-876	Diarylquinoline	ATP synthesis inhibition	[233, 234]
 Clofazimine	Riminophenazine	Clofazimine is a competitive inhibitor of menaquinone (MK-4) and checks the point of entry of electrons into the respiratory chain, which leads to energy depletion.	[235–240]
 TBI-166	Riminophenazine	TBI-166 presumably competes with menaquinone (MK-4), an essential cofactor in the mycobacterial ETC, and checks the electron's entry point into the respiratory chain	[241, 242]
 Q203	Imidazopyridine amide	Cytochrome bc1 complex inhibition	[243–246]
 Delamanid	Nitro-dihydro-imidazooxazole	Delamanid exhibits anti-mycobacterial activity by inhibiting methoxy-mycolic acid and ketomycolic acid.	[102, 247, 248]

Table 2 (continued)

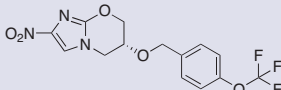
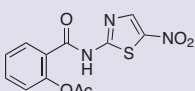
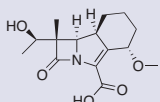
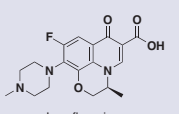
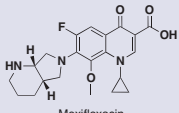
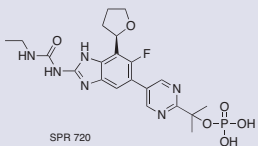
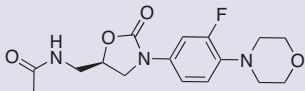
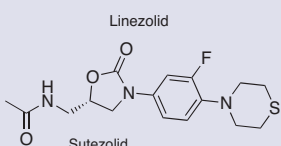
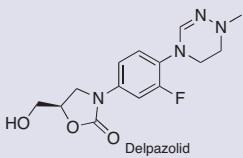
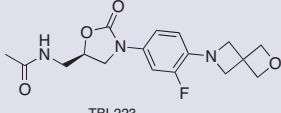
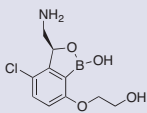
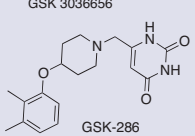
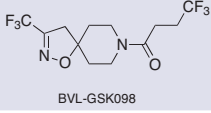
Anti-TB Agents/Drugs	Chemical Class	Mode of Action	Ref.
 Pretomanid	Nitroimidazooxazine	Pretomanid inhibits cell wall lipids and proteins.	[248–250]
 Nitroxanide	Nitrothiazolyl salicylamide	Nitroxanide displays activity against <i>M.tb</i> by disrupting membrane potential and pH homeostasis. Moreover, Nitroxanide also targets human quinone oxidoreductase (NQO1)-mediated mTORC1 signalling that promotes autophagy of <i>M.tb</i> inside host cells.	[251–254]
 Sanfetrinem = R = H Sanfetrinem cilexetil = R = $-\text{C}(\text{CH}_3)\text{HOCOOC}-\text{C}_6\text{H}_{11}$	Tricyclic β -lactam	Cell wall inhibitors	[255–258]
 Levofloxacin	Fluoroquinolone	Inhibition of DNA replication by binding to topoisomerase II (DNA gyrase).	[259, 260]
 Moxifloxacin			[259, 260]
 SPR 720	Aminobenzimidazole	Inhibition of gyrase B	[44, 96]
 Linezolid	Oxazolidinone	Inhibition of protein synthesis by binding to the 50S ribosome subunit	[261]
 Sutezolid			[262]
 Delpazolid			[263–265]
 TBI-223			[266]
 GSK 3036656	Benzoxaborole	Leucyl-tRNA synthetase (LeuRS)	[36]
 GSK-286	Pyrimidine-2,4-dione	Cholesterol catabolism	[36]
 BVL-GSK098	Spiroisoxazoline	Antagonizes the resistance mechanism of ethionamide	[267]

Table 2 (continued)

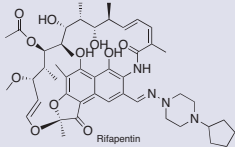
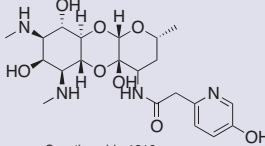
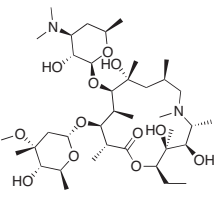
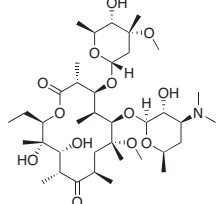
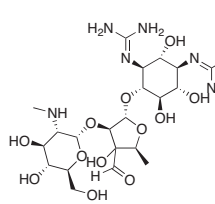
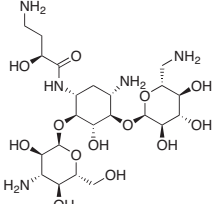
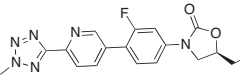
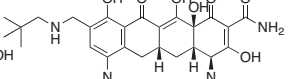
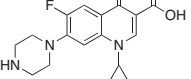
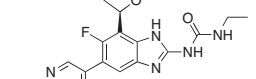
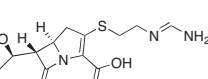
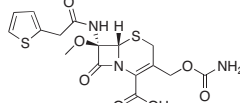
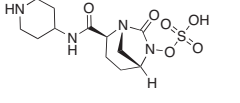
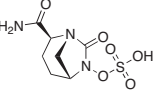
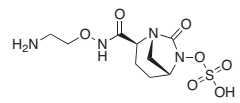
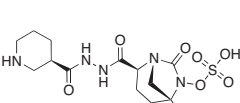
Anti-TB Agents/Drugs	Chemical Class	Mode of Action	Ref.
	Macrolactam	Inhibition of DNA-dependent RNA polymerase activity	[268–271]
	Aminoglycoside	Ribosome protein synthesis inhibition	[272–275]
			
			
			
			
			
			
			
			
			
			
			
			
			
			

Figure 2 Chemical structures of compounds acting against NTM species.**Table 3** Major Drug-Resistant *M.tb* Strains

Type of Drug-resistant <i>M.tb</i> Strains	Resistant to Drug
I-R	<i>M.tb</i> shows resistance to isoniazid (INH)
RR	<i>M.tb</i> shows resistance to rifampicin (RIF)
MDR	<i>M.tb</i> shows resistance to INH + RIF
Pre-XDR	<i>M.tb</i> shows resistance to RIF + fluoroquinolone (a class of second-line anti-TB drugs)
XDR	<i>M.tb</i> shows resistance to RIF + any fluoroquinolone, + at least one of bedaquiline or linezolid
TDR	<i>M.tb</i> shows <i>in vitro</i> resistance to all first- and second-line drugs.

resistance to RIF. The MDR-TB strain is resistant to INH and RIF. Pre-extensively drug-resistant (Pre-XDR) TB strains are resistant to RIF and fluoroquinolones. XDR TB strains are resistant to RIF, fluoroquinolones, and BDQ or linezolid. Finally, TDR TB strains are resistant to all first- and second-line anti-TB drugs [58]. MDR-TB poses a significant challenge that has made conventional TB treatment ineffective, which warrants immediate attention and innovative solutions. A list of drug-resistant *M.tb* strains is given in **Table 3**.

MDR in *M.tb* strains primarily evolved due to the initial ineffectiveness of TB treatments, including first- and second-line antibiotics and exposure of bacilli to several

antibiotics [56]. Antibiotic resistance primarily arises from factors, such as inadequate antibiotic concentration, extended periods of monotherapy, poor prescribing practices, and variations in the pharmacogenomics, pharmacodynamics, and pharmacokinetics of the administered drugs [59, 60].

Antibiotic resistance in mycobacteria can be divided into two categories: acquired resistance; and intrinsic resistance. Intrinsic resistance refers to the natural ability of bacteria to resist antibiotics, whereas acquired resistance develops through specific chromosomal mutations or the horizontal transfer of genes [61]. There are no reports of horizontal gene transfer (HGT) of resistance genes through plasmids or transposable elements in *Mycobacterium* spp. This indicates

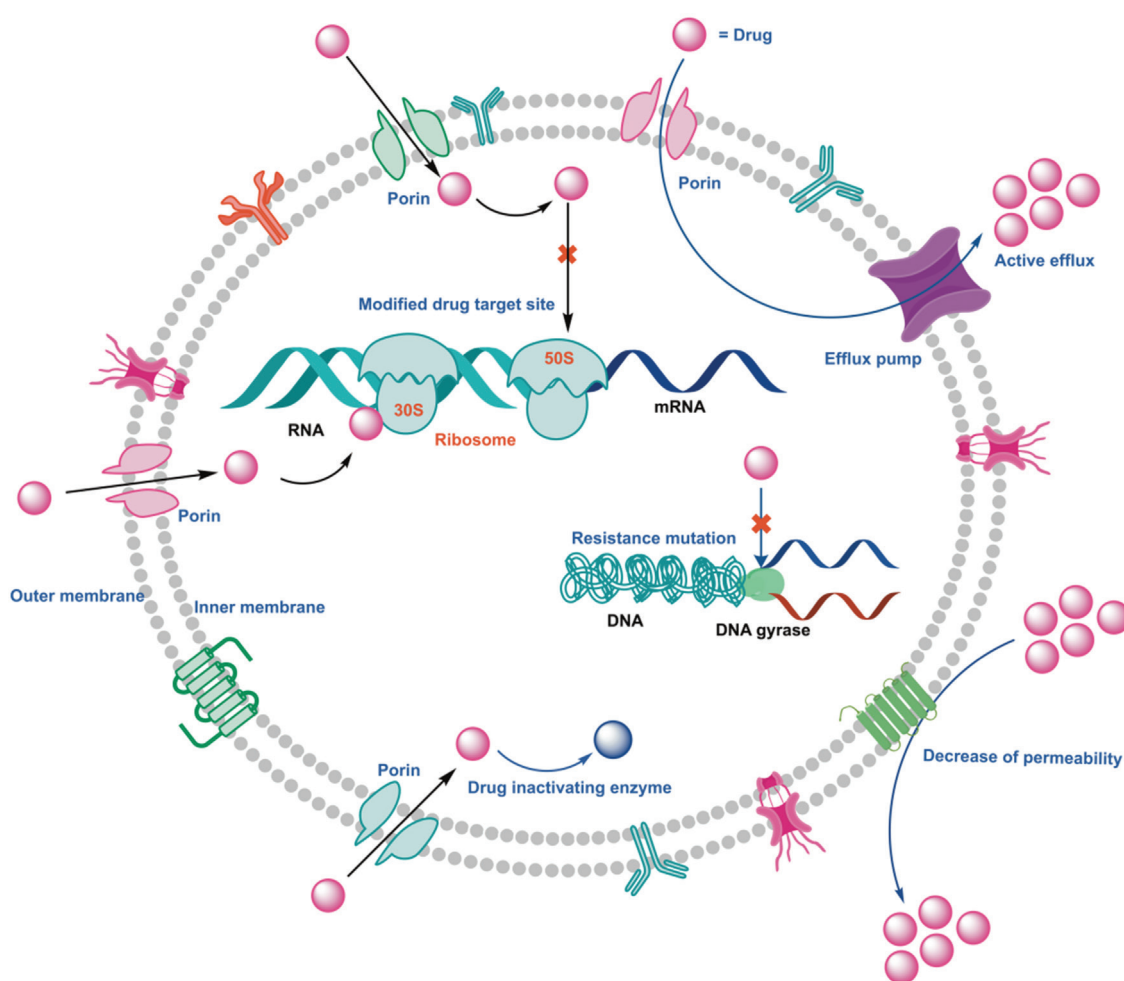


Figure 3 A graphical representation depicting bacterial modes of resistance, including antibiotic degrading enzyme, antibiotic altering enzyme, antibiotic decrease to permeability, and efflux pump.

that the acquired resistance results from chromosomal mutations [61, 62]. Single-nucleotide polymorphisms (SNPs), mutations involving changes in nucleotide sequences, insertions of new genetic material, and deletions cause changes in the genomic DNA of *M.tb* [12, 63, 64]. *Mycobacterium* bacilli exhibit several modes of antibiotic resistance, including low permeability of the cell wall, overactive efflux pumps, enzymatic degradation of antibiotics, and modifications at the target site (Figure 3) [57, 65]. Recent data have revealed that *M.tb* and NTM exhibit acquired resistance against most antibiotics and develop complementary mechanisms to counteract their effects [66]. Therefore, there is an urgent need to control MTBC and NTM infections and address *Mycobacterium* resistance issues [28].

Genes-mediated antibiotic resistance in *Mycobacterium* spp.

MTBC acquires antibiotic resistance due to spontaneous gene alterations. For example, the antibiotic-dependent spontaneous mutation rate that leads to resistance varies, as

follows: RIF, approximately 1 in 10^8 bacilli; and INH, STR, and EMB, approximately 1 in 10^6 bacilli [67]. Sequential changes in additional genes make the strains unresponsive to various targets [63, 68]. MspA, the *M. smegmatis* membrane porin, transfers hydrophilic substances via the outer membrane to the cytoplasm. Additionally, MspA is responsible for introducing small hydrophilic antibiotics into *M. smegmatis*. Studies have shown that deleting MspA porin results in resistance to various hydrophilic antibiotics. The porin mutant of *M. smegmatis*, ML10 (a deletion of MspA and MspC) exhibits β -lactam antibiotic resistance [69]. Similarly, CpnT is an outer membrane protein in *M. bovis* BCG and *M.tb* that is involved in food absorption. Strains with a CpnT mutation are unresponsive to antibiotics, such as RIF, EMB, clarithromycin, tetracycline, and ampicillin, and to nitric oxide [70].

