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Review article

Verapamil as an Efflux Inhibitor Against Drug Resistant Mycobacterium Tuberculosis: A Review

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ABSTRACT

Resistance to antituberculosis (anti-TB) drugs is a growing global problem, which is not only related to the high level of HIV co-infection. However, it can also be caused by the presence of multi-, extensively-, and totally drug-resistant *Mycobacterium tuberculosis* strain, hence the choice of using anti-TB drugs is getting smaller. The mechanism of emergence of drug-resistant bacterial strains is due to the mutation of drug target genes, decreased barrier permeability, and increased efflux rate. Drug resistance due to increased efflux pump activity is caused by overexpression of efflux pump genes, and amino acid substitution in protein, making efflux pump activity more efficient. Both mechanisms cause a reduction in intracellular anti-TB concentration so that the organism becomes less susceptible to the drug component. Number of studies have been conducted to explore components that can inhibit the action of bacterial efflux pump. Verapamil is a blocker of Ca^{2+}, a prototype of the phenylalkalylamine group that has the potential to inhibit the efflux pump of *M. tuberculosis* bacteria to be more susceptible to anti-TB drugs both *in vivo* and *in vitro*. The study of new components or a combination of adjuvant components and anti-TB drugs to treat drug-resistant *M. tuberculosis* infections is the main goal in the development of more effective TB treatment strategies.

Keywords: Antibiotics, drug-resistant, Efflux inhibitors, *M. tuberculosis*, Verapamil.

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INTRODUCTION

Mycobacterium tuberculosis is a pathogen causing tuberculosis (TB) which is difficult to control. This is because this bacterium has a complex impermeable cell wall structure, long cell generation time, and the potential to change the aerobic metabolic pathway into anaerobes. This flexibility is one of the ability determinants to adapt in bacteria to survive in the human body which at any time can change from oxygen-rich conditions in the lung alveolar region to microaerophilic/anaerobic conditions in the tuberculous granuloma region.[1]

It requires at least six months for antibiotic therapy for TB patients to be declared cured, which is confirmed by the results of TB microscopic examination.[2-5] However, the long period of therapeutic drug regimens was the main obstacle to the elimination of TB. It was reported that *M. tuberculosis* strains showed in vitro resistance to isoniazid (INH) and rifampicin (RIF) in ~ 480,000 cases and 250,000 cases of death.[6] Extensively drug-resistant tuberculosis (XDR-TB) strains show additional resistance to fluoroquinolone (FQ) and second-line injection drug agents, and are reported to cause infection in 106 countries.[6,7] With a high mortality rate, the XDR-TB strain is a threat to public health in general, plus an epidemic of HIV / AIDS infection.

One of the factors causing intrinsic resistance of *M. tuberculosis* to anti-tuberculosis drugs is due to the mechanism of the efflux pump which works synergistically with the permeable cell wall barrier so that the antimicrobial molecules do not enter bacterial cells.[8] Efflux pump causes low level drug resistance, but plays an important role in the process of evolution of drug-resistant *M. tuberculosis* strains.[9] Long-term exposure to subinhibitory concentrations of anti-TB drugs causes chromosomal mutations and is the basis for the formation of *M. tuberculosis* strains with high-level drug-resistant phenotypes due to acquisition of drug target genes.[10]

About > 90% of *M. tuberculosis* RIF-resistant strains having mutation in the *Rifampicin resistance-determining region* (RRDR) in *rpoB* gene which encodes the RNA polymerase β subunit enzyme and plays an important role in bacterial protein synthesis. A systematic review showed that mutations in *katG* induced about 64.2% of INH resistance cases. The combination of *katG*, *inhA* (especially in the promoter region), and *ahpC-oxyR* gene mutations could cause of ~ 84% of INH resistant phenotype globally.[11]

An approach is carried out to overcome resistance due to the mechanism of the efflux pump through the addition of inhibitor compounds called efflux pump inhibitors (EPIs) which work as adjuvants to maximize antibiotic function.[12] Efflux inhibitors are considered as a putative supporting component of anti-TB drug regimens, because they can restore *M. tuberculosis* susceptibility to antibiotics at sub-inhibitory concentrations. This review will discuss the efflux pump, the efflux inhibitor verapamil which is effective against *M. tuberculosis*, the effect of verapamil efflux inhibitors on mycobacterial growth, and the significance of the pre-clinical testing of standard TB treatment combined with verapamil efflux pump inhibitors.

