Implications of Chromosomal Mutations for Mycobacterial Drug Resistance

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Abstract Tuberculosis (TB) remains a global health concern, despite availability of antituberculosis drugs. Drug-resistant *Mycobacterium tuberculosis* strains were identified shortly after the discovery and introduction of streptomycin for the treatment of this disease. Subsequently, multidrug therapy was implemented for TB treatment; however, this was soon followed by reports of multi-, extensively, and totally drug-resistant tuberculosis cases globally. The amplification of this drug resistance is due to the sequential accumulation of chromosomal alterations in target genes in the *Mycobacterium tuberculosis* genome. It is also evident that the presence of mutations that confer drug resistance results in the emergence of compensatory mechanisms which restore bacterial fitness. The recent approval by the Food and Drug Administration for bedaquiline as an antituberculosis drug provided some hope. However, clinical resistance to this new drug has already been reported. This underscores that it is imperative to understand drug resistance and its associated mechanisms in order to direct research efforts to the development of antituberculosis regimens with novel mechanisms of actions.

1 Introduction

In 2015 the World Health Organization (WHO) reported 9.6 million new cases of tuberculosis (TB), with 3.3 % of these and 20 % of previously treated cases infected with a multidrug-resistant (MDR) strain of *Mycobacterium tuberculosis* (WHO

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2015). More alarmingly, the average proportion of MDR-TB cases with extensively drug-resistant TB (XDR-TB) is 9.7 % (WHO 2015). Resistance to the first effective anti-TB drug, streptomycin (STR), was observed shortly after its introduction in 1944 (Nachega and Chaisson 2003; Keshavjee and Farmer 2012), and this trend had continued for many other TB drugs (Fig. 1). Numerous MDR-TB outbreaks were identified in the early 1990s, emphasizing TB as a global health problem (Nachega and Chaisson 2003) (Fig. 1). MDR-TB is characterized by a mycobacterial infection with *M. tuberculosis* strains that are resistant to rifampicin (RIF) and isoniazid (INH) (Gupta et al. 2003). Outbreaks of XDR-TB have been reported globally (Gandhi et al. 2006; Migliori et al. 2007b; Masjedi et al. 2010; Klopper et al. 2013; Cohen et al. 2015), with XDR-TB defined as an infection with an MDR-TB strain with further resistance to a fluoroquinolone (FQ) and one injectable drug, amikacin (AMI), kanamycin (KANA), and capreomycin (CAP) (Holtz 2007; Holtz and Cegielski 2007; Louw et al. 2009). Recently, *M. tuberculosis* strains resistant to all available anti-TB drugs have been identified globally and have been named totally drug-resistant TB (TDR-TB) (Migliori et al. 2007a; Velayati et al. 2009; Udwadia 2012; Udwadia et al. 2012; Klopper et al. 2013; Udwadia and Vendoti 2013) (Fig. 1). Although this term is somewhat controversial, TDR-TB has been defined as *M. tuberculosis* strains with in vitro resistance to all available first- and second-line drugs tested (INH, RIF, STR, EMB, PZA, ETH, PAS, DCS, OFL, AMI, CIP, CAP, KANA) (Parida et al. 2015). Factors fueling the drug-resistant TB epidemic include the inadequacies of TB control in combination with HIV coinfection.

The WHO recommends that new patients with pulmonary TB receive intensive phase treatment (2 months duration) which consists of INH, RIF, PZA, and EMB. Subsequently, a patient infected with a drug-sensitive *M. tuberculosis* strain is treated with INH and RIF during the 4-month continuation phase treatment (WHO 1997). Patient noncompliance is a consequence of the long treatment duration, and these factors fuel the development of drug resistance. An 8-month

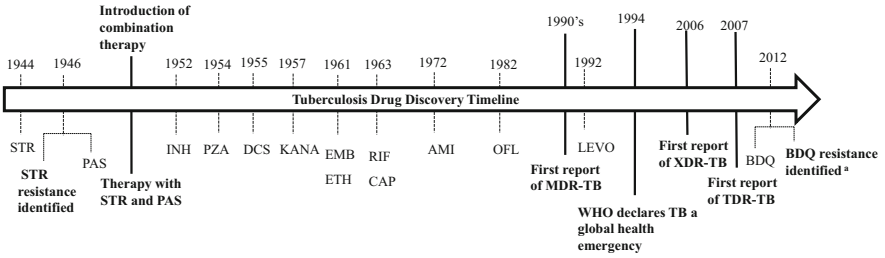

Fig. 1 Illustration of the tuberculosis drug discovery timeline and drug resistance development reports: *STR* streptomycin, *PAS* para-aminosalicylic acid, *INH* isoniazid, *PZA* pyrazinamide, *DCS* D-cycloserine, *KANA* kanamycin, *EMB* ethambutol, *ETH* ethionamide, *RIF* rifampicin, *CAP* capreomycin, *AMI* amikacin, *OFL* ofloxacin, *LEVO* levofloxacin, *BDQ* bedaquiline, *MDR-TB* multidrug-resistant tuberculosis, *WHO* World Health Organization, *XDR-TB* extensively drug-resistant tuberculosis, *TDR-TB* totally drug-resistant tuberculosis. [a]First report of BDQ resistance identified in a TB patient (Bloemberg et al. 2015)

Table 1 Treatment for the various forms of TB (WHO 2008)

Resistance pattern	Treatment
Sensitive	INH-RIF-PZA-EMB
INH	RIF-PZA-EMB
INH-RIF	PZA-STR-LEVO-ETH-DCS-PAS
INH-RIF-EMB	
INH-RIF-PZA-EMB	STR-LEVO-ETH-DCS-PAS
INH-RIF-STR	KANA-LEVO-ETH-DCS-PAS
INH-RIF-EMB-STR	
INH-RIF-EMB-PZA-STR	

INH isoniazid, *RIF* rifampicin, *EMB* ethambutol, *PZA* pyrazinamide, *STR* streptomycin, *LEVO* levofloxacin, *ETH* ethionamide, *DCS* cycloserine, *PAS* para-aminosalicylic acid, *KANA* anamycin

retreatment regimen with first-line anti-TB drugs for previously treated patients awaiting DST results consists of 2 months with INH, RIF, PZA, EMB, and STR; 1 month with INH, RIF, PZA, and EMB; and 5 months with INH, RIF, and EMB. Treatment of MDR-TB requires a regimen with second-line drugs administered over 18–24 months (Mukherjee et al. 2004). Recommendations for the treatment of various forms of drug-resistant TB are tabulated in Table 1. Current drugs used for TB treatment have limited efficacy against drug-resistant *M. tuberculosis* strains. However, new anti-TB drugs in development, specifically drugs with different modes of actions than the current drugs, could be effective against both drug-sensitive and drug-resistant TB.

