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EXPERT REVIEWS

Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs

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Tuberculosis is one of the major causes of infection across the world. The emergence of multi-, extensively- and totally drug-resistant strains of *Mycobacterium tuberculosis* contributes to the lack of therapeutic options available. The mechanisms associated with this resistance could involve mutations in genes coding for target proteins, decreased permeability, increased efflux and so on. Resistance mediated by efflux systems has become more relevant, since these systems help the bacteria to extrude antibiotics until relevant mutations emerge and become established in the population. Therefore, compounds that inhibit these transport systems are of major importance and have been studied in the last few years. Not only do these compounds act on the bacterial efflux systems but they have also been explored for their dual role as boosters of the macrophage-infected cells. The search for novel compounds or combinations of adjuvant compounds and antibiotics to treat mycobacterial multidrug-resistant infections has become a major goal in the treatment of these diseases.

Keywords: drug resistance • efflux pump inhibitors • efflux pumps • macrophage killing activity • multidrug-resistant tuberculosis • mycobacteria • phenothiazines • tuberculosis

The dissemination of multidrug-resistant (MDR) *Mycobacterium tuberculosis* has reduced the therapeutic efficacy of many anti-tubercular drugs, and is now one of the major human healthcare emergencies causing thousands of deaths in developed countries [1]. To efficiently treat infections caused by MDR strains of *M. tuberculosis*, it is necessary to clearly define the molecular basis of the resistance in order to judiciously design strategies to circumvent it. In a general and simplistic way, antibiotic resistance mechanisms of bacteria involve either the modification of the antibiotic target, usually caused by a genetic alteration of the coding genes, or the hindrance of the antibiotic to inhibit the intended target [2,3]. This latter mechanism includes direct inactivation of the antibiotic or decreased access of the antibiotic to its target, via decreased membrane permeability or active efflux pumps, which reduce the intracellular concentration of antimicrobial drugs [2–4].

In tuberculosis (TB), clinical drug resistance is classified as: acquired resistance, when drug-resistant mutants are selected as result of ineffective treatment; or primary resistance, when a patient is infected with a resistant *M. tuberculosis* strain. The selection of naturally occurring drug-resistant mutants happens when the therapeutic regimen is not correctly followed. This is intimately associated with the long generation time of *M. tuberculosis*, its low metabolic activity and its ability to enter dormancy [5]. The localization of the infection increases the probability of exposure to monotherapy, since *M. tuberculosis* may be located inside pulmonary cavities, which restrictthe access of the antibiotics [6]. This effect is enhanced in the presence of an insufficient dosage of anti-TB drugs; a result of inadequate prescription by the physician or nonadherence associated with pharmacokinetic variability among TB patients [4]. In addition to vaccination and timely diagnostics, effective public health control measures to prevent the dissemination of TB and the emergence of drug-resistant strains should focus on the prevention of transmission and implementation of effective therapies [7–10].

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The first anti-TB drug, streptomycin, was discovered in 1943 and its therapeutic introduction, together with the general improvement in public health in Europe and in the USA, helped reduce the burden of TB. The establishment of TB control programs, along with the introduction of a successful anti-TB treatment, resulted in an evident decrease of infection and mortality. In the second half of the 20th century, the disease was considered close to eradication, causing a decreased interest in industrialized countries and consequently, the abandonment of the TB control programs. Despite the decrease of TB cases, the disease never disappeared completely due to the decline of the TB control programs and the spread of the HIV/AIDS [8,9]. In 1993 WHO declared TB a global health emergency and in 1998, together with the International Union Against Tuberculosis and Lung Disease and other international partners, they formed the Stop TB Initiative that has evolved into the global Stop TB Partnership [10]. Despite these efforts, TB is still a major threat. The WHO estimates that in 2010 there were over 8 million new cases of *Mycobacterium tuberculosis* infection and approximately 1.5 million deaths caused by TB [1].

The global resurgence of TB is closely related to the emergence of *M. tuberculosis* strains that are increasingly resistant to the four first-line drugs; isoniazid, rifampicin, ethambutol and pyrazinamide; and a small group of second-line drugs, comprising of aminoglycosides, polypeptides, fluoroquinolones and thioamides. Initially, the control of MDR-TB, defined as resistance to isoniazid and rifampicin with or without resistance to other first-line drugs, was sufficient and satisfactory from the clinical, laboratory and public health view points, to implement effective control measures. In 2006, WHO reported the emergence of extensively drug-resistant tuberculosis (XDR-TB) strains, that are resistant to isoniazid or rifampicin, in addition to any fluoroquinolone and to at least one of the three second-line injectable drugs (amikacin, capreomycin or kanamycin). Within a year of the first reports of XDR-TB in South Africa, isolated cases were also described in Europe [11,12]. In 2009, a group of 15 Iranian TB patients were reported as being infected by a strain resistant to all anti-TB drugs tested. In 2011, in India, a similar situation was reported and named as totally drug-resistant tuberculosis (TDR-TB) [13,14].

It has now become generally accepted that the overall mycobacterial resistance to any antimicrobial agent is not due to just one single resistance mechanism, but to a synergy between intrinsic and genetic resistance [4,15–18]. In mycobacteria, and in particular following isoniazid and ethambutol exposure, it has been shown that efflux emerges prior to acquisition of mutations in the drug target genes and that overexpression of efflux pump genes results in an increase in antibiotic resistance, conferring a low-level resistance phenotype [4,18–20]. Most likely, this prolonged exposure to subinhibitory antibiotic concentrations may increase the probability of acquiring mutations in genes encoding the target protein. This will result in the emergence of a new subpopulation of mycobacteria presenting a high-level resistance phenotype, as already seen in other groups of bacteria [21–23]. This fact may be particularly relevant in the case of long-term therapy, such as that used in TB treatment, where a sustained pressure of subinhibitory concentrations of an antibiotic can result in an increased efflux activity, allowing the selection of spontaneous mutants. A way to prevent these events from occurring could be the inhibition of these efflux systems. This would restore the activity of antibiotics that are subject to efflux. If efflux pumps play a role in the selection/stabilization of mutants, these events should appear with decreased frequency in the presence of an efflux pump inhibitor. For that reason, it is necessary to understand the molecular and functional mechanisms behind efflux-mediated resistance in *M. tuberculosis* and how this knowledge can be used to prevent their consequences.

It is the aim of this review to provide a comprehensive description of the mechanisms associated with antibiotic resistance in MDR-, XDR- and TDR-TB strains. The use of mycobacterial efflux pump inhibitors as potential boosters for anti-tubercular drugs will be explored as a new therapeutic approach for the treatment of TB. A list of compounds as well as their mechanism of action will be discussed. Additionally, the use of these compounds in infected macrophages to promote the killing of the intracellular mycobacteria will be highlighted and a model explaining the compound's actions on the infected cells will be presented. Taken together, the combined use of mycobacterial efflux pump inhibitors and antibiotics could constitute a novel approach that should be considered in the future to control and prevent drug-resistant TB.