EFFLUX PUMP SYSTEM (PROTEIN MEMBRANE TRANSPORTER)

The mechanism of the efflux pump in bacteria is known to be related to the nature of resistance. The presence of efflux pump in bacterial cells is already there compared to the development of antibiotics. So that the physiological process of bacterial cells has no correlation with the use of antibiotics, but is related to the destruction process of harmful agents to cells, allowing bacterial cells to be able to withstand extreme environmental conditions.[13,14] For example, the natural process of efflux pump is related to the secretion of intracellular metabolites and protection of bile salt and fatty acids for enteric bacteria which is a response to environmental changes.

Antimicrobial resistance due to increased efflux pump activity is caused by over genetic expression of the efflux pump, or due to amino acid

substitution on the protein itself and results in more efficient efflux pump activity. Both mechanisms cause the reduction of intracellular antimicrobial concentrations so that the organism becomes less susceptible to these agents. Efflux pump may be substrate specific or transport several molecules with various variations (including antibiotics from all groups), so it can be associated with resistance to anti-tuberculosis drugs. Genes that encode efflux pump can be found on bacterial chromosomes or on transmission elements such as plasmids.[13,14]

The efflux pumps system can be divided into 5 families based on energy sources and structures, namely: ATP binding cassette family (ABC); Major Facilitator Superfamily (MFS); Multidrug and toxic compound extrusion family (MATE); Small Multidrug Resistance family (SMR); Resistance Nodulation Division family (RND). Efflux pump, including the ABC family, is considered as the primary transporter because the group hydrolyzes ATP as its energy source, while the other efflux pump families use proton gradients namely membrane energy and PMF (ΔpH and ΔΨ) as their energy sources, therefore they are called secondary transporters.[12-15] Efflux pump sometimes acts as a symporter for drug / proton metabolites, antiporters and uniporters.

ATP BINDING CASSETTE SUPERFAMILY

ATP Binding Cassette Superfamily (ABC) transporters are related to various transport functions such as efflux toxins, metabolites, and drugs. This system is divided into two cytoplasmic parts that bind ATP and two hydrophobic transmembrane domains.[16,17] Nucleotide binding domains are very homologous and have the motive of Walker A and B, which are commonly found in all ATP binding proteins, and specific motives for ABC transporters.[16,18] The gene encoding ABC transporters composes 2.5% of the *M. tuberculosis* genome, and 37 complete and incomplete transporters have been identified.[19] But only a few of these transporters are related to resistance mechanisms. *M. tuberculosis* has a doxorubicin resistant operon, *drr*BC.[19] *drr*AB gene is present in *M. smegmatis* which causes resistance to a broad-spectrum antibiotic, including TET, erythromycin, ethambutol, norfloxacin, STR and chloramphenicol. The resistant phenotype can be reversed by the addition of reserpine or verapamil, a component that inhibits the efflux mechanism.[20] The results showed that Drr protein in *M. tuberculosis* has a major role in exporting lipids to the exterior of cells, and specifically DrrC has an association with transport of phthiocerol dimycocerosate.[21] *M. tuberculosis* operon *Rv2686c-Rv2688c* encodes ABC transporters responsible for the fluoroquinolone efflux when produced from plasmid multiplication. When *M. smegmatis* overexpressed, the operon increased 8-fold from the ciprofloxacin MIC and 2-fold the norfloxacin MIC. Resistance levels decreased with the presence of reserpine, carbonylcyanide m-chlorophenylhydrazone (CCCP) and verapamil.[22]

Major Facilitator Superfamily

Major Facilitator Superfamily (MFS) is a superfamily transporter involved in import, uniport or antiport in a variety of substrate. All proteins are 400-600 amino acid residues and have 12 or 14 important trans-membrane domains.[23-25] *Rv1634* is a family of MFS efflux pump which is suspected to be the newest fluoroquinolone transporter in *M. tuberculosis*.[26] It is known that this efflux pump decreases the susceptibility to broad-spectrum fluoroquinolones when over-expression of *M. smegmatis* occurred. So, the accumulation indicates that *Rv1634* is also involved in norfloxacin and ciprofloxacin efflux.[26]

