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Targeting mycobacterial efflux system to enhance tuberculosis therapy: Review article

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Abstract--The worldwide health epidemic of tuberculosis (TB) persists. It still claims millions of lives each year despite medical breakthroughs. Long-term therapies, drug-resistant TB strains, and co-infections with conditions like HIV necessitate novel approaches. The use of Mycobacterium tuberculosis (Mtb) efflux pumps, which actively expel drugs and decrease their effectiveness, is one such approach. EPIs (Efflux Pump Inhibitors) have the potential to treat tuberculosis. This review offers insights into Mtb's immune evasion mechanisms, including its strong cell wall, granuloma formation, immunological modulation, and dormancy, as well as TB epidemiology, worldwide eradication efforts, and those processes. Dissecting Efflux Pumps highlights their significance in drug resistance, particularly against first-line TB medications. Efflux pumps are notorious for causing antibiotic resistance. EPIs may have benefits such as increased drug efficacy, resistance reversal, shortened treatment durations, less toxicity, improved adherence, and targeting of latent TB. The effectiveness of various EPI classes, including phenylalkylamines, protonophores, phenothiazines, and plant-derived derivatives, against Mtb growth and efflux pumps is evaluated. The creation of effective EPIs, a clearer understanding of efflux pump control, and essential clinical trials for validation remain obstacles.

Keywords---drug resistance, efflux pump inhibitors, efflux Pump, MDR, mycobacterium tuberculosis.

1 Introduction

In the past, tuberculosis (TB) was commonly referred to as the "white plague" and the "captain of death." Even today, tuberculosis remains a significant contributor to illness and mortality on a global scale. An estimated 10.4 million people had active TB in 2015, which led to 1.8 million fatalities. By 2019, these numbers had reached 1.4 million deaths worldwide, solidifying its position as the leading infectious disease-related cause of death globally for that year. While the WHO Global Tuberculosis Report for 2020 officially records 7.1 million new tuberculosis diagnoses, it suggests that the actual number might be closer to 10 million, acknowledging the likelihood of underreporting and underdiagnosis[1].

An estimated 504,000 individuals in India passed away from tuberculosis or TB in 2021. India bears the burden of over 25% of all the estimated TB cases globally. The United Nations has committed to This approach strives to eliminate the financial hardship faced by families affected by TB and aims to decrease TB incidence by 80% and TB-related deaths by 90%. India declared that it would work to eradicate tuberculosis in India by 2025, five years before the UN's deadline[2, 3]. There has been a steady increase in the number of new cases of tuberculosis diagnosed each year, with 206,030 cases confirmed to be multidrug-resistant (MDR-TB) or resistant to rifampicin. Even if the occurrence of tuberculosis has decreased worldwide, the disease continues to pose a substantial public health concern. This is attributed to various factors, including the lengthy and complex treatment regimens currently in use, the coexistence of other health issues such as HIV and diabetes, which exacerbate the TB problem, and the growth in drug-resistant forms of tuberculosis and its severe symptoms, such as TB meningitis. New medications and treatment regimens may improve results for TB patients. Just two medications, bedaquiline, and delamanid, have recently been brought to market, indicating that minimal progress has been made in the past few decades in the development of new anti-TB medications. Bedaquiline was the first novel anti-TB medication with a different mode of action in almost 40 years when it was licensed in 2012 as part of combination therapy for MDR-TB. However, efflux-mediated resistance has been observed in certain cases of bedaquiline recently[3, 4]. This underscores the urgent need to address the issue of efflux-mediated resistance to anti-TB drugs. However, no efflux pump inhibitors (EPIs) have been approved for clinical use to date. Therefore, there is a pressing need for action in this area. Researchers are increasingly recognizing the importance of understanding drug transport mechanisms to develop effective TB treatments, as a key obstacle in the treatment of tuberculosis is medication transfer across human and mycobacterial membranes[1, 2].

In order to increase treatment efficacy while new medications are discovered, utilizing drug transporter inhibitors in conjunction with existing Anti-TB treatments may be a successful strategy.

1.1 Tuberculosis

Tuberculosis, caused by *Mycobacterium tuberculosis* (*Mtb*), is an infectious disease. It predominantly impacts the respiratory system, often resulting in symptoms like persistent coughing, fever, and chest discomfort. But it may also

impact other organ systems, such as the liver, bones, genitourinary tract, gastrointestinal tract, lymph nodes, and central nervous system[5]. Respiratory droplets are a major route of TB transmission. To lessen the impact of TB on the general public's health, identified mycobacterium cases must be treated[4, 6]. Different types of Tuberculosis are depicted in Figure 1. Latent tuberculosis (LTB) and active tuberculosis are the two primary forms of the disease.

- 1) Latent TB: This type of TB is not contagious, and the person infected does not have any symptoms. The bacteria are in a dormant state, and the immune system can keep them under control. However, if the immune system weakens, the bacteria can become active, leading to active TB[4, 7].
- 2) Active TB: Active tuberculosis represents a widespread infection affecting multiple organs. Either a primary infection or the resurgence of latent TB can cause it. This form of TB is contagious and typically manifests with symptoms like persistent cough, weight loss, fever, and night sweats. This TB impacts the lungs, known as pulmonary TB, or other areas of the body, referred to as extra-pulmonary TB[4, 8].

Further Active TB can be of majorly four types[4].

- 1) Pulmonary TB: This form of active TB is the most prevalent. It has an impact on the lungs and can result in symptoms including a chronic cough, chest pain, fever, and weight loss.
- 2) Extra-pulmonary TB: Apart from the lungs, extra-pulmonary TB can affect various organs such as the lymph nodes, bones, joints, kidneys, and even the brain when it becomes an active infection. Symptoms can differ based on the specific organ involved.
- 3) Multi-drug-resistant TB (MDR-TB): MDR-TB, which stands for multidrug-resistant tuberculosis, represents a formidable variant of active TB. This form of the disease is resistant to the two most powerful first-line medications used for TB treatment, namely rifampicin and isoniazid.
- 4) Extensively drug-resistant TB (XDR-TB): Typically, rare and exceptionally grave sort of TB that is resistant to most of the antibiotics utilized to treat TB. XDR-TB requires a long and complex treatment regimen that is often less effective than regular TB treatment. A major characteristic of the disease is resistance to fluoroquinolone antibiotics and at least any one of the three second-line injectable drugs (amikacin, kanamycin, or capreomycin).