Molecular & functional aspects of efflux pumps in mycobacteria

The intrinsic resistance of mycobacteria to antimicrobial drugs is mainly attributed to the permeability barrier provided by the lipid-rich cell wall in synergy with the active efflux of drugs [4,15,24]. Peptidoglycan and arabinogalactan layers limit the entry of hydrophobic molecules, whereas the mycolic acid layer limits the access of both hydrophobic and hydrophilic molecules [15,24]. Relatively hydrophobic antibiotics, such as rifampicin and fluoroquinolones, may enter the cell by diffusion through the hydrophobic bilayer, whereas hydrophilic antibiotics and nutrients use porin-like channels [16]. These channel-forming proteins are functionally similar to the porins of Gram-negative bacteria and have been described in many mycobacteria (e.g., MspA of *Mycobacterium smegmatis*, and Rv0899 [*ompATb*] and Rv1698 of *M. tuberculosis*) [25,26]. Mycobacterial porins are much less abundant than in Gram-negative bacteria and only allow low rates of uptake for small hydrophilic nutrients and antibiotics [24]. Nevertheless, they have been associated with drug susceptibility. It was demonstrated that deletion of MspA and MspC increased resistance of *M. smegmatis* to β-lactams, chloramphenicol and norfloxacin. Furthermore, expression of *mspA* in *M. tuberculosis* and *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) promoted glucose uptake and increased the growth rate and the susceptibility to hydrophilic antibiotics, such as β-lactams, isoniazid, ethambutol and streptomycin [27].

Working synergistically with mycobacterial cell wall impermeability are efflux pumps; membrane-spanning proteins involved

in the outward transport of a wide variety of substrates to the exterior of the cell in an energy-dependent manner. Similar to other bacterial pathogens, in mycobacteria, the intracellular concentration of a given drug depends on the balance between its influx and efflux; it is therefore of great importance to increase the understanding of the processes of drug influx through porins, as it has been outlined above, and drug efflux via efflux pumps, in order to prevent drug resistance [25,28]. Efflux pumps can be organized in several families according to their energy source and phylogenetic relationship (Box 1).

Several studies in the past 10 years have demonstrated the association of efflux pumps of mycobacteria with transport and low-level resistance to antibiotics such as tetracycline, aminoglycosides, fluoroquinolones, rifampicin, isoniazid and chloramphenicol [17,29–37]. Table 1 summarizes most of these pumps, and the most well-characterized transporters are further discussed.

The gene coding for the tetracycline efflux pump, Tet(V), was isolated from *M. smegmatis* and, when overexpressed it increases the minimum inhibitory concentration (MIC) of tetracycline from two to fourfold. The distribution of the *tet(V)* gene among the genus *Mycobacterium* has been investigated by PCR, and *M. smegmatis* and *Mycobacterium fortuitum* were the only species tested that revealed this gene [30].

The LfrA protein of *M. smegmatis* was the first efflux pump to be described in the genus *Mycobacterium* and is responsible for low-level resistance to fluoroquinolones, acridine and quaternary ammonium compounds [38–40]. When overexpressed in plasmids, LfrA seems to play an important role in the resistance to ciprofloxacin in *M. smegmatis*. However, in the absence of overexpression, LfrA is thought to have no effect on the susceptibility to fluoroquinolones. In fact, disruption of the *lfrA* gene decreased the MIC of ethidium bromide and acriflavine eightfold and the MICs of ciprofloxacin, doxorubicin and rhodamine twofold, revealing more affinity to quaternary compounds [31]. The region upstream from the *lfrA* gene revealed the presence of an open-reading frame encoding a putative polypeptide of 195 amino acids, LfrR, homologous to several transcriptional regulators of the TetR family [41]. The *lfrR* and *lfrA* genes are organized into an operon that is most likely controlled by LfrR. It has been demonstrated that deletion of the *lfrR* gene enhances *lfrA* expression, increasing the resistance to ciprofloxacin, norfloxacin, ethidium bromide and acriflavine [31]. No known homolog of the *lfrA* gene has been found in the genome of *M. tuberculosis* [42].

The characterization of the *M. fortuitum* Tap efflux pump led to the identification of its *M. tuberculosis* homolog Rv1258c, which was the first functional efflux pump reported in *M. tuberculosis* [29]. These two ortholog efflux pumps confer resistance to tetracycline and aminoglycosides, including streptomycin. When cloned on a plasmid, Tap increased the resistance of *M. smegmatis* mc^2155 to gentamicin, streptomycin and tetracycline [29,42] and its expression increased in the presence of rifampicin and ofloxacin in a *M. tuberculosis* clinical strain [43]. Furthermore, tetracycline accumulation experiments showed that the efflux activity of Tap from *M. fortuitum* is inhibited by carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine, two commonly used efflux pump inhibitors; a result consistent with the decrease of the MIC of this antibiotic in the presence of these compounds. In addition, CCCP, reserpine and also chlorpromazine reduced the MIC of tetracycline in a *M. smegmatis* strain expressing the Tap protein [44].

Disruption and overexpression of *tap* in *M. bovis* BCG revealed an altered susceptibility to many clinically approved antibiotics associated with different levels of efflux of tetracycline and acriflavine, as well as a different expression pattern along the different growth phases. In stationary phase, Tap inactivation caused a general stress response and the repression of genes involved in cell wall biosynthesis [45]. Another study associated the activity of Tap with drug tolerance using *Mycobacterium marinum*-infected zebrafish larvae as an infection model, demonstrating the effect of this efflux pump on tolerance to rifampicin, one of the main anti-TB drugs [46].

Another putative efflux pump of the major facilitator superfamily found in *M. tuberculosis* that has been suggested as a fluoroquinolone efflux transporter was Rv1634, whose activity was related to the decreased susceptibility to various fluoroquinolones when overexpressed in *M. smegmatis*. Furthermore,

Box 1. General characteristics of efflux pumps.

- Efflux pumps are involved in several physiological processes, such as cell wall division, maintenance of the pH homeostasis and secretion of intracellular metabolites [22]
- Based on bioenergetics criteria, efflux pumps can be categorized into primary and secondary transporters [22,23,74,78]
- Primary transporters are energized by the hydrolysis of ATP and constitute the ATP-binding cassette (ABC) family [23,74,78]
- Secondary transporters harness energy stored in an electrochemical gradient generated by protons that are transported and distributed to the surface of the cell, also known as proton motive force [74,78]
- Secondary transporters are further classified into four families: the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion family; the small multidrug resistance family; and the resistance nodulation division family [23,74,75,78]
- Efflux pumps can contribute to the development of drug resistance in several ways: bacterial cells have basal levels of efflux activity that cause a natural decreased susceptibility to one or more drugs (intrinsic resistance); increased expression of genes that code for efflux pumps may be the first step in the development of clinically-relevant drug resistance; the diversity of compounds that can be extruded by efflux pumps allows them to confer a low-level multidrug-resistant phenotype; the decrease of the intracellular concentration of a given antimicrobial through the activity of efflux pumps allows the bacteria to survive for a greater length of time, until chromosomal mutation(s) arise conferring high-level resistance to that particular drug [4,18,22]

Table 1. List of efflux pumps reported in mycobacteria.