Multidrug and Toxic compound Extrusion Family

The Multidrug and Toxic compound Extrusion Family (MATE) family contains 400-550 residues with 12 trans-membrane helixes. Although it was never sequenced to find out the MATE family components, all these proteins had 40% similarity. There are two energy sources used by MATE transporters: proton motive force and sodium ion gradient.[27] MATE transporters in mycobacteria have not been reported, but are commonly found in *Escherichia coli* and *Vibrio sp*.[28]

Small Multidrug Resistance Family

The second smallest transporter from the Small Multidrug Resistance Family (SMR) group. This protein has a length of 110 amino acid residues with four trans-membrane helixes. Mmr is the only family protein of SMR that has been studied in *M. tuberculosis*.[29] The *mmr* chromosomal gene, when inserted into the cloned plasmid, causes a decrease in *M. smegmatis* susceptibility to TPP, EtBr, erythromycin, acriflavine, safranin O, and pyronin Y. Accumulative studies showed Mmr secretes TPP molecules, the process of which depends on the energy of the proton. The presence of a similar *mmr* gene in mycobacteria species (*M. simiae*, *M. gordonae*, *M. marinum*, and *M. bovis*) has been studied with the Southern Hybridization method.[29]

Resistance Nodulation Division Family

These transporters can transport molecules from various substrate types and can emit positive, negative, or neutral molecules, hydrophilic and hydrophobic components.[13,14] Almost all Resistance Nodulation Division Family (RND) transporters consist of polypeptid chain components with 700-1300 amino acid residues and are thought to stretch 12 times the membrane with two periplasmic regions located between transmembrane helix 1, 2, 7, and 8.[30] The *M. tuberculosis* genome contains several genes that encode changes in transport protein in the RND superfamily. This protein works in MmpL (*Mycobacterial* membrane protein, large) and is thought to be involved in the mechanism of transport of fatty acids.[31] In *M. tuberculosis* MmpL7 produces dimycocerosate phthiocerol (PDIM), a lipid component of the outer membrane of bacterial cells.[32] At a location opposite the *mmpL7* gene there is the *fadD*28 gene, which encodes acyl-CoA synthase that is involved in the process of releasing and transferring mycocerosic acid from mycocerosic acid synthase. Bacterial strains that experience *mmpL7* insertion produce dimycocerosate molecules (DIM), which will be in the cytoplasm or cytoplasmic membrane. *MmpL7* production in *M. smegmatis* triggered 32 times the increase in minimum inhibitory concentration (MIC) of INH. However, this phenotype can change if *FadD*28 and *mmpL7* are expressed simultaneously, so it is assumed that DIM and INH compete for the same MmpL7 transporter.[33]

ENERGY METABOLISM IN EFFLUX PUMPS OF *M. TUBERCULOSIS*

The sub-optimal intracellular concentration of antibiotics can lead to bacterial tolerability of antibiotics, which are encoded by chromosomes and become permanent characteristics of bacterial cells. Several findings have been suggested that *M. tuberculosis* bacteria can extrude antibiotic molecules out of their cells, create an antibiotic resistance. In many cases, this process occurs in the mechanism of proton motive force (PMF) as an energy source of transporter proteins in extruding antibiotic molecules from within the cell, as well as the presence of number of ATP at the right concentration in the cell. This indicates that the electron transport cycle (ETC) of mycobacteria that are affected by different conditions can affect the susceptibility of *M. tuberculosis* to antibiotics.[34]

The electron transport cycle (ETC) is integrally involved in energy formation through the process of oxidative phosphorylation. Electrons enter bacterial cells and are dispersed through ETC via several pathways, which are influenced by the source of growth or nutrient substrates and the presence of terminal electron acceptors. In aerobic conditions, oxygen is used in the final stages of electron transfer, whereas in anaerobic conditions, nitrate and fumarate can be used instead of oxygen. Electron transport in mycobacteria is initiated through the diverse activities of NADH dehydrogenase (NDH) and succinate

dehydrogenase (SDH), which can transfer electrons to menaquinone which is a lipophilic redox carrier. Then the electrons are transmitted to various cytochrome oxidases, whose functions are affected by the presence of oxygen.[34-38] ETC mycobacteria, such as bacterial ETC in general, have branches and various pathways in terms of the use of electron and acceptor donors to reduce oxygen level tension and different decreasing equivalent availability.