Extrapulmonary TB can be classified as[9]-

- 1) Lymphadenitis TB: It's a variation of extra-pulmonary tuberculosis that impacts the lymph nodes. It can cause painless swelling in the neck or other parts of the body.
- 2) Skeletal TB: This type of extra-pulmonary TB affects the bones and joints. It can cause bone pain, joint swelling, and limited mobility.
- 3) Miliary TB: This severe type of disease develops when the bacteria enter the bloodstream and travel throughout the body, where they cause numerous tiny lesions in different organs. Symptoms may include fever, weight loss, and night sweats.

- 4) Genitourinary TB: This type of extra-pulmonary TB affects the urinary tract and reproductive organs. Symptoms may include pain during urination, blood in the urine, and infertility.
- 5) Liver TB: This is a rare form of extra-pulmonary TB that affects the liver. It may result in symptoms like weight loss, jaundice, and stomach pain
- 6) Gastrointestinal TB: This type of extra-pulmonary TB affects the digestive system. It can cause abdominal pain, diarrhoea, and weight loss.
- 7) TB meningitis: This uncommon but dangerous extra-pulmonary TB infection damages the membranes that shield the brain and spinal cord. Headache, fever, and stiff neck may be symptoms.
- 8) TB peritonitis: The lining of the abdominal cavity is impacted by this kind of extra-pulmonary tuberculosis. It may result in ascites (abdominal fluid buildup), weight loss, and abdominal pain.
- 9) TB pericarditis: The lining around the heart is impacted by this uncommon but severe form of extra-pulmonary TB. It may result in symptoms like coughing, shortness of breath, and chest pain.
- 10) Cutaneous TB: This type of extra-pulmonary TB affects the skin. It can cause skin lesions, ulcers, and nodules. It is usually acquired through direct contact with an infected person or animal.

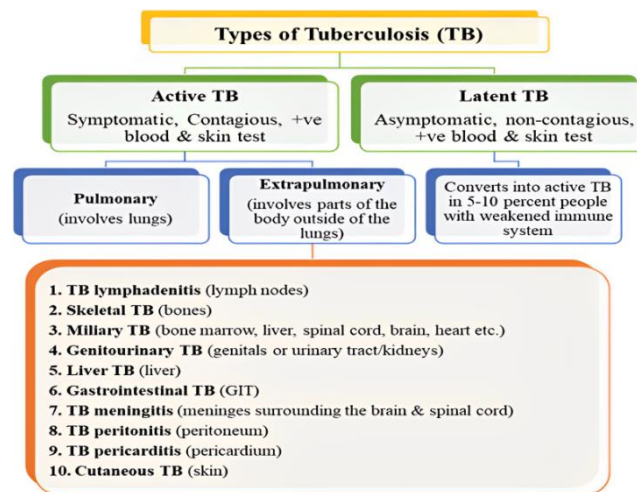


Figure 1. Types of Tuberculosis

Mycobacterium Tuberculosis

Mtb is a large, immobile, rod-shaped bacterium that relies on oxygen and thrives in aerobic conditions. It is usually found in the well-ventilated upper lobes of the lungs, often entering the body when droplet nuclei are inhaled. The bacterium lives inside macrophages as a facultative intracellular parasite, where it multiplies. They are expelled from dead macrophages into the alveolar environment as the bacteria proliferate. The immune system of the host determines the myco- bacterium's fate. A strong immune system may be able to eradicate the bacteria, but exposure can potentially cause LTBI or even primary TB. Successful TB containment is facilitated by the cellular immune system[9, 10].Helper T cells are involved in facilitating this response. T cells and

macrophages have the capacity to form granulomas, which contain a central region of necrotic cells and are encircled by lymphocytes and macrophages. This process hinders the proliferation and dissemination of mycobacteria[1, 9].

1.2 History of Tuberculosis

In recent years, there has been considerable interest in determining the source of *M. tuberculosis*, the bacterium responsible for tuberculosis (TB). TB can present itself in various forms, including one that affects the bones, leading to skeletal deformities, which will be explored in the upcoming section. Notably, skeletons from ancient Egypt often exhibit evident signs of tubercular abnormalities, suggesting the prevalence of the disease in that region[10]. In the seventh century B.C., Assyrian clay tablets documented cases of individuals experiencing symptoms resembling tuberculosis. Some theories suggest that TB might have been introduced to Europe through the movement of Indo-European cattle herders who contracted the disease from infected livestock. An illustrative case of how enhanced living conditions and sanitation measures led to a decrease in TB-related deaths during the latter part of the 19th century is the urban redevelopment of Paris in the 1850s[10, 11]. Due to the implementation of enhanced public health programs and the widespread administration of the *M. bovis* BCG vaccination, tuberculosis (TB) rates of illness and death experienced a gradual decline throughout the 20th century in industrialized nations. However, this positive trend was interrupted in the mid-1980s, when the incidence of new TB cases started to increase.

This "mini epidemic" of new TB cases has been reversed since AIDS first emerged, with its catastrophic breakdown of the cell-mediated immune response in coinfecting individuals [8, 10, 12-14].

In an effort to comprehend the disease's genesis, medical professionals and researchers have described TB in all of its manifestations. Aristotle emphasized the disease's contagious aspect, although Hippocrates believed it was mostly inherited. Conversely, medical practitioners and experts in Northern countries tended to favor constitutional or genetic factors as potential causes of tuberculosis. The 19th century saw the height of this philosophical divide [1, 12, 15]. TB was definitively caused by a bacteria by the time Robert Koch published a report on it 17 years later. The French medical community, notably Herman Pidoux, vigorously contested the findings, asserting that the solution to the tuberculosis issue required more "contemporary" and societal reforms. Thoughts on TB's social causes persisted throughout the early 20th century. An intriguing and educational review has been written about the Trudeau Institute's history of TB research and treatment. The advent of antibiotics in the 1940s with the discovery of streptomycin did not completely eradicate the illness. Despite the widespread adoption of BCG, an attenuated vaccine derived from the progressive passage of a virulent *M. bovis* strain in Paris during the 1920s, the prevalence of tuberculosis has not seen a significant reduction [10, 12]. For the prevention and treatment of tuberculosis, new vaccinations and medications are required. Nonetheless, it's essential to continuously heed Dubos' cautions, which underscored the social dimension of tuberculosis.