Efflux pumps	*Mycobacterium* species	Substrates	Family	Ref.
LfrA	*M. smegmatis*	FQs, EtBr, ACR	MFS	[31,38–40]
Tet(V)	*M. smegmatis*	TET	MFS	[30]
Tap	*M. fortuitum*	AGs, TET	MFS	[29,44]
Rv1258c (Tap homolog)	*M. tuberculosis*	INH, RIF, EMB, OFL	MFS	[29,43,45]
Rv1877	*M. tuberculosis*	TET, KAN, ERY	MFS	[31]
Rv1634	*M. tuberculosis*	FQs	MFS	[42]
EfpA	*M. tuberculosis*	Possibly INH	MFS	[31,50,51]
P55	*M. tuberculosis, M. bovis*	AGs, TET, RIF	MFS	[33,36,47,48]
Rv2333c (Stp protein)	*M. tuberculosis, M. bovis*	TET	MFS	[53]
MmpL5	*M. tuberculosis, M. paratuberculosis*	Azole	RND	[58]
MmpL7	*M. tuberculosis*	INH	RND	[32,55,56]
Mmr	*M. tuberculosis*	TPP, EtBr, ERY, ACR	SMR	[59]
IniA–IniB–IniC	*M. tuberculosis*	INH	Membrane protein	[35,62]
PstB	*M. smegmatis, M. tuberculosis*	INH, RIF, EMB, CIP	ABC	[127]
Rv2686c, Rv2687c, Rv2688c	*M. tuberculosis*	FQs	ABC	[65]
Rv1747	*M. tuberculosis*	INH	ABC	[63]
DrrA–DrrB–DrrC	*M. tuberculosis*	TET, STR, EMB	ABC	[64]
Rv0194	*M. tuberculosis, M. bovis*	β-lactams, STR, TET, chloramphenicol, vancomycin	ABC	[66]
Rv1218c	*M. tuberculosis*	Novobiocins, biarylpiperazines, pyridines, bisanilinopyrimidines, pyrroles, pyrazolones	ABC	[67]

ABC: ATP-binding cassette; ACR: Acriflavine; AG: Aminoglycoside; CIP: Ciprofloxacin; EMB: Ethambutol; ERY: Erythromycin; EtBr: Ethidium bromide; FQ: Fluoroquinolone; INH: Isoniazid; KAN: Kanamycin; MFS: Major facilitator superfamily; OFL: Ofloxacin; RIF: Rifampicin; RND: Resistance nodulation division; SMR: Small multidrug resistance; STR: Streptomycin; TET: Tetracycline; TPP: Tetraphenylphosphonium.

accumulation assays suggested that Rv1634 is also involved in the efflux of norfloxacin and ciprofloxacin [42].

The P55 efflux pump from *M. bovis* and its *M. tuberculosis* homolog Rv1410c has been well characterized in several studies that demonstrated their association with low-level resistance to several drugs, including tetracycline, aminoglycosides and rifampicin [33,36]. In *M. tuberculosis*, Rv1410c forms an operon with Rv1411c, encoding the lipoprotein LprG. It is thought that both genes support the *in vivo* growth of *M. tuberculosis*, and studies performed in *M. smegmatis* have shown that this operon is required for survival in the presence of noxious agents, such as ethidium bromide [47,48]. Recently, it was demonstrated that P55 plays a role in at least three important processes: it extrudes and provides resistance to several drugs (including rifampicin); it is part of the oxidative stress response; and it is needed to maintain normal growth characteristics on solid and in liquid media [3,36,49].

A less well-characterized efflux pump is the *M. tuberculosis* putative efflux protein EfpA, which presents the transporter motifs characteristic of QacA of *Staphylococcus aureus*, including those associated with proton antiporter function and those specific to drug transporters [50]. It was shown that expression of *efpA* increases in the presence of isoniazid, which could suggest that the protein encoded by this gene transports molecules involved in the synthesis of mycolic acids [51]. The deletion of the *efpA* homolog in *M. smegmatis* resulted in a twofold increase in susceptibility to ethidium bromide, gentamicin and fluoroquinolones, and a eightfold increase in susceptibility to acriflavine. However, it also resulted in a fourfold decrease in susceptibility to rifamycins and chloramphenicol and a twofold decrease in susceptibility to isoniazid and erythromycin [31].

Mutations in *efpA* have been found in *M. tuberculosis* clinical isolates that are resistant to isoniazid [52], but more studies are needed in order to completely clarify the relevance of EfpA in drug resistance.

In *M. bovis*, the efflux pump Stp (Rv2333c) conferring low-level resistance to tetracycline and spectinomycin was characterized [53]. The expression of this efflux pump increased upon infection of human macrophages, reinforcing the role of efflux pumps in other processes in addition to drug resistance.

The genome of *M. tuberculosis* contains several genes that code for putative transport proteins of the resistance nodulation division (RND) superfamily. These proteins have been designated

mycobacterial membrane proteins, large (MmpL), and are thought to be involved in the transport of fatty acids [54]. In *M. tuberculosis*, MmpL4, MmpL7, MmpL8 and MmpL1 play an important role maintaining in the virulence of the tubercle bacilli in mice [55]. This contribution to virulence may be associated with the wide range of substrates of MmpL proteins that include the lipids and mycolic acids that assemble in the mycobacterial cell wall, some of which are released from the mycobacterial envelope within the phagosomes of infected macrophages [55].

In particular, MmpL7 and MmpL8 are involved in transport of methyl-branched lipids containing carbohydrates or polyketides present in the cell wall of *M. tuberculosis*. Interestingly, MmpL7 exports phthiocerol dimycocerosate, a lipid component of the outer membrane and a related, but structurally distinct, phenolic glycolipid expressed only in a subset of highly virulent *M. tuberculosis* strains [55,56]. Upstream from the *mmpL7* gene is the *fadD28* gene, which encodes an acyl-CoA synthase that is probably involved in the release and transfer of mycocerosic acid from mycocerosic acid synthase to diols. The isoniazid resistance level shown by *M. smegmatis* cells expressing the *mmpL7* gene (MIC >512 mg/l) was more than 16-times higher than the wild-type strain (MIC = 32 mg/l). The MmpL7 protein in *M. tuberculosis* can utilize isoniazid as a substrate when it is expressed in *M. smegmatis*. In *M. tuberculosis*, isoniazid can compete with phthiocerol dimycocerosate (the natural substrate of MmpL7) since its principal physiological role appears to be the export of complex lipids to the cell exterior [32]. It has also been demonstrated that *mmpL7* is among the efflux pump genes overexpressed after exposure to isoniazid in *M. tuberculosis* [18,57].

Another important putative membrane protein of the MmpL family is MmpL5. In *M. tuberculosis*, this protein is involved in resistance to azole. Azoles are potent inhibitors of mycobacterial cell growth and have demonstrated anti-tubercular activity in mice; therefore, these drugs could constitute a novel strategy against TB. In *M. tuberculosis* strains, the resistance to azole and consequently the upregulation of *mmpS5–mmpL5* genes has been reported. This upregulation was linked to mutations in the *Rv0678* gene, hypothesized to be involved in either the transcriptional regulation of this efflux system or in its putative promoter/operator region [58].

Mmr is the only protein of the small multidrug resistance family that has been described in *M. tuberculosis* [59]. The chromosomal gene *mmr*, when inserted into a multicopy plasmid, decreases the susceptibility of *M. smegmatis* to tetraphenylphosphonium, ethidium bromide, erythromycin, acriflavine, safranin O and pyronin Y, and is present in isoforms in other Mycobacterium species (*Mycobacterium simiae*, *Mycobacterium gordonae*, *M. marinum* and *M. bovis*) [59]. Other studies have shown that *mmr* is overexpressed after exposure to isoniazid alone or in combination with ethambutol [57,60]. A recent study has shown that Mmr appears to be involved in the efflux of potential drug candidates of the pyrrole class in *M. tuberculosis* [61].

The operon Rv0341-Rv0342-Rv0343 was demonstrated to be induced by treatment with isoniazid [62]. The three genes that form this operon were designated as *iniB*, *iniA* and *iniC* (for isoniazid inducible gene) in the order they appear in the operon. From these three genes, the most studied is *iniA*, and it has been shown that it may be involved in the development of tolerance to isoniazid and ethambutol [35]. In fact, deletion of *iniA* from *M. tuberculosis* increased the susceptibility to isoniazid, whereas the overexpression of this gene in *M. bovis* allowed the survival of the organism for a longer period of time in the presence of isoniazid and ethambutol, also resulting in resistance to ethidium bromide. The exposure of the *iniA*-overexpressing *M. bovis* BCG strain to reserpine reversed both tolerance to isoniazid and resistance to ethidium bromide. The fact that IniA forms multimeric structures containing a central pore suggests that this protein could be a pump component. By this manner, IniA may function through an efflux pump-like mechanism, although it does not seem to directly transport isoniazid from the bacterial cell [35].