EFFLUX PUMP INHIBITOR: VERAPAMIL

Verapamil is a synthetic papaverine derivative, which was first known as smooth muscle relaxant which has the ability of peripheral vasodilator and potent coronary in animals.[39,40] Verapamil is used to treat heart disorders, headaches, and migraine,[41] in mammalian cells, verapamil acts to inhibit vesicular transporters of monoamine and P-glycoprotein.[42] Electrophysiological studies using different tissues showed that verapamil has a unique cellular action mechanism that selectively inhibits transmembrane calcium efflux.[43,44] Verapamil shows the influence of primary pharmacology through the mechanism of antagonism in cell membranes, by modifying calcium uptake, binding or exchanging calcium concentrations, and influencing calcium levels that activate ATP-ase work.[45]

Verapamil is a Ca^{2+} channel blocker, a prototype of the phenylalkalylamine group that inhibits multidrug ATP-dependent transporters and MDR pumps, inhibiting proton motive force transport processes, acting as inhibitors of efflux pump activity in prokaryotic cells.[46,47] In several studies, verapamil showed a significant inhibitory effect on the activity of mycobacteria efflux pump.[48,50] Blocking the Ca^{2+} can limit the growth of *M. tuberculosis* bacteria through intervention in cell metabolic processes, especially in the MDR and XDR strains of *M. tuberculosis*. The effect of bactericidal verapamil on *M. tuberculosis* strains has an association with bacterial cell energy metabolism. A study was conducted on the effect of adding verapamil as a Ca^{2+} channel blocker to the determination of ATP levels[50-52] which showed that verapamil caused a decrease in intracellular ATP accompanied by an increase in bactericidal effects on *M. tuberculosis* strains which are sensitive and resistant to INH and RIF antibiotics at the same concentration. The decrease in the amount of ATP in bacterial cells is due to verapamil exposure which directly affects the condition of bacterial cell metabolism that provides a certain amount of ATP for the active efflux process. Efflux pump in prokaryotic and eukaryotic cells uses energy sources in the form of ATP or PMF to extrude ions, secondary metabolites, or toxic compounds through oxidative phosphorylation as the main source of energy.[53,54] The energy production cycle starts with NADH dehydrogenase and ends in the F1-F0 ATP-synthase cycle. Enzymes that play a role in ETC which are directly or indirectly inhibited by verapamil.[55]

A study showed that verapamil could restore partial susceptibility to rifampicin in mono-resistant rifampicin on *M. tuberculosis* strains and MDR strains.[56] Resistance to rifampicin can induce resistance to ofloxacin, so the addition of verapamil causes resistance ofloxacin decreasing 2-fold in *M. tuberculosis*.[57] The same result is shown by in vitro studies, where verapamil can restore susceptibility to isoniazid, rifampicin, ofloxacin, streptomycin, ethidium bromide, and macrolide in *M. tuberculosis* and *M. avium* strains.[47,58-60] Verapamil can also restore changes in the tolerance of *M. tuberculosis* drugs in macrophages. Its aim was to look at the performance of inhibitors at the eukaryotic intracellular level. It is known that the mechanism of action of macrophages depends on the presence of potassium and calcium ions in cells.[61,62] Gupta *et al*.[63] showed that inhibition of type L ion channels causes an increase in intracellular calcium in macrophages which causes decreasing in the number of *Mycobacteria* cells in macrophages. This means that verapamil as a block of Ca^{2+} can limit the growth of *M. tuberculosis* even though it is inside the macrophage. Verapamil has long been known as an antipsychotic, antihypertensive, easily available, can be taken orally, and has few side effects.[64] According to Machado D *et al*.[65] the mechanism of blocking Ca^{2+} channel such as verapamil works through: a) on bacterial cells, after the verapamil component enters the cell, causing a sequence of events involving the inhibition of complex respiration cycles and energy production that support efflux activity. Thus, indirectly causing a decrease in the level of bacterial resistance to antibiotics, due to the main focus of cell work to improve the mechanism of inhibition in the initial event (in vitro); b) on the host cell, treatment using verapamil causes acidification of the phagosome and increases the hydrolase transcription, causing the growth of *M. tuberculosis* bacteria to be inhibited, regardless of the resistance properties possessed by *M. tuberculosis*. The two mechanisms work together so that the bactericidal effect of anti-TB drugs on *M. tuberculosis* can be increased by a combination of verapamil. The combination of antibiotics and verapamil can maximize drug function by increasing the number of mycobacteria cells that are turned off, preventing the emergence of bacterial resistance, and can shorten the duration of drug therapy (Figure 1).[66] The strategy for treating tuberculosis in the future emphasizes the inhibitory mechanism of efflux pump and the ability of drugs or compounds to kill *M. tuberculosis* cells, but has a low toxicity effect on humans, so that the clinical potential of drugs can be maximized.[67]

