Molecular Basis of Drug Resistance in Mycobacterium tuberculosis

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ABSTRACT In this chapter we review the molecular mechanisms of drug resistance to the major first- and second-line antibiotics used to treat tuberculosis.

In 2011, the World Health Organization (WHO) reported nearly 60,000 new cases of multidrug-resistant tuberculosis (MDR-TB) (1), and estimates of the annual global incidence are much higher. The emergence of drug-resistant strains has made the treatment of TB complex, costly, toxic, time-intensive, and less efficacious. Design of a treatment regimen for drug-resistant TB includes the administration of first-line drugs to which the strains remain susceptible together with second-line drugs. These second-line agents are more expensive, more difficult to administer (several require intravenous administration), and are often associated with severe toxicities, including hepatic and renal dysfunction. In comparison to the 6 months required to treat drug-susceptible TB, drug-resistant TB requires a prolonged treatment duration of 18 to 24 months. These logistics constitute considerable hardships for patients as well as for overburdened public health services. Too frequently, premature discontinuation of therapy occurs, leading to treatment failure and the emergence of Mycobacterium tuberculosis strains with additional drug resistance.

DEFINITIONS OF DRUG-RESISTANT TB

MDR-TB is defined as M. tuberculosis with in vitro resistance to two first-line medications: isoniazid (isonicotinic acid hydrazide, INH) and rifampin (RIF).

Extensively drug-resistant TB (XDR-TB) is defined as M. tuberculosis that is resistant to not only INH and RIF, but also to other medication classes that comprise the backbone of drug-resistant TB therapy, namely a quinolone and one of the second-line injectable drugs (kanamycin [KAN], amikacin [AMK], or capreomycin [CAP]) (2).

Totally drug-resistant TB (TDR-TB) refers to strains that are resistant to all available TB drugs, although the number and degree of resistance to each drug has not yet been precisely defined. The emergence of these strains further underscores the medical and public health urgency to control drug-resistant TB (3, 4).

Received: 17 September 2013, Accepted: 27 November 2013, Published: 13 June 2014
Editors: Graham F. Hatfull, University of Pittsburgh, Pittsburgh, PA, and William R. Jacobs, Jr., Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY
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MECHANISMS OF DRUG RESISTANCE

The traditional mechanisms by which bacteria achieve antimicrobial resistance are (i) barrier mechanisms (decreased permeability/efflux), (ii) degrading/inactivating enzymes, (iii) modification of pathways involved in drug activation/metabolism, and (iv) drug target modification or target amplification. As will be discussed below, *M. tuberculosis* uses all of these mechanisms to achieve resistance. For the purposes of this chapter we define drug resistance as genetic changes that alter the phenotypic resistance levels. Antibiotic tolerance (not discussed herein) addresses mechanisms that do not require genetic changes in order to achieve an altered phenotypic resistance level and is usually reversible after removal of the drug or a change in the growth conditions.

Table 1 delineates the commonly used TB drugs with the genes associated with their respective resistance and major mechanism of resistance.

### FIRST-LINE AGENTS

**Rifamycins**

Rifamycins are critical to sterilization and are the key drugs associated with the development of modern short-course therapy for human TB. Currently, three rifamycins are used for the treatment of TB: RIF, rifapentine, and rifabutin (5). These agents differ primarily in their human pharmacology, but with respect to their antimycobacterial mechanisms of action and resistance they are similar.