Genes encoding ATP-binding cassette (ABC) transporters comprise approximately 2.5% of the *M. tuberculosis* genome and at least 37 complete and incomplete ABC transporters have been identified [2,3,63]. However, only a few of these transporters have been characterized and shown to be involved in drug resistance. *M. tuberculosis* contains a putative doxorubicin-resistance operon, *drrABC* [64]. The *drrAB* genes expressed in *M. smegmatis* confer resistance to a broad range of antibiotics, including tetracycline, erythromycin, ethambutol, norfloxacin, streptomycin and chloramphenicol. The resistant phenotype is reversed by treatment with reserpine or verapamil, compounds known to inhibit efflux [64]. The *M. tuberculosis* Rv2686c-Rv2687c-Rv2688c operon encodes an ABC transporter responsible for fluoroquinolone efflux when produced from a multicopy plasmid. When overexpressed in *M. smegmatis*, this operon increases the MIC of ciprofloxacin eightfold and the MIC of norfloxacin twofold. The level of resistance decreases in the presence of reserpine, CCCP and verapamil [65]. Interestingly, given that *M. tuberculosis* has an active β-lactamase enzyme, an ABC transporter was found to be associated with resistance to β-lactams in addition to streptomycin, chloramphenicol, vancomycin and tetracycline in *M. tuberculosis* and *M. bovis* [66]. Another ABC transporter that has been associated with drug resistance is Rv1218c; it was shown to play a role in mediating efflux to a wide variety of chemical classes, including novobiocins, biarylpiperazines, pyridines, bisanilinopyrimidines, pyrroles and, to a smaller extent, pyrazolones [67].

The contribution of efflux pumps to the increase of multidrug resistance has been shown when exposure of drug-susceptible mycobacteria to one or more antibiotics that are efflux pumps substrates results in the development of an MDR phenotype, mediated by the overexpression of efflux pumps [17,57]. This phenomenon was also observed in other bacteria such as *Escherichia coli* following exposure to tetracycline, which resulted in the overexpression of the AcrAB-TolC pump system [68,69]. This response was also observed in *S. aureus* which, when exposed to the quaternary compound ethidium bromide, resulted in the overexpression of the NorA pump [70]. Furthermore, the multisubstrate activity of many efflux pumps confers an extended resistance phenotype that favors the acquisition and stabilization,

in the bacterial population, of additional mechanisms of antibiotic resistance, such as mutations [4,18]. Therefore, a strategy to prevent the continuous increase of MDR clinical bacteria showing efflux-mediated resistance is the combination of an antibiotic with an efflux inhibitor [71,72].

Inhibition of efflux pump activity in mycobacteria

Given the contribution of efflux systems to the development of drug resistance, there is a need for compounds that are able to circumvent the efflux activity. To date, several molecules have been described as potential efflux inhibitors by our group and others; however, their precise mechanism of action within the cell remains unknown [57,72–74]. To enhance the activity of antimicrobials subjected to efflux, the antibiotic should be coadministered with an inhibitor of efflux, thus rendering the compound once again effective, even in resistant organisms [73,75]. The blocking of the efflux of an antimicrobial compound by efflux inhibitors will decrease the MIC of that compound. Several classes of compounds that inhibit RND efflux systems have been identified in screens of chemical libraries or in biodiversity-issued compounds. These molecules can be further optimized by structure–activity relationship studies and therefore potentiate the activity of antibiotics that are substrates of RND efflux systems [74,75]. Nevertheless, while it is very compelling scientifically and has clear potential clinical benefit, this approach brings in many challenges. Besides the obvious issues common to all the infectious diseases drug development programs (appropriate potency, spectrum of activity, bioavailability, clearance, toxicity and so on), clinical trials must be designed to demonstrate improvement of a combination product over existing therapies [76]. So far, many putative inhibitors of bacterial efflux pumps have been tested and reported in literature, but none has evolved toward clinical usage, with the exception of omeprazole [76–78].

Compounds with inhibitory activity against efflux in mycobacteria (Table 2) also show antimicrobial activity. Some of these have antipsychotic properties and are used in clinical practice as antidepressants, anxiolytics and/or antihypertensives. The discovery that antipsychotic drugs could have anti-TB activity began in 1951 with the resynthesis of isoniazid and the synthesis of iproniazid, an isoniazid derivative, as a part of a deliberate search for new anti-TB drugs [79]. Together with isoniazid, iproniazid was found to possess anti-TB activity and was subsequently used to treat TB. However, iproniazid has greater toxicity when used at clinically effective concentrations compared with the parental compound . For this reason, its use as anti-TB drug was not feasible [80]. In 1952, iproniazid was demonstrated to inhibit monoamine oxidase, the enzyme that breaks down the neurotransmitter, serotonin [81]. This showed that hydrazine derivatives have antidepressant properties [82]. These heterocyclic compounds (Figure 1) are dopamine agonists and calcium channel blockers, blocking D_2 receptors in the dopamine pathways [83]. These compounds possess calmodulin-blocking properties and inhibit voltage-activated calcium channels and calcium-activated potassium channels [84]. In eukaryotic cells, L-type calcium channels need cell depolarization for activation [85]. Membrane depolarization occurs as a consequence of cell trauma leading to intracellular calcium overload [83]. Once membrane depolarization occurs, calcium enters and initiates several damage mechanisms. The role of calcium channel blockers is to restore the homeostasis at the membrane level, inhibiting the influx of calcium and retention of dopamine. The primary mode of action of these compounds in prokaryotic cells, although far from being completely elucidated, seems to be quite similar, that is, through their interference with calcium

Table 2. Compilation of the main compounds reported to have inhibitory activity against efflux in mycobacteria.

Substrates/inhibitor (drug class)	Pharmacological/physiological mode of action	Organism	Ref.
Verapamil (phenylalkylamine)	Antihypertensive; calcium channel blocker	*M. smegmatis*; *M. avium* complex; *M. tuberculosis* complex	[18,57,90,91,122,128]
Thioridazine, chlorpromazine (phenothiazine)	Antipsychotic; calcium channel blocker	*M. smegmatis*; *M. avium* complex; *M. tuberculosis* complex	[18,57,90,91,122]
Cis-(Z)-flupentixol (thioxanthene)	Antipsychotic; calcium channel blocker	*M. avium* complex	[99, Machado *et al.*, Unpublished Data]
Bromperidol, haloperidol (butyrophenone)	Antipsychotic; calcium channel blocker	*M. smegmatis*; *M. tuberculosis* complex; *M. avium* complex	[102, Machado *et al.*, Unpublished Data]
Reserpine (plant alkaloid)	Antihypertensive; calcium channel blocker	*M. smegmatis*; *M. avium* complex; *M. tuberculosis* complex	[34,35,128]
Farnesol (natural plant metabolite)	Antipsychotic; calcium channel blocker	*M. smegmatis*	[112]
Carbonyl cyanide-*m*-chlorophenylhydrazone (protonophore)	Uncoupler of the proton motive force	*M. avium; M. smegmatis; M. fortuitum*	[44,65,90]
L-phenylalanyl-L-arginyl-β-naphthylamide (dipeptide amide)	Putative efflux-substrate competitor and outer membrane permeabilizer	*M. tuberculosis*	[61]

transport at the membrane level, resulting in the intracellular accumulation of the compounds [86]. A description of the major compounds for which inhibitory activity against mycobacterial efflux pumps has been demonstrated is made in the following sections.