Chen C *et al*.[68] stated that the action mechanism of verapamil does not directly affect intracellular drug uptake and accumulation through direct inhibition of the efflux pump process in *M. tuberculosis*. However, through physiological and microbiological approaches showed that the biological influence of verapamil was significantly found in cell membrane bioenergetics and induction in cell membrane stress response. Verapamil is proven to cause an increase in intracellular EtBr accumulation (a common substrate used to evaluate the mechanism of efflux pump in mammalian cells), where verapamil is synergistic with EtBr. [53,69] Verapamil induces EtBr accumulation as a result of verapamil's indirect influence on changes in efflux pump function due to interference with the energetic balance of cell membranes.[53] The limitations of all physiological tests on drugs uptake using verapamil, should limit the incubation period to only 24 hours, to avoid artifacts (dead bacterial cells) associated with envelope cell integrity and *M. tuberculosis* culturability in agar medium, which will affect enumeration of CFU (colony form unit) data required for the normalization of measurements of intracellular concentrations of drugs.[68]

Xu *et al*.[70] stated that adjunctive verapamil activity in vivo is caused by the presence of systemic exposure that accompanies anti-tuberculosis drugs. This is influenced by the presence of transporters in mammalian cells, not because of the inhibition mechanism of bacterial efflux pumps. Proportional doses of verapamil that can affect cell membrane energetics is in the range of 8 M – 1 mM, in accordance with the potentiation of bedaquiline and clofamizine *in vitro*. In order to provide inhibitory growth effects on *M. tuberculosis*, the concentration of verapamil used ranges from 512 μM or more, which can be rationalized based on the fact that proton motive force is a combination of two parameters namely (electric potential) and pH (transmembrane proton gradient), and bacteria tend to be able to show partial tolerance either a decrease or increase in one of these parameters.[71,72] Allegedly, besides the function of verapamil which is already quite known as an efflux inhibitor in mammalian cells, the research data showed that the effect of verapamil to maximize antimycobacterial function in vitro was not caused by the ability of verapamil to accumulate intracellular concentrations of drugs, but mainly due to the ability of verapamil which can directly affect the energetics of cell membranes. This discovery is a new solution from the therapeutic side, where *M. tuberculosis* membrane can be a new target in the field of pharmacology. Some cationic amphiphilic antimycobacterial drugs such as SQ109, are believed to work on target membrane proteins