1.3 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis, the microorganism responsible for tuberculosis, utilizes several strategies to avoid the host's immune defenses and successfully establish an infection. Here are some of the key mechanisms of action[1, 6]:

- 1) Cell wall composition: *Mycobacterium tuberculosis* possesses an intricate cell wall structure that makes it resistant to numerous common antibiotics and the immune-mediated reaction of the host. Mycolic acids are present in the cell wall, which are long, fatty acids that provide a hydrophobic barrier to the entry of antimicrobial agents and prevent the bacterium from being easily engulfed by immune cells.
- 2) Granuloma formation: *Mycobacterium tuberculosis* can evade the host immune response by forming granulomas, which are small nodules that surround the infected cells and contain the immune system's T cells and macrophages. The granulomas help to contain the infection and prevent the spread of the bacterium to other parts of the body
- 3) Immune system modulation: Numerous methods have been evolved by *Mycobacterium tuberculosis* to manipulate the host immune response in its favor. For instance, it may prevent host immune cells from producing reactive oxygen and nitrogen species, which are vital for killing bacteria. Additionally, it possesses the capability to inhibit the fusion of lysosomes with phagosomes, the areas where immune cells engulf and kill bacteria.
- 4) Dormancy and reactivation: *Mycobacterium tuberculosis* can enter a dormant state within the host, which makes it difficult to detect and treat. In this state, the bacterium is not actively replicating, but it is still viable and can reactivate later when conditions are favorable.

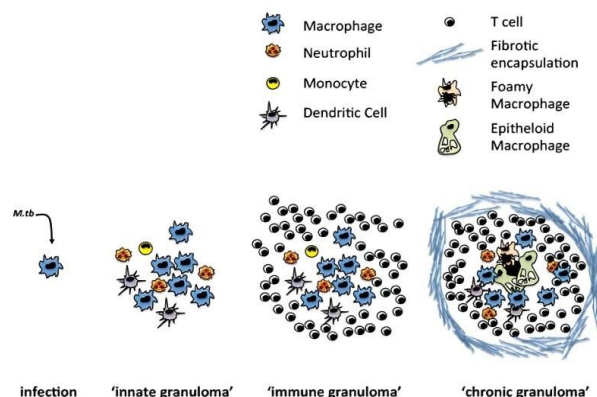


Figure 2- Granuloma Formation after tuberculosis infection

Overall, *Mycobacterium tuberculosis* is a complex bacterium with several mechanisms that enable it to evade the host immune response and establish chronic infection. Understanding the biology and pathogenesis of this bacterium is critical for developing effective treatments and strategies for controlling the spread of tuberculosis[1, 16].

Inhalation of *Mycobacterium tuberculosis* can result in one of three distinct outcomes: the elimination of the bacteria, the establishment of latent TB infection (LTBI), or the progression to active TB disease. It's worth noting that around one-third of the world's population has been infected with *M. tuberculosis*, often leading to asymptomatic LTBI. A 10% probability exists for LTBI patients to develop active TB, with the risk being higher for individuals with a compromised immune system.

Chronic coughing, blood in the sputum, fever, night sweats, weight loss, and exhaustion are all signs of pulmonary TB. The disease's severity is partially influenced by the host's immune response, and tuberculosis (TB) can also manifest in locations outside the lungs, referred to as extrapulmonary TB[6, 7]. The characteristic histopathological feature of TB is the granuloma, where infected and uninfected phagocytes surround bacteria, forming an expanded and interdigitated structure called "epithelioid". The granuloma's center is composed of dead phagocytes (dead bacteria) and a material called caseum. More recent research suggests that granuloma is more of a dynamic structure that "walls off" the infectious agent. Mycobacterial antigens are taken up by macrophages and presented to MHC class II to activate CD4+ T cells of the T-helper 1 type (Th1) that are specific for *M. tuberculosis*. These T cells fight illness by destroying infected macrophages and stimulating macrophages with cytokines like interferon γ (IFN- γ). Granulysin and perforin, which T cells use, can directly destroy mycobacteria[1, 16, 17].

Tuberculosis is a disease that requires a balance between an insufficient inflammatory response and an excessive inflammatory response. Granulomas play a pivotal role in controlling the proliferation of mycobacteria by facilitating the interaction between cytokines and chemokines, but dysregulation of the inflammatory response can lead to increased mycobacterial proliferation[1, 18, 19].

A gene called *lta4h*, which modulates TNF production by pro-inflammatory eicosanoids, has been shown to correlate with susceptibility to TB in humans. The lack or suppression of this gene stops macrophages' ability to inhibit intracellular proliferation, but uncontrolled activity can result in necrosis and the growth of bacteria.

2 The function of efflux pumps in resistance to antimicrobial drugs

Bacteria can counter the effects of antibiotic mixtures using a variety of reciprocal mechanisms. These include changing or eliminating the antibiotic, altering the antibiotic's target, reducing the antibiotic's membrane permeability, and overexpressing efflux pumps[13, 20]. Efflux is a common self-defense mechanism in both prokaryotic and eukaryotic cells. Extrusion of foreign substances and endogenous metabolic waste is a physiological mechanism that keeps regular cell function[7, 13, 21].

One class of transport proteins that helps move potentially hazardous substances from inside cells to the outside environment is the efflux pump protein. This class of protein includes almost all classes of therapeutically significant antibiotics[20,

22]. Gram-positive and gram-negative bacteria, along with eukaryotes, are known to contain efflux pump proteins. Antibiotics from different classes are examples of the structurally diverse molecules they can transport, or they can show selectivity for a specific substrate. Multidrug resistance (MDR) and these pumps may be related. Efflux pumps based on sequence homology, bacteria are grouped into five superfamilies. (Figure 3)

- 1) ATP-binding cassette superfamily (ABC) [23, 24].
- 2) Major facilitator superfamily (MFS) [8, 18, 25].
- 3) Multidrug and toxic compound extrusion family (MATE) [26].
- 4) Resistance-nodulation-cell-division superfamily (RND)[27].
- 5) Small multidrug resistance family (SMR) [28].

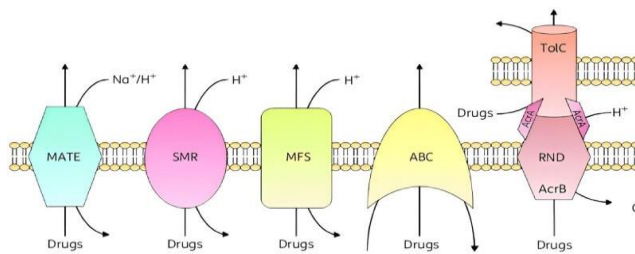


Figure 3. Efflux Pumps in Bacteria

These pumps belong to five families, and each family is involved in antibiotic resistance. The other systems rely on the proton motive force as their energy source, with the exception of the ABC family, which uses ATP hydrolysis to drive substrate export. [21, 29].