2 Mode of Action and Mycobacterial Drug Resistance Mechanism

2.1 Cell Wall Synthesis Inhibitors

2.1.1 Isoniazid

INH is a prodrug that inhibits mycolic acid biosynthesis (Vilcheze and Jacobs 2007). This inhibition occurs via multiple mechanisms and results in the loss of trehalose monomycolate, trehalose dimycolate, and mycolates (Vilcheze and Jacobs 2007). INH is activated by KatG, which is a catalase-peroxidase, encoded by the *katG* gene. Upon activation, INH forms an adduct with NAD (Rozwarski et al. 1998) and binds and inhibits *inhA*, encoded by the enoyl-acyl carrier protein reductase InhA (NADH dependent), which is part of the fatty acid synthase type II system (Marrakchi et al. 2000). The INH-NAD adducts inhibit the activity of InhA, thereby resulting in intracellular accumulation of long-chain fatty acids, decreased mycolic acid biosynthesis, and subsequent cell death.

The loss of activation of INH by KatG is one of the mechanisms of INH resistance in mycobacteria. Mutations in the *katG* gene lead to a reduction in catalase activity. This results in a decrease in activated INH and a decreased capacity to form the INH-NAD adduct to inhibit InhA and subsequent high-level INH resistance (Heym et al. 1999; Ramaswamy et al. 2003). The Ser315Thr mutation in the *katG* gene is reported to be the most frequent mutation found in clinical *M. tuberculosis* strains resistant to INH (Seifert et al. 2015). Mutations within the *inhA* promoter (−15T and −8A loci) result in overexpression or modification of *inhA* and subsequently confer low-level INH resistance and ETH cross-resistance (Banerjee et al. 1994). Mutations in the structural gene are less frequent, but the Ser94Ala *inhA* mutation has been reported to be associated with low-level INH resistance (Quemard et al. 1995). Approximately 10 % of INH resistance is not attributed to mutations in *katG* and *inhA*, suggesting that additional resistance mechanisms contribute to INH resistance in mycobacteria. Additional genes (*kasA*, *ahpC*, *ndh*, and the *ahpC-oxyR* intergenic region) have been implicated in INH resistance; however, their direct impact on clinical INH resistance is not fully understood (Vilcheze et al. 2005; Vilcheze and Jacobs 2007; Campbell et al. 2011).

2.1.2 Ethionamide

The second-line drug, ETH, has a common molecular target to INH, namely, InhA of the FAS II system (Banerjee et al. 1994; Marrakchi et al. 2000). ETH is a prodrug and INH structural analog, which also inhibits mycolic acid biosynthesis. It was shown that *M. tuberculosis* strains with low-level INH resistance also exhibit resistance to ETH (Banerjee et al. 1994). ETH is activated by the monooxygenase, *ethA*, with subsequent formation of an ETH-NAD adduct. Even though the ETH-NAD adduct inhibits InhA, in the same manner as the INH-NAD adduct, the activating enzymes of the different compounds are distinct.

Numerous mutations in the *ethA* gene, resulting in a failure to activate ETH, have been reported to contribute to ETH resistance (Morlock et al. 2003; Brossier et al. 2011). The TetR-like repressor, EthR, negatively regulates the expression of *ethA* and interacts directly with the *ethA* promoter region, and EthR overexpression leads to ETH resistance (Baulard et al. 2000; DeBarber et al. 2000). Intragenic *inhA* mutations (Ser94Ala, Ser94Trp, Leu11Val) in addition to *inhA* promoter mutations (−102A and −47C) have also been identified in ETH-resistant *M. tuberculosis* isolates (Morlock et al. 2003; Brossier et al. 2011).

Approximately 50 % of ETH-resistant *M. tuberculosis* strains exhibit an absence of mutations in *inhA* or *ethA*, suggesting an alternative resistance mechanism (Boonaiam et al. 2010). Recently, mutations in the *mshA* gene (including a Val171Gly-Ala187Val double mutation) were identified in ETH-resistant isolates (Vilcheze et al. 2008; Brossier et al. 2011). MshA is a glycosyltransferase that is involved in mycothiol biosynthesis, and mutations in *mshA* have been proposed to result in the failure to activate ETH (Vilcheze et al. 2008). Interestingly, it was also observed that mutations in *ndh* resulted in defects in NdhII activity, subsequently

leading to increased intracellular NADH/NAD^+ ratio (Vilcheze et al. 2005). The increase in the NADH levels protects against InhA inhibition by either the INH-NAD or ETH-NAD formed when INH and ETH is activated, subsequently leading to ETH and INH co-resistance (Vilcheze et al. 2005). Even with the identification of the additional gene mutations, it is evident that additional resistance mechanisms exist that could contribute to ETH resistance.

2.1.3 Ethambutol

EMB is a bacteriostatic agent that targets the integral membrane arabinosyltransferases involved in polymerizing arabinose into arabinan components of arabinogalactan (Takayama and Kilburn 1989; Zhu et al. 2004; Wolucka 2008; Xu et al. 2015). Resistance to EMB is primarily attributed to mutations in the arabinosyltransferases encoded by *embB*, with 60 % of EMB-resistant isolates carrying a mutation at *embB*306 (Ramaswamy et al. 2000; Zhang and Yew 2009; Safi et al. 2013; Xu et al. 2015). However, several studies report discordance between genotypic and phenotypic resistance testing; this could be due to inaccurate diagnostic tests that are dependent on the medium used (Sreevatsan et al. 1997; Johnson et al. 2006a; Plinke et al. 2010; Xu et al. 2015).