Phenylalkylamines

The prototype of this class, verapamil, is a calcium channel blocker used for the treatment of various disorders, such as angina, hypertension and cardiac arrhythmia [87,88]. Verapamil is also an inhibitor of P-glycoprotein in mammalian cells [89]. To date, several studies demonstrated that verapamil possesses the strongest capacity to inhibit efflux of ethidium bromide by *M. smegmatis*, *Mycobacterium avium* complex [90–92] and *M. tuberculosis*, when compared with other efflux inhibitors like thioridazine and chlorpromazine [18,57]. Also, verapamil was able to significantly reduce the MIC values for isoniazid in the same *M. tuberculosis* strains [18,57].

Phenothiazines & related compounds

The phenothiazines are antipsychotic drugs that have long been known to have *in vitro* and *in vivo* antimycobacterial activity. Phenothiazines are divided into three groups: the aminoalkyl compounds, like chlorpromazine; piperidine compounds, like thioridazine and; piperazine compounds, like fluphenazine. Chlorpromazine was the first antipsychotic drug discovered [93]. The phenothiazines have long been known to have *in vitro*, *ex vivo* and *in vivo* antimycobacterial activity [94–96]. In eukaryotic cells, the principal mode of action of phenothiazine derivatives is the inducement of changes in the membrane structure with consequent inhibition of electron transport [93]. This class of drugs is known to inhibit the transport of calcium by preventing its binding to calcium-dependent enzymes [93,97]. The ability of thioridazine and chlorpromazine to inhibit the efflux of ethidium bromide, accompanied by the reduction of the MIC of several compounds, has been well demonstrated in *M. smegmatis* and *M. avium* complex [90–92], but in *M. tuberculosis*, these two compounds have shown a less evident *in vitro* inhibitory effect [18,57]. However, improvements in the phenothiazine basic structure can make these compounds more efficient. The phenothiazines possess a three-ring structure, in which a sulfur atom and a nitrogen atom are linked to two benzene rings. With a substitution of the nitrogen atom by a carbon atom in the central ring it becomes a thioxanthene [97]. The thioxanthenes can be represented as two geometric isomers, cis(Z) and trans(E), but only the cis(Z) possess antipsychotic properties [97]. Among these compounds, flupenthixol is a high-potency thioxanthene with well-established antipsychotic properties [98]. It was first tested against mycobacteria in 1986, revealing that *M. tuberculosis*, *M. avium* and

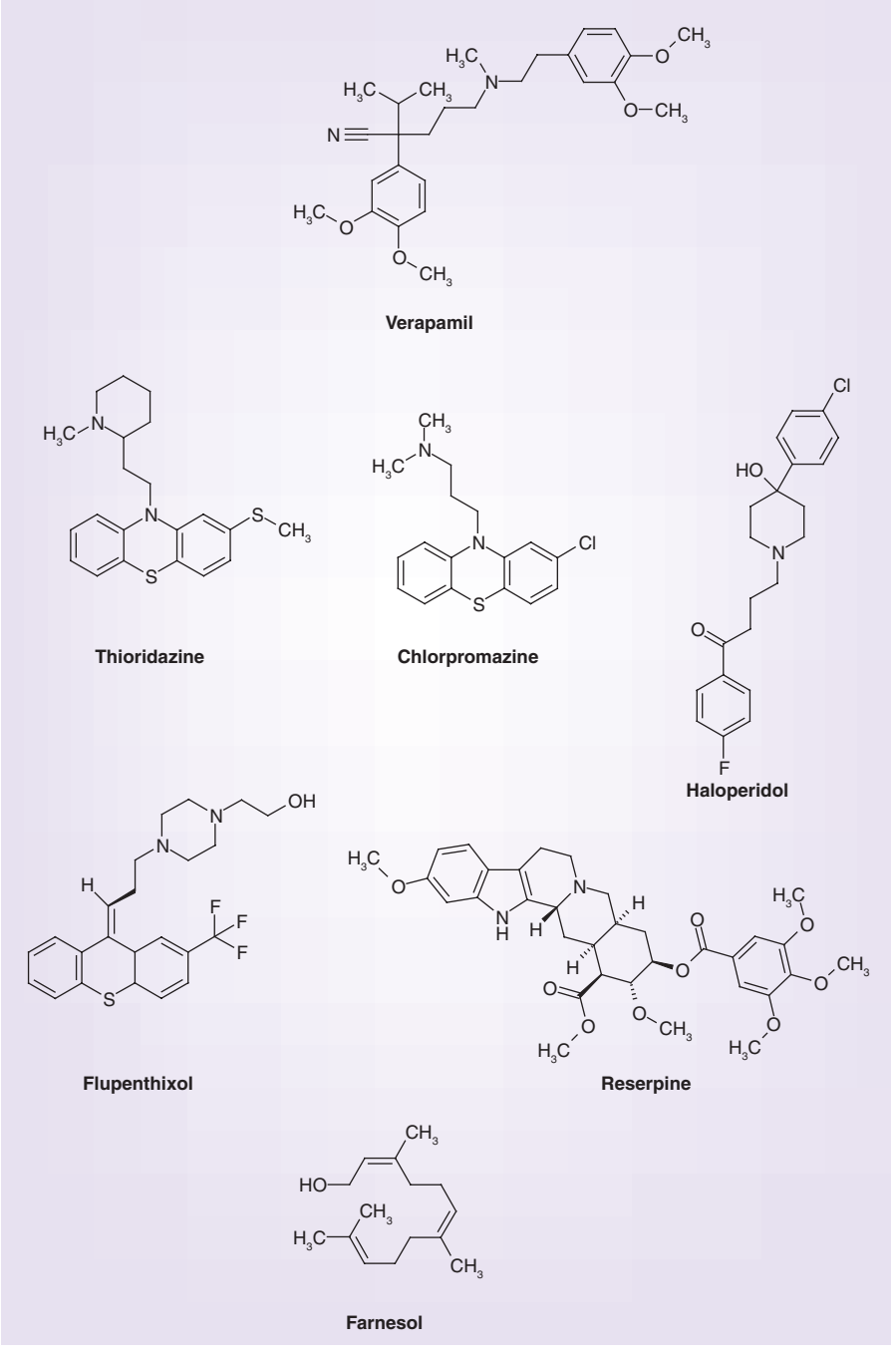

Figure 1. Structures of the most important compounds reported to have inhibitory activity against mycobacteria.

Mycobacterium intracellulare are highly sensitive to this drug [99]. Besides the thioxanthenes, the butyrophenones, structurally and clinically related to phenothiazines and whose representative is haloperidol, are also used as antipsychotic drugs [100]. The chemically related compound bromperidol [101] has been demonstrated to possess antimycobacterial activity against *M. smegmatis* and *M. tuberculosis*, as described by Ramón-García *et al.* [102]. The similarities between butyrophenones and phenothiazines suggest their usefulness as adjuvants in the therapy of TB [102]. Regrettably, the butyrophenones seem to have poor *in vitro* activity against *M. tuberculosis* and reduced capacity to decrease the efflux of ethidium bromide by *M. avium* and *M. intracellulare* [Machado *et al.*, Unpublished Data].

Recently, a pyrrole derivative, BM212 [1,5-diaryl-2-methyl-3-(4-methylpiperazin-1-yl)methyl-pyrrole], was revisited and shown to be active against MDR *M. tuberculosis* clinical isolates *in vitro* and within the macrophage, as well as against atypical mycobacteria [103,104]. These data suggest that BM212 is a potential anti-TB drug that targets the RND transporter MmpL3 [104].