Efflux pump genes are frequently components of operons, and their expression is controlled by the regulatory gene. Resistance to the transported chemicals is linked to increased expression. For example, overexpression of *acrAB* in *E. coli* results in resistance to specific antibiotics and bile salts. Mutants may have efflux pump genes on plasmids, but This is because the bacteria has an innate mechanism that allows it to survive in harsh circumstances, such as the presence of antibiotics, thanks to the efflux pump genes on its chromosome[30].

These pumps most likely evolved to allow the removal of harmful substances from the bacteria, which allowed them to survive. In fact, it is generally acknowledged that the "intrinsic resistance" that distinguishes Gram-negative bacteria from Gram-positive bacteria with regard to resistance to certain antibiotics is caused by the efflux systems in place. [3, 13, 21, 31].

The broad spectrum of substrates that efflux systems can transport poses a challenge. Often, when a pump is overexpressed, it can lead to resistance against a range of substances, including dyes, detergents, disinfectants (including commonly used biocides), and antibiotics from various classes. Another concern is cross-resistance, where exposure to a single chemical from a pump's substrate profile may promote the overexpression of that pump, thereafter leading to cross-resistance against all other substrates of the pump[2, 13, 20, 30, 32].

Efflux pump inhibitors have been explored for their potential to augment the effectiveness of exported antibiotics. This method has been used by researchers to create inhibitors that lessen the effect of efflux pumps on the activity of fluoroquinolones[20].

It is expected that a single inhibitor substance could effectively target a range of efflux pumps found in different bacterial species due to the substantial structural similarity shared by many efflux pumps. Efflux pumps are involved in many different physiological processes by nature. For example, Rv1258c appears to be important during the stationary growth phase and is necessary for appropriate growth kinetics and cell shape. Rv1410c (P55) is associated with maintaining normal growth traits and the response to oxidative stress[8, 19, 33].

Other transporters, including the massive mycobacterial membrane proteins (MmpL), facilitate the transfer of lipids and stimulate the formation of cell walls. In addition to their main roles in the cell, the majority of efflux pumps can move a wide range of unrelated compounds. For instance, MmpL3 plays a role in exporting mycolates, a crucial component of the cell wall, while MmpL7 is responsible for exporting phthiocerol dimycocerosate, a lipid component of the outer membrane. For instance, Rv1217c-Rv1218c is connected to the efflux of different substances and resistance against biaryl piperazines, bisanilinopyrimidines, novobiocins, pyrazolones, pyridines, pyrroles, whereas Mmr is involved in resisting tetraphenylphosphonium, ethidium bromide, erythromycin, acriflavine, safranin O, pyronin Y, and cetyltrimethylammonium bromide[7]. Two strategies are currently being developed to address efflux-mediated resistance:

- 1) Modifying current drugs to find variations that are least affected by efflux pumps.
- 2) The exploration and progress of therapeutic medicines that block the efflux pumps'
- 3) Transport activity and could be combined with currently available antibiotics.

The *M. tuberculosis* genome continues to house many counterparts of the primary families of PMF-dependent efflux pumps. Additionally, it encodes a substantial number of ABC-type transporters and hypothetical proteins exhibiting transporter-like characteristics. This intricate assortment of efflux pumps underscores the tubercle bacillus's capacity to transport various harmful substances or medications, with noteworthy implications for drug resistance in tuberculosis infections[21, 22].

It's crucial to note that a significant number of these transportation systems rely on either the proton motive force (PMF) or ATP, linking their function to metabolic flow through the electron transport chain (ETC) and the upkeep of an energetically active membrane[21, 34].

The efflux pumps (EPs) that rely on both ATP and PMF are listed below. The electron transport chain (ETC) in mycobacteria produces a membrane potential ($\Delta\psi$) and a proton gradient (ΔpH) across the membrane; these two factors combined make up the proton motive force (PMF). EPs expel drugs by

transporting protons into the pseudo-periplasmic region. Major facilitator superfamily (MFS) EPs are integral membrane proteins with 12–14 transmembrane segments, in contrast to small multidrug resistance (SMR) EPs, which have four to six transmembrane domains. Both systems rely on periplasmic protons, although they have varying dependencies on ΔpH and $\Delta\psi$ [31].

Gram-negative bacteria harbor members of the resistance nodulation-cell division (RND) efflux pump family, which combine with other proteins to create multi-subunit complexes. These complexes span both the inner and outer membranes. RND division efflux pumps are crucial membrane proteins[35]. Therefore, it's possible that mycobacteria have a similar structure, in which the EPs from this group bridge the pseudo periplasmic region and associate with the mycolic acid layer, likely via OmpA (outer membrane protein A)- like homologs. Protons and the proton motive force (PMF), though these can be obtained outside, are also necessary for RND proteins. In contrast, ABC transporters depend on cellular energy production as they require ATP to actively expel drugs[22, 36].

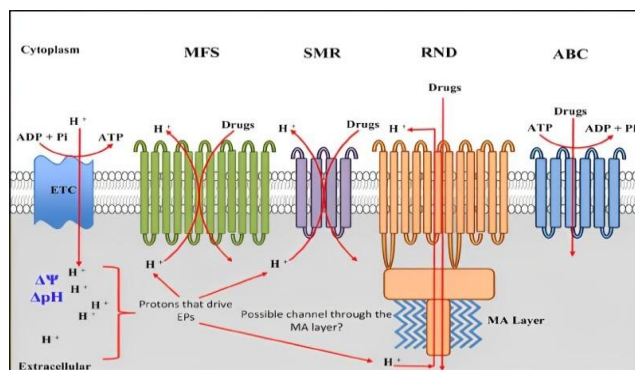


Figure 4- Efflux Pumps In *M.tuberculosis*

The efflux pumps of *M. tuberculosis* are shown in Figure 4. The multidrug and toxic compound extrusion (MATE) proteins, the fifth type of efflux pumps, are not shown in the image. Among these members, while some rely on the proton motive force (PMF) and protons, the majority of them operate by facilitating sodium influx[24]. Because efflux pumps are associated with resistance to antimicrobials, particularly first-line anti-TB medications, there has been an increasing amount of interest in them recently. Rv1258c has been shown to provide rifampicin tolerance during macrophage infection, whereas Rv1410c, Rv2936, and Rv0783 may be the source of rifampicin low-level resistance[37].