Mutations in the *embC*, *embA*, and *embR* genes have also been implicated in EMB resistance, with alterations located in the embC-*embA* intergenic region conferring high-level EMB resistance (Cui et al. 2014; Xu et al. 2015). *embR* has been reported to modulate the level of arabinosyltransferase activity in vitro in a phosphorylation-dependent manner, acting downstream of the Ser/Thr-kinase PknH (Belanger et al. 1996). Interestingly, mutations were identified in the *ubiA* gene in EMB-resistant XDR-TB isolates lacking shared *embB* mutations (Motiwala et al. 2010; He et al. 2015), and these mutations were associated with high-level EMB resistance (Safi et al. 2013). The *ubiA* gene is essential for growth of *M. tuberculosis* and is involved in the synthesis of decaprenylphosphoryl-D-arabinose (Huang et al. 2005). It was recently reported that overexpression of wild-type *ubiA* gene resulted in an increase in EMB resistance in *M. tuberculosis* (He et al. 2015). This indicates that multiple mechanisms could result in the EMB resistance phenotype in mycobacteria.

2.1.4 SQ109

One of the newer anti-TB drugs, SQ109, was identified by screening a library of EMB derivatives based on the upregulation of the *iniBAC* operon promoter (Lee et al. 2003; Protopopova et al. 2005). Exposure of mycobacteria to SQ109 leads to the inhibition of trehalose dimycolate production and concomitant upregulation of trehalose monomycolate levels (Li et al. 2014b). This results in failure to attach mycolic acids to the cell wall arabinogalactan (Grzegorzewicz et al. 2012; Tahlan et al. 2012). The MIC for SQ109 ranges from 0.16 to 0.78 μg/ml for all

M. tuberculosis strains tested (Jia et al. 2005), and synergy was observed between INH/RIF and SQ109 in in vitro and in vivo analysis (Nikonenko et al. 2007). *M. tuberculosis* has a low spontaneous mutation rate of 2.55×10^{-11} for SQ109 resistance (Sacksteder et al. 2012).

The mycobacterial transport protein responsible for trehalose dimycolate transport, MmpL3, has been identified as the target of SQ109 (Sacksteder et al. 2012; Tahlan et al. 2012). Attempts to generate mutants against SQ109 have been unsuccessful. However, whole genome sequencing of in vitro mutants generated against analogs of SQ109 revealed that mutations in the *mmpL3* gene led to SQ109 and SQ109 analog resistance without cross-resistance to EMB (Tahlan et al. 2012). Mmpl3 mutations (Ala700Thr, Gln40Arg, and Leu567Pro) were reported to result in a greater than fourfold increase in SQ109 resistance level (Tahlan et al. 2012), with cross-resistance being observed between other MmpL3 inhibitors (Li et al. 2014b). Recently, it was observed that SQ109 inhibits enzymes involved in menaquinone synthesis, respiration, and therefore ATP synthesis (Li et al. 2014a). Additionally, SQ109 disrupts the proton motive force, thereby acting as an uncoupler (Li et al. 2014b). This effect on the proton motive force may also impact MmpL proteins, since it is suggested that the resistance-nodulation-division transporters catalyze the export of substrates via a proton anti-port mechanism (Li et al. 2014b).

2.1.5 D-Cycloserine

DCS is recommended by the WHO for the treatment of drug-resistant TB, despite severe side effects (WHO 2000). Resistance to DCS is attributed to overexpression of *alrA* in *M. smegmatis* (Caceres et al. 1997). AlrA encodes for D-alanine racemase that is involved in D-alanine synthesis. D-Alanine is an integral component of peptidoglycan which is an essential component of the cell wall. L-Alanine is converted to D-alanine by the catalytic activity of AlrA (Chacon et al. 2002). Subsequently, the D-alanine/D-alanine ligase (Ddl) catalyzes the dimerization of D-alanine into D-alanyl-D-alanine (Chacon et al. 2002). Studies indicate that *alrA* overexpression is a result of a G→T transversion in the *alrA* promoter (Caceres et al. 1997). These reports also show that *M. smegmatis alrA* null mutants have the ability to grow in the absence of D-alanine, suggesting the presence of another pathway of D-alanine biosynthesis (Chacon et al. 2002). Moreover, these *alrA* null mutants were more susceptible to DCS. It was also observed that a mutation (Gly122Ala) in the *cycA* gene, which encodes a D-serine/alanine/glycine transporter, partially contributes to the DCS resistance phenotype in *M. bovis* BCG vaccine strains (Chen et al. 2012). From these reports it is evident that more research needs to be done on DCS in order to elucidate and understand its resistance mechanisms fully.

2.2 Inhibitors of DNA Replication

2.2.1 Fluoroquinolones

Quinolones are synthetic compounds active on the enzymes essential for DNA replication, the DNA gyrases (Ginsburg et al. 2003). By interfering with DNA gyrase activity, the FQs disrupt DNA supercoiling, thereby inhibiting cell division and gene expression. DNA gyrase is comprised of two alpha and two beta subunits, encoded by the *gyrA* and *gyrB* genes, respectively (Takiff et al. 1994). Scientific reports indicate that spontaneous mutations develop at a frequency of 2×10^{-6} to 10^{-8} (Alangaden et al. 1995).