INH is a prodrug used in treating TB that requires metabolic activation by catalase-peroxidase. INH resistance has been observed in *M.tb* with *katG* and *inhA* gene mutations. *Mycobacterium* strains with *katG* gene mutations have decreased INH activation because there is a pronounced decrease or loss in catalase-peroxidase activity. *inhA* gene mutations may influence construction of the bacillus cell wall. The minimum inhibitory concentration (MIC) of INH is significantly higher than *M.tb* isolates with *katG*

mutations. Moreover, structural alterations in the INH target subsequently results in INH resistance [71].

Additional mutational genes in other clinical INH-resistant isolates have been identified, which include *furA*, *ndh* (encodes NADH dehydrogenase), *ahpC*, *Rv0340-0343*, *fadE24*, *efpA*, and *kasA* [72, 73]. *M.tb* exposed to INH shows variance in ManLam-related gene expression. The *hspX* gene is increased in RIF-resistant and MDR-TB strains in response to INH, whereas *tgsI* and *sigE* are strongly upregulated in MDR-TB and drug-sensitive TB strains, respectively [74]. RIF exhibits anti-mycobacterial activity by acting on the β -subunit of DNA-dependent RNA polymerase. The *Escherichia coli* model was used to study RIF resistance, which demonstrated that the *rpoB* gene mutations are the primary cause of RIF resistance [75]. It has been shown that *rpoB* gene mutations are responsible for cross-resistance to several rifamycins. In addition, *rpoB* gene mutations are present in the 81 bp RIF-resistance determining region (RDDR), which affects the RNA polymerase β -subunit. These modifications prevent rifamycins from binding to the RNA polymerase β -subunit, leading *M.tb* to be less susceptible to the RIF, and ultimately RIF-resistant strains emerged [76]. The most common mutated codons in the RDDR are 531, 526, and 516 [77]. Moreover, homoplastic SNP analysis revealed that the compensatory effects of *rpoC* and *rpoA* significantly contribute to RIF resistance [78].

PZA is a prodrug that requires pyrazinamidase for conversion to the metabolically active form. A phylogenetic analysis of the MTBC tree revealed that PZA resistance could be attributed to the *pncA* H57D mutation [79]. Along with *pncA*, other genes, such as *rpsA* and *panD*, contribute significantly to PZA resistance in *M.tb*. Mutations, such as NK11K, P69T, and D126N, are present in the *pncA* gene. According to a recent study, *pncA* gene mutations reduce the active site volume, making it more difficult for PZA to bind to the active site. Additionally, these modifications lead to a low binding affinity for PZA [80, 81]. Interestingly, *M. bovis*, which resists PZA therapy, carried the *pncA* H57D mutation [79]. Notably, mutations in the *pncA* gene do not always result in PZA resistance and PZAse critical failure. It has been verified that *pncA* gene mutations lead to a limited degree of PZA resistance by partially decreasing PZAse activity. In addition to the above-mentioned mutations other mutations e.g. D12A, T135P, and D49N are implicated in PZA resistance [82].

EMB exhibits anti-mycobacterial activity by acting on the target Emb proteins responsible for cell wall biosynthesis. The Emb proteins include EmbA, EmbB, and EmbC, and EMB resistance mutations are mainly located near the Emb binding site [83]. The mutations are primarily present in the *embCAB* operon that encodes arabinosyl transferases, which are responsible for EMB resistance. Among the arabinosyl transferases, alterations are most frequently noted in the *embB* gene and less often in the *embC*, *embA*, *embR*, and *ubiA* genes [84]. Moreover, *ubiA* gene mutations lead to enhanced production of decaprenylphosphoryl-D-arabinose, which is the building block used in cell wall synthesis and is the likely cause of EMB resistance [85]. Most mutations have been noted in the 576 bp *embB* region, which is known as the ETB resistance-determining region (ERDR).

The canonical mutations of *embB* codons are 306, 406, and 497 [86, 87]. Interestingly, it has been shown that EMB resistance in *M.tb* isolates have slight variations according to geographic regions [86, 87]. STR hinders translational proofreading and stops protein synthesis in *Mycobacterium* spp. *rrs*, *rpsL*, and *gidB* gene mutations encode 16S rRNA, protein S12, and 7-methylguanosine methyltransferase, which are linked to STR resistance. *gidB* inactivation methylates 16S rRNA, resulting in the development of STR resistance [88]. The three *rpsL* gene mutations are present in Lys43Arg, Lys88Arg, and Lys88Thr residues, and two *gidB* mutations are mainly observed in Trp45Ter and Gly69Asp residues. The most frequent STR mutation among *rpsL* gene mutations is Lys43Arg. Notably, *Mycobacterium* spp. devoid of *rpsL* and *rrs* mutations do not exhibit whiB7-mediated STR resistance [89]. Insignificant resistance to kanamycin and STR has been reported to be due to eight different mutations in the 5' untranslated region of the transcriptional activator whiB7 [90]. There is resistance to kanamycin and STR due to acquisition of unlinked chromosomal mutations. Notably, mutations increased whiB7 transcripts by a factor of 23–145, which enhances *eis* (*Rv2416c*) and *tap* (*Rv1258c*) expression. Increased expression of *tap* mediates STR resistance via overexpression of the efflux pump and *eis* is responsible for kanamycin resistance in mutated *Mycobacterium* spp. [90].

D-cycloserine exhibits anti-mycobacterial activity by acting on the enzymes (D-alanine racemase and D-alanine:D-alanine ligase), which are involved in peptidoglycan metabolism. Notably, overexpression of D-alanine racemase and D-alanine:D-alanine ligase are responsible for D-cycloserine resistance in *M. smegmatis*. A point mutation in the *cycA* gene causes *M. bovis* BCG resistance to D-cycloserine [91]. A mutation in the alanine racemase-encoding gene (*rv3423c*) is responsible for cycloserine resistance in *M.tb* [92].

FQs exhibit anti-mycobacterial action by acting on DNA gyrase and topoisomerase IV [43]. Mutations causing resistance to quinolone antibiotics occur in a specific segment of *gyrA* known as the quinolone resistance-determining region (QRDR). Codons 90 and 94 of gyrase A are referred to as the QRDR [93]. Moreover, mutations in *gyrA* and *gyrB* may cause the development of cross-resistance to many FQs [94]. *M.tb* isolates exposed to ofloxacin had a primary target mutation in *gyrA* and a minor mutation in *gyrB* [94]. In addition, the ESX-5 membrane complex secretion system protein, Rv1783, exhibited a V762G mutation, which is responsible for ofloxacin resistance [94]. This V762G mutation increased the MIC of ofloxacin from 1 to 32 mg/L in the *M.tb* strain. Notably, the MIC of ofloxacin in the *M.tb* strain did not show any change in the use of efflux pump inhibitors (EPIs), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and reserpine. This finding implies that the *gyrA* target mutation and the V762G mutation in Rv1783 reduced the efficacy of ofloxacin against *M.tb* [94]. Other studies have suggested that *M. smegmatis*, *M.tb*, and *M. avium* possess the *mfpA* gene responsible for quinolone resistance. The *mfpA* gene encodes a pentapeptide repeated pentapeptide protein. This peptide binds to DNA gyrase, preventing FQs from reaching the target [94]. SPR719 belongs to an aminobenzimidazole class, which exhibits anti-TB activity by

blocking gyrase B enzyme activity [95]. SPR720, a prodrug of SPR719, demonstrates anti-mycobacterial activity against MDR-TB, drug-susceptible *M.tb*, and MDR-TB strains. Moreover, SPR720 is active against NTM, such as MAC and *M. abscessus* [43, 44, 96]. A recent investigation revealed that mutation in the gyrase B ATPase domain is associated with low-frequency resistance in *M. avium* and *M. abscessus*. Furthermore, frameshift mutations in the transcriptional repressor, MAB_4384, a regulator of MmpS5/MmpL5 expression, are responsible for SPR719 resistance in *M. abscessus* [95].

M.tb uses proton motive force (PMF) generated across the plasma membrane to synthesize ATP. Inhibiting ATP synthesis results in an imbalance in cellular pH and PMF. BDQ selectively inhibits *M.tb* ATP synthase [97]. The *atpE* gene encodes ATP synthase, which is responsible for BDQ resistance in *M.tb* strains. In addition, it has been shown that the development of BDQ resistance in *M.tb* leads to clofazimine cross-resistance. Rv0678 is a negative regulator of mmpL5 and mmpS5. This mutation is also responsible for BDQ resistance. Significantly, there was a > 4-fold increase in BDQ MIC due to this mutation. Verapamil and reserpine are well-known EPIs that reduce the MIC of BDQ and clofazimine against *M.tb* with the Rv0678 mutation. Additionally, reserpine enhanced the bactericidal effects of BDQ, whereas verapamil had no such effect on mice infected with *M.tb*. Therefore, reserpine may inhibit the Rv0678 gene mutation *in vivo* [98].

Another study showed that loss of mutations in *pepQ* (Rv2535c), corresponding to the putative Xaa-Pro aminopeptidase, is responsible for clofazimine cross-resistance in *M.tb*. Further analysis of mice infected with *M.tb* H37Rv (*pepQ* mutants) showed a 4-fold higher BDQ and clofazimine MIC. The concomitant use of verapamil and reserpine reduces and restores the BDQ and clofazimine MIC in mutant and parent *M.tb* strains. This result implies that *pepQ* function loss decreases *M.tb* susceptibility to BDQ and clofazimine [99]. The anti-TB program recently included BDQ for treating MDR-TB. Involvement of the mutation in Rv0678 and Rv2535c reduces the effectiveness of BDQ. Therefore, adjuvant therapy may be required to treat TB.

Delamanid is a prodrug belonging to the nitro-dihydroimidazooxazole class used in treating MDR-TB [100, 101]. *M.tb* bacilli activate delamanid and catalyze reduction of the nitro to the active form. Delamanid activation requires reduced deazaflavin co-factor, F₄₂₀, F₄₂₀-dependent glucose-6-phosphate dehydrogenase, and the nitroreductase gene product, Rv3547 [102]. Pretomanid is also a prodrug belonging to the nitroimidazoles class. Mycobacterial glucose-6-phosphate dehydrogenase converts pretomanid into the metabolically active form. Delamanid and pretomanid resistance have been linked to gene mutations (*ddn*, *fgdI*, *fbiA*, *fbiB*, *fbiC*, and *fbiD*) involved in the F₄₂₀-dependent bioactivation pathway [103, 104]. A structural investigation showed that several point mutations in the gene encoding the delamanid-activating enzyme impact the ability to bind with delamanid [105]. The gene, Rv2983 (*fbiD*), is required to synthesize the co-factor, F₄₂₀. Notably, *fbiB* and Rv2983 mutants have minor alterations in delamanid sensitivity but substantial levels of pretomanid resistance. Additionally,

nitroimidazole-resistant, F₄₂₀ H₂-deficient *M.tb* mutants are hypersensitive to malachite green [106].

DprE1 is an enzyme crucial for the biosynthesis of decaprenylphosphoryl-D-arabinofuranose (DPA). D-arabinofuranose (Araf) residues and arabinosyltransferase biosynthesis use DPA as a substrate. D-arabinofuranose is the building block for lipoarabinomannan and arabinogalactan [107]. Inhibition of the DprE1 enzyme prevents DPA biosynthesis. Therefore, the DprE1 enzyme is an essential anti-TB target [108]. BTZ043 and PBTZ169 have anti-mycobacterial activity by blocking the DprE1 enzyme, which causes arabinogalactan depletion. These two drugs have undergone clinical evaluation and completed phase II clinical studies. Interestingly, mutations in Rv0678 exhibit negligible cross-resistance to medicines of the benzothiazinones class, including BTZ043 and PBTZ169. Further studies are required to confirm that Rv0678 mutations will impair the efficacy of BTZ043 and PBTZ169 in treating TB in humans [109].

LZD belongs to the oxazolidinone class and demonstrates antibacterial action by interfering with bacterial protein synthesis in *M. abscessus* [38]. Earlier studies have confirmed the involvement of efflux pumps, including *drdABC*, *rv0987*, *lmrS*, *acrAB*, *mmpL9*, *acrF*, and *optrA*, in extrusion of LZD. The molecular analysis of LZD-resistant *M. abscessus* harbors 23S rRNA mutations. Also, there are enhanced *lmrS* and *mmpL9* transcriptional levels in resistance strains compared to LZD-susceptible strains [110]. The anti-TB drugs/agents and the mutation-induced resistance is summarised in Table 4.

Enzymes mediate antibiotic resistance in *Mycobacterium* spp.