Table 1 enlists the different efflux pumps as per transporter families and the associated drug resistance with these efflux pumps[38]. It was recently shown that point mutations in Rv1258c have the ability to cause clinical isolates to develop drug resistance that is clinically important, especially when it comes to pyrazinamide, streptomycin, and isoniazid. When *M. tuberculosis* was exposed to isoniazid, it overexpressed *mmpL7* and *mmr*, the latter of which was also present when ethambutol was present[21]. Ethambutol resistance has also been linked to the doxorubicin-resistance operon, *drrABC*. Rv0191, Rv3756c, Rv3008, and

Rv1667c were found to be four potential efflux proteins in *M. tuberculosis* in 2017. They displayed that resistance to pyrazinamide was caused by overexpression of the genes that code for these proteins, which was alleviated by efflux inhibitors[39].

Efflux pumps are recognized to be resistant to all classes of antibiotics, including drug-resistant antibiotics used to treat drug-resistant tuberculosis (DRTB). The operons Rv2686c, Rv2687c, and Rv2688c encode the ABC transporter, which is responsible for removing fluoroquinolones from cells. Furthermore, DrrABC contributes to the conferment of resistance to many antibiotic classes, including erythromycins (erythromycin), tetracyclines (tetracycline), streptomycins (streptomycin), norfloxacin's (norfloxacin), and chloramphenicol[40]

Table 1: Efflux pumps and associated drug resistance

Transporter Family	Efflux pump	Drugs related	References	
ABC	Rv0194	β -lactams, STR, TET, CHL, VAN	[36]	
	Rv1217c- Rv1218c	INH, RIF, AZI-533 (pyrrole), AZI-219 (pyrazolone)	[21]	
	Rv1473	Macrolides	[3]	
	Rv1667c	PZA	[39]	
	Rv2477	OFX, STR	[36]	
	Rv3756	PZA	[39]	
	Rv2686c- Rv2687c- Rv2688c	FQs	[3]	
	Rv2936-Rv2937- Rv2938 (DrrABC)	CHL, RIF, STR, TET, EMB, ERY, FQs	[3, 41]	
	MFS	Rv0191	CHL, EtBr, methylene blue, PZA	[39]
		Rv0783	INH, RIF	[3]
Rv0849		AZI-533 (pyrrole), AMK	[21]	
Rv1410c (P55)		INH, EMB, RIF, CFZ	[42]	
Rv1634		FQs	[3]	
Rv2333c (Stp)		SP, TET	[3]	
Rv2459 (JefA)		INH, EMB	[43]	
Rv2846c (EfpA)		INH, ETH	[44, 45]	
Rv1258c (Tap)		Aminoglycosides, AZI-533 (pyrrole), INH, OFX, PZA, RIF, STR, TET	[21, 39]	
Rv2994		STR, FQs, CIP	[3, 45]	
Rv3008		PZA	[39]	
Rv3728		INH, EMB	[45]	
RND	Rv2942 (MmpL7)	INH	[46]	
	Rv0676c- Rv0677c(MmpL5- MmpS5)	BDQ, CFZ, azole	[3, 47]	
SMR	Rv3065 (Mmr)	INH, EMB, EtBr, TPP, CTAB, AZI-533 (pyrrole)	[3, 21, 43]	

ABC (ATP-binding cassette), AMK (Amikacin), BDQ (Bedaquiline), CFZ (Clofazimine), CHL (Chloramphenicol), CIP (Ciprofloxacin), CTAB (Cetyltrimethylammonium bromide), EMB (Ethambutol), EtBr (Ethidium bromide), ERY (Erythromycin), ETH (Ethionamide), FQs (Fluoroquinolones), INH (Isoniazid), MFS (Major facilitator superfamily), OFX (Ofloxacin), PZA (Pyrazinamide), RIF (Rifampicin), RND (Resistance nodulation division), SMR (Small multidrug resistance), SP (Spectinomycin), STR (Streptomycin), TET (Tetracycline), TPP (Tetraphenylphosphonium), and VAN (Vancomycin);

2.1 Pharmacological action of anti-tuberculosis drugs

When administering drugs to combat tuberculosis, they encounter various transporters in humans and mycobacteria that play a significant role in determining their effectiveness and distribution. Drug pharmacokinetics are impacted by the presence of these transporters and enzymes involved in drug metabolism in organs such as the intestines, liver, and kidneys. On the endothelial cell membranes of specific organs, particularly the brain, reside efflux transporters. These transporters act as gatekeepers, forming "sanctuary sites" that limit or completely impede the entry of certain foreign chemicals (xenobiotics) like anti-tuberculosis medications. This mechanism of efflux transporter-mediated sanctuary site formation poses a significant challenge to effectively treating tuberculous meningitis, the most severe manifestation of *Mycobacterium tuberculosis* (*MTB*) infection[38]

Efflux transporters at the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) significantly influence the delivery of anti-tuberculosis drugs to the brain and cerebrospinal fluid (CSF). Therefore, targeting these efflux mechanisms has the potential to enhance the therapeutic efficacy of TB meningitis treatment. The last human barrier that anti-TB medications must get past in order to reach the infection site is the macrophage membrane. Unlike typical bacterial infections, *MTB* has the ability to persist and reside within macrophages. This unique characteristic creates an additional obstacle between anti-TB medications and their targets within the mycobacteria[48].

Drugs' physiochemical characteristics and the presence of efflux transporters deposited on the macrophage outer membrane determine their capacity to pass this membrane. The efficiency of the medications may be hampered by lower and possibly insufficient intracellular drug concentrations caused by the inhibition of these transporters[21].

The effectiveness of anti-TB medications faces its final challenges at the *MTB* cell wall and plasma membrane. Mycobacterial efflux transporters are believed to play a pivotal role in the development of resistance to anti-TB medications. Recent research suggests that inhibiting these mycobacterial drug efflux mechanisms could serve as a unique supplementary treatment approach to enhance the concentrations of anti-TB medications within mycobacteria. This approach holds the potential to counteract resistance induced by *MTB* efflux pumps. In conclusion, the intricate interplay between various transporters and enzymes found in both humans and mycobacteria significantly influences the effectuality of anti-TB drugs and the ability to overcome drug resistance[1].

