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Efflux Pump Mediated Second-Line Tuberculosis Drug Resistance

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Abstract

The burden and spread of drug-resistant tuberculosis disease is a major public health problem worldwide. The causative agent, *Mycobacterium tuberculosis* uses several mechanisms to counteract therapy through drug-resistance. A major and most common mechanism of drug-resistance is mediated through target mutations. Efflux pumps are emerging as potential agents of drug-resistance and treatment failure. In this review we explore the origin and principles of efflux pump-mediated resistance and determine their impact on second-line drugs used against extensively drug resistant tuberculosis. Inhibition of efflux pumps as a therapeutic intervention is also discussed.

Keywords: Efflux pumps; Second-line drugs; Mutations; Efflux pump inhibitors; Minimal inhibitory concentration

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a serious problem worldwide, with about 9.6 and 13 million incident and prevalent cases reported in 2014 respectively [1]. The burden of TB continues to be a global threat and has shown to be difficult to control in regions with high prevalence to human immune deficiency virus (HIV) infection [2]. There were 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4 million among HIV-positive people), of which approximately 890 000 were men, 480 000 were women and 140 000 were children [1]. The global resurgence of TB due to progression of antibiotic resistance *M. tuberculosis* from mono-resistant through multi-drug resistant (MDR), extensively drug-resistant (XDR) and now totally drug-resistant (TDR) forms is worrisome [3,4]. Resistance to at least rifampicin (RIF) and isoniazid (INH) the two most effective TB drugs used in first line treatment is defined as MDR-TB [5]. Further resistance of MDR-TB strains to fluoroquinolone (FLQ) and at least one of the three following injectable drugs namely capreomycin (CAP), kanamycin (KAN), and amikacin (AMK) leads to XDR-TB [6].

Status of XDR-TB Treatment

As early as 2006, XDR-TB has been reported in 17 countries and now found throughout the world is difficult to treat [6]. Primary XDR-TB patients are treated with second-line drugs (SLDs) and the duration of the intensive phase of treatment with an injectable drug (i.e., CAP) is at least 6 months while the continuation phase (without the injectable drug) should last until 18 months after culture conversion [7]. According to treatment guidelines, registered chronic TB patients are started on a standard, Green Light Project-approved, category IV regimen, which consist of an intensive phase of 6 months with pyrazinamide, ofloxacin (OFL), KAN, ethionamide and cycloserine, followed by a 15-month continuation phase with OFL, ethionamide and cycloserine [8]. In South Africa, CAP has replaced AMK/KAN, while p-aminosalicylic acid replaced pyrazinamide and moxifloxacin replaced OFL for the treatment of XDR-TB [9].

Classification of Second-Line Drugs

Most of the treatment regimens against XDR-TB require the use of SLDs which are less effective and toxic. Several factors are considered when choosing the appropriate drug, including availability, rationale, the cost of the drug, and the possibility of toxic adverse events [7]. Drugs used for treatment of drug-resistant TB are classified into five groups; the first group (RIF and INH) is reserved for TB treatment while the last four groups are used for MDR and XDR-TB treatment. The second group of drugs is also mainstay in the treatment of MDR and XDR-TB and includes the aminoglycosides (streptomycin (STR), KAN, and AMK) and polypeptides (CAP) [7]. The FLQ drugs are classified under group three and deliver better clinical outcomes than drugs in the other groups [7]. Main drugs that belong to FLQ group (i.e., OFL, levofloxacin, moxifloxacin and gatifloxacin) are the most effective SLDs recommended for treatment of MDR and XDR-TB patients [10]. However, moxifloxacin a new-generation drug, has been recommended by the World Health Organization (WHO) for the treatment of XDR-TB [11]. The p-aminosalicyclic acid, cycloserine and ethionamide are bacteriostatic agents and classified under group four drugs [7]. Linezolid and clofazimine classified under group five are considered as third-line may be an important option for the treatment of XDR-TB; however are associated with adverse events [12,13]. Other drugs in these groups include bedaquiline (BDQ), a diarylquinoline, approved for the treatment of MDR and XDR-TB in combination therapy with at least three other active drugs [14].