Mycobacterial enzymes transform drugs, rendering drugs inactive and unable to bind to targets. *Mycobacterium* spp. synthesize several enzymes that can cleave and degrade several classes of antibiotics, including β -lactams, aminoglycosides, and macrolides [111]. *Mycobacterium* produces ambler class-A β -lactamase, which is encoded by the *blaC* gene and cleaves β -lactam antibiotics into an inactive form. *Mycobacterium* spp., such as *M. smegmatis* and *M. fortuitum*, have been reported to possess ambler class-A β -lactamase [112]. Aminoglycosides, such as neomycin, kanamycin, gentamycin, tobramycin, and ribostamycin, have amino groups. The mycobacterial N-acetyltransferase enzyme carries out acetylation to these amino groups and renders the amino acid-containing antibiotics inactive. The *M. abscessus* MAB_4395 gene encodes 2' N- acetyltransferase, which is responsible for acetylating gentamicin C, dibekacin, tobramycin, and kanamycin B, and rendering the antibiotics inactive [111]. Notably, several *Mycobacterium* spp., such as *M.tb*, *M. kansasii*, *M. fortuitum*, and *M. smegmatis*, have been shown to produce N-acetyltransferase. In contrast, the MAB_2385 gene encodes 3'-O-phosphotransferase, which is responsible for inactivating STR and resistance in *M. abscessus*. The binding sites for aminoglycoside antibiotics,

Table 4 Anti-Tubercular Drugs/Agents and Mutation-Associated Resistant Mechanism

Drug/agent	Mutation	Ref.
Isoniazid	Suppression of KatG leads to decreased activation of INH.	[71, 72]
Rifampicin	The <i>rpoB</i> mutation causes a conformational change in the β -subunit of RNA polymerase, leading to decreased binding affinity for rifampicin.	[75, 76]
Pyrazinamide	A mutation in the <i>pncA</i> gene prevents pyrazinamide from being converted into its active acid form.	[79–81]
Ethambutol	Mutations in the <i>embCAB</i> operon encode arabinosyl transferases, which are responsible for EMB resistance.	[84, 86, 87]
Amikacin/Kanamycin	Modulation of the 16S rRNA target site (1400 and 1401 <i>rrs</i> gene) and overexpression of aminoglycoside acetyltransferase results in increased drug inactivation.	[36, 37]
Capreomycin	A mutation in the <i>tlyA</i> gene reduces the activity of rRNA methyltransferase and is associated with cross-resistance to aminoglycosides.	[276]
Streptomycin	Mutations in the <i>rrs</i> , <i>rpsL</i> , and <i>gidB</i> genes encode 16S rRNA, protein S12, and 7-methylguanosine methyltransferase, which are linked to STR resistance.	[88]
Fluoroquinolones	Mutations in the <i>gyrA</i> and <i>gyrB</i> genes alter the binding of DNA gyrase A/B to fluoroquinolones.	[94]
Ethionamide	Mutations in the <i>ethA</i> and <i>inhA</i> genes result in reduced prodrug activation and overexpression of <i>InhA</i> , which also contributes to cross-resistance with INH.	[277]
Cycloserine	The overexpression of <i>alrA</i> leads to a reduction in the effectiveness of cycloserine. A point mutation in a <i>cycA</i> gene causes resistance to D-cycloserine.	[91]
<i>P</i> -aminosalicylic acid	<i>thyA</i> mutations reduce the activated concentrations of <i>P</i> -aminosalicylic acid, while <i>folC</i> mutations change the binding site of <i>P</i> -aminosalicylic acid.	[276]
Clofazimine	A mutation in <i>Rv0678</i> leads to upregulation of <i>MmpL5</i> .	[235, 236]
Clarithromycin	Expression of <i>emr37</i> leads to modulation of the 23S rRNA site.	[111]
Bedaquiline	<i>atpE</i> mutations cause modulation in the binding site for bedaquiline.	[98]
Q203	Mutation in the b subunit of the cytochrome bc1 complex.	[278]
Delamanid and pretomanid	Gene mutations (<i>ddn</i> , <i>fgd1</i> , <i>fbiA</i> , <i>fbiB</i> , <i>fbiC</i> , and <i>fbiD</i>) are involved in the F_{420} -dependent bioactivation pathway.	[103, 104]

such as capreomycin and viomycin, are located within the ribosomal subunit interface of 16S rRNA helix 44 and 23S rRNA helix 69. Nucleotides, including C1409 of 16S rRNA and C1920 of 23S rRNA, are methylated at the 2'-hydroxyl. Methyltransferase *tlyA* deactivation leads to unmethylated ribosomes, inactivating capreomycin and viomycin [113, 114].

Azithromycin and clarithromycin belong to the macrolide chemical class and interfere with antibacterial protein synthesis by inhibiting 50S ribosomal subunits [34, 35]. *M. chelonae*, *M. abscessus*, and *M. fortuitum* exhibit methylase gene resistance. The transcriptional regulator, WhiB7, regulates expression of the *erm* (41) gene, which is responsible for macrolide-induced resistance. Azithromycin exposure is more accountable for rapid macrolide resistance in *M. abscessus* than clarithromycin [115]. Also, the *erm* gene is responsible for explicit methylation of the 23S rRNA A2058 nucleotide, which leads to reduced ribosome affinity to macrolides and thus leads to macrolide resistance [111, 116]. Interestingly, deleting *erm 41* genes in *M. massiliense* and *M. abscessus* leads to increased susceptibility to the macrolide and lowers the MIC [111]. In addition to the above-mentioned enzymes, *Mycobacterium* also biosynthesizes RIF ADP-ribosyltransferase, which attaches an ADP-ribose unit to a hydroxyl residue at position 23 of RIF, which leads to rifamycin resistance in *Mycobacterium* spp. [117].

M.tb Rv1026 (*ppx2*) is a novel exopolyphosphatase responsible for hydrolytic activity in the long-chain poly(P). *M.tb* knockdown with *Rv1026* expression displays slow growth, poly(P) accumulation, and increased cell wall thickness. These factors lead to decreased INH activity

and enhanced resilience towards heat and acidic pH, and *Mycobacterium* spp. can increase intracellular macrophage infection at a speedy rate [118].

Biofilm-mediated antibiotic resistance in *Mycobacterium* spp.

Biofilms are a three-dimensional extracellular matrix made up of polymeric substances (EPSs) used to cover the microorganisms. This matrix is also known as a “safe haven” for microorganisms because the matrix provides a biological barrier against antimicrobial agents and hosts immune component cells. Notably, under biofilm protection, the microorganism not only survives the effects of antibiotics but can resume growth after withdrawal of antibiotics [119]. Several studies have suggested that efflux pumps are critical in intrinsic drug resistance, acquired drug resistance, and biofilm formation [120]. Also, efflux pump overexpression enhances biofilm formation [121]. For example, Rv1877, a protein belonging to *M.tb* major facilitator superfamily (MFS). Rv1877 expression in *Escherichia coli* results in an increase in resistance to several classes of antibiotics. Additionally, Rv1877 expression increases biofilm formation in *E. coli*. The above finding suggests that EPIs, potentially inhibiting biofilm formation and impairing the activity of the efflux pump, can be a promising strategy to restore the activity of existing antibiotics [121].

Efflux pumps and gene involvement in antibiotic resistance among *Mycobacterium* spp.

Efflux is a physiologic procedure that expels foreign substances and endogenous metabolic waste through an efflux pump to maintain normal cell function [122]. The increased bacterial resistance to several antibiotic classes is primarily due to enhanced expression of multi-drug efflux mechanisms [123]. Efflux can transport numerous substrates out of cells or specific types of antibiotics [124].

The bacterial efflux pumps are classified into five different superfamilies of proteins, including ATP-binding cassette (ABC), small multi-drug resistance (SMR), multi-drug and toxic compound extrusion (MATE), MFS, and resistance-nodulation-division (RND) [66, 125, 126]. Efflux pumps belonging to ABC, MFS, RND, and SMR families are present in *Mycobacterium* [122]. Transporters require energy to extrude the substrates outside cells. The ABC superfamily uses ATP as an energy source and other transporters utilise the transmembrane PMF or an Na^+/H^+ ion electrochemical gradient to move substrates from the pumps [66, 125, 126].

A recent study conducted by Kanji et al. discovered SNPs in efflux pump genes. According to a comparative analysis, the detected alleles are either exclusive to DS-TB strains or found in XDR strains. Importantly, XDR-TB expresses more *Rv2688* and *rrrB* than DS-TB strains [127]. Another study reported epistatic relations between the drug-resistance gene in MDR and XDR-TB [128].

ABC transporters

Eukaryotes and prokaryotes have ABC protein transporters. These transporters extrude substances across biological membranes, including ions, antibiotics, polysaccharides, peptides, amino acids, and proteins [129]. The ABC superfamily transporter-encoding genes are *Rv1456c*, *Rv1457c*, *Rv1458c*, *Rv1217c*, *Rv1218c*, and *Rv1473*. *M.tb* clinical isolates overexpressing *Rv1456c*, *Rv1457c*, and *Rv1458c* are responsible for RIF, INH, STR, and EMB resistance. In contrast, *Rv1473* is involved in *M. smegmatis* macrolide resistance via efflux and the transcription factor, *WhiB7*, positively regulates *Rv1473* on exposure to macrolides [129]. *MSMEG-3762* and *Rv1687c* are annotated as ATP-binding proteins of the ABC efflux pump in *M. smegmatis* and *M.tb*, respectively. De Siena et al. demonstrated that *M. smegmatis* contains the *MSMEG-3762/63* protein complex, which acts as an efflux pump. This efflux pump has been shown to be responsible for effluxing RIF and ciprofloxacin, and biofilm development [130]. *M.tb* and *M. smegmatis* with the *Rv2686c-Rv2687c-Rv2688c* locus are involved in ciprofloxacin resistance, and to some extent, other FQs, such as norfloxacin, moxifloxacin, and sparfloxacin. Notably, FQ resistance can be decreased by reserpine, CCCP, and verapamil. *Rv1217c* and *Rv1218c* overexpression in *M.tb* results in higher RIF MICs, whereas overexpression of *Rv1218c*

alone leads to a higher INH MIC in *M.tb* [131]. *Rv1217c* and *Rv1218c* in *M.tb* are significantly upregulated during biofilm formation. Compounds, such as piperine and 1-(1-naphthylmethyl)-piperazine (NMP), inhibit the dose-dependent expression of *Rv1217c* and *Rv1218c* genes. This finding suggests that EPIs, such as piperine and NMP, can be used as adjuvants to primary anti-TB drugs for biofilm inhibition [132]. Liu et al. carried out point mutations in *M.tb* (V219A and S292L) in *Rv1258c*. Interestingly, mutated V219A and S292L are responsible for PZA, INH, and STR resistance in *M.tb*. Notably, the V219A point mutation is responsible for low PZA, INH, and STR resistance, whereas the S292L mutation is associated with increased PZA, INH, and STR resistance. Piperine reduces INH and PZA resistance in the S292L mutant but not in the V219A mutant [133].

The *Rv1258c* (TAP-2) efflux pump encodes tetracycline/aminoglycosides resistance in *M.tb* and is implicated in drug resistance to RIF, INH, FQs, and aminoglycosides [134]. EPIs, such as verapamil and piperine, inhibit *Rv1258c*. Cloete et al. performed molecular modeling and simulation studies of *Rv1258c* and reported the probable binding sites of the substrate and inhibitors. The substrate, which has similar binding, was effluxed by the *Rv1258c* pump and inhibited efflux by piperine. The modeling studies revealed that spectinomide binds differently than other substrates, suggesting spectinomide can escape *Rv1258c* efflux pump activity [134].

MFS transporters

MFS proteins identified in bacteria include *Rv2209*, *Rv3331*, *Rv1902c*, *Rv1672c*, *Rv0191*, *Rv0849*, *Rv2456c*, *Rv0037c*, *Rv0842*, *Rv0876c*, *Rv1258c*, *Rv2508c*, *Rv0783c*, *Rv1250*, *Rv1410c*, *Rv1634*, *Rv1877*, *Rv2333c*, *Rv2459*, *Rv2846c*, *Rv3239c*, *Rv3728*, *Rv2265*, *Rv1200*, *Rv3476c*, *Rv0261c*, *Rv0267*, *Rv1737c*, *Rv2329c*, and *Rv2994* [121].

EfpA is part of the QacA transporter family, which belongs to the MFS. Several studies have confirmed upregulation of EfpA in several clinical *M.tb* strains, including the *M.tb* strains resistant to INH, RIF, OFX, or multidrug treatments. BRD-8000.3 and BRD-9327 are potent inhibitors of EfpA [135, 136]. Recently, Wang et al. reported that the cryo-EM structures of EfpA in an outward open conformation bind to endogenous lipids or inhibitor BRD-8000.3. Three lipids in EfpA extend from the inner to the outer leaflet of the membrane. BRD-8000.3 occupies a lipid site within the inner membrane leaflet, which competitively inhibits lipid binding. Based on this study, Wang et al. stated that the EfpA structure is similar to the lysophospholipid transporter, MFSD2A, and the lipid-binding site functions as lipid flippase. Notably, the inhibitor, BRD-8000.3, prevents lipid entry into the pathway, while BRD-9327 likely disrupts the lipid flipping process between site A and site B [137].