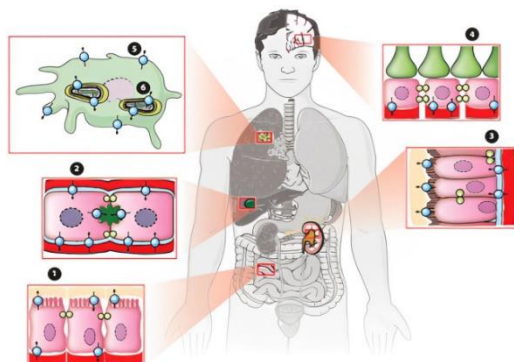


Figure 5: Illustrative overview of human and mycobacterial transporters

Overview of the relevant human and mycobacterial transporters for the treatment of tuberculosis (TB)[48].

- 1) Transporters located in the intestines are vital for drug absorption.
- 2) Hepatic transporters are essential for drug disposition, mediating both the metabolism and biliary excretion of xenobiotics.
- 3) Kidneys are essential for drug excretion and are influenced by transporters, contributing to drug-drug interactions and pharmacokinetics.
- 4) Efflux transporters at the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) play a critical role in controlling the delivery of antituberculosis drugs to the brain and CSF, thereby significantly impacting the efficacy of TB meningitis treatment.
- 5) Local concentrations of TB drugs are controlled by transporters within the membranes of macrophages and bacteria, influencing drug efficacy.

2.2 Efflux pump inhibitors and their potential to enhance TB therapy

One of the key contributors to medication resistance is the efflux of antimicrobial substances by bacterial efflux pumps. Drug efflux is prevented by Efflux Pump Inhibitors (EPs), which also increase drug effect and lessen drug resistance. Efflux is a common self-defense mechanism in both bacterial and eukaryotic cells. Extrusion of foreign substances and endogenous metabolic waste is a physiological mechanism that keeps regular cell function[48]. One of the unsolvable issues with anti-TB therapy is *Mycobacterium tuberculosis (Mtb)* resistance to antituberculosis (Anti-TB) medications. The causes of resistance are not fully comprehended, and a number of potential causes are being researched right now, including[21]:

- 1) Mutations in *Mtb* genes associated with drug resistance, which encode vital enzymes or transcription factors.
- 2) Overexpression of *Mtb* efflux pumps.
- 3) Alterations in *Mtb* cell wall permeability.

- 4) Elevated expression of the *Mtb* two-component system, regulating *Mtb*'s adaptation to both intracellular and extracellular environments.

Efflux pump inhibitors (EPIs) are a class of molecules that target bacterial efflux pumps, hindering their ability to expel drugs. This action by EPIs can lead to several potential benefits in TB treatment: increased killing of *Mycobacterium tuberculosis* (*Mtb*), reversal of drug resistance, and synergistic effects when combined with first-line anti-TB drugs[49]. Two categories of potential anti-TB medication exist: synthetic medications and medications derived from plant extracts. One or more efflux pumps may be the target of each medication[50].

A. Synthesized drugs

- 1) Phenylalkylamines- Calcium channel blockers, specifically phenylalkylamines, are commonly prescribed to manage a range of medical conditions such as angina, hypertension, and cardiac arrhythmia. The two most well-known members of this category are Verapamil (VP) and Norverpamil (NVP)[51].
 - a) Verapamil- Studies on verapamil (VP) in combination with isoniazid (INH)- or rifampicin (RIF)-resistant *Mycobacterium tuberculosis* (*Mtb*) strains or clinical samples have demonstrated that VP co-treatment reduces the minimum inhibitory concentration (MIC) of both INH and RIF. Furthermore, this combination treatment has been effective in reversing drug resistance in *Mtb* against both INH and RIF[52]. Levofloxacin's MIC was reduced by 2–8 fold in clinical samples that were resistant to the drug when combined with VER, but not in samples that were susceptible to the drug. It has been demonstrated that VER has a stronger effect than CCCP, RES, and chlorpromazine in increasing the retention of medicines in *Mtb* cells by inhibiting efflux pumps[11, 53].
- 2) Protonophores- Ionophores known as protonophores are used to transport protons across cell membranes. Ionophores, which carry ions across the cell membrane, are lipid-soluble complexes. Ionophores come in two ionophores. [49]. In contrast to carrier ionophores, which act as mobile shuttles for specific ions, channel formers are typically larger proteins that create hydrophilic pores within the membrane. These pores allow for the passive diffusion of ions across the otherwise hydrophobic membrane barrier[49]. The protonophores - CCCP, 2,4-dinitrophenol (DNP), and VLM may have anti-TB effects.
 - a) Carbonylcyanide- The synergistic effects of m-chlorophenyl hydrazone (CCCP) with other anti-TB medications are emphasized, particularly the inhibitory or fatal impacts on *Mtb* of the combined therapy. Research has revealed that various ABC and MFS family efflux pumps play a role in *Mtb* resistance to INH and RIF. In instances of INH- or RIF-resistant *Mtb*, the overexpression of these efflux pumps has been observed when exposed to CCCP, suggesting that CCCP induces the expression of efflux pumps. Furthermore, CCCP has exhibited inhibitory effects not only on the MFS and ABC families but also on the RND family of efflux pumps[4, 54].
 - b) DNP- Similar to CCCP, DNP (Dinitrophenol) acts as an Efflux Pump Inhibitor (EPI). Research involving clinical isolates of *Mtb* resistant to ofloxacin has shown that DNP reduced the minimum inhibitory concentration (MIC) of ofloxacin, rendering *Mtb* more susceptible to this

drug. However, no such effect was observed in *Mtb* that was sensitive to ofloxacin.