Approximately 90 % of FQ resistance in *M. tuberculosis* is attributed to mutations in a region named the quinolone-resistance-determining region (QRDR) in the *gyrA* and the *gyrB* gene (Takiff et al. 1994; Aubry et al. 2006). Mutations at codons 90 and 94 in the *gyrA* gene are most commonly observed among clinical isolates (Aubry et al. 2006), along with a Ser95Thr polymorphism in *gyrA* that is also present in FQ-sensitive clinical isolates (Maruri et al. 2012). Double mutations in *gyrA* and *gyrB* have been reported to exhibit high-level OFL resistance (Isaeva et al. 2013; Nosova et al. 2013). Mutations in *gyrA* (e.g., Ser91Pro, Asp94Ala, Ala90Val) also result in OFL, MOXI, and LFX cross-resistance with MIC90 > 4 μg/ml (Kambli et al. 2015; Willby et al. 2015). Although the majority of clinical FQ resistance is attributed to mutations in the *gyrA* and *gyrB* genes, additional mechanisms that can contribute to FQ resistance include efflux and DNA mimicry (Pasca et al. 2004). The clinical significance of these mechanisms has not been extensively investigated yet.

2.3 Inhibitors of Transcription

2.3.1 Rifampicin

RIF is a highly effective rifamycin that interferes with transcription by inhibiting the DNA-dependent RNA polymerase (RNAP) enzyme (McClure and Cech 1978). The majority of RIF-resistant *M. tuberculosis* strains harbor mutations in an 81 bp RIF resistance-determining region (RRDR) of the *rpoβ* gene, which encodes the β-subunit of RNAP (Telenti et al. 1993). Mutations at different loci in the RRDR of the *rpoβ* gene result in different RIF resistance levels (Louw et al. 2011), with His526Arg, His526Asp, His526Pro, His526Tyr, and Ser531Leu mutations being among the most common among RIF-resistant *M. tuberculosis* isolates (Telenti et al. 1993; Bodmer et al. 1995). Mutations in the RRDR are not the sole contributors to RIF resistance; mutations outside of the RRDR (Heep et al. 2001; Siu et al. 2011), along with the significant upregulation of efflux pumps upon RIF exposure (Louw et al. 2011), have been associated with RIF resistance. In 2011, the WHO endorsed the implementation of an automated test, Xpert® MTB/RIF assay, to

rapidly detect TB and RIF-resistant TB (Friedrich et al. 2013). Assessments of the assay indicates that despite the cost limitations, it does provide rapid results, and it significantly increases detection of TB and RIF resistance in culture-confirmed cases, compared to smear microscopy (Steingart et al. 2014).

2.4 Inhibitors of Translation

2.4.1 Aminoglycosides

2.4.1.1 Streptomycin, Amikacin, and Kanamycin

The aminoglycosides inhibit protein synthesis by binding to the 30S subunit of the mycobacterial ribosome (Ramaswamy and Musser 1998), with mutations in the *rpsL*, *rrs*, *gidB*, and *eis* genes implicated in aminoglycoside resistance (Maus et al. 2005a; Zaunbrecher et al. 2009; Georghiou et al. 2012; Reeves et al. 2013). Mutations in the essential *rpsL* gene, which encodes the 12S protein, result in resistance to STR, with the most common *rpsL* mutations being K43R and K88R (Ali et al. 2015). Mutations in the *rrs* gene, encoding for 16S rRNA, result in high-level resistance to STR, AMI, and KANA, with the A1401G mutation being the most frequently observed in AMI and KANA co-resistance (Campbell et al. 2011). Various different mutations in the *gidB* gene, which encodes a 7-methylguanosine methyltransferase that specifically modifies residues on 16S rRNA, have been identified in STR-resistant *M. tuberculosis* strains. These mutations result in the failure to methylate specific residues on the 16S rRNA molecule, thereby leading to resistance conferred by loss-of-function mutations (Ali et al. 2015). It was reported that promoter mutations in the 5′ untranslated region of the *eis* gene, encoding an aminoglycoside acetyltransferase, confer clinical low-level resistance to KANA. This acetyltransferase acetylates KANA, thereby leading to its inactivation, which subsequently prevents the drug from binding to the 30S ribosome (Zaunbrecher et al. 2009). To date, these mutations have been relatively selective for KANA resistance; therefore many strains with *eis* mutations would be classified as AMI susceptible. Interestingly, it has recently been reported that mutations in the 5′ untranslated region of the *eis* transcriptional activator, *whiB7*, also results in KANA resistance. These mutations in *whiB7* lead to an upregulation of *eis*, thereby resulting in KANA degradation and subsequent resistance (Reeves et al. 2013).

2.4.2 Cyclic Peptides

2.4.2.1 Capreomycin and Viomycin

CAP and VIO are cyclic peptides that inhibit protein synthesis. VIO has been shown to bind both the 30S and 50S ribosome subunits and to inhibit ribosomal

translocation by interference with the peptidyl tRNA acceptor site (Yamada et al. 1978). VIO and CAP cross-resistance occurs in *M. tuberculosis*. Cross-resistance between CAP and AMI/KANA has been reported, but cross-resistance between CAP and STR is rare (Maus et al. 2005a). Mutations at A1401G, C1402T, and G1484T are associated with CAP resistance, with additional mutations at various positions in the *tlyA* gene, an rRNA methyltransferase reported to exhibit VIO and CAP resistance (Maus et al. 2005a, b).

2.4.3 Oxazolidinones

2.4.3.1 Linezolid

Linezolid (LIN) was first introduced to treat gram-positive infections, including staphylococcal and streptococcal infections (Perry and Jarvis 2001). In vitro linezolid MICs for susceptible *M. tuberculosis* strains ranged from 0.25 to 1 μg/ml with an MIC90 of 0.5 μg/ml. Development of resistance against linezolid was considered to be rare (Richter et al. 2007). Reported in vitro frequencies for linezolid resistant mutants were 2×10^{-8} to 5×10^{-9} (Hillemann et al. 2008). Sequencing of the 23S rRNA gene in linezolid resistant mutants revealed the presence of a G to T nucleotide substitution at either position 2061 or position 2576 (Richter et al. 2007). The level of resistance for LIN mutants with the nucleotide substitution at position 2061 was 32 μg/ml, whereas those with a nucleotide substitution at position 2576 had a resistance level of 16 μg/ml (Richter et al. 2007). Interestingly, the predominant mutation identified in clinical and in vitro selected LIN mutants was in the *rplC* gene, encoding the L3 ribosomal protein, at T460C (Beckert et al. 2012).