Newer drugs under this group include delamanid (a nitroimidazole) that received accelerated approvals based on small trials showing sputum culture conversion [15]. A new and more potent ethylenediamine derivative (SQ109) is active against ethambutol (EMB)-resistant *M. tuberculosis* strains and targets MmpL3, a membrane transporter involved in mycolic acid synthesis and cell wall assembly [16,17].

Drug Resistant Mechanisms of Second-Line Drugs

The main mechanism in development of FLQ resistance in *M. tuberculosis* is by chromosomal mutations within the quinolone resistance-determining region (QRDR) of gyrA or gyrB genes [18]. However, only 60-70% of *M. tuberculosis* strains with FLQ resistance can be accounted for by these mutations within QRDR [19]. Most of the mutations are mostly found in positions 90, 91 and 94 in the gyrA gene [19]. In addition, novel mutations within gyrA such as Met81Thr, Leu109Pro, and Gln113Leu substitutions have been detected in FLQ resistant strains [20]. Other factors such as active efflux mechanisms could contribute to the rest of FLQ resistance [21].

Aminoglycosides (i.e., AMK, KAN) and cyclic peptides (i.e., CAP) group referred to as injectables inhibit protein synthesis through modification of ribosomal structures at 16S rRNA and formation of 30S ribosomal subunit respectively [22]. Appropriate use of "second-line" injectable drugs of AMK, KAN and/or CAP is critical for the effective treatment of MDR-TB and prevention of XDR-TB [23]. Point mutations of A1401G and G1484T within 16S rRNA gene (rrs) are responsible for high level resistance to AMK and KAN drugs [24]. Resistance to KAN drug is further caused by C14T, G37T and G10A mutations found within the promoter region of enhanced intracellular survival (eis) gene [25]. While CAP resistance is mainly caused by rrs C1402T and tlyA gene mutations, however they have been detected with low frequency [26]. Therefore, only 70-80% of global *M. tuberculosis* strains with injectable resistance harbour rrs, eis and tlyA mutations [23]. The remaining resistance cannot be explained on the basis of these target site mutations and other mechanism could be involved.

While major resistance (60-95%) is caused by drug target mutations, mechanisms to SLD drugs are not as well understood as that of first line drugs [23,27,28]. Efflux pumps (EPs) play a role in drug resistance however their influence in the presence and absence target mutations is not well understood. Our lack of complete understanding of the resistance mechanisms to SLD makes it difficult to diagnose and treat XDR-TB timeously. Missed XDR-TB cases are mostly fatal and can lead to amplification of resistance potentially resulting in TDR-TB which is resistant to all known drugs with high mortality in the cases reported [2].

In this review we provide an overview on current knowledge on efflux mediated drug-resistant mechanisms to SLDs.

The Evolution of Drug Resistance in *Mycobacterium tuberculosis*

Drug resistance in *M. tuberculosis*, as in any other bacterium, is an outcome of multiple mechanisms operating simultaneously within the bacteria [29]. Changes within cell permeability, target gene mutations and drug efflux contribute to acquired and intrinsic drug resistance in *M. tuberculosis* [30,31]. Mutations in target genes are major contributors, but when in combination with efflux pump system they can make an organism hard to treat. Efflux pumps (EPs) are membrane transporters that can extrude a broad range of small molecules from the bacterial cytoplasm including drugs to the external environment [32]. They could be activated by immune or drug pressures when *M. tuberculosis* bacterium enters the human macrophage [33]. Once activated they decrease accumulation of antimycobacterial drugs and reduce their cytoplasmic concentration to sub-inhibitory levels [34]. The EPs systems can confer resistance to single drug or multiple classes of drugs leading to multidrug resistant phenotype [35].