- c) DNP has the potential to inhibit efflux pumps belonging to the MFS and ABC family, and this inhibitory effect may extend to all quinolone drugs.
 - d) VLM - The depsipeptide ion transporter VLM exhibits a strong affinity for potassium ions. Studies have shown that VLM effectively hinders the efflux of pyrazinoic acid, thereby promoting its intracellular accumulation in pyrazinamide-resistant *Mycobacterium smegmatis* and *M. tuberculosis* (*Mtb*). This observation suggests that VLM may fully or partially inhibit proton-driven efflux mechanisms. By disrupting the transmembrane proton and electrochemical gradients that energize the P55 efflux pump, VLM potentiates the susceptibility of *Mtb* to both rifampicin (RIF) and novobiocin[49].
- 3) Phenothiazines- Long recognized to have antimycobacterial activity both in vitro and in vivo, phenothiazines are antipsychotic medications. In anti-TB research, chlorpromazine and thioridazine are the two medicines that are widely used[2, 49].
- a) Chlorpromazine [55]- Several anti-TB medications, including INH, RIF, STM, pyrazinamide, and rifabutin, have exhibited a synergistic effect when combined with chlorpromazine. This synergy has led to the enhanced effectiveness of these medications in killing intracellular *MTb*.
 - b) Thioridazine (TZ) [56]-The most promising drug was thioridazine, which had two effects on *M. tuberculosis*:
 - [1] Reduction of efflux pumps' activity
 - [2] Enhances the killing ability of macrophages by blocking potassium and calcium channels.
 This action leads to a decrease in pH within the phagolysosome, activation of hydrolases, and subsequent mycobacterial elimination.

B. Plant extracts and derivatives

In *M. tuberculosis*, some of the commonly used drugs include reserpine, piperine, farnesol, luteolin, and biochanin A[49].

- 1) Piperine- Piperine, a common alkaloid found in the fruits of various pepper plants, has been shown to have an anti-TB effect. Studies have revealed that dietary intake of piperine can inhibit the enzyme responsible for drug breakdown, leading to increased levels of P-gp substrates like phenytoin and RIF in the plasma. Piperine also has the potential to enhance the Th-1 cell factor, promote T-cell and B-cell development, and boost macrophage activity[57].
- 2) Reserpine- When *M. smegmatis* expressing mmpL7 was studied, reserpine was found to reduce ciprofloxacin resistance mediated by Rv2686 and lower the minimum inhibitory concentration (MIC) of isoniazid. Additionally, reserpine increased the susceptibility of both *M. tuberculosis* and *M. bovis* BCG to isoniazid[58].
- 3) Quercetin- Quercetin has exhibited strong binding affinity with the efflux pumps found in *MTb* Mmr and *E. coli* EmrE. Notably, the molecular interaction between quercetin and these pumps appeared to be combined with

chlorpromazine. This synergy is more stable compared to interactions with VER, RES, and chlorpromazine.

- 4) Tetradrine- When combined with INH or EMB, tetradrine has the potential to enhance the anti-TB action of these medications. This combination approach may contribute to reducing the required dosage of the drugs and mitigating their adverse effects[49].

Table-2- Anti-Tb drugs (EPI's)

Drug Type	Drug Name	Putative Drug Targets	Main Drug Effects	References
Synthesized Drugs	Norverapamil And Verapamil	Abc: Drrab, Pstb, Rv2686c-2687c-2688c, Mfs:Lfra, Rv1634, Rv1258c, Rv0678 Smr:Rv3065(Mmr) Rv1877,Rv2846 CRnd:Rv1145,Rv1146,	Inhibit <i>Mtb</i> growth and tolerance. Exhibit a synergistic effect when combined with anti-TB medications. Enhance the retention of anti-TB medications within <i>Mtb</i> . Allow for reduced drug dosages. Lower the minimum inhibitory concentration (MIC) of anti-TB medications. Improve the elimination of <i>Mtb</i> in macrophages. Potentially reduce the duration of TB treatment.	[49, 51-53]
	Protonophore : CCCP	ABC: Rv2936- Rv2937(DrrAB),Rv0933(PstB) Rv1877,Rv2846c RND:Rv1145,Rv1146, Rv0676c- Rv0677c(MmpS5-MmpL5) SMR:Rv3065(mmr) Rv2686c-2687c 2688cMFS:lfrA,Rv2459(jefA),Rv1410c(P55), Rv1634,Rv1258c,Rv1410c,	Decrease <i>Mtb</i> resistance to anti-TB medications. Lower the minimum inhibitory concentration (MIC) of anti-TB drugs. Exhibit a synergistic effect when used in combination with anti-TB drugs.	[49, 54]
Protonophore : DNP	ABC:Rv2936- Rv2937(Drrab), Rv0933(Pstb) , Rv2686c-2687c-2688c MFS:Lfra,Rv1634,Rv125	Reduce the minimum inhibitory concentration (MIC) of anti-TB drugs.	[49, 59]	

	8c		
	Protonophore : Valinomycin	MFS:Rv1410c(P55)	Increase the retention of anti-TB drugs within <i>Mtb</i> . Enhance <i>Mtb</i> 's sensitivity to anti-TB drugs. [49, 60]
	Phenothiazine: Chlorpromazine	Rnd:Rv1145,Rv1146Mfs: Rv1877, Rv2846c Smr:Rv3065(Mmr)	Demonstrate a killing effect against both drug-sensitive and drug-resistant <i>Mtb</i> . Exhibit a synergistic effect when used alongside anti-TB medications. [55, 61]
	Phenothiazine: Thioridazine	RND: Rv3160c-Rv3161c	Exhibit killing activity against drug-sensitive and resistant <i>Mtb</i> [49, 55, 62]
Plant Extracts or Derivatives	Reserpine	ABC:Rv2936-Rv2937-Rv2938(DrrABC), Rv0933(PstB),Rv2686c-Rv2687c-Rv2688c RND:Rv0678,Rv1145, Rv1146,Rv2942(mmpL7) MFS:Rv1410c(P55),Rv1877, Rv2846c SMR:Rv3065(mmr)	Lower the minimum inhibitory concentration (MIC) of anti-TB medications. Reduce or reverse medication resistance in <i>Mtb</i> . Increase the concentrations of anti-TB drugs within <i>Mtb</i> . [49, 58]
	Piperine	MFS: Rv1258c	Reduce the MIC of anti-TB medications Mice with reduced CFU Enhanced cellular immunity [49, 57, 63]
	Quercetin	SMR:Rv3065(mmr),Isocitrate lyase	Limit the development of <i>Mtb</i> [49, 64]
	Tetrandrine	MFS:Rv2459(jefA),Rv3728 SMR:Rv3065(mmr)	Reduce the MIC of anti-TB medications Report a synergistic interaction with anti-TB medications [49, 65]