2.5 Anti-TB Drugs That Target Energy Metabolism

2.5.1 Pyrazinamide

Pyrazinamide (PZA) susceptibility testing is technically difficult due to the acidic medium required for DST tests (Hoffner et al. 2013). PZA-resistant *M. tuberculosis* strains emerge due to a lack of pyrazinamidase (PZase) activity. PZase is required to convert PZA to its active form pyrazinoic acid (POA) (Konno et al. 1967). The protonated form, HPOA, enters the cell, accumulates, and eventually kills the cell (Zhang and Mitchison 2003). The PZA MIC of M. *tuberculosis* ranges from 6.25 to 50 μg/ml at pH 5.5 (Stottmeier et al. 1967). However, a PZA MIC > 2000 μg/ml has been reported for *M. avium* and *M. smegmatis* due to intrinsic PZA resistance as a result of efflux. *M. bovis* is also naturally resistant to PZA due to C→Gnt169 in *pncA*, whereas *M. kansasii* has weak PZase activity and exhibits an MIC of 250 μg/ml (Ramirez-Busby and Valafar 2015). PZA resistance in *M. tuberculosis* is mostly due to mutations in the *pncA* gene (Whitfield et al. 2015a); however, *pncA*

polymorphisms that do not confer the PZA-resistant phenotype have also been identified (Whitfield et al. 2015b). Mutations in *rspA*, involved in trans-translation, have also been identified in PZA-resistant strains (Louw et al. 2006; Shi et al. 2011; Feuerriegel et al. 2013; Simons et al. 2013b; Tan et al. 2014). Interestingly, *M. canetti* is naturally resistant to PZA due to a mutation (Met117Thr) in *panD* (Zhang et al. 2013). Subsequently, *panD* mutations in PZA-resistant *M. tuberculosis* strains lacking *rpsA* or *pncA* mutations have also been identified (Shi et al. 2014). POA inhibits enzymatic activity of *panD*, and it was observed that anti-TB activity of POA could be antagonized by B-alanine or pantothenate (Dillon et al. 2014).

2.5.2 Bedaquiline

Bedaquiline (BDQ) (Sirturo or TMC207) is the first anti-TB drug in 40 years to be FDA approved for treatment of sensitive and MDR-TB. The use of BDQ in addition to the standard TB therapy in the murine model accelerated the bactericidal effect (Andries et al. 2005; Lounis et al. 2006; Ibrahim et al. 2007). The minimum inhibitory concentrations of BDQ for *M. tuberculosis* H37Rv and drug-susceptible strains ranged from 0.03 to 0.12 μg/ml (Table 1) (Andries et al. 2005). Computational models suggest that BDQ restricts the rotational activity of ATP synthase, thereby inhibiting ATP production (deJonge et al. 2007). Spontaneous mutant selection and subsequent whole genome sequence analysis of the resistant *M. tuberculosis* and *M. smegmatis* mutants identified mutations (Ala63Pro and Asp32Val) in the c-subunit of ATP synthase encoded by the *atpE* gene (Andries et al. 2005; Koul et al. 2007). Mutations in *atpE* partially account for the BDQ resistance phenotype, with the report of spontaneous mutants without *atpE* gene mutations (Andries et al. 2005; Huitric et al. 2007, 2010). Recently, clofazimine (CFZ)-BDQ cross-resistance was observed in CFZ-resistant in vitro mutants. In the absence of *atpE* mutations, these mutants harbored mutations in the transcriptional repressor, Rv0678, which subsequently resulted in the upregulation of the *Rv0678* and the *mmpL5-mmpS5* efflux system (Milano et al. 2009; Hartkoorn et al. 2014). This upregulation led to a four- to eightfold increase in the level of resistance for CFZ and BDQ, which could be reversed with the addition of verapamil and reserpine (Andries et al. 2014; Hartkoorn et al. 2014).

2.6 Multi-target Drugs

2.6.1 PA-824/Pretomanid

PA-824 is a member of the nitroimidazole family containing a nitroimidazopyran nucleus. The MIC for PA-824 ranges from 0.039 to 0.25 μg/ml for sensitive strains compared to 0.015–0.513 μg/ml for drug-resistant strains, with a mutation frequency of 1.9×10^{-5} to 6.38×10^{-7} (Stover et al. 2000). PA-824 is a prodrug

that is activated to its toxic form, by the mycobacterial membrane-bound nitroreductase Ddn, a deazaflavin F420-dependent enzyme. This activation leads to the inhibition of mycolic acid synthesis, resulting in cell death (Singh et al. 2008). Investigation on the modes of action of PA-824 has shown that intermediate metabolites of PA-824 act as intracellular nitric oxide donors, therefore encouraging intracellular killing of *M. tuberculosis* in anaerobic conditions (Singh et al. 2008; Manjunatha et al. 2009). When bacteria are in a hypoxic nonreplicating state, PA-824 kills as a nitrous donor (Manjunatha et al. 2009). Interestingly, *M. leprae* is intrinsically resistant to PA-824 due to the lack of the *ddn* gene (Manjunatha et al. 2006).

Another mode of action for PA-824 is suggested by the observation that an *fbiC* knockout mutant in H37Rv, which is deficient for F420 production, is hypersensitive to oxidative stress and INH, moxifloxacin, and CFZ (Gurumurthy et al. 2013). By isolating PA-824-resistant mutants from the H37Rv *M. tuberculosis* background, it was observed that 29 % of isolates harbored mutations in the *ddn* gene and 26 % (*fbiC*), 19 % (*fbiA*), 7 % (*fgd1*), and 2 % in the *fbiA* gene. The mutation Ser11STOP in *ddn* gene conferred high-level PA-824 resistance; however, approximately 17 % of mutants lacked mutations in target genes screened, suggesting a different resistance mechanism (Haver et al. 2015).