Apart from chromosomal mutations, *M. tuberculosis* can increase its drug resistance by preventing the drug from entering through the cell wall and reaching site of action [4]. Activation EPs is a simple process but it does entail multiple coordinated processes which requires the binding of substrate, provision of energy, translocation of the substrate across the membrane and resetting of the transporter [36]. Multidrug resistant EPs actively extrude drugs out of the cell with a remarkably broad range of substrate specificity, and are typically most effective when combined with other resistance mechanisms such as mutations [2]. The action of EPs could prevent drugs from reaching concentrations lethal to the bug leading to intrinsic resistance [37]. A population of mycobacteria within the lung can contain members with different susceptibilities and drug concentrations fluctuate during therapy thus may favour the induction of EPs [34]. The over-expression of EPs through physiological induction and spontaneous mutation formation can significantly lower the intracellular concentration of drugs causing an impact on their clinical efficacy [38]. During patient treatment sub-inhibitory drug exposures normally reaches the site of infection and the bacterial EPs can be activated within few bacillary replication cycles leading to low level resistance [39,40]. The development drug resistance through activation of EPs is an important mechanism in *M. tuberculosis*. Below we discuss activation of EPs that could impact on the development of resistance.

Activation of Efflux Pumps and Accumulation of Mutations

There is substantial variability in the response to TB therapy, even in those patients infected with fully drug-sensitive strains [41]. Given the pharmacokinetic (e.g. time course of drug levels in body fluids) and pharmacodynamics (e.g. rate and extent of bactericidal action) variability of drug concentrations at the site of infection, optimal microbial killing may not be achieved and resistance may ensues [42]. Schmalstieg et al. proposed that the induction of EPs is not due to the immune system but is specifically in response to sub therapeutic drug stress within the bacteria [39]. It is proposed that induction of EPs which transports two or more drugs is the first step to the emergence of resistance [43]. During INH treatment, greater than 99% of the initial sputum bacillary load is killed during the first 2 days of treatment, after which the rate of killing drops off markedly [44]. The residual bacteria are a phenotypically resistant, "drug-tolerant" population; and their minimum inhibitory concentrations (MICs) remain unchanged throughout treatment [44]. Empirical studies have shown that it takes months of therapy to eradicate drug-tolerant bacteria and produce a stable cure [45]. Adams et al. proposed that EPs induced by macrophages lead to drug-tolerance, an important barrier *in vivo* to shorten TB treatment [46]. Drug-tolerant bacteria originate in macrophages and their survival is dependent on the activation of bacterial EPs used to transport drugs out of the cytoplasm [46].

The drug-tolerant bacteria continue to replicate under the protection of EPs and can generate chromosomal mutations associated with high level resistance [47]. Once mutations within drug target genes are acquired high level drug resistance in mycobacteria is initiated. Schmalstieg et al. also hypothesized that this pathway could lead to the development of drug resistance in mycobacteria [39]. Development of drug resistance leading to MDR and XDR-TB is a stepwise process and evolution from susceptible to resistant strain occurs [48].

Metabolic Changes and Efflux Pumps Activation

During infection, the *M. tuberculosis* bacilli reside in different micro environmental conditions that include lung cavities or host macrophages that are characterized by nutrient starvation, oxidative stress, and acidic pH all of which affect their metabolic statuses [49]. Such varied conditions constitute the basis for producing heterogeneous bacterial populations, including nonreplicating persisters and growing bacteria with different capacities for persister formation [49]. Persisters may lead to a slower metabolism and transcription rate in cells without specific drug resistant mutation [50]. In the presence of drugs, the average transcription rate decreases and efflux pump activity increases [51]. Moreover persistent bacteria with slow growth rate had decreased replication rate that reduced the expression of ribosomal proteins and FLQ target genes (gyrA and gyrB) [52]. Transcription studies on *M. tuberculosis* strains that lacked mutations in drug target genes had activated drug efflux pumps (i.e., DrrA) that may promote the persistence [53]. During treatment of XDR-TB patient, iniBAC operon coded by EPs was over-expressed on the fifth month of treatment and despite lobectomy procedure the infection still persisted [48]. Therefore, it is reasonable to assume that reduced cell growth and over-expression of EPs could lead to high levels of resistance [51]. Thus EP mediated resistance might be an important mechanism in persistent bacteria of patients on treatment [21].