Natural plant metabolites

Reserpine is an alkaloid extracted from the root of the climbing shrub *Rauwolfia* and has been used for decades as an antihypertensive agent [105]. Previous studies indicate that reserpine may have a calcium channel blocker-like activity [106]. Another study showed that reserpine inhibits L-type calcium channels in pituitary cells [107]. Reserpine has been shown to be an effective inhibitor of the pyrazinoic acid efflux pump, an active derivative of pyrazinamide; therefore, increasing the susceptibility to pyrazinamide in *M. tuberculosis* [108] and isoniazid efflux in *M. tuberculosis* and *M. bovis* BCG [34,35]. A recent study indicates that reserpine and its derivatives have antioxidant and antimycobacterial activities [109]. Although reserpine is no longer extensively used, its use as an efflux inhibitor is compromised due to its carcinogenicity [110]. However, another natural plant metabolite, farnesol, has been described to block smooth muscle L-type calcium channels [111]. Farnesol is also an antipsychotic substance and a recent study indicates that farnesol is effective in blocking the efflux of ethidium bromide in *M. smegmatis* [112]. Unlike reserpine, farnesol was shown to be devoid of toxic effects and nonmutagenic *in vitro* and *in vivo* [113].

Excluding phenothiazines, the other drugs mentioned above have been less studied in mycobacteria, although verapamil and flupenthixol have demonstrated increased inhibitory efflux activity and antimycobacterial properties. Moreover, atypical antipsychotics designated as selective serotonin reuptake inhibitors (SSRIs) are drugs that, in eukaryotic cells, work by modifying the activity of serotonin. They act by inhibiting the pump that exists in the synaptic space that directs serotonin toward the presynaptic neuron, increasing the amount of serotonin at the synapticspace [114]. The SSRIs can be looked at with renewed interest, not only due to their surprising antibacterial activity (mainly against Gram-positive bacteria [115]) and as inhibitors of Red-Nile efflux by *E. coli* strains overproducing several RND efflux pumps [116], but also because verapamil, despite its D_2 agonist profile, also demonstrates SSRI properties. These atypical antipsychotics have fewer side effects than older antipsychotics, making their study as potential antimicrobials quite appealing.

Another compound widely used in mycobacterial efflux assays is CCCP. This nitrile, hydrazone and ionophore compound is known as a chemical inhibitor of oxidative phosphorylation. CCCP affects the protein synthesis in seedling mitochondria, by causing an uncoupling of the proton gradient established during the normal activity of electron carriers in the electron transport chain. This compound acts as an ionophore, which affects the energy level of the bacterial membrane, reducing the ability of ATP synthase to function optimally, and is used to dissipate the proton motive force and inhibit the efflux of several drugs [3,29,36,45,58]. However, this compound also reduces the viability of the bacterium and causes cell death, and therefore, the observed effect on the efflux activity may also be due to causes other than efflux inhibition depending on the concentration used. Recently, Phe-Arg-β-naphtylamide, a broad-spectrum efflux inhibitor in a variety of Gram-negative bacteria [73,74,78], was reported to be active against *M. tuberculosis* [61]. Regrettably, these compounds are highly toxic in humans, and there is evidence that they can also be substrates of efflux pumps [73,74,76,78].

The potential use of some of these compounds that are devoid of toxic effects as inhibitors of mycobacterial efflux pumps can constitute an important alternative as adjuvants in the conventional therapeutic regime of TB [2,71,117,118]. The advantage of this strategy is that they are normally employed in clinical practice and are approved by the US FDA, shortening the time required for the endorsement of novel proposals. However, the therapeutic utility of compounds with the ability to inhibit bacterial drug efflux pumps and therein potentiate the activity of coadministered antibiotics remains to be validated in the clinical setting. Nevertheless, this is a strategy that safeguards the future of TB treatment by improving the efficacy and extending the clinical utility of existing antibiotics. Additionally, if some of these compounds also have an enhancer or immunomodulatory effect on *M. tuberculosis*-infected macrophages, their utility can be expanded and novel treatment regimens explored. This approach will be the subject of the following section.

The dual role of phenothiazines in mycobacteria: efflux inhibitors & macrophage killing activity booster compounds

Pulmonary TB is considered mainly an intracellular infection of the alveolar macrophage. Therefore, drugs that seem to be effective against *M. tuberculosis* must have activity where the mycobacteria are usually sequestered, that is, at its intracellular site. Moreover, a drug that is able to stimulate or induce the macrophage to kill the internalized bacteria will bypass the problem of antibiotic resistance of the bacterium itself. Consequently, the antimicrobial effect of the drug and/or its enhancer or immunomodulatory effect on the infected macrophage can be correlated. On the basis of this premise, instead of designing a drug that is only active against the bacteria itself, it may be wiser to design drugs that promote intracellular killing of the bacteria, regardless of its