Umar et al. evaluated the impact of anti-tubercular drugs on the mRNA efflux pump gene, *Rv1250*, in *M.tb* patients. A polymerase chain reaction revealed that the susceptible ($n = 11$) and resistant ($n = 40$) groups had different levels of *Rv1250* mRNA expression (5.961 ± 0.414 and $10.192 \pm$

1.978 fold changes; $P < 0.05$). The extent of *Rv1250* expression depends upon exposure to antimicrobial drug treatments [138]. Chloramphenicol resistance is linked to *Rv0191* of the MFS transporter. Notably, *Rv1353c* controls *Rv0191* transcription. Hence, when *Rv1353c* is overexpressed, transcription of *Rv0191* is downregulated, making the strain more susceptible to chloramphenicol [139]. The TetR-related transcriptional repressor, *LfrR*, regulates the primary facilitator family gene *LfrA* expression, which actively extrudes FQs, cationic dyes, and anthracyclines out of the cell and promotes innate resistance to antibiotics. The increased expression of *LfrA* has been shown to be responsible for FQ resistance [139].

EfpA belongs to the MFS family and is highly conserved in *M.tb* and slow- and fast-growing *Mycobacterium* spp. *M.tb* with *EfpA* overexpression is responsible for resistance to FQs, INH, RIF, tetracycline, and clofazimine [140]. A recent study demonstrated that controlled expression of the *EfpA* gene in *M. smegmatis* increases drug tolerance by 32-fold to RIF, INH, STR, and amikacin. At the same time, *M. smegmatis* and *M. bovis* BCG overexpressing *EfpA* have higher drug tolerance to moxifloxacin by >180- and 8-fold, respectively [141].

There are 112 *M. smegmatis* mc²155 MFS members, which typically include 6–15 conserved transmembrane domains. The *MSMEG_3705* gene of *M. smegmatis* mc²155 encodes a transporter protein containing 12 alpha-helical transmembrane domains. Zhang et al. deleted *MSMEG_3705*, which was designated as *M. smegmatis* mc²155 Δ*MSMEG_3705*. Interestingly, the mutant strain, *M. smegmatis* mc²155 Δ*MSMEG_3705*, is more sensitive to capreomycin and accumulates more EtBr than the wild strain. Deleting *MSMEG_3705* also influences and affects bacterial growth. Deleting *MSMEG_3705* causes upregulation of *MSMEG_3706*, a gene that encodes an isocitrate lyase (ICL), and downstream *MSMEG_3705*, which might be responsible for faster growth in the mutant strain (*M. smegmatis* mc²155 Δ*MSMEG_3705*) [142].

Small multidrug resistance (SMR) family

M.tb TBsmr (previously *mmr* [*Rv3065*]) of the SMR superfamily has approximately 43% sequence homology to *E. coli* EmrE. Antibiotics and fluorescent dyes are among the substances that TBsmr, a secondary active multi-drug transporter, transports [143]. *Rv3066* controls *Mmr* expression [144]. Additionally, it has been shown that *Mmr* is significantly overexpressed in *M.tb* strains exposed to INH. Overexpressed *mmr* reduces sensitivity to safranin O, acriflavine, and EtBr in *M.tb*, and simultaneously verapamil and CCCP restores responsiveness [145].

Resistance-nodulation-division (RND) Superfamily

The RND family efflux pumps, such as membrane proteins large (MmpL) and mycobacterial small membrane protein

(MmpS), are present in *M.tb*. The genomic sequence of *M.tb* H37Rv reveals 14 types of *mmpL* genes. Different species have different numbers of *mmpL* genes. For example, *M. leprae* has 5 *mmpL* genes, whereas *M. abscessus* has 31 *mmpL* genes. Specifically, *MmpS5/MmpL5* is responsible for an efflux of azoles, clofazimine, BDQ, and ionophores [125]. Notably, alteration in the *Rv0678* gene is involved in upregulating the *mmpS5-mmpL5* genes. Importantly, *M.tb* with *MmpS5-MmpL5* overexpression shows resistance to INH, RIF, and diarylquinolines [140]. *M. smegmatis* cell overexpression of the *mmpL7* gene leads to INH efflux and is responsible for INH resistance. The level of INH resistance is reduced in the presence of reserpine and CCCP [140].

Previous work has shown that the *Mmr* transporter is overexpressed in strains exposed to INH and *mmr* knockout mutants have enhanced sensitivity to ethidium bromide, tetraphenylphosphonium, and cetyltrimethylammonium bromide (CTAB). Overexpressed *mmr* reduces sensitivity to EtBr, acriflavine, and safranin O, and this effect is decreased in the presence of verapamil and CCCP. Importantly, INH susceptibility in *M.tb* is independent of *mmr* expression [145].

The role of *MmpL3*, *MmpL7*, and critical genes, such as *katG*, *inhA*, and *nat*, was studied to understand INH resistance in *M.tb*. The investigation suggested that the *katG* gene has a substitution mutation AGC-ACC at codon 315, the *inhA* promoter expresses C replacement at nucleotide position 15, and a silent mutation GGA to GGG at codon 207 in the *nat* gene. Taken together, these mutations are thought to be the factors responsible for INH resistance. Remarkably, no isolates exhibited mutations in *MmpL3* and *MmpL7* [146]. The TetR family transcription factor, *MAB_2299c* gene, which codes for *MmpS-MmpL* efflux pumps, controls *MAB_2300-MAB_2301* and *MAB_1135c-MAB_1134c* expression. Deleting *MAB_1135c-1134c* in *M.tb* increases susceptibility to clofazimine and BDQ. *MAB_1135c-1134c* encodes *MmpS-MmpL* in *M. abscessus*. Interestingly, deletion of *MAB_2300-MAB_2301* and *MAB_1135c-MAB_1134c* genes in a mutant lacking *MAB_2299c* and *MAB_2300-MAB_2301* exacerbate strain sensitivity to clofazimine and BDQ [147].

Several studies have demonstrated that *MmpS4/MmpL4* and *MmpS5/MmpL5* constitute part of the siderophore export system in *M.tb*. The *MmpS4/MmpL4* and *MmpS5/MmpL5* systems export and recycle siderophores, including mycobactins and carboxymycobactins, in *M.tb* [125]. A study was conducted using green fluorescent protein (GFP), which revealed that *MmpL5* forms a homotrimeric functional complex in the inner membrane in the presence of *MmpS5*. *MmpS5* expression facilitates assembly of monomeric *MmpL5* into a homotrimer, which promotes extrusion of several antimycobacterial drugs [148]. The intracellular concentration of ³H-radiolabelled econazole was measured in *M. bovis*. The *MmpS5/MmpL5* system was responsible for decreasing the concentration of econazole in the *M. bovis*, and after the addition of CCCP, the concentration was maintained inside *M.bovis*. This finding suggested that econazole is a substrate for the *MmpS5/MmpL5* system in *M. bovis* [125]. Farina et al. studied the role of *MmpS5/MmpL5* and its gene regulator (*Rv0678*) in MDR isolates.

Genetic mutations in MmpL5 (Rv0676) due to insertion, deletion, and substitution could change the functionality of the efflux pump and cause a significant reduction in drug susceptibility, thus developing drug resistance in *M.tb* strains [149]. In another study, clofazimine-resistant *M.tb* strains were isolated, unexpectedly showing cross-resistance to BDQ. Furthermore, genomic analysis revealed that mutations in the transcriptional regulator, Rv0678, were shown to be responsible for the concomitant upregulation of the efflux pump, MmpL5, and thus were responsible for BDQ resistance [150]. Additionally, other studies have confirmed the role of the MmpS5-MmpL5 efflux system thiacetazones, azoles, and imidazo[1,2-*b*][1,2,4,5]tetrazines, and spiroketal indolyl Mannich bases [151, 152].

Overexpression of genes and transporters

The T28 *erm*(41) gene, positive regulatory gene, *whiB7*, and efflux genes, including *MAB_2355c*, *MAB_1409c*, and *MAB_1846*, are responsible for enhanced clarithromycin resistance [153]. Notably, *MAB_2355c* belongs to the ABC-F family protein and is involved in ribosome protection. *MAB_2355c*-deleted strains have increased sensitivity to macrolide [154]. In addition, *M. abscessus* strains exposed to clarithromycin had efflux pump gene (*MAB_3142* and *MAB_1409*) overexpression. However, verapamil increases clarithromycin susceptibility to *M. abscessus* by 4- to ≥64-fold. The above finding suggests that T28 *erm*(41) polymorphisms and overexpressed efflux genes are responsible for macrolide resistance in *M. abscessus* subsp. *abscessus* and *bolletii* [155].

Rodrigues et al. exposed *M. bovis* BCG to INH to induce INH-resistant strains, which demonstrated an increased extrusion activity and enhanced expression of *efpA*, *mmpL7*, *mmr*, *p55*, and the Tap-like gene, *Rv1258c*. Notably, EPIs (chlorpromazine, thioridazine, and verapamil) decreased INH resistance in INH-induced resistance [156]. Overexpression of *jefA* (Rv2459) in *M.tb* showed resistance to INH and EMB [157].

Overexpression of genes in *Mycobacterium*, including *Rv0191*, *Rv3756c*, *Rv3008*, and *Rv1667c*, are responsible for resistance to PZA and pyrazinoic acid. Notably, reserpine, piperine, and verapamil increase *M.tb* strain susceptibility to PZA, having overexpressed *Rv0191*, *Rv3756c*, *Rv3008*, and *Rv1667c* [158]. Zimic et al. determined the vulnerability of *M.tb* and *M. smegmatis* based on the efflux of pyrazinoic acid. Zimic et al. reported the efflux of pyrazinoic acid was within the range and resistant *M.tb* strains exhibited below this range. Also, pyrazinoic acid efflux was higher in *M. smegmatis* than *M.tb* [159].

Caleffi-Ferracioli et al. exposed *M.tb* H37Rv to RIF, verapamil, and RIF + verapamil for 16 h and 72 h. Expression of efflux genes was determined after 72 h, which included *Rv1456*, *Rv3065*, *Rv1458*, *Rv1457*, *Rv2846*, *Rv1258*, *Rv2942*, *Rv1217*, *Rv2459* and *Rv1410* for RIF, *Rv3065*, *Rv1457*, and *Rv1410* for verapamil, and *Rv1457*, *Rv2846*, *Rv1258* and *Rv2942* for RIF + verapamil. Treatment with RIF + verapamil lowered all the efflux pump genes, except

for *Rv2846*, compared to RIF. Additionally, combining these drugs leads to cumulative morphologic changes (wrinkles and rounding) to *M.tb* [160].

In another study, Narang et al. evaluated the role of MmpL2 (Rv0507), MmpL5 (Rv0676c), *Rv0194*, and *Rv1250* in RIF-resistant *M.tb*. The RIF-resistant strain had an *Rv0194* induction of 50%, *mmpL5* induction of 44%, *Rv1250* induction of 25%, and *mmpL2* induction of 12.5%. RIF-susceptible isolates exhibited *Rv0194* induction of 18%, *mmpL5* induction of 9%, *Rv1250* induction of 18%, and *mmpL2* induction of 9%. This study found There was no combination role for *Rv0194*, *MmpL2*, *MmpL5*, and *Rv1250* genes in RIF resistance. However, the RIF-resistant isolates had more *Rv0194* and *mmpL5* overexpression than RIF-susceptible isolates [161].

THP-1 infected with *M.tb* H37Rv were treated with RIF and verapamil alone and in combination. RIF exposure led to enhanced expression of efflux pump genes in the SMR, ABC, and MFS families. In association with verapamil, RIF led to downregulation of eight efflux genes, which suggests that verapamil exhibits efflux pump inhibitory activity inside the macrophage and thus could be beneficial in treating TB in humans [162].

Li et al. reported that RIF-monoresistant *M.tb* isolates had mutations in *rpoB* overexpressed with one or two of efflux pump genes, such as *Rv2333*, *rrrB*, *rrrC*, *Rv0842*, *bacA*, and *efpA*. Notably, CCCP, VP, TZ, and CPZ modulated RIF activity in clinical strains [163].

Machado et al. investigated the relationship between mutations and the role of efflux pump genes in drug resistance. After exposure of anti-TB drugs to *M.tb* isolates, the efflux pump genes (*mmr*, *mmpL7*, *Rv1258c*, *p55*, and *efpA*) were shown to be overexpressed. Additionally, these strains had mutations, including C-15T (*inhA* prom), S94A (*inhA* ORF), I194T (*inhA* ORF), S315T (*katG*), D735A (*katG*), S315T (*katG*), S531L (*rpoB* RRDR), D516Y (*rpoB* RRDR), A1401G (*rrs* 1400 region), wt/A1401G* (*rrs* 1400 region), G-10A (*eis* prom), ins GT at POS 755/756 (*tly*), D94N (*gyrA* QRDR), A90V (*gyrA* QRDR), and S91P (*gyrA* QRDR). Efflux of the substrates was reduced using EPIs. However, these EPIs were not able to reverse antibiotic resistance [164]. Therefore, the level of drug resistance seen in these strains resulted from efflux activity and a mutation in a target gene for drug resistance [164]. Figure 4 depicts the *Mycobacterium* membrane and efflux pumps. The efflux pump superfamily and the efflux pumps with substrate and inhibitors are listed in Table 5.

EPIs

EPIs target bacterial efflux proteins to prevent the extrusion of substrates from the bacterial membrane. When used in combination with antibiotics, EPIs offer a promising strategy to combat efflux pump activity. The benefits of EPIs include the following: (i) inhibiting the efflux of anti-TB drugs, thereby reversing drug resistance in *M.tb*; (ii) producing synergistic effects with anti-TB medications; and (iii) reducing the required dosage of the drugs [165, 166].