### TABLE 1

The commonly used TB drugs with the genes associated with their respective resistance and major mechanism of resistance

<table>
<thead>
<tr>
<th>Drug or drug class</th>
<th>Resistance genes</th>
<th>Rv number</th>
<th>Gene function</th>
<th>Mechanism of drug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifamycins</td>
<td>rpoB</td>
<td>Rv0667</td>
<td>RNA polymerase B</td>
<td>Target modification</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>katG</td>
<td>Rv1908c</td>
<td>Catalase-peroxidase enzyme</td>
<td>Decreased drug activation</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Rv1484</td>
<td>NADH-dependent enoyl-acyl carrier protein</td>
<td>Target amplification or modification</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>Rv2043c</td>
<td>Pyrazinamidase</td>
<td>Decreased drug activation</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embCAB operon</td>
<td>Rv3793-5</td>
<td>Arabinosyltransferases</td>
<td>Target modification</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>rpsL</td>
<td>Rv0682</td>
<td>12S ribosomal protein</td>
<td>Target modification</td>
</tr>
<tr>
<td></td>
<td>rrs</td>
<td>n/a</td>
<td>16S rRNA</td>
<td>Target modification</td>
</tr>
<tr>
<td></td>
<td>gidB</td>
<td>Rv3919c</td>
<td>7-Methylguanosine methyltransferase</td>
<td>Target modification</td>
</tr>
<tr>
<td>Kanamycin/amikacin</td>
<td>rs</td>
<td>n/a</td>
<td>165 rRNA</td>
<td>Target modification</td>
</tr>
<tr>
<td></td>
<td>eis</td>
<td>Rv2416c</td>
<td>Aminoglycoside acetyltransferase</td>
<td>Inactivating mutation</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>rs</td>
<td>n/a</td>
<td>165 rRNA</td>
<td>Target modification</td>
</tr>
<tr>
<td></td>
<td>tylA</td>
<td>Rv1694</td>
<td>rRNA methyltransferase</td>
<td>Target modification</td>
</tr>
<tr>
<td>Quinolones</td>
<td>gyrA</td>
<td>Rv0006</td>
<td>DNA gyrase A</td>
<td>Target modification</td>
</tr>
<tr>
<td></td>
<td>gyrB</td>
<td>Rv0005</td>
<td>DNA gyrase B</td>
<td>Target modification</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>ethA</td>
<td>Rv3854c</td>
<td>Mono-oxygenase</td>
<td>Decreased drug activation</td>
</tr>
<tr>
<td></td>
<td>ethR</td>
<td>Rv3855</td>
<td>Transcriptional regulatory repressor protein (TetR family)</td>
<td>Decreased drug activation</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Rv1484</td>
<td>NADH-dependent enoyl-acyl carrier protein</td>
<td>Target amplification and modification</td>
</tr>
<tr>
<td>Para-aminosalicylic acid</td>
<td>thyA</td>
<td>Rv2764c</td>
<td>Thymidylate synthase</td>
<td></td>
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<tr>
<td></td>
<td>nbd</td>
<td>Rv2671</td>
<td>Enzyme in riboflavin biosynthesis</td>
<td></td>
</tr>
<tr>
<td>Cycloserine</td>
<td>alr</td>
<td>Rv3423c</td>
<td>Alanine racemase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ddl</td>
<td>Rv2981c</td>
<td>d-Alanine-d-alanine ligase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cycA</td>
<td>Rv1704c</td>
<td>Bacterial d-serine/L- and d-alanine/glycine/d-cycloserine proton symporter</td>
<td></td>
</tr>
<tr>
<td>Bedaquiline</td>
<td>atpE</td>
<td>Rv1305</td>
<td>ATP synthase</td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td>rli</td>
<td>n/a</td>
<td>23S rRNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rplC</td>
<td>Rv0701</td>
<td>50S ribosomal protein L3</td>
<td>Target modification</td>
</tr>
</tbody>
</table>
The principal target of rifamycins is the β subunit of RNA polymerase (RNAP), which is encoded by the gene rpoB (Rv0667 in M. tuberculosis H37Rv or MT0695 in M. tuberculosis CDC1551). Binding of a rifamycin to the RpoB protein inhibits the activity of RNAP, thereby preventing transcription. Because a basal level of transcription occurs in stationary growth phases, rifamycins are active against mycobacterial quiescent states. It is this activity that is thought to underpin the sterilizing activity that has effectively shortened the duration of TB chemotherapy from 18 months to 9 months with the introduction of RIF.