2.6.2 OPC67683/Delamanid

Delamanid belongs to the nitro-dihydro-imidazooxazole class of antibiotics that inhibit mycolic acid synthesis (Barry and O'Connor 2007). Delamanid has an MIC90 of 0.006–0.05 μg/ml (Diacon et al. 2011), with an in vitro mutation frequency of 6.44×10^{-6} to 4.19×10^{-5} (Szumowski and Lynch 2015). Mutations in F420 biosynthetic genes also result in PA-824-delamanid cross-resistance.

2.6.3 Clofazimine

CFZ is lipophilic riminophenazine developed in 1957 for the treatment of MR-TB (Van Deun et al. 2010). It is a prodrug that is reduced by NADH dehydrogenase (Ndh2), and subsequently re-oxidized by O_2, to release reactive oxygen species (ROS). The production of ROS and subsequent cell death have been reported in *M. smegmatis* treated with CFZ and CFZ analogs (Yano et al. 2011). In vitro isolation of CFZ mutants reported cross-resistance to BDQ due to the presence of mutations in the transcriptional repressor, *Rv0678*, and subsequent upregulation of efflux pumps *mmpL5-mmpS5* (Hartkoorn et al. 2014). Recently, whole genome sequence analysis of spontaneous CFZ mutants revealed mutations in two additional genes that conferred the CFZ-resistant phenotype. These mutations were Glu89STOP in the putative peptidase, PepQ, resulting in the inactivation of this protein (Zhang et al. 2015a). The authors suggest that PepQ could be involved in CFZ activation. The additional mutation, Val351Ala, was identified in a possible

permease, Rv1979c, which is involved in amino acid transport (Zhang et al. 2015a). Although it is suggested that this protein could be involved in CFZ uptake and transport, it is evident that the direct effect of these two additional genes on CFZ resistance should be investigated further.

2.7 Anti-TB Drugs That Target Pathways

2.7.1 Para-aminosalicylic Acid

PAS is used as a second-line drug that targets the mycobacterial folate pathway (WHO 2000; Chakraborty et al. 2013). This prodrug is a structural analog of para-aminobenzoic acid (PABA) that is the substrate of the dihydropteroate synthase, encoded by *folP1*/*folP2*. The condensation of PABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate to 7,8-dihydropteroate is catalyzed by dihydropteroate synthase. This is subsequently converted to dihydrofolate and reduced by dihydrofolate reductase, encoded by *dfrA*, to produce tetrahydrofolate (Table 2).

Rengarajan and colleagues showed that PAS resistance is attributed to mutations in the *thyA* gene, encoded for by thymidylate synthase A, which is essential for thymine synthesis. In addition, *thyA* gene mutations were also present in clinical *M. tuberculosis* isolates resistant to PAS, indicating that PAS functions as a folate antagonist (Rengarajan et al. 2004; Fivian-Hughes et al. 2012). The dihydrofolate synthase, FolC, is essential for the activation of PAS, and mutations in *folC* have been reported to result in the PAS-resistant phenotype (Zhao et al. 2014). In addition, mutations in *ribD*, encoded for by the alternate dihydrofolate reductase, have been reported to result in its overexpression, thereby leading to PAS resistance (Zheng et al. 2013; Zhao et al. 2014; Zhang et al. 2015b). It was suggested that overexpression of *ribD* confers resistance by compensating for the inhibition of DfrA function.

3 Drug Resistance Mechanisms Other Than Chromosomal Mutations

Drug resistance in *M. tuberculosis* is not attributed to horizontal gene transfer, due to the lack of plasmids in this bacillus (Zainuddin and Dale 1990). Alternative mechanisms that contribute to mycobacterial drug resistance include (a) the production of drug-modifying enzymes, (b) the production of enzymes that inactivated the drug, (c) low cell wall permeability resulting in a decrease in drug influx, and (d) efflux-related mechanisms leading to a reduction in intracellular drug concentration (Davies and Courvalin 1977; Dabbs et al. 1995; Liu et al. 1996; Takiff et al.

Table 2 Characteristics of the anti-TB drugs, its associated MICs, and drug targets

Mode of action	Drug	MIC range for *M. tuberculosis* (μg/ml)		In vitro mutation frequency	Target	References
		Sensitive	Resistant			
Cell wall synthesis inhibitors	INH	0.5	>100	3.5×10^{-6}	*katG*, *inhA*, *furA*, *ahpC*, *kasA*, *ndh*	Vilcheze et al. (2005), Siddiqi et al. (2007), Vilcheze and Jacobs (2007), Zhang and Yew (2009)
	ETH	0.25	>100	$1–4 \times 10^{-8}$	*ethA*, *ethR*, *inhA*, *ndh*	Rastogi et al. (1996), Baulard et al. (2000), DeBarber et al. (2000), Morlock et al. (2003), Vilcheze et al. (2005), Vilcheze et al. (2008), Boonaiam et al. (2010), Brossier et al. (2011)
	EMB	5–15	4–40	3.1×10^{-5}	*embR*, *embC*, *embA*, *embB*, *ubiA*	Sreevatsan et al. (1997), Zhu et al. (2004), Safi et al. (2013), Cui et al. (2014)
	SQ109	0.16–0.78	2–4	10^{-7} (for analogs)	*mmpL3*	Jia et al. (2005), Sacksteder et al. (2012), Tahlan et al. (2012), Li et al. (2014b)
	DCS	8–25	>100	10^{-6} to 10^{-7}	*cycA, ddlA, alr*	Rastogi et al. (1996), Caceres et al. (1997), Chacon et al. (2002), Chen et al. (2012)
Transcriptional inhibitors	RIF	>0.5	>256	3.1×10^{-8}	*rpoβ*, *rpoA*, *rpoC*	Johnson et al. (2006b), Louw et al. (2011)
Translational inhibitors	STR, CAP, AMI, KANA, VIO	1	>16	3.80×10^{-6}	*rpsL*, *rrs*, *rrl*, *tlyA*, *eis*, *whiB7*, *gidB*	Rastogi et al. (1996), Campbell et al. (2011), Reeves et al. (2013)
	LIN, STZ	0.25–1	>64	2×10^{-8} to 5×10^{-9}	*rplC*, *rrl*	Richter et al. (2007), Hillemann et al. (2008), Beckert et al. (2012), Adams et al. (2014)
DNA gyrase inhibitors	CIP, MOXI, OFL, GATI	0.5	>1	2×10^{-6} to 10^{-8}	*gyrA*, *gyrB*	Avalos et al. (2015), Singh et al. (2015), Willby et al. (2015)
Energy metabolism	BDQ	0.030–0.12	0.12–3.84	2×10^{-8}	*atpE*	Andries et al. (2005), Petrella et al. (2006), Huitric et al. (2007)
	PZA	<100	>400	1×10^{-5}	*pncA*, *panD*, *rspA*	Louw et al. (2006)