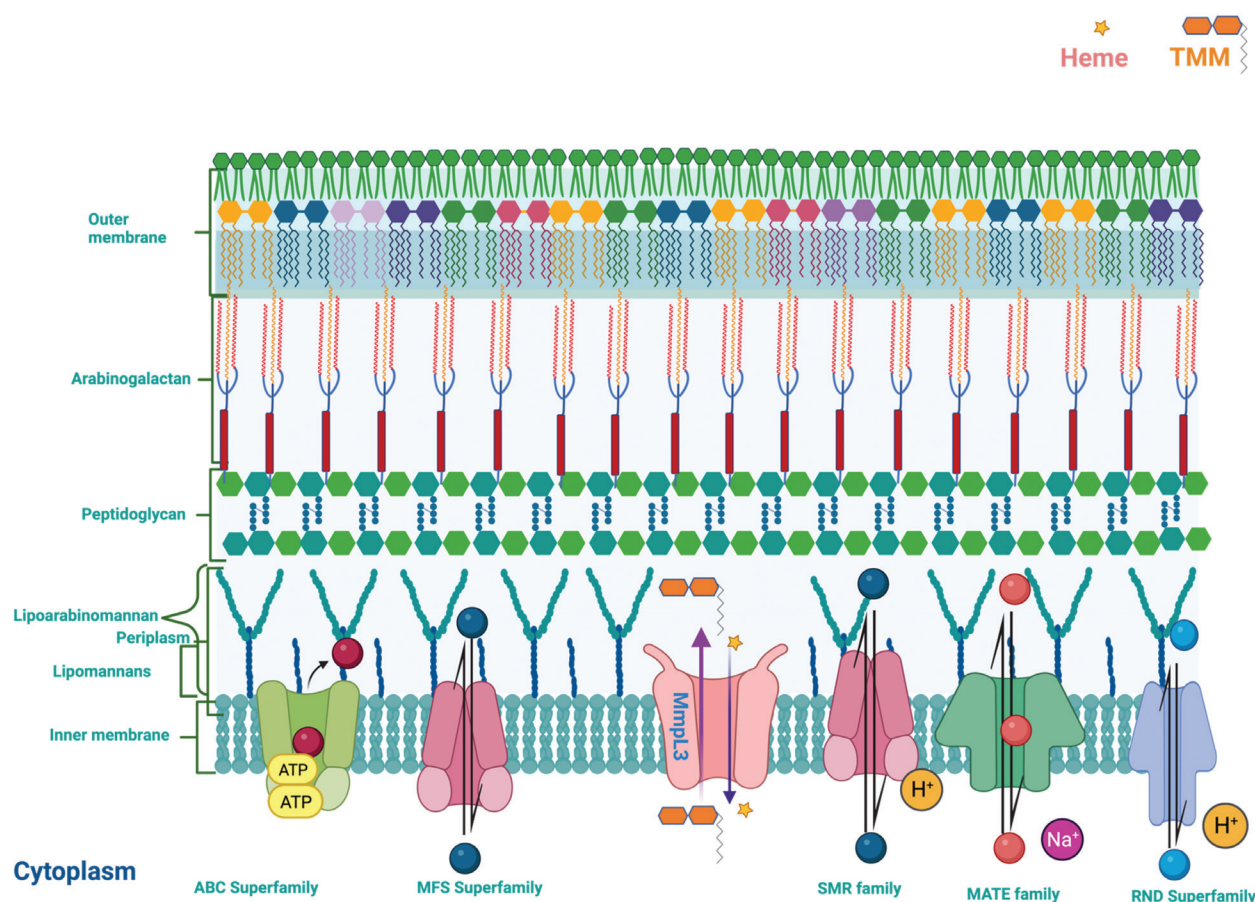


Figure 4 A schematic representation depicting the *Mycobacterium* membrane and the efflux pump families.

Natural products and analogs acting as *Mycobacterium* efflux pump inhibitors

Piperine (**1**) is an alkaloid that exhibits moderate antimycobacterial activity against *M.tb* H37Rv with an MIC value of 128 µg/mL. Immunomodulatory activity, and in combination with rifampicin, improved efficacy and decreased bacterial loads in the lungs of infected mice were shown [167]. Moreover, piperine showed inhibitory activity against Rv1258c, a putative multidrug efflux pump of *M.tb*. Piperine reduced the MIC and the mutation prevention rifampicin concentration in *M.tb* H37Rv and MDR *M.tb* clinical isolates. Furthermore, piperine enhanced the bactericidal activity of rifampicin and significantly extended the post-antibiotic effect [168]. In another study, piperine showed synergistic activity with streptomycin against *M.tb* H37Rv [169].

Farnesol (**2** [32 µg/mL]), in combination with ethidium bromide (EtBr), modulates farnesol (**2**) activity, reduces the MIC by 4-fold, and enhances the accumulation of EtBr in *M. smegmatis*. Moreover, farnesol (**2**) exhibits synergism with RIF against *M. smegmatis* [170].

Reserpine (**3**; **Figure 5**) has anti-mycobacterial activity against *M.tb* H37Rv with an MIC value of 128 µg/mL [171]. Reserpine inhibits the efflux pump of *M.tb* and increases the intracellular concentration of the ciprofloxacin (MIC value of 4 µg/mL against MDR *M.tb*) [171]. The reserpine mode

of action reduces drug excretion and decreases the energy supply through ATP hydrolysis. Reserpine increases the intracellular concentration of ciprofloxacin in *M.tb* and susceptibility to bacilli [172].

Gröblacher et al. isolated diarylheptanoid scaffold compounds, including trans,trans-1,7-diphenylhepta-4,6-dien-3-one (**4**; **Figure 5**), (5R)-trans-1,7-diphenyl-5-hydroxyhept-6-en-3-one (**5**; **Figure 5**), (3S,5S)-trans-1,7-diphenylhept-1-ene-3,5-diol (**6**; **Figure 5**), and in the flavonoid category, pinocembrin (**7**; **Figure 5**), from *Alpinia katsumadai* belonging to family Zingiberaceae [173]. The identified compoundsefflux pump inhibitory and anti-mycobacterial activities were assessed in *M. smegmatis* mc² 155. Compounds (**4–7**) inhibited *M. smegmatis* mc² 155 with MIC values of ≥128, 64, ≥128, and 128 mg/L, respectively. Compound **4** (64 mg/L), **5** (32 mg/L), **6** (64 mg/L), and **7** (64 mg/L) modulated RIF activity by 4- to 8-, 4-, 4-, and 2-fold, EMB activity by 1-, 1- to 2-, 2-, and 2- to 4-fold, INH by 1-, 1-, 2-, and 1-fold, ciprofloxacin activity by 1-, 2-, 2-, and 1- to 2-fold, and EtBr activity by 4-, 8-, 8-, and 4-fold, respectively [173]. Although diarylheptanoids and pinocembrin exhibited weak anti-mycobacterial activity, these compounds accumulated EtBr and decreased the RIF MIC. Thus, the diarylheptanoids class of compounds could be developed to reverse anti-mycobacterial drug resistance. Furthermore, compounds **4–7** should be evaluated in other *Mycobacterium* spp. and *in vivo* models of TB infection. Also, the safety should be considered in the cell model of TB infection [173].

Table 5 Efflux Pump Transporter Family and Efflux Pumps with their Substrate and known Inhibitors

Efflux Pump Transporter Family	Efflux Pump	Antibiotic (substrates)	Efflux Pump Inhibitors	Ref.
ABC superfamily	Rv2686c/87c/88c	Floroquinolones (ciprofloxacin, norfloxacin, moxifloxacin, and sparfloxacin)	Carbonyl cyanide m-chlorophenyl hydrazone, reserpine, and verapamil	[279]
	MSMEG_3762/63/65	Rifampicin and ciprofloxacin		
	drvA/drrB	Rifampicin and isoniazid	Reserpin and, verapamil	[130]
	Rv1473	Roxithromycin, erythromycin, and azithromycin	Carbonyl cyanide m-chlorophenyl hydrazone, reserpine, an verapamil	[280]
	Rv1218c	Isoniazid, rifampicin, moxifloxacin, linezolid, bedaquiline, PA-824, and lofazimine	Piperine and 1-(1-naphthylmethyl)-piperazine	[129]
				[132]
	Rv1217c	Rifampicin	Piperine ane 1-(1-naphthylmethyl)-piperazine	[131]
	Rv1218c	Isoniazid	Piperine and 1-(1-naphthylmethyl)-piperazine	[131]
	MAV_3306	Azithromycin		[281]
	Rv0191	Pyrazinamide	Reserpine, verapamil, and piperine	[158]
	Rv1667c	Pyrazinamide	Reserpine, verapamil, and piperine	[158]
	Rv3756c	Pyrazinamide and pyrazinoic acid	Reserpine, verapamil, an piperine	[158, 159]
	Rv0194	RIF	Reserpine and verapamil	[161]
	MAV_1406	Azithromycin		[281]
	Rv0849			
MFS superfamily	Rv0191	Pyrazinamide, pyrazinoic acid, and chloramphenicol	Reserpine, verapamil, and piperine	[139]
	Rv0783			[156, 167]
	Rv0849			[156, 167, 282]
	Rv1258c	Rifampicin, isoniazid, and kanamycin		[133, 156, 167, 282]
			Piperine and verapamil	
				[121]
	Rv1634	Floroquinolones		[121]
	Rv2333c	Tetracyclines		[121]
	Rv2459	Isoniazid,	Verapamil	[121, 157, 160]
	Rv2846c			[121]
SMR	Rv2994			[121]
	Rv3008	Pyrazinamide and pyrazinoic acid		[158]
	Rv3728			[121]
	EfpA		Verapamil	[156]
	IfrA			[283]
RND superfamily	Rv3065	Safranin O, acriflavine, and ethidium bromide	Verapamil and carbonyl cyanide m-chlorophenyl hydrazone	[123]
	Mmr	Ethidium bromide, acriflavine, and safranin O	Verapamil and carbonyl cyanide m-chlorophenyl hydrazone	[145]
	MmpS5/MmpL5 (Rv0678)	Azoles, clofazimine, bedaquiline, ionophores isoniazid, and rifampicin,	BM212, SQ109, indolcarboxamides, THPPs, and adamantyl ureas	[125]
	MmpL7	Isoniazid		
	Rv2942	Rifampicin	Carbonyl cyanide m-chlorophenyl hydrazone, and reserpine	[284]
			Verapamil	[160]

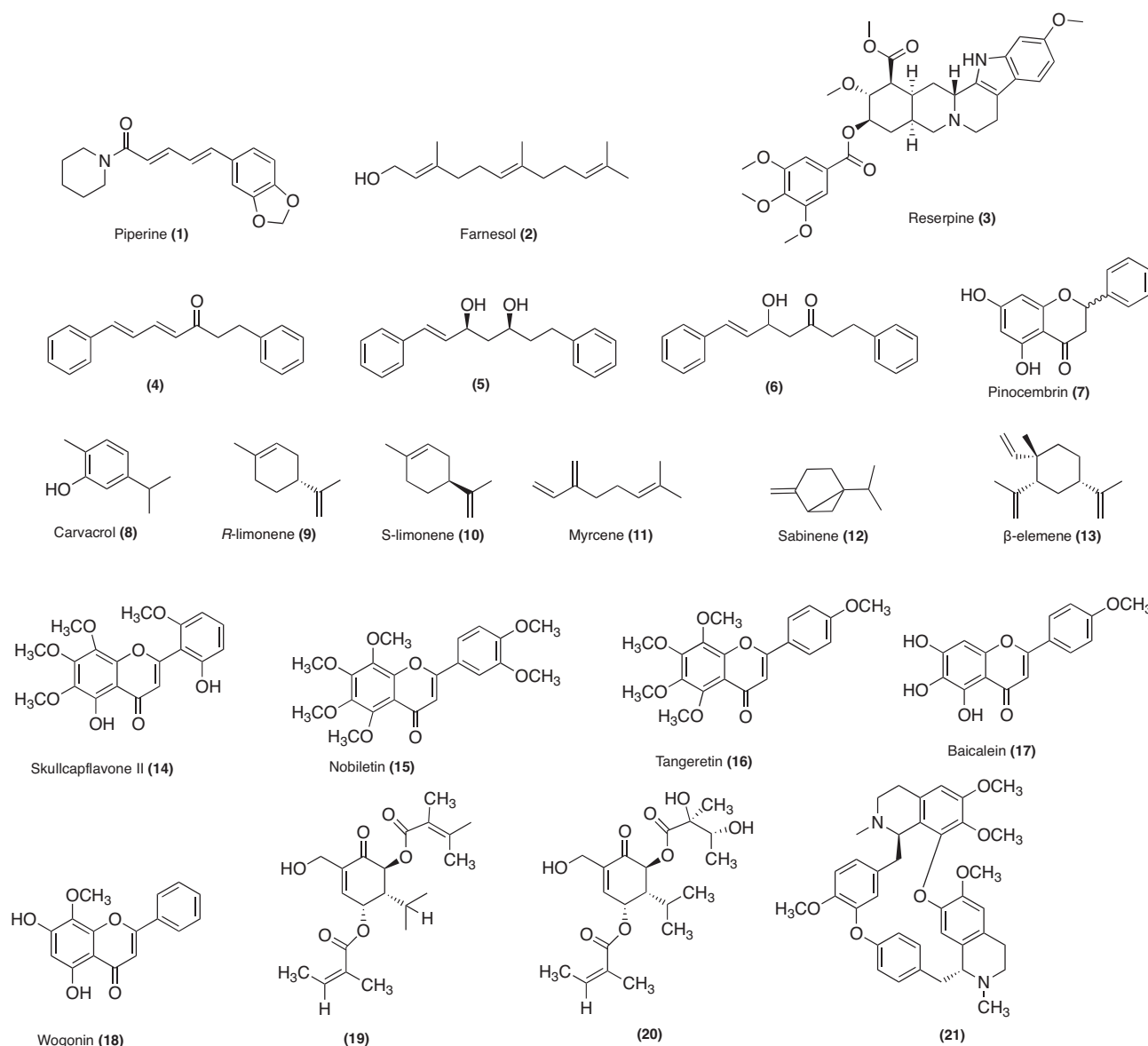


Figure 5 Chemical structures of natural products as EPIs.