Drug Efflux Pumps in *Mycobacterium tuberculosis*

Drug efflux pump genes code for transporter proteins involved in the natural removal of toxic substances from the interior of the cell to the exterior environment requires energy [54-56]. In terms of energy requirements and structural criteria EPs are divided into primary and secondary transporters. Bacterial EPs, including *M. tuberculosis* have been classified into five superfamilies: ATP-binding cassette (ABC), major facilitator super-family (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) (Table 1) and the multidrug and toxic compound extrusion (MATE) family [57,58]. The efflux pump proteins of MFS, SMR, RND and MATE families uses proton motive force (H+ or Na+) provided by trans-membrane electrochemical gradient of proton or sodium ion to drive the extrusion of drugs from the cell [54]. The primary ABC superfamily uses ATP as an energy source to pump drugs out of the cell and cause multidrug efflux [54,59]. Secondary transporters (i.e., MFS, RND, SMR, MATE) are the most dominant while ABC transporters are mainly substrate specific [35]. In *M. tuberculosis* genome, genes encoding mainly for drug efflux transporters belong to ABC, MFS, SMR and RND families. Multidrug efflux systems are present on bacterial cell walls and limit the access of antimicrobial agents to their targets. Moreover EPs appear to be one of the most widespread antibiotic resistance mechanisms among most microorganisms [60].

Efflux Pump Family	Efflux pump Genes	Resistance to drugs	Inhibitors of Efflux pumps	Phenotypes	Reference(s)
	Rv2936 (ddrA)			MDR-TB	[53]
	Rv1687			MDR-TB	[53]
	Rv1686			MDR-TB	[53]
	Rv2937 (ddrB)	RIF INH,STR,EMB		MDR-TB XDR-TB	[68, 2, 21, 62, 98, 99]
	Rv1456/57/58	FLQ	CCCP, TDZ,VER		[62]
	Rv2686/87/88	OFL			[62]
	Rv2477	OFL, STR			[79]
	Rv2938	STR	RES		[59, 79, 80]
	Rv0194	STR	VER, RES, CCCP		[61, 62]
	Rv1217/18	OFL			[62]
ABC	Rv2209				[62]
	Rv1258	STR,RIF, INH, FLQ	CCCP, Piperine, VER	MDR-TB	[2, 60, 65, 76, 86]
	Rv1410c			MDR-TB	[100]
	Rv3728	CFZ	CCCP	MDR-TB	[59, 80]
	Rv0783c	INH, RIF			[101]
	Rv2459	INH, EMB,STR			[55, 60]
MFS	Rv2994	STR, FLQ,CIP			[62]
SMR	Rv3065 (mmr)	INH, EMB,STR			[53, 74, 77, 80, 85]

RND	Rv2942	BDQ	CCCP		[85]

ABC-ATP binding cassette, MFS-Major facilitator superfamily, RND-Resistance Nodulation Division, SMR-small multidrug resistance, CIP-ciprofloxacin; EMB-ethambutol; INH-isoniazid; KAN-kanamycin; OFL-ofloxacin; RIF-rifampin; STR-streptomycin, BDQ-Bedaquiline, CFZ-clofazamine, FLQ-fluoroquinolone, VER-verapamil, RES-reserpine, TDZ-thriodazine, CCCP- carbon cyanide m-chlorophenylhydrazone. MDR-multidrug resistant, XDR-extensively drug resistant

Table 1: Efflux pump genes in *Mycobacterium tuberculosis* involved in drug-resistance.

Over-expression of Efflux Pumps causing Drug Resistance *in vitro*

The over-expression of EPs decreases the susceptibility of bacteria to specific drug or multiple drugs in clinical isolates [61,62]. In many cases, EPs are part of an operon, with a regulatory gene controlling expression and increased expression is associated with resistance to the substrates [63]. Over-expression or the increased activity of existing EPs in response to prolonged exposure to the sub-effective levels of anti-mycobacterial compounds, such as in the case of ineffective management of the TB patient, may render an organism increasingly resistant to one or more drugs employed in therapy [34]. Moreover, treatment of MDR and XDR-TB can be more than 24 months and *M. tuberculosis* organism under toxic pressure may over-express EPs proteins leading to resistance. Thus even in drug compliant patients, over-expression of EPs and slow metabolic processes in the case of persistent bacteria may be responsible for low level resistance to multiple drugs without the presence of drug resistant mutations with consequent treatment failure [53].