(continued)

Table 2 (continued)

Mode of action	Drug	MIC range for *M. tuberculosis* (μg/ml)		In vitro mutation frequency	Target	References
		Sensitive	Resistant			
Multi-targeted	PA-824, delaminid	0.039–0.25	0.36–1.79	1.9×10^{-5} to 6.38×10^{-7}	*ddn*, *fbiA*, *fbiB*, *fbiC*, *fgd1*	Stover et al. (2000), Haver et al. (2015)
	CFZ	0.44–0.25	0.108–8	5×10^{-6}	*ndh2*, *Rv0678*, *mmpL5-mmpS5*, *Rv1979c*, *Rv2535c*	Reddy et al. (1999), Lu et al. (2011), Gupta et al. (2014), Zhang et al. (2015a)
Folate pathway inhibition	PAS	<0.125	>128	2×10^{-7} to 2×10^{-8}	*folC*, *folP1*, *thyA*, *ribD*, *folP2*	Rengarajan et al. (2004), Zheng et al. (2013), Minato et al. (2015)

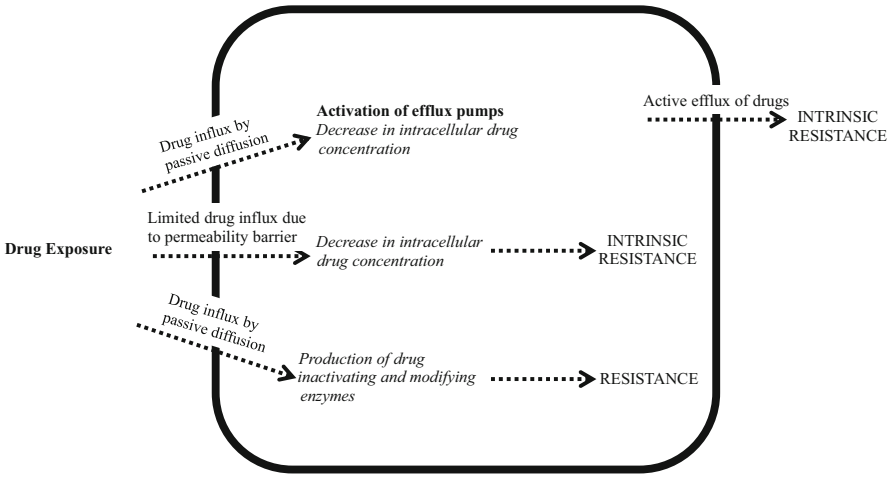

Fig. 2 Mycobacterial drug resistance mechanisms other than chromosomal alterations. Mechanisms such as the activation of efflux pumps and limited drug influx due to the decreased drug permeability lead to a reduction in the intracellular drug concentration and subsequent intrinsic resistance. The production of drug-inactivating and drug-modifying enzymes also results in drug resistance

1996; Davies and Wright 1997; Quan et al. 1997; Imai et al. 1999; Nikaido 2001; Brennan 2003; Draker et al. 2003; Li et al. 2004; Ashenafi et al. 2014) (Fig. 2).

3.1 Permeability Barrier and Activation of Efflux Pumps

Certain mycobacterial species exhibit an intrinsic drug-resistant phenotype that is not the result of antibiotic exposure (Fajardo et al. 2008). Intrinsic drug resistance is attributed to the activation of efflux pumps and an inherently low permeability of the mycobacterial cell wall (Nikaido 2001; Borges-Walmsley et al. 2003; Louw et al. 2009). Recently, knockdown of Rv1026 (*ppx2*), an exopolyphosphatase, was shown to result in increased bacterial cell wall thickness and decreased INH permeability (Nikaido 2001; Brennan 2003; Chuang et al. 2015). This indicated a molecular basis contributing to decreased permeability and intrinsic drug resistance.

Whole genome sequencing of *M. tuberculosis* revealed the presence of various efflux pumps that may enable the bacilli to evade the antimycobacterial killing action. Efflux pumps export various toxic compounds including antibiotics and metabolites, resulting in a decrease in intracellular concentration (Pages et al. 2005; Gupta et al. 2006). This phenomenon has been extensively studied in mycobacteria recently (Li et al. 2004; Morris et al. 2005; Buroni et al. 2006; Zechini and Versace 2009; Adams et al. 2011; Louw et al. 2011; Rodrigues et al. 2011, 2012; Balganesh et al. 2012; Hartkoorn et al. 2014).