Nakamura de Vasconcelos et al. evaluated carvacrol (**8**; **Figure 5**) and its derivatives for efflux pump inhibitory activity. Compound **8** promoted the accumulation of EtBr in *M.tb* H37Rv, which exhibits a relative final fluorescence (RFF) of 0.38 (similar to verapamil with an RFF of 0.20). Carvacrol displayed anti-mycobacterial activity against *M.tb* H37Rv with an MIC value of 76 $\mu\text{g/mL}$. Carvacrol demonstrated synergistic activity with RIF. However, carvacrol derivatives showed no anti-mycobacterial activity [174]. Additionally, compound **8** demonstrated EPI activity in several strains, including *Salmonella enteritidis* 1129 strains, *S. typhimurium* LT2 DT104, *E. coli* ATCC^R 35218, and *Staphylococcus aureus* ATCC^R 25923 [175]. Sieniawska et al. evaluated natural terpenes (*R*-limonene [**9**; **Figure 5**], *S*-limonene [**10**; **Figure 5**], myrcene (**11**; **Figure 5**), sabinene (**12**; **Figure 5**), and β -elemene (**13**; **Figure 5**) in combination with first-line anti-TB drugs [176]. compounds **9–13** showed anti-mycobacterial activity against *M.tb* with MIC values of 64, 64, 32, 32, and 2 $\mu\text{g/mL}$. Of all the compounds examined, compound **10** showed the most synergistic efficacy against

M. tb with all first-line anti-TB drugs. Compound **10** modulated the EMB MIC from 16 to 0.475 $\mu\text{g/mL}$, the RIF MIC from 16 to 0.237 $\mu\text{g/mL}$, and the INH MIC from 32 to 0.475 $\mu\text{g/mL}$. Compounds **9** and **11–13**, and **12**, in association with first-line antibiotics, modulated the antibiotic MIC in *M.tb* (from 3.9 to 0.475 $\mu\text{g/mL}$ for EMB, 15 to 0.475 $\mu\text{g/mL}$ for INH, and 0.475 to 0.237 $\mu\text{g/mL}$ for RIF). However, α -pinene in combination with EMB and INH showed the opposite effect in *M.tb* and increased MIC values from 16 to 125 $\mu\text{g/mL}$ for EMB and from 32 to 125 $\mu\text{g/mL}$ for INH [176].

Solnier et al. evaluated flavonoids (skullcapflavone II [**14**; **Figure 5**], nobiletin [**15**; **Figure 5**], tangeretin [**16**; **Figure 5**], baicalein [**17**; **Figure 5**], and wogonin [**18**; **Figure 5**]) against *M. smegmatis* mc² 155, *M. aurum* ATCC 23366, and *M. bovis* BCG ATCC 35734 for anti-mycobacterial and efflux pump inhibition activity [177]. The anti-mycobacterial activity results indicated that compounds **15–18** had an MIC₉₉ value $\leq$ 31.25 mg/L against *M. aurum* and compound **14** had an MIC₉₉ value of 7.8 mg/L. Compounds **14**, **15**, and **18** had anti-mycobacterial activity against *M. smegmatis*

mc² 155 with an MIC₉₉ value ≤128 mg/L and compound **17** inhibits *M. smegmatis* mc² 155 with an MIC₉₉ value of 31.25 mg/L. Compounds **14–18** had activities against *M. bovis* BCG ATCC 35734 with MIC₉₉ values of 31.25, 62.5, 31.25, 250, and 500 mg/L, respectively. Among these flavonoids, compounds **14** (32 mg/L), **15** (32 mg/L), **16** (32 mg/L), **17** (8 mg/L), and **18** (32 mg/L) modulated the activity of EtBr and RIF in *M. smegmatis* mc² 155 with modulation factors of 128 and 8, 2 and 32, 2 and 32, 8 and 32, and 4 and 16, respectively. These results suggested that flavonoids could be used as adjuvants in combination with RIF to reverse drug resistance [177].

Seven carvotacetones were extracted from *Sphaeranthus africanus* by Tran et al. and screened against the mycobacterial model strains *Mycobacterium smegmatis* mc² 155, *M. aurum* ATCC 23366, and *M. bovis* BCG ATCC 35734 for determination of anti-mycobacterial and EPI activities [178]. All carvotacetones exhibited moderate anti-mycobacterial activity against *M. smegmatis* mc² 155, ranging between 32 and 128 µg/mL. Representative carvotacetones (compound **19**; Figure 5) and (compound **20**; Figure 5) strongly modulated the activity of RIF and EtBr in *M. smegmatis* mc² 155. Compound **19** (at 1 and 16 mg/L concentrations) modulated EtBr activity by 8-fold and RIF activity by 2-fold in *M. smegmatis* mc² 155. Compound **20** (1 mg/mL) was only able to modulate EtBr activity 8-fold in *M. smegmatis* mc² 155. Moreover, compounds **19** and **20** inhibited *M. aurum* ATCC 23366 with MIC values of 85.9 and 78.5 µM and *M. bovis* BCG ATCC 35734 with MIC values of 42.9 and 78.5 µM, respectively [178].

Stephania tetrandra S. Moore's root contains tetrandrine (compound **21**; Figure 5). Tetrandrine is used in traditional Chinese medicine to treat angina pectoris, cardiac arrhythmia, and hypertension. Tetrandrine has calcium channel antagonist properties and interacts with the slowly gated K (Ca²⁺) channel and the voltage-activated L- and T-type Ca²⁺ channels with variable degrees of specificity and affinity [179]. Zhang et al. evaluated compound **21** against 200 isolates resistant to isoniazid and ethambutol. Incubation of compound **21** with *M.tb* strains (resistant to INH and EMB) showed no activity against growth at 30 µg/mL. Compound **21**, in association with EMB, modulated EMB activity and reduced the MIC by > 64-fold from 80 µg/mL to 1.25 µg/mL. Also, Compound **21** reduced the MIC of INH by > 32-fold from 8 µg/mL to 0.25 µg/mL. Notably, compound **21** showed a reversal in MIC in 82% resistant *M.tb* strains, which was more potent than verapamil. This finding suggested that compound **21** could be developed as an adjuvant in combination with first-line anti-TB drugs [180].

Synthetic compounds as *Mycobacterium* spp. efflux pump modulators

Proton influx into the cytosol is induced by the protonophore, CCCP (**22**; Figure 6). Compound **22** controls proton influx by generating electrochemical potentials from CCCP and CCCP⁻ anions. This process is not dependent on the

electrochemical H⁺ gradient. Furthermore, this action leads to disruption of energy sources in the membrane [181]. As a result of the diminished F₁F₀-ATP synthase activity, the principal active ABC transporters are also affected. All transport processes are hampered by a lack of energy, including importing and exporting nutrients through the cell membrane components. CCCP treatment decreases overexpression of the *mmpL7* gene and can reverse the resistance associated with the *mmpL7* gene [182].

Clinically, verapamil (**23**; Figure 6) alleviates heart conditions. Verapamil moderately inhibits *M.tb* H37Rv with an MIC value of 128 µg/mL. Verapamil enhances the effectiveness of several anti-tubercular drugs *in vivo* and *in vitro*. The cationic amphiphile nature disrupts the bacterial membrane and creates a stress response in *Mycobacterium* spp. [183]. The homo-tetrameric bacterial Ca²⁺ channel inhibits ATP-binding cassette transporters and Ca²⁺ selectivity filter from the intracellular side [160, 184]. Moreover, verapamil modulates the potency of anti-TB drugs. Moreover, in association with RIF, verapamil helps lower the fractional inhibitory concentration index (FICI), reduces viable cell counts, and decrease efflux pump genes [160]. Verapamil also regulates downregulation of efflux pump genes in *M.tb* inside the macrophages and enhances the accumulation of ethidium bromide in the cells [162, 185]. The BDQ and clofazimine MICs were lowered by verapamil against drug-susceptible and -resistant *M.tb* strains [186]. Additionally, verapamil increases systemic BDQ exposure, which boosts the effectiveness of BDQ in treating murine TB infection. In contrast, verapamil does not influence the oral bioavailability or effectiveness of clofazimine in mice [187]. Moreover, verapamil increases Q203 activity against *M.tb* *in vitro* and *ex vivo* in an unidentified overexpressed efflux pump [188, 189]. Verapamil and its analogues (compounds **24** and **25**; Figure 6) exhibit improved synergy with RIF and comparable anti-TB activity [190, 191].

Two lines of evidence indicate that the *M.tb* drug efflux pumps have a role in the drug tolerance induced by macrophages. In a virulent laboratory strain (CDC1551), the genetic knockdown of the MFS efflux pump Tap (Rv1258c) leads to loss of RIF tolerance, which is usually observed when *M.tb* infects macrophages. It has also been observed that drug tolerance induced by macrophages is inhibited by various structurally distinct drugs that are known to block bacterial efflux pumps [192]. Lake et al. reported that verapamil reduces *M.tb* RIF efflux and macrophage-induced RIF tolerance. Notably, verapamil and proton pump inhibitors (PPIs), including omeprazole, pantoprazole, lansoprazole, and lansoprazole sulfide, decrease RIF efflux, intramacrophage proliferation, and macrophage-induced tolerance. Most anti-TB drugs have shown macrophage-induced tolerance. It will be interesting to determine if anti-TB agents and PPIs reverse drug resistance and tolerance. Further, *in vivo* studies should be conducted to assess the translation value [193].

Initially, phenothiazines were identified as anti-psychotic, but scientists were subsequently curious about potential anti-mycobacterial effects [194]. According to the investigations, phenothiazines selectively block *M.tb* type II NADH dehydrogenases. As a result, phenothiazines hinder oxidative phosphorylation and the respiratory chain

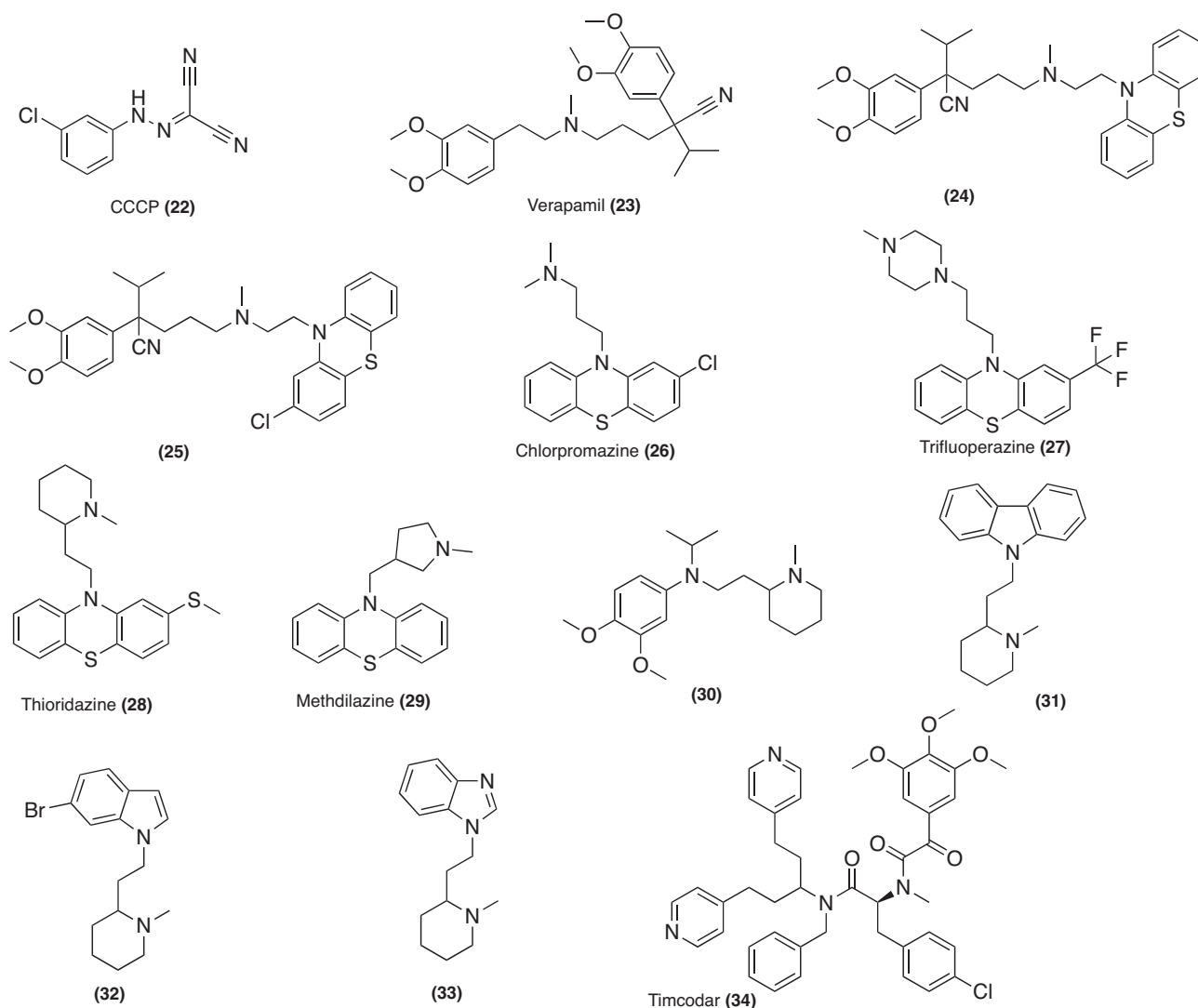


Figure 6 Chemical structures of synthetic compounds acting as EPIs.