The over-expression of EPs results in sub-therapeutic intracellular concentration of drugs and may cause subsequent treatment failure of two or more unrelated drugs [64]. This was shown by simultaneous over-expression of Rv2459, Rv2728 and Rv3065 EPs associated with drug resistance to a combination of INH, EMB and STR drugs [55]. For instance, Rv1258c over-expression was shown to play a role in INH phenotypically resistant but genetically susceptible isolates [46]. Transcriptome analysis in *M. bovis* revealed that disruption of the Rv1258c gene, encoding the Tap protein, led to an extensive change in gene expression patterns during stationary phase, with no changes during exponential growth [65]. It is possible that Rv1258c may play a role in the progression from low to high level resistance through elevated MIC levels [66]. Most of the pumps studied, are Rv1218 and Rv3065 which belong to the ABC and SMR families respectively and appear to play important roles in efflux of different antibiotics [67]. Over-expression of ABC transporters of Rv1456c, Rv1457c and Rv1458c appeared in all the clinical isolates resistant to at least one of four first-line drugs, RIF, INH, STR and EMB [68]. Below we focus more specifically on overexpression of EPs that may lead to resistance to SLDs and therapeutic implications.

Fluoroquinolone Efflux Pump Mediated Resistance and Inhibition

Over-expression of Rv1634 and Rv2686c-Rv2687c-Rv2688c EPs conferred resistance to FLQ by increasing MICs values by eight fold when expressed in *M. smegmatis* [2,21]. One of these putative pumps, Rv1634 decreases susceptibility to FLQs of the same class namely norfloxacin and ciprofloxacin [60]. Several mycobacterial EPs associated with FLQ resistance have been described, including pumps of the MFS family (lfrA and Rv1258c) and ABC transporters (DrrAB, PstB and Rv2686c-2687c-2688c) [60].The FLQ resistance due to absence of DNA gyrase mutations were found to have over-expressed EPs leading to increased MICs [2]. Sparfloxacin is a more hydrophobic FLQ and strong interaction with MfpA protein coded by Rv3661 occurs, leading to intrinsic resistance [69]. Using gene expression based analysis a tenfold increase was revealed in the Rv1258c transcript level in the presence of RIF and a sixfold increase in the presence of OFL drug [70]. Moreover OFL stress altered the expression of two more EPs namely, Rv2477 and Rv2209 of ABC family [62]. Apart from mutations within target genes, EPs are recognized as causes of resistance to FLQ group, however it remains to be seen if newer drugs of moxifloxacin and gatifloxacin are similarly affected. To counteract the effects of resistance by EPs, an efflux pump inhibitor (EPI) is used.

Other FLQ drugs such as ciprofloxacin had MICs decreased in 30% of resistant strains that over-expressed EPs [71]. While the MIC levels of linezolid (a protein synthesis inhibitor) were slightly reduced in the presence of reserpine (RES) [72]. Efflux pump inhibitors such reserpine, verapamil (VER), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and thioridazine (TDZ) have been shown to inhibit overexpression of Rv2686c-Rv2687c-Rv2688c operon involved in drug resistance [21]. The ABC transporters were inhibited by VER in the development of OFL resistance in *M. tuberculosis* isolates [73]. Over-expression of Rv1634, used by the pathogen as a potential mechanism to resist drug activity is inhibited by TDZ [74]. Moreover FLQ resistant isolates without mutation in the DNA gyrase region, had their MICs reduced by reserpine, VER and CCCP indicating the expression of efflux mediated resistance [75].

Aminoglycosides and Cyclic Peptides Efflux Pump Mediated Resistance and Inhibition

The Rv1258c EP was shown to confer low-level resistance to aminoglycosides when expressed in *M. smegmatis* [76]. For instance an increase in whiB7 (Rv3197) expression leads to upregulation of at least two different antibiotic resistance genes in the whiB7 regulon namely eis and Rv1258c [25]. Furthermore a decrease in the MIC of KAN drug was observed in the knockout mutant of Rv3065 gene of the SMR family [77]. Transport proteins of the ABC and MFS families, i.e., Rv2688, Rv2938, and Rv2994 have been observed to be over-expressed causing STR drug extrusion in *M. tuberculosis* [57,62].