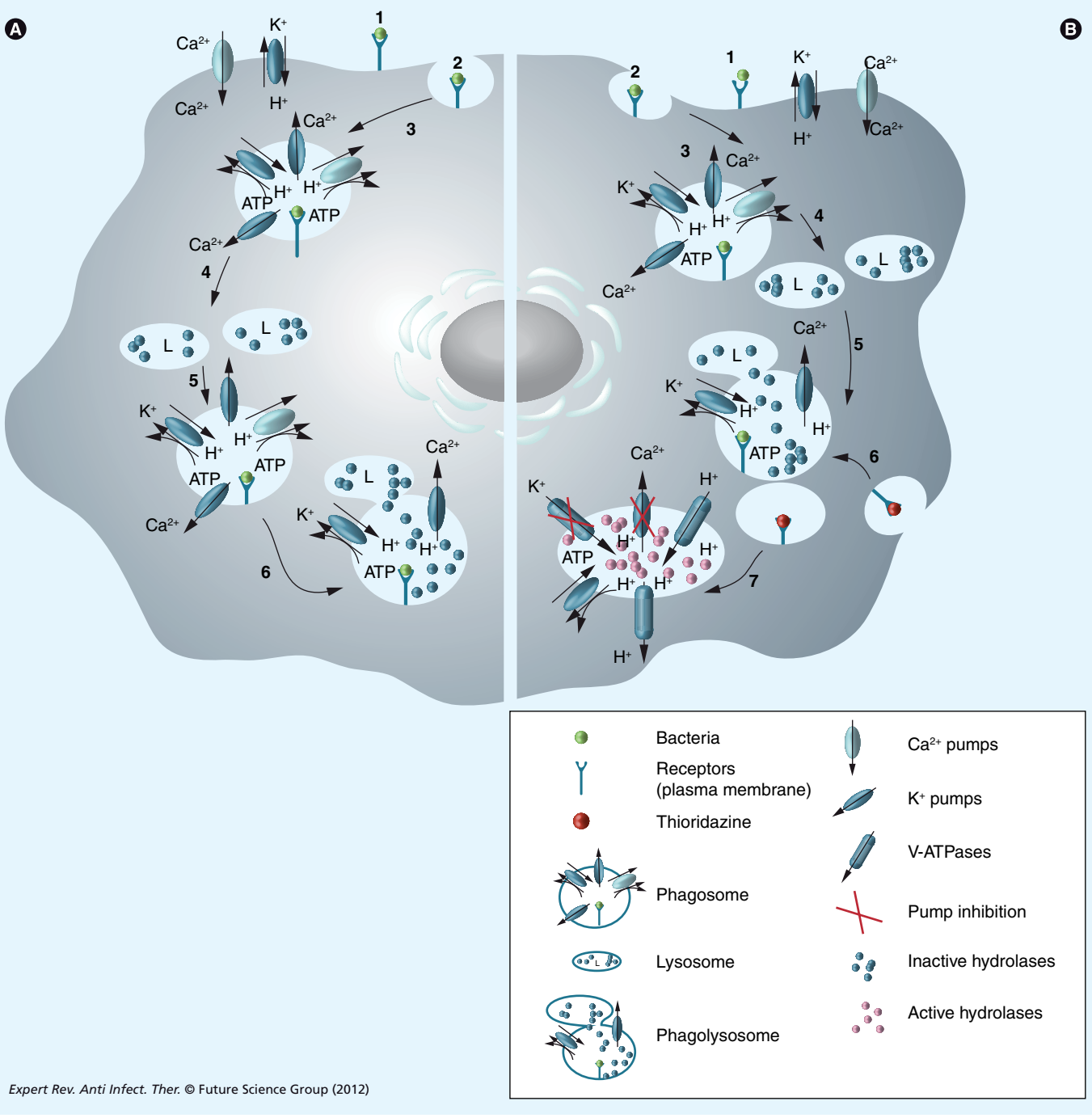

Figure 2. Theoretical model proposed for the enhancement of the macrophage killing activity by the efflux pump inhibitors. **(A)** Manipulation of the macrophage apparatus by *Mycobacterium tuberculosis*. **(A1)** The bacterium is recognized by receptors present on the plasma membrane of the macrophage. The mycobacterium is internalized by the invagination of the plasma membrane **(A2)** into a phagosome **(A3)**. The pumps present in the plasma membrane of the macrophage transport potassium (K^+) into the macrophage. Due to the invagination, these pumps will extrude potassium from the macrophage lumen to the cytoplasmic compartment. When the phagolysosome is formed, these pumps obviate or reduce the availability of potassium and calcium (Ca^{2+}), and hence, the process of acidification needed for the activation of the hydrolases does not take place and the bacteria are not killed **(A4–6)**. **(B)** Infected macrophage treated with inhibitors of efflux pumps. **(B1–5)** The mycobacterium is internalized by the macrophage and resides in the phagolysosome of the cell. **(B6–7)** When the inhibitors of potassium and calcium flux are added to the infected cultures, the calcium-activated potassium pumps of the phagolysosome are inhibited, as well as the calcium channels. The concentration of potassium is increased by diffusion from the cytoplasm into the phagolysosome and the vacuolar proton (H^+)-ATPases are activated to compensate the hypertonicity of the organelle, pumping protons into the lumen. **(B7)** This rise of protons causes the decrease of the pH in the phagolysosome, activating the hydrolases that consequently kill the mycobacteria.
Data taken from [117,118].

antibiotic resistance status, such as the case of *M. tuberculosis*. Consequently, the therapeutic regimens could be shortened and the drug's efficacy significantly increased.

In a situation of infection, the mycobacterium is recognized by receptors on the plasma membrane of the macrophage (Figure 2A1) and internalized, inducing an invagination of the plasma membrane (Figure 2A2), called a phagosome (Figure 2A3). The pumps, present in the plasma membrane, that usually transport potassium into the macrophage will still be present on the membrane of the phagosome. However, these pumps extrude potassium from the macrophage lumen to the cytoplasmic compartment. When the phagosome fuses with the lysosome (forming the phagolysosome), these pumps obviate or reduce the availability of potassium and calcium, and hence, the process of acidification needed for the activation of the hydrolases does not take place [117–120], and the mycobacteria are not killed (Figure 2A4–6). This is one of several mechanisms that *M. tuberculosis* has to persist in the interior of the phagocytic cell. Additionally, the ability to block the maturation of the phagosome or just simply avoid the fusion of it with lysosomes contribute to its success as an intracellular pathogen. Taken together, the ability to manipulate the macrophage destruction mechanisms is an ideal situation for the mycobacteria and leads to persistence. This persistence of *M. tuberculosis* inside human macrophages has been identified as one of the factors that contribute to the failure of many therapeutic regimens.

In the last few years, several strategies have been pursued and explored in order to overcome this problem. One of the strategies relies on the action of inhibitors of bacterial efflux systems, such as the phenothiazines. These compounds show a dual-role approach, that is, they are active against the sequestered bacteria as well as acting as activators of the infected macrophages. Therefore, they are excellent candidates to treat intracellular infections. The mechanism by which these efflux inhibitors modulate the killing of intracellular MDR-TB/XDR-TB by nonkilling human macrophages has been suggested by several studies [46,117,118]. The killing activity of macrophages is known to be dependent upon the availability of potassium and calcium ions. These ions contribute to the process of acidification of the phagolysosome and the subsequent activation of its hydrolases, which in a final step degrade the bacterium [119,120]. As a result, it is anticipated that the effect of inhibitors of potassium and calcium transport/efflux, such as ouabain, verapamil or the phenothiazines, on the killing of phagocytosed MDR-TB/XDR-TB be enhanced [118]. The phenothiazines induce changes in the structure of the membrane with consequent inhibition of electron transport. These compounds inhibit the transport of calcium by preventing its binding to calcium-dependent enzymes [97,117,118]. In this way, the enhancement of killing stresses its effect on the transport of calcium and potassium along a theoretical model that describes the sequence of events that result in the promoted killing of the intracellular mycobacteria (Figure 2B).

After the mycobacterium is internalized by the macrophage, as previously described, it resides in the phagolysosome of the cell (Figure 2B1–5). When the inhibitors of efflux/potassium and calcium flux (e.g., phenothiazines) are added to macrophage cultures that contain the phagocytosed bacteria, the calcium-activated potassium pumps of the phagolysosome are inhibited, as well as the calcium channels (Figure 2B6–7). The concentration of potassium is increased by diffusion from the cytoplasm into the phagolysosome, and the vacuolar proton-ATPases (also known as V-ATPases) are activated to compensate the hypertonicity of the organelle by pumping protons into the lumen [118]. This rise of protons is responsible for the pH decrease in the phagolysosome and therefore, for the activation of the hydrolases and consequent killing of the mycobacteria (Figure 2B) [118–120].

The demonstration that ion-dependent efflux pump inhibitors promote the killing of intracellular bacteria by having an effect on the killing apparatus of the nonkilling macrophage results in a totally new concept for the therapy of pulmonary TB infections caused by MDR-TB, XDR-TB and now TDR-TB [121]. This novel concept focuses on targeting the macrophage rather than the bacterium itself. Because the killing is independent of the bacterium, the antibiotic resistance status of the bacterium is not relevant. Whereas drugs that target the bacterium itself will eventually lose their effectiveness due to spontaneous mutations that result in the modification of the intended target, the activity of an agent that targets the macrophage evades the problem of bacterial resistance. This means that the problem of bacterial resistance may no longer result in problematic therapy.

Additionally, these compounds reduce or reverse multidrug resistance in bacteria [77,78], particularly in mycobacteria [18,61,122], as described in the previous sections. These compounds are able to inhibit the transport of ions by preventing their binding to ion-binding proteins and, as a consequence, enzyme systems, such as those involved in generating cellular energy obtained from the hydrolysis of ATP, are inhibited [123,124]. Considering that overexpression of efflux pumps is associated with multidrug resistance and that most of these systems are driven by the proton-motive force, which is dependent of ion-dependent enzyme systems, their inhibition will render the bacteria susceptible to an antibiotic to which they were initially resistant [71,76–78].