electron transfer from NADH to menaquinone. PMF and ATP depletion are caused by respiratory chain disturbance. Additionally, thioridazine enhances cell wall permeability by changing plasma membrane composition [184]. Among phenothiazines, chlorpromazine (26), trifluoperazine (27), thioridazine (28), and methdilazine (29; Figure 6) were shown to have anti-mycobacterial activity [195]. Kumar et al. synthesised hybrid compounds (having verapamil and tricyclic chemo-sensitizer core of phenothiazines), which exhibited intrinsic anti-mycobacterial and intracellular activities in macrophages [196]. In free form, thioridazine (28) is toxic to cells and zebrafish embryos. The encapsulation of thioridazine in poly(lactic-co-glycolic) acid nanoparticles results in no toxicity. Combined with RIF nanoparticles, the encapsulated thioridazine showed a slight boost in the ability of macrophages to kill *M. bovis* BCG and *M.tb* [197]. Thioridazine exhibits bactericidal against *M. avium* by inhibiting intracellular growth at 2X MIC by blocking EtBr efflux [198].

A panel of drug-resistant *M.tb* was used to assess the mycobacterial potential of EPIs with ion channel blocking capabilities, such as verapamil, thioridazine, chlorpromazine, flupenthixol, and haloperidol. These compounds demonstrate

synergistic activity with INH and RIF and inhibit efflux pumps. In addition, these compounds in combination displayed activity against *M.tb*. The bactericidal action of compounds was due to blocking of ion channels, which decreased intracellular ATP levels. These substances also decreased the intracellular mycobacterial burden by activating lysosomal hydrolase and phagosome acidification. Therefore, ion channel inhibitors could be developed as adjuvants for treating TB caused by susceptible and resistant isolates [199].

Furthermore, Pieroni et al. synthesized hybrid scaffolds containing thioridazine, verapamil, and the representative synthesized compounds (30–33; Figure 6), demonstrated higher efflux inhibitory activity, less cytotoxicity, and a synergistic effect with anti-TB drugs *in vitro* and in intracellular macrophages [200]. Timcodar (34; Figure 6) showed weak inhibition against *M.tb* with an MIC value of 18.7 µg/mL. Synergistic activity with RIF, BDQ, and clofazimine was shown but not with other anti-TB agents. In addition, timcodar exhibited a 10-fold increase in growth inhibition against *M.tb* cultured in host macrophages and demonstrated synergy with RIF, moxifloxacin, and BDQ [201]. Moreover, the efficacy of RIF and INH was demonstrated in a mouse model of *M.tb* infection than each drug alone [163].

Li et al. reported lowering the RIF MIC in combination with EPIs (carbonyl cyanide *m*-chlorophenyl hydrazine, verapamil, thioridazine, and chlorpromazine) on RIF-monoresistant isolates (*rpoB*) overexpressed with one or two putative efflux pump genes (*Rv2333*, *rrrB*, *rrrC*, *Rv0842*, *bacA*, and *effA*) [163].

M. abscessus subsp. *abscessus* was exposed to antibiotics, such as amikacin, cefoxitin, clarithromycin, clofazimine, and tigecycline, for 24 h in another study. RNA sequencing was evaluated after antibiotic exposure. All five antibiotics (amikacin, cefoxitin, clarithromycin, clofazimine, and tigecycline) induced expression of one or more genes responsible for efflux transporter systems. In addition, antibiotics acting through ribosomes exhibited the most pronounced effects in expressing genes responsible for efflux pumps. A time-kill kinetics study showed that amikacin, cefoxitin, clarithromycin, clofazimine, and tigecycline in combination with EPIs (thioridazine and verapamil) had synergistic activity. One interesting observation from this study was that ribosome-targeting antibiotics resulted in pronounced efflux transporter expression. This finding suggested that antibiotics targeting ribosomes could be explored for expression of efflux pumps and possible efflux pump inhibition activity [202].

Thirty-one phenotypically and genotypically identified *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* clinical isolates were screened for BDQ activity in the presence and absence of verapamil and reserpine. Interestingly, verapamil reduced the BDQ MIC in all 100% isolates of *M. abscessus* subsp. by modulating BDQ activity from 4- to 32-fold and achieved maximum activity in *M. abscessus* subsp. *bolletii*. While reserpine reduced the BDQ MIC in all 54.8% isolates of *M. abscessus* subsp. and modulated the activity by 2- and 4-fold. This finding suggested that BDQ could be developed as combination therapy with verapamil as an adjuvant for treating *M. abscessus*-associated infections in humans [203].

Menichini et al. evaluated clarithromycin in combination with berberine or CCCP, piperine, or tetrandrine in 12 MAC clinical isolates. Mutations were present in domain V of the 23S RNA gene that affect the activity of EPIs on clarithromycin-resistant strains of MAC. Specifically, it was observed that the nucleotide change (A→C at position 2059) in strains, such as MAH 2587, MAH 2854, and MAH 2915, maintains a resistant phenotype despite a significant reduction in the MIC of clarithromycin when EPIs (berberine, CCCP, and piperine) are present. In contrast, strain MAH 1565 exhibited a point mutation at position 2058, with a nucleotide change from A→G, which restored sensitivity to clarithromycin in the presence of tetrandrine. Additionally, strain MI 2895 exhibited a nucleotide change (A→T), resulting in an intermediate phenotype when treated with berberine and tetrandrine. The overall finding suggested that on treatment with EPIs (berberine, CCCP, piperine, or tetrandrine) AC-resistant strain was made susceptible to clarithromycin [204].

Disulfides are obtained from *Allium stipitatum* and possess antibacterial properties. Disulfide analogues (35–37; Figure 7) showed anti-mycobacterial effects by suppressing the drug efflux pump and biofilm formation [205]. The category of synthetic compounds included tetrahydropyridine derivatives, which possess calcium channel regulators. With this

in view, Halicki et al. evaluated 2,2,2-trifluoro-1-(1,4,5,6-tetrahydropyridin-3-yl) ethanone (NUNL02) [compound 38; Figure 7] in combination with anti-TB drugs against *M.tb*-resistant strains. Compound 38 inhibited susceptible *M.tb* H37Rv and FURG-2 (MDR) with MIC values of 25 and 12.5 µg/mL, respectively. Also, compound 38 (1.56 µg/mL) in association with RIF reduced the RIF MIC by 16-fold (from 256 to 16 µg/mL). Consequently, compound 38 makes the MDR-TB strains more susceptible to RIF. Notably, compound 38 increases EtBr accumulation in the FURG-2 strain more than susceptible *M.tb* H37Rv and the RFF value was 4-fold higher than verapamil. Interestingly, compound 38 exhibited sterilizing activity against *M.tb* H37Rv and was non-toxic to VERO cells. Furthermore, compound 38 should be evaluated in combination with RIF and another first-line anti-TB antibiotic in an animal model of TB to assess compound 38 efficacy [206].

Biochanin A (39; Figure 7) belongs to isoflavone, which shows promising efflux pump inhibition in *M. smegmatis*. Cannalire et al. performed bioisosteric replacement by structural modification in biochanin A replacing oxygen with nitrogen [207]. In addition, the phenolic group was removed from the parent compound 39 and the 4'-methoxy group was substituted by a propyloxy, which resulted in the formation of a 2-phenylquinoline scaffold. Additionally, several 3-phenylquinoline derivatives were synthesized and evaluated for the ability to block the efflux pump in *M. smegmatis* mc² 155 and *M. avium*. The representative 3-phenyl quinoline derivatives (40; Figure 7) and (41; Figure 7) displayed the highest efflux pump activity. Compounds 40 and 41 modulated the activity of macrolides, including clarithromycin, erythromycin, azithromycin, and fluoroquinolones, such as ciprofloxacin and ofloxacin, and EtBr in *M. avium* strains. Importantly, modulation activity was better than standard verapamil, chlorpromazine, and thioridazine. Furthermore, preclinical and clinical studies are required to evaluate the safety and potency of 3-phenylquinoline to use as an adjuvant with antibiotics to reverse drug resistance in *M. avium*. This finding also suggested the combination of 3-phenylquinoline and FQs for treating *M.tb* infections in humans [207].

The 3-phenylquinolone compound class showed antibacterial activity against *M. smegmatis* and *M. avium*. 3-phenylquinolone also demonstrated activity against *S. aureus*, which overexpresses the NorA pump. Felicetti et al. modified the C6 and C7 positions of 3-phenylquinolone to improve the toxicity profile of the EPIs [208]. Representative compounds (42–44; Figure 7) showed synergistic activity with clarithromycin, ciprofloxacin, and EtBr at low concentrations against *M. avium*. A substantial synergistic action was demonstrated by compound 44 (4 µg/mL [1/32 MIC]) with clarithromycin, which also caused a 6-fold modulation in the MIC. In an *ex-vivo* animal harbouring *M. avium* 104 infections compound 44 demonstrated an 80% reduction in bacterial viability. Additionally, these compounds did not display toxicity to human macrophages. This finding suggested that these compounds can be developed as potential EPIs against *M. avium* [208].

Sen et al. synthesized quinazolinone analogs and evaluated quinazolinone analogs for efflux pump modulation activity in *M. smegmatis*. All quinazolinone analogs

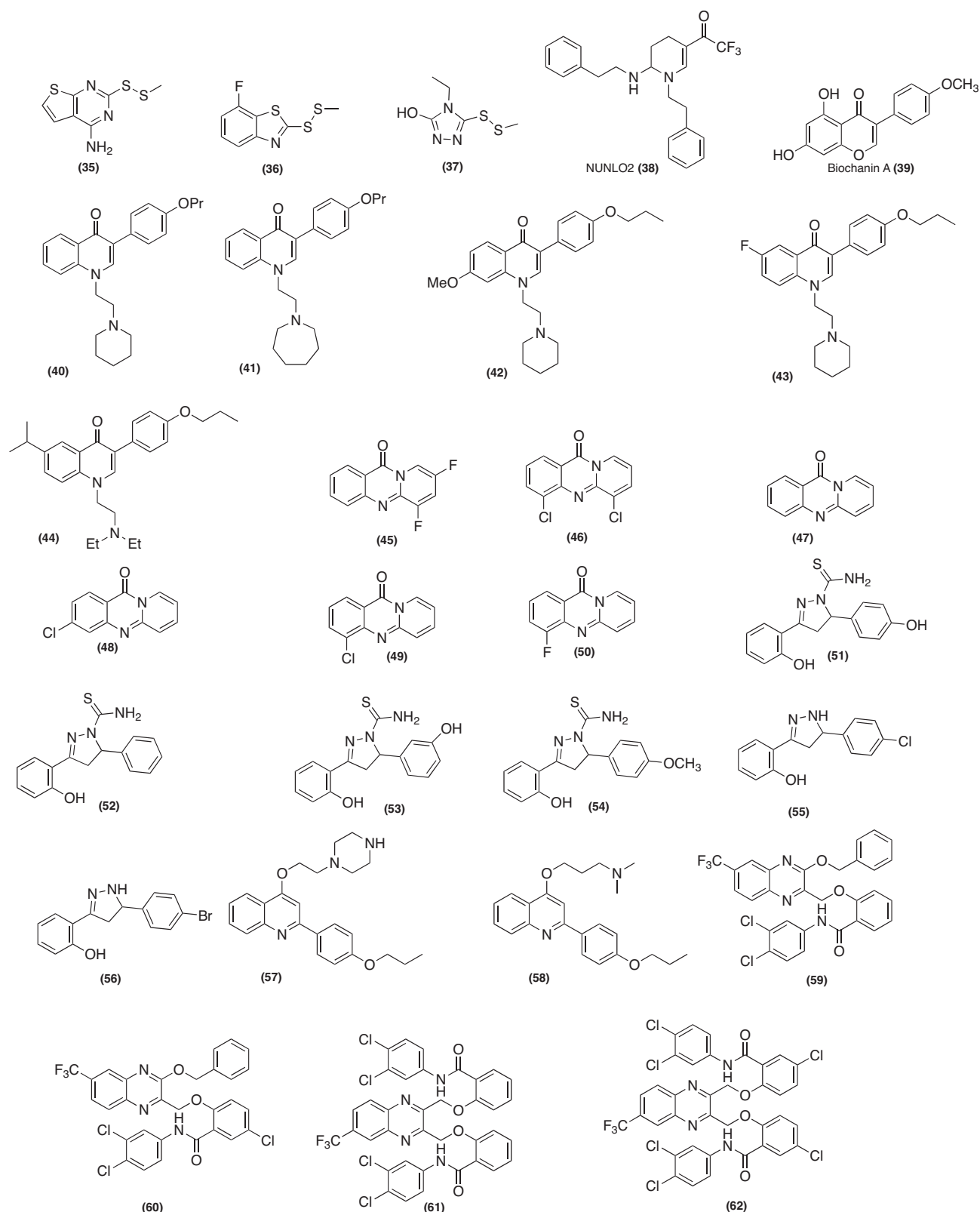


Figure 7 Chemical structures of synthetic compounds acting as EPIs.