The aminoglycoside spectinomycin (a STR analog) was effluxed by Rv2333c EP when expressed in *M. bovis* [61]. However a new class of spectinomycin analog, spectinamide evaded Rv1258c over-expression through structural modification and restored MICs to susceptible levels by binding to 16S bacterial ribosomal subunit [78]. Over-expression of ABC transporter Rv0194 lead to an increased resistance of both *M. smegmatis* and *M. bovis* to multiple drugs including ampicillin, chloramphenicol, tetracyclin, vancomycin (VAC), erythromycin, novobiocin and STR [79]. The cyclic peptide, VAC was also effluxed by Rv1258c, Rv0849, Rv1218c and Rv3065 genes [80].

It has been noted that some isolates of *M.tuberculosis* with a low level resistance to aminoglycosides did not present with any mutations in the rpsL, rrs, and gidB gene sequences but show decreased MICs in the presence of an EPI [81]. A knockout mutant of Rv1410c was studied and the levels of resistance to bacitracin, clofazimine (CFZ), econazole, novobiocin, PA-824, rifampin, valinomycin, and VAC returned to the wild-type suggesting that it contributes to the intrinsic resistance to these drugs [82].

In essence Rv1410c provides low level intrinsic resistance to a range of different antimicrobial compounds in *M. bovis* BCG [83]. The Rv1410c mutant was shown to have reduced resistance to rifampicin, amikacin, moxifloxacin, linezolid, and rifabutin [84]. Clearly this demonstrates that Rv1410c of *M. tuberculosis* is involved in multiple drug resistance.

The p-aminosalicylic acid drug (a second-line oral anti-TB agent) together with spectinomycin, and tetracycline were identified as specific Rv1258c substrates in *M. bovis* since a four-fold change in sensitivity was observed in resistant strains as compared to the wild-type in the presence of EPI [65]. More recently the MIC levels of recently approved BDQ drug were increased four-fold together with overexpression of MmpL5 protein in resistant strain when compared to the control strain [85].

Moreover BDQ was shown to inactivate EPs through the inhibition of ATP energy source [32]. Cross-resistance in second-line injectable drugs is a common phenomenon and this could lead to poor patient treatment outcomes. Newer drugs such as BDQ that could inhibit EPs are crucial and should be considered in new treatment regimens.

Aminoglycosides MIC levels were reduced in the presence of CCCP, suggesting that a decrease in their extrusion was predominant [76]. Since aminoglycosides are known to enter cells by an energy-dependent mechanism; CCCP can affect the levels of resistance to this group by decreasing both the uptake and extrusion through Rv1258c [79]. Furthermore piperine, an EPI was shown to play a significant role in the inhibition of Rv1258c [86]. While VER reduces drug tolerance by inhibiting Rv1258c induced upon entry of the drug into intracellular matrix [46].

On the contrary it was found that VER had an antagonistic effect when ribosome-targeting drugs such as STR, KAN, and CAP, as well as the cell wall agent cycloserine were used in macrophages [87]. The Rv1258c EP is well characterized and may play a significant role in aminoglycoside resistance and the effects can be reversed by EPI.

Mutations within Drug Efflux Pump Genes

The introduction of whole genome sequencing (WGS) in *M. tuberculosis* genome analysis is revealing novel mutations within EPs. Earlier work has indicated that mutantion causing an over-expression of EPs are a potential threat to overcoming drug resistance [63]. Liu and Xie identified 20 known or putative EPs with non-synonymous mutations in MDR, pre-XDR and XDR-TB *M. tuberculosis* isolates but none in H37Rv strain [88]. Non-synonymous mutations of M74T, R426H and I948V belonging to Rv0194, Rv0507 and Rv0676c respectively of the transporter families have been found in clinical isolates [88].