**Mechanism of action**

RIF has long been known to have its impact after RNAP binds to DNA and initiates transcription. Studies have shown that rather than inhibiting RNAP initiation, RIF prevents the elongation of RNA when the transcript becomes 2 to 3 nucleotides in length. In 1999 Darst and colleagues completed the first crystal structure of an RNAP, namely, that for Thermus aquaticus (6), and in 2001 the structure of RIF-bound RNAP was reported (7). These structures showed that RIF binds deep within the DNA/RNA channel of RNAP but more than 12 Å away from the active site.

More recent studies using the crystal structure of Escherichia coli RNAP have revealed a yet more complex, multistep mechanism of RNAP action: (i) RNAP binds to promoter DNA, producing an RNAP-promoter closed complex where the DNA remains double-stranded and is not loaded into the RNAP active-center cleft; (ii) RNAP loads DNA into, and unwinds DNA in, the RNAP active-center cleft, yielding an RNAP-promoter open complex (RPoc); (iii) RNAP synthesizes the first ~10 nucleotides of RNA using a “scrunching” mechanism whereby RNAP remains stationary on promoter DNA, pulling downstream DNA into the cleft (called the RNAP promoter initial transcribing complex [RPitc]); and (iv) RNAP escapes the promoter and synthesizes the rest of the RNA using a “stepping” mechanism, in which RNAP translocates relative to DNA, as a transcription elongation complex. RIF has been shown to exert its action by preventing step iii, namely, the formation of the RNAP initial transcribing complex RPitc. These properties led to the proposal that rifamycins inhibit the formation of RPitc through a steric-occlusion mechanism, whereby RIF binds adjacent to the RNAP active center, along the path of the RNA product, and physically prevents synthesis or retention of RNA products >2 to 3 nucleotides in length (8, 9).

RIF bound within its β subunit pocket is 12 Å away from the active site of RNAP, where ribonucleotides complementary to the DNA template strand are added to the growing RNA chain. However, the RIF-binding pocket within the β subunit is situated in the main DNA/RNA channel of RNAP. The binding of RIF to its cognate pocket obstructs this channel, preventing the elongation of RNA molecules beyond 2 to 3 nucleotides in length.

**Mechanism of resistance: target modification: rpoB**

RNAP in M. tuberculosis, as in most bacterial species, is comprised of four polypeptides: α, β, β′, and σ, arranged in a five-subunit enzyme, α2ββ′σ. Single genes (rpoA, rpoB, and rpoC) encode the α, β, and β′ subunits, respectively, while 13 sigma factors may serve as the promoter-recognition subunits (10).

The mechanism by which these mutations confer RIF resistance has been inferred from X-ray crystallography studies of RNAP from other bacterial species, in particular T. aquaticus and E. coli. Greater than 95% of RIF-resistant strains of M. tuberculosis harbor non-synonymous mutations in the rpoB gene encoding the β subunit of RNAP (11). These mutations cluster in an 81-base-pair region of the rpoB gene known as the RIF resistance-determining region (RRDR) (11, 12). Across multiple prokaryotic species, numerous mutations in the RRDR have been identified (13), although mutations G498Q, G498E, D516V, D516Y, N518deletion, H526R, H526D, H526P, H526Y, and S531L (corresponding to E. coli RNAP codons) are among the most common.

RIF binds between two structural domains of the RNAP β subunit. The mutations in the RRDR all occur in residues in or near this pocket. All of these mutations prevent effective binding of RIF within the β subunit pocket. Approximately half of the mutations accounting for RIF resistance occur in amino acids within this binding pocket that are in close enough proximity to directly interact with RIF when it is complexed with RNAP. Other residues known to account for RIF resistance occur one layer removed from the binding pocket and can lead to distortions of the binding pocket that also prevent RIF binding (7).

**Fitness of RIF-resistant