In vivo and in vitro *studies* have revealed that antibiotic exposure of mycobacterial cells resulted in the significant upregulation of efflux pumps. It was shown that exposure to RIF resulted in an increase in expression of *Rv1258c*, which is a tap-like efflux pump (Adams et al. 2011, 2014), and arise in the RIF resistance level. Treatment with efflux pump inhibitors, verapamil, reserpine, and tetrandrine, along with RIF, INH, and EMB, could reverse the resistance phenotype of these anti-TB drugs (Adams et al. 2011, 2014; Louw et al. 2011). Studies have also shown that the exposure of *M. tuberculosis* to anti-TB drugs such as EMB, INH, RIF, OFL, STR, and PAS results in the upregulation of efflux pumps like *drrA*, *drrB*, *efpA*, *mmr*, *jefA*, *Rv1634*, *whiB7*, *Rv1456c-Rv1457c-Rv1458c*, *Rv1258c*, and *pstB* (Morris et al. 2005; Ramon-Garcia et al. 2012; Gupta et al. 2014; Hartkoorn et al. 2014; Garima et al. 2015; Li et al. 2015; Zhang et al. 2015c). The upregulation of the efflux pumps results in an MDR phenotype. Interestingly, the organosilicon compound, SILA-421 and thioradazine, both shown to have efflux pump inhibitory activity, demonstrated time- and concentration-dependent activity against *M. tuberculosis* as well as the enhanced killing of intracellular XDR-TB (Martins et al. 2009; Simons et al. 2013a; de Knegt et al. 2014, 2015). These compounds also enhanced the activity of INH and RIF in vitro and prevented the emergence of INH- and RIF-resistant mutants. However, they did not show in vivo activity enhancement of INH and RIF in *M. tuberculosis*-infected mice treated with INH-RIF-PZA for 13 weeks (de Knegt et al. 2014, 2015).

3.2 Production of Drug-Modifying and Inactivating Enzymes

M. smegmatis has been confirmed to be naturally resistant to RIF due the rifampin ADP-ribosyltransferase (Arr-ms), encoded by the chromosome, which assists in covalently adding a ribose group to RIF. This addition modifies and inactivates RIF, thus resulting in intrinsic resistance in *M. smegmatis* to RIF (Dabbs et al. 1995; Imai et al. 1999; Quan et al. 1997; Baysarowich et al. 2008).

The production of inactivating enzymes, e.g., the acetyltransferase AAC (2′)—Ic and the phosphotransferase encoded by the *Rv3225c* gene, APH (6)-la and APH (6)-ld from producer strain *Streptomyces griseus*, has been associated with STR resistance (Davies and Courvalin 1977; Davies and Wright 1997; Draker et al. 2003; Ashenafi et al. 2014). Similarly, the lack of antimicrobial activity in *M. abscessus* of aminoglycosides could be reversed by disruption of the chromosomally encoded *aac(2′)* gene (Maurer et al. 2014, 2015) (Fig. 2). By using *M. smegmatis*, it was shown that the activity of acetyltransferase was significantly induced in response to aminoglycoside, thereby resulting in the inhibition of protein synthesis.

4 Compensatory Mechanisms, Fitness, and Drug Resistance

Some resistance-causing mutations have been found to incur a fitness cost (Gagneux et al. 2006). The fitness cost may be compensated for by the acquisition of secondary mutations at a different site during the evolution of resistant bacteria (Bjorkman et al. 2000). The mutant carrying the chromosomal alteration can become extinct, or the mutations might be fixed in the population by means of compensatory evolution (Bottger and Springer 2008). These compensatory mechanisms can reduce the cost by restoring physiological functions impaired by the resistance mutations without altering the level of bacterial resistance (Schrag and Perrot 1996).

Recently, whole genome sequencing of RIF-resistant *M. tuberculosis* strains with *rpoB* mutations revealed novel mutations in *rpoA* and *rpoC* that emerged over time. Strains with these mutations exhibit high competitive fitness in vitro and in vivo and lead to MDR strains with high fitness (Comas et al. 2012). Previously, it was shown by in vitro pair-wise competition experiments that the wild-type *rpoB M. tuberculosis* strains outcompeted strains harboring the Ser522Leu, His526Tyr, and Ser531Trp mutations (Billington et al. 1999; Mariam et al. 2004). The extent of fitness loss was dependent on the specific *rpoB* mutation, with the Ser531Leu *rpoB* mutation only exhibiting a minor fitness defect compared to other mutations (Billington et al. 1999; Gagneux et al. 2006; Mariam et al. 2004). Additionally, mutations in *rpoC* illustrated that epistatic interactions between mutations that confer drug resistance, compensatory mutations, and diverse strain genetic background might influence compensatory evolution (de Vos et al. 2012).

In INH resistance, mutations in *katG* eliminate catalase-peroxidase activity, thereby preventing the activation of INH (Heym et al. 1999). It was shown that the expression of KatG or the alkyl hydroperoxidase, AhpC, exhibited a protective effect against organic peroxides in bacilli. The overexpression of AhpC, due to the presence of a mutation in *ahpC*, enabled INH-resistant *katG* mutants to survive during infection (Sherman et al. 1996).

These alternative mechanisms compensating for the loss of fitness caused by genetic mutations are difficult to detect using PCR-based methods as these methods only target mutation hotspots associated with drug resistance. Thus, it is imperative to also consider these compensatory mechanisms upon designing and developing new drugs and treatment regimens.

5 Perspectives

The history of TB drug development and use provides numerous examples of chromosomally encoded resistance, which often emerges very rapidly after the introduction of new drugs. This highlights the need for a diverse product portfolio

entering the TB drug development pipeline. Fortunately, there are several promising new drugs at various stages within the TB drug development pipeline. These include bactericidal compounds in the benzothiazinone class, targeting the enzyme decaprenylphosphoryl-β-D-ribose 2′-oxidase (DprE1), which is essential for cell wall synthesis (Makarov et al. 2015). However, as with all TB drugs, there is a need for a better understanding of mechanisms of drug resistance and consequences of mutations that confer drug resistance. The emergence of compensatory mechanisms following the evolution of drug resistance-conferring mutations, after selective pressure, is an additional factor to consider upon rational drug design. Recently, bacterial collateral resistance and sensitivity to various combinations of anti-TB drugs have been reported. However, it is evident that the collateral sensitivity and resistance networks are complex, thereby complicating tailoring specific treatment regimens based on existing drug treatments. It would be desirable to explore alternative approaches to treatment, including the inclusion of efflux pump inhibitors or immunomodulators. Ideal treatment regimens would eliminate the formation of bacterial persisters, reduce the selection of resistant mutants, and ultimately offer a much-reduced treatment regime, to increase compliance.

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