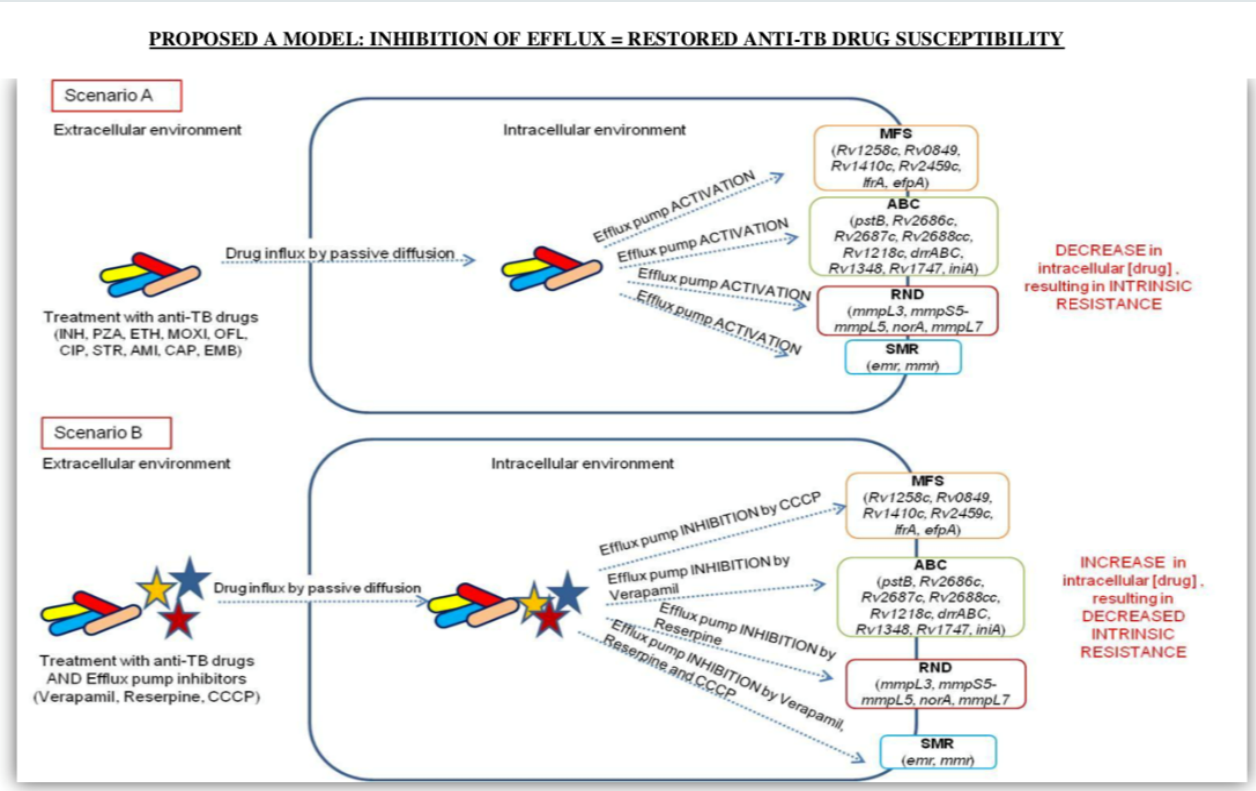

Figure 1. Diagrammatic illustration of inhibition efflux = restored anti-TB drug susceptibility. The inhibition of efflux pumps responsible for intrinsic resistance by efflux pumps inhibitors (EPIs) usage, results in restored anti-TB drugs (substrate to the EP).[66]

(MmpL3 transporters). SQ109 was found to be able to interact with membrane lipids through certain mechanisms that cause structural and electrical perturbations.[73] Due to the biophysical properties of SQ109, the bacterial membrane acts as a cationic amphiphilic container, so the use of membranes inserted into small amphiphilic cationic molecules is considered as a new approach to antimycobacterial therapy against dormant *M. tuberculosis* and actively replicate that have a phenotype resistant to conventional antimycobacterial drugs.[74,75] The ability of persistent organisms such as *M. tuberculosis* to maintain membrane energetics during extended cell dormancy is a major factor in adaptation.[76] It requires a high concentration of verapamil in order to provide bactericidal effects on *M. tuberculosis*, but it can be toxic to mammalian cell membranes, so it needs more potent verapamil analog design for *M. tuberculosis* to obtain therapy using a new adjuvant that synergizes with antimycobacterial drugs, and targeting *M. tuberculosis* cells that are either actively replicating or dormant cells.[71]

CONCLUSION

Antimicrobial resistance is caused by the activity of the efflux pump, over genetic expression of the efflux pump, and substitution of amino acids in proteins. The mechanism causes a reduction in intracellular drug concentration so that *M. tuberculosis* becomes less susceptible to the drug. Efflux pump may be specific substrate and transport a number of molecules with various variations (including antibiotics from all groups), so that it can be associated with resistance to anti-tuberculosis drugs. Verapamil is a blocker of Ca^{2+}, a prototype of the phenylalkalylamine group that limits the growth of *M. tuberculosis* through intervention in cell metabolic processes, causing a decrease in intracellular ATP, which can directly affect cell membrane energetics, especially in MDR and XDR *M. tuberculosis* strains. The combination of antimycobacterial and adjuvant verapamil can maximize drug function as measured by the increasing number of mycobacteria cells that are turned off, prevent the emergence of bacterial resistance, and can shorten the duration of drug therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

TB: Tuberculosis; MDR: Multi drug resistant; XDR: Extensively drug resistant; ATP: Adenosine tri-phosphate; Ca: Calcium; EtBr: Ethidium bromide; NADH: Nicotinamide adenosine dinucleotide hydrogen; ETC: Electron transport cycle; PMF: Proton motive force; INH: Isoniazid; RIF: Rifampicin; FQ: Fluoroquinolone; ABC: ATP binding cassette; MFS: Major Facilitator Superfamily; MATE: Multidrug and toxic compound extrusion; SMR: Small Multidrug Resistance; RND: Resistance nodulation cell division; MIC: Minimum inhibitory concentration; NAD: NADH dehydrogenase; SDH: succinate dehydrogenase.

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