However, to develop these agents for a clinical application in the therapy of TB, they have to be devoid of serious side effects at concentrations that target the killing apparatus of the pulmonary macrophages. Although hundreds of compounds have *in vitro* activity against antibiotic-susceptible and -resistant strains of *M. tuberculosis*, few have activity against the bacterium at the intracellular site where it normally resides – the nonkilling macrophage of the pulmonary alveolar unit [121]. Recently, the proposed model for activation of the nonkilling macrophages by the efflux pump inhibitor thioridazine, and its transformation into an effective killer of phagocytosed strains of mycobacteria regardless of the bacterium's antibiotic resistance profile (MDR-TB or XDR-TB) has been substantiated by clinical evidence from MDR-TB patients who were cured in India and Argentina with combined antibiotic regimens that included thioridazine [125,126].

In summary, various compounds have been used to inhibit the efflux activity *in vitro*; their dual role as antimicrobial agents and enhancers of the macrophage activity has been demonstrated, but none of them is currently used in clinical practice for this

purpose. These inhibitors could be used as adjuvant compounds, administered in combination with conventional antibiotics to which the bacteria was initially resistant, taking advantage of their holistic activity, as recently demonstrated with MDR-TB-infected patients [125,126]. However, clinical trials are still needed to demonstrate the importance of efflux inhibitors as adjuvant compounds in the therapy of drug-resistant bacterial infections. Special concern with the selectivity of these compounds should be taken, since they can inhibit both eukaryotic and bacterial efflux systems, and its safe usage has to first be guaranteed in analysis [78,125]. In conclusion, so far the search for efflux pump inhibitors has proven to be a challenge and more studies are required until effective and specific inhibitors are found, and in the future, seriously incorporated in antidrug-resistant TB regimens.

Conclusion

M. tuberculosis continues to be a major threat for public health worldwide, and only a scarce number of antibiotics are active against this pathogen, as it easily acquires resistance to these drugs, leaving a very limited therapeutic option to the clinician. For this reason, treatment of TB consists of a combination of three or more relatively effective drugs that must be given simultaneously for a long period of time in order to potentiate the synergistic activity, prevent the selection of resistant strains and guarantee a successful outcome. In addition to the mutation-mediated acquisition of drug resistance, *M. tuberculosis* is provided with a diversity of intrinsic drug resistance mechanisms, such as the impermeability of the cell wall and the activity of drug efflux pumps that contribute to resistance and act in synergy with drug resistance mutations, allowing the bacteria to survive for a longer period of time in the presence of subinhibitory concentrations of antibiotics until mutations emerge. One of the main reasons why several antibiotics and regimens have not been successful in the treatment of TB is the fact that they target solely the bacteria, not taking into account that, in the particular case of TB, the bacteria reside inside the pulmonary macrophage. Several approaches have been pursued in the last few years to discover compounds that are able to kill the internalized bacteria. One of the strategies is to use compounds that target the infected cell of the pulmonary system – the macrophage of the alveoli. A group of compounds, known as efflux pumps inhibitors, has been studied in the past few years because of their dual role as antimicrobial agents and enhancers of the macrophage activity [118]. However, despite all the efforts made, none of them have been implemented or used in clinical practice. If these compounds can be administered in combination with conventional antibiotics to which the bacteria was initially resistant, they could target the infected cell by modulating its killing activity, and in this manner, overcome the status of antibiotic resistance shown by the bacteria. Preliminary studies were already conducted in MDR-TB-infected patients, with very promising results [125,126]. We should bear in mind that there is still the need for clinical trials to demonstrate once and for all the importance of efflux inhibitors as adjuvant compounds for the therapy of MDR bacterial infections. Specific issues such as the compound's selectivity and its safe usage have to be addressed, since these compounds have the ability to inhibit both eukaryotic and bacterial efflux systems due to their structural similarity. Even so, the 'proof of concept' is on its way and the fact that several studies report the successful use of these compounds to treat MDR infections opens new avenues for their possible implementation in the clinical practice.

Expert commentary

Evidence has recently been gathered that shows efflux mechanisms play a significant role in the acquisition of resistance in *M. tuberculosis*. This is a particularly relevant phenomena in the case of long-term therapies such as that used in TB treatment and easily potentiated by the patient's nonadherence to the therapeutic regimen [4,18–20]. An obvious way to prevent these events from occurring would be the inhibition of efflux pumps using efflux inhibitors to increase the activity of antibiotics that are subject to efflux and decrease the frequency of selection of drug-resistant mutants. Unfortunately, this approach has been hampered by the fact that the majority of the efflux inhibitors have *in vitro* activities at concentrations that are toxic *in vivo*. From the group of known mycobacterial efflux inhibitors, there are a few that have been used for decades as neuroleptics or antihypertensive drugs, which not only have *in vitro* activity against *M. tuberculosis* regardless of antibiotic resistance, but also boost the killing of these bacteria in the macrophage [46,117–121]. Targeting the non-killing macrophage to kill intracellular MDR/XDR/TDR-TB in this dual perspective avoids the problem of mutational responses by the bacterium, which result in resistance and enhances the successful outcome. This new therapeutic concept on the usefulness of nonantibiotic agents that can act as efflux inhibitors in mycobacteria, as adjuvants of the conventional antimycobacterial therapy, would not only restore the activity of antibiotics that are subject to efflux but also prevent the selection of drug-resistant strains. The control of these infections by enhancing the activity of existing anti-TB drugs and simultaneously boosting the killing activity of the macrophages, must be wisely explored as a potential new strategy to avoid the ongoing deadly spiral of drug resistance in TB and overcome the desperate scarcity of new therapeutic approaches.

Five-year view

The global resurgence of pulmonary TB, coupled with the development of MDR/XDR/TDR-TB infections, is mainly due to the failure of the TB control policies. WHO recommends the implementation and application of new molecular methods for the direct screening of active TB, demanding rapid, aggressive and effective therapeutic regimens. During the next 5 years, we are likely to see more MDR/XDR/TDR-TB cases being diagnosed early without a really effective therapeutic regimen available to be implemented. A deeper understanding on how drug-resistant TB develops and how treatment regimens that are available today can be made more effective is urgently needed. The use of therapeutic adjuvants, to be employed for those situations where standard antibiotic regimens fail, might be a judicious option in the future and therefore studies

to provide recommendations for their use in human patients are needed and should be seriously considered [118,125].

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Key issues

- Tuberculosis (TB) and drug-resistant TB is a major health problem worldwide, and drug-resistant forms of *Mycobacterium tuberculosis* demand effective therapeutic regimens to be implemented as soon as possible.
- Drug-resistant forms of *M. tuberculosis* are selected and potentiated during standard TB treatment failure and the early detection of the multi-, extensively and totally drug-resistant TB-infected patients using molecular diagnosis is a determinant in the contention of transmission and in the implementation of effective therapy.
- Efflux-pump mechanisms play a significant role in the acquisition of resistance *in M. tuberculosis* during treatment and an obvious way to prevent these events from occurring would be the inhibition of efflux pumps by using efflux inhibitors.
- The use of efflux inhibitors as therapeutic adjuvants, to be employed for those situations where standard antibiotic regimen has failed, has already gathered enough scientific evidence to support the clinical study of these compounds for the effective therapy in the infected patient.
- Efflux inhibitors that also affect the transport of potassium and calcium from the phagolysosome containing the bacteria, promoting its acidification and the consequent activation of hydrolases, like the phenothiazine thioridazine, show high activity against intracellular multi- and extensively drug-resistant TB.
- Clinical studies that provide recommendations for its use in human patients are needed and should be seriously considered in the near future.

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