Detection of XDR-TB strains with non-synonymous mutations of P1098L, G198A and C213A within Rv0194, Rv1634, and Rv2688c EPs respectively in contrast to MDR-TB strains has been reported [89]. Resistant FLQ strains lacking DNA gyrase genetic changes showed various mutations in five EPs genes of Rv1217c, Rv0783c, Rv0849, Rv1877 and Rv2459 [90]. Moreover mutations were identified in transport proteins in 11% of samples that underwent WGS [91]. Using WGS we detected number of mutations within Rv0987, Rv2039, Rv0402 in an OFX resistant isolate without gyrA mutation [92].

Cross-resistance to STR and KAN due to over-expression of Rv1258c has been associated with a G133C mutation [93]. Rv3226 and Rv0849 had R26G and T403I mutations respectively in XDR-TB as compared to sensitive strains [94]. Several EPs, including the ABC transporters of Rv0194 and Rv1463, were affected by a larger number of independent mutations in resistant strains relative to sensitive strains during WGS bioinformatic analysis [95].

Genetic mutations in the form of insertions or deletions within Rv0678 caused MmpL5 protein over-expression [96]. Other mutations within 5' untranslated region of whiB7 that regulates Rv1258c, lead to KAN and STR cross-resistance in *M. tuberculosis* in the absence of rrs gene mutations [93].

As previously stated a non-synonymous caused by single nucleotide polymorphism was observed in Rv0678 gene (A202G leading to S68G) causing an increased MIC in BDQ [85]. Mutations within EPs mediating resistance over-expressed Rv1258c and treatment with inhibitors lead to reverse of resistance [46]. Furthermore drug candidate, SQ109 was inhibited by non-synonymous mutation Q40R of Rv0206c gene (MmpL) [97]. A summary of mutations in EPs is found in (Table 2).

Efflux Pump Family	Efflux pump gene	Mutation	Drugs	Reference(s)
ABC	Rv0194	M74T, P1098L, A277V, V398M, G431R,L486M, F705I		[88], [89, 95, 102]
	Rv2688c	C213A		[89]
	Rv1217c	V463C		[90]
	Rv1463	198E		[95]
	Rv1704	93L	Fluoroquinolones	[90]
	Rv1272c	H613N		[102]
	Rv1273	G416V, C142K		[102]

	ddrA/B/C	H309N, G253A, G158A		[102]
RND	Rv0507 (mmpL2)	R426H		[88]
	Rv0676c (mmpL5)	I948V		[88]
	Rv0678	A202G/S68G C189A/S63R	Bedaquiline Clofazimine	[85]
	Rv0206c (mmpL3)	A644L, A677C	SQ 109 (Sequella)	[103]
MFS	Rv1634	G198A, T31GI47Y,G54C, V296A		[89, 102]
	Rv0849	V206G, T403C	Fluoroquinolones	[94]
	Rv1877	T5P	Fluoroquinolones	[90]
	Rv2549	L484T, A487P	Fluoroquinolones	[90]
	Rv2333c	69Y, I 202H, D68N, D21E		[95, 102]
	Rv1250	R278G,F549L		[102]
	Rv 0783c	Q527H, G405D, R219G		[102]
	Rv2846c (EfpA)	N12K		[102]
ABC-ATP binding cassette, MFS-Major facilitator superfamily, RND-Resistance nodulation				

Table 2: Mutations within efflux pumps gene causing drug-resistance in *Mycobacterium tuberculosis.*

Summary and Conclusions

Drug resistance in *M. tuberculosis* is a complicated phenomenon involving both intrinsic and acquired mechanisms. Intrinsic mechanisms in the form of EPs activation is regarded as the first step towards higher levels of resistance. Once drugs enter the organisms, over-expression and mutations in either EPs or target regions may lead to high level resistance. To counteract the effects of EPs activation, EPI in combination with drug regimens can be used in chemotherapy. Ideally they will be combined with less effective SLDs to improve treatment outcomes. Clinically approved inhibitor, VER has therapeutic effects against most of the EPs that use ATP as an energy source used in combination with BDQ it is highly effective since they both block ATP energy source. Moreover, SQ109 (Sequella) that blocks EPs currently undergoing phase II clinical trials is showing great promise. Indeed, EPs are becoming an attractive area of research in terms of drug development and diagnosis through mutations.

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