Efflux pump inhibitors (EPIs) are substances that act to hinder or inhibit the operation of efflux pumps, which are responsible for pumping out toxic substances from bacterial cells, including antibiotics. In the case of *Mycobacterium tuberculosis (Mtb)*, EPIs have been proposed as potential adjuvants for tuberculosis (TB) therapy. Here are some potential merits of efflux pump inhibitors in *Mtb* [32, 34, 50, 66]:

1. Enhance the efficacy of current TB drugs: Through the inhibition of efflux pumps, EPIs have the potential to elevate the intracellular concentration of

- TB drugs, resulting in improved effectiveness against *Mycobacterium tuberculosis* (*Mtb*).
2. Overcome drug resistance: One of the major challenges in treating tuberculosis (TB) is the emergence of drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*). EPIs could potentially help overcome this problem by reducing the ability of *Mtb* to pump out drugs, making it easier for antibiotics to kill the bacteria.
 3. Reduce TB treatment duration: By enhancing the efficacy of TB drugs, EPIs could definitively help reduce the duration of TB treatment, which currently takes at least six months.
 4. Reduce drug toxicity: EPIs may possibly lessen drug toxicity and side effects by enabling the use of lower doses of TB drugs.
 5. Increase treatment compliance: The extended duration of TB treatment can pose challenges for patients in maintaining treatment adherence. Shortening the duration of treatment with the help of EPIs could potentially increase treatment compliance and reduce the risk of treatment failure.
 6. Improve treatment outcomes in HIV-positive patients: HIV-positive individuals are at a higher risk of developing TB, and TB treatment is less effective in this population. EPIs could potentially improve treatment outcomes in HIV-positive individuals by enhancing the efficacy of TB drugs.
 7. Potentially effective against latent TB: Latent TB refers to a form of disease where the bacteria exist within the body but are not actively causing illness. Current TB drugs are not effective against latent TB. EPIs could potentially help activate dormant *Mtb* bacteria and make them susceptible to treatment with TB drugs.
 8. It is crucial to emphasize that while EPIs show promise as potential adjuncts in TB therapy, further research is necessary to comprehensively assess their safety and effectiveness in addressing multidrug-resistant TB (MDR-TB). MDR-TB is a specific type of TB that demonstrates resistance to two or more of the most potent TB drugs. EPIs have shown promise in preclinical studies as a potential strategy to combat MDR-TB by inhibiting the efflux pumps that contribute to resistance.
 9. Potentially effective against extensively drug-resistant TB: Many of the most potent TB drugs, including those employed in MDR-TB treatment, are ineffective against extensively drug-resistant TB (XDR-TB). In order to address resistance and improve treatment outcomes for XDR-TB patients, EPIs may be utilized in conjunction with other medications.
 10. Synergistic effects with other drugs: EPIs have demonstrated synergistic effects when combined with other drugs utilized in TB treatment, such as rifampicin and bedaquiline. This means that when used together, the drugs have a greater effect than when used alone, which could improve treatment outcomes[47].
 11. Potential to reduce transmission of TB: When an infected person coughs, sneezes, or interacts, TB propagates through the air. By enhancing the efficacy of TB drugs, EPIs could potentially reduce the bacterial load in patients, making them less infectious and reducing the spread of TB in the community.
 12. Broad-spectrum activity: *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* are only a few of the bacterial pathogens that EPIs have demonstrated effectiveness against. This shows that EPIs may not only be utilized to treat TB but also a variety of bacterial diseases.

13. Combination therapy with new TB drugs: EPIs may be combined with newly developed anti-TB medications to boost their effectiveness and bypass any potential resistance mechanisms that may develop.
14. Repurposing existing drugs: Some EPIs are compounds that have already been approved by regulatory agencies for other indications, such as verapamil and thioridazine. The development of novel TB drugs could be sped up and drug development costs.

3 Conclusion

- 1) Many studies on the relationship between mycobacterial efflux pumps and drug resistance only indirectly suggest their role. For instance, they examine efflux pump expression in isolates that are resistant to drugs or track differences in drug sensitivity following exposure to drug/EPI combos.
- 2) In some studies where intracellular drug concentrations were measured, there were no differences in drug accumulation between the presence and absence of EPIs, or it was difficult to link increased drug accumulation with improved drug susceptibility.
- 3) In addition, limited has been discovered about the mechanisms underlying drug transporter upregulation, and the majority of the data that are available merely describe how it impacts the phenotype of the organism without clearly defining the external stimuli or cellular pathways at play.
- 4) The possibility that multiple transporters may have overlapping triggers and substrate specificities makes it difficult to interpret the results.
- 5) Studies frequently document the upregulation of multiple transporter genes in response to single or combined drug stimulation. However, there is no clear pattern that has been identified so far.
- 6) For instance, different genes within an operon can have various expression patterns even though they all encode the same transporter. Additionally, the expression of efflux pumps has only been associated with a small number of transcriptional regulators, including *Lsr2*, *whiB7*, *MmpLR5* (Rv0687), *Rv0302*, *MarR*, and perhaps *NapM*.
- 7) Nevertheless, there is limited understanding of their environmental triggers, signaling mechanisms, and their role in efflux pump-mediated resistance.
- 8) Numerous research endeavors have underscored the pivotal role played by human and mycobacterial membrane transporters in the pharmacokinetics-pharmacodynamics interaction of anti-TB drugs. These discoveries hold implications for TB treatment across various dimensions, including the potential for suboptimal drug exposure.
- 9) Gaining insight into their environmental triggers, signaling mechanisms, and their role in efflux pump-mediated resistance is crucial.
- 10) Given the slow pace of developing new anti-TB drugs, the inhibition of efflux pumps has been proposed as an adjunct to existing treatments.
- 11) EPIs offer a complementary approach by enhancing the effectiveness of existing antibiotics. This is essential when dealing with drug-resistant tuberculosis because there are few effective treatments available and it can be expensive and time-consuming to create new medications.
- 12) EPIs may also be able to overcome multidrug resistance, which is a significant obstacle in the treatment of many bacterial illnesses. Multidrug-resistant bacteria are resistant to several different classes of antibiotics,

making treatment challenging and raising the possibility of treatment failure and infection spread. EPIs have the ability to overcome drug resistance to multiple classes of medication and improve the effectiveness of antibiotics by preventing the action of efflux pumps.

- 13) Overall, EPIs represent a promising approach to combatting antibiotic resistance in bacteria, particularly in the context of drug-resistant tuberculosis. However, the development of effective EPIs for clinical use is still in the early stages and more research is needed to determine their safety and efficacy.

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