(45–50; **Figure 7**) had a higher MIC value (200 µg/mL) against *M. smegmatis* [209]. Representative (compounds **45** [100 µg/mL] and **46** [100 µg/mL]) modulated EtBr activity in *M. smegmatis* by 16-fold. In contrast, compounds **47** (25 µg/mL), **48** (100 µg/mL), and **49** (25 µg/mL) modulated norfloxacin activity in *M. smegmatis* by a modulation factor of 8. In comparison, compound **50** (12.5 µg/mL)

showed the best modulation activity against *M. smegmatis* and modulated norfloxacin activity by a modulation factor of 16. Furthermore, these compounds exhibited the least moderate toxicity in a hepatocyte cell line (CC-1) based on a cell-based cytotoxicity assay [209]. Mycobactin analogs with diaryl-substituted pyrazoline were synthesized. Under iron-deprived conditions, compounds **51–54** (**Figure 7**)

Table 6 *Mycobacterium* Species Efflux Pump Inhibitors

Compounds	Modulation of antibiotic activity by efflux pump inhibition	Strain	Additional information	Ref.
Piperine	Piperine modulated the activity of RIF.	<i>M.tb</i> H37Rv	Piperine demonstrated synergistic activity with RIF and STR.	[167]
Farnesol	Farnesol modulated the activity of EtBr.	<i>M. smegmatis</i>	Farnesol exhibited synergism with RIF.	[170]
Reserpine	Reserpine modulated the activity of ciprofloxacin.	<i>M. tuberculosis</i>	Reserpine increases the intracellular concentration of ciprofloxacin in <i>M.tb.</i> and increases susceptibility to antibiotics.	[171, 172]
Compounds 4, 5, 6, and 7	Compounds 4, 5, 6, and 7 modulated the activity of RIF, EMB, and EtBr.	<i>M. smegmatis</i> mc ²		[173]
Carvacrol	Carvacrol modulated the activity of EtBr.	<i>M.tb</i> H37Rv	Carvacrol causes the accumulation activity of EtBr.	[174]
S-limonene	S-limonene modulated the activity of EMB, RIF, and INH.	<i>M. tuberculosis</i>		[176]
R-limonene, myrcene, sabinene, and β-elemene	R-limonene, myrcene, sabinene, and β-elemene modulated the activity of EMB, RIF, and INH.	<i>M. tuberculosis</i>		[176]
Skullcapflavone II, nobiletin, tangeretin, baicalein, and wogonin	Skullcapflavone II, nobiletin, tangeretin, baicalein, and wogonin modulated the activity of EMB RIF.	<i>M. smegmatis</i> mc ² 155		[177]
Carvotacetone	Carvotacetone modulated the activity of EMB and RIF.	<i>M. smegmatis</i> mc ² 155	Carvotacetone significantly inhibits <i>M. aurum</i> ATCC 23366 with an MIC value of 85.9 μM.	[177]
Tetrandrine	Tetrandrine modulated the activity of EMB and INH.	<i>M. tuberculosis</i>	Tetrandrine has calcium channel antagonist activity.	[178]
Verapamil	Verapamil modulated the activity of EtBr.	<i>M. tuberculosis</i>	In association with RIF, verapamil causes a decrease in the overexpression of the efflux pump gene in <i>M.tb.</i>	[182–184]
	Verapamil modulated the activity of BDQ.	<i>M. abscessus</i> subsp. <i>bolletii</i>		[186]
PPIs (omeprazole, pantoprazole, lansoprazole, and lansoprazole sulphide)	PPIs modulated the activity of RIF.	<i>M. tuberculosis</i>	PPIs reduced the RIF efflux.	[192]
Thioridazine	Thioridazine modulated the activity of EtBr.	<i>M. avium</i>	Thioridazine blocks the efflux of EtBr.	[183]
Timcodar	Timcodar modulated the activity of RIF and INH.	<i>M. tuberculosis</i>	Timcodar showed synergistic activity with RIF, moxifloxacin, and BDQ in a mouse model of <i>M.tb</i> infection.	[200]
2,2,2-trifluoro-1-(1,4,5,6-tetrahydropyridin-3-yl)	It modulated the activity of RIF.	<i>M.tb</i> H37Rv	2,2,2-trifluoro-1-(1,4,5,6-tetrahydropyridin-30yl) also increases the EtBr accumulation in the FURG-2 (MDR <i>M.tb</i>) strain.	[205]
Biochanin A and its derivatives 40 and 41.	These compounds modulated the activity of macrolides (clarithromycin, erythromycin, and azithromycin), fluoroquinolones (ciprofloxacin and ofloxacin), and EtBr.	<i>M.avium</i>		[206]
3-phenylquinolone derivative (44)	44 modulated the activity of clarithromycin.	<i>M. avium</i> 104		[207]
Quinazolinone analogs (45–50)	Quinazolinone analogs modulated the activity of EtBr and norfloxacin	<i>M. smegmatis</i>		[208]
Phenyl quinoline derivatives (54 and 55)	Phenyl quinoline derivatives modulated the activity of clarithromycin.	<i>M. smegmatis</i> mc2 155	54 showed synergistic activity with clarithromycin in an ex vivo macrophage model infected with <i>M. avium</i> 104.	[210, 211]
Quinoxaline derivatives (59–62)	Quinoxaline derivatives modulated the activity of azithromycin.	<i>M. abscessus</i>		[212]

exhibited activity against *M.tb* but were inactive against *M. smegmatis*. Notably, pyrazoline with diaryl acetyl failed to show any activity against *M.tb* and *M. smegmatis*. Pyrazoline (**55** and **56**; **Figure 7**) demonstrated activity in *M. smegmatis* and *M.tb* without any substitution at the N1 position. The mode of action for pyrazoline is MbtA inhibition and efflux pump inhibition in *M. smegmatis* [210].

The 2-phenyl quinoline derivatives (**57** and **58**; **Figure 7**) have NorA efflux pump inhibition activity [211, 212]. Compounds **57** and **58** displayed anti-mycobacterial activity against *M. smegmatis* mc² 155 and *M. avium* 104 with the same MIC value (32 µg/mL). All compounds (at a concentration of 1/2 MIC) increased the accumulation of EtBr in *M. smegmatis* and *M. avium*. The representative phenyl quinoline (**57** and **58**; **Figure 7**) showed potent efflux pump activity with RFF values of 5.91 and 5.84, respectively ($P < 0.01$). Compound **57** (1/2 MIC) modulates clarithromycin activity and decreases the MIC from 8 µg/mL to < 0.125 µg/mL (> 64 -fold reduction) and to 4 µg/mL (2-fold drop) at one-fourth the MIC against the *M. smegmatis* mc² 155 strain. In contrast, compound **58** decreases the half-MIC of CLA from 8 to 1 µg/mL (8-fold reduction) and alters clarithromycin activity. Furthermore, compound **57** shows potent EtBr efflux inhibitory activity against *M. avium* 104 (RFF of 1.23; $P < 0.01$) comparable to verapamil (RFF of 1.77; $P < 0.01$) and better than thioridazine and chlorpromazine. Compound **57** exhibits superior EtBr efflux inhibitory action compared to verapamil against the *M. avium* 104_{CLA3} strain with an RFF index of 2.11 ($P < 0.05$). Moreover, compound **57** (1/4th MIC) lowers the clarithromycin MIC against *M. avium* 104 by 8-fold. Simultaneously, MIC reductions of 32- and 16-fold were noted when erythromycin and azithromycin were combined with compound **57**. Additionally, compound **57** has synergistic activity with clarithromycin in an *ex vivo* macrophage model infected with *M. avium* 104 [212].

Quinoxaline derivatives exhibit a high MIC value (> 256 µg/mL) against *M. abscessus* and *M. simiae* [213]. Furthermore, these derivatives were assessed for EPI activity against *M. abscessus* and *M. simiae*. Compounds **59–62** (**Figure 7**) modulate azithromycin activity in *M. abscessus* by 8-, 8-, 8-, and 2-fold, respectively. In addition, compounds **59** and **62** modulate amikacin activity at a similar 2-fold value. Among these synthesized compounds, compound **59** was the most potent and able to modulate azithromycin and amikacin activity against *M. simiae*. The SAR study suggested that introducing the side chain and chlorine atoms to quinoxaline did not improve EPI activity of the compound. Instead, quinoxaline had deleterious effects on the efflux pump inhibition activity. The above finding is based on the *in vitro* studies. Furthermore, *in vivo* studies are needed to determine efficacy and safety [212]. The compounds with potential efflux pump inhibition activity are summarised in **Table 6**.

Conclusions and future perspective

The inappropriate use of antibiotics for non-microbial conditions in humans, animals, and plants has led to the concerning

emergence of MDR strains. In light of this challenge, one promising avenue to address antibiotic resistance is through the inhibition of efflux pumps. The use of EPIs as adjuvants alongside antibiotics has the potential to enhance effectiveness and broaden the antimicrobial spectrum, thereby restoring sensitivity and extending the duration of use. Moreover, incorporating EPIs into treatment protocols may also contribute to decelerating the development of antibiotic resistance. EPIs, like verapamil, reserpine, CCCP, piperine, and phenothiazines, have immense potential to be used as adjuvants because EPIs have shown great promise as adjuvants and exhibit potent activities in macrophages and *in vivo* mouse models of MDR infection. The robust efficacy in macrophages and *in vivo* mouse models of MDR *Mycobacterium* spp. infection underscores the potential to significantly enhance treatment strategies. Moreover, the use of adjuvants has demonstrated the potential to reduce the dose of existing antibiotics that are known to exhibit additional side effects, cytotoxicity, and low efficacy. Furthermore, to use EPIs as an adjuvant to existing antibiotics, in-depth biological studies are needed to develop treating *Mycobacterium* spp. diseases.

There are several technical challenges associated with the discovery of EPIs. One significant issue is the lack of available crystal structures for *Mycobacterium* spp. efflux pumps, as well as the absence of co-crystallized proteins with ligands. This limits our ability to conduct a thorough investigation of these proteins and the binding residues. To accelerate the identification of EPIs, it is essential to screen larger chemical libraries through high-throughput screening methods. These screenings should utilize *in silico* technologies and complementary *in vitro* and *in vivo* biological studies to develop effective EPIs against MDR strains.

Acknowledgements

GK thanks Director BITS Pilani for the research facilities. The authors also acknowledge the support of Dr. Nisha Rathor, Professor, Department of Microbiology, Nims Institute of Medical, and Dr. Suresh Kumar, Professor, Department of Humanity of Nims University Rajasthan, for their kind technical review of the manuscript.

Conflicts of Interest

The authors declare no competing financial interests.

Abbreviations

AhpC, alkyl hydroperoxide reductase; ABC, ATP-binding cassette; AMI, amikacin; Araf, D-arabinofuranose; CAP, capreomycin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; COPD, chronic obstructive pulmonary disease; CTAB, cetyltrimethylammonium bromide; DPA, decaprenylphosphoryl-β-D-arabinofuranose; DprE1,

decaprenylphosphoryl- β -D-ribose 2'-epimerase 1; DNA, deoxyribonucleic acid; EMB, ethambutol; EPIs, efflux pump inhibitors; ETH, ethionamide; ERDR, ETB resistance-determining region; FIC, fractional inhibitory concentration; FQs, fluoroquinolones; HGT, horizontal gene transfer; ICL, isocitrate lyase; INH, isoniazid; KAN, kanamycin; LAM, lipoarabinomannan; LZD, linezolid; MABA, microplate alamar blue assay; MAC, *Mycobacterium avium* complex; MATE, multidrug and toxic compound extrusion; MDR-TB, multidrug-resistant TB; MFS, major facilitator superfamily; MmpL, membrane proteins large; MmpS, mycobacterial small membrane protein; mRNA, messenger RNA; *M.sm*, *Mycobacterium smegmatis*; MTBC, *Mycobacterium*

tuberculosis complex; *M.tb*, *Mycobacterium tuberculosis*; MXF, moxifloxacin; NMP, 1-(1-naphthylmethyl)-piperazine; NTM, non-tuberculous mycobacteria; OM, outer membrane; PMF, proton-motive force; PPIs, proton pump inhibitors; PZA, pyrazinamide; QRDR, quinolone resistance determining region; RFF, relative final fluorescence; RIF, rifampicin; RDDR, RIF-resistance determining region; RNA, ribonucleic acid; RT-qPCR, quantitative real-time PCR; SMR, small multidrug resistance; RND, resistance-nodulation-division; SAR, structure-activity relationship; SNPs, single-nucleotide polymorphisms; STR, streptomycin; TB, tuberculosis; XDR TB, extensively drug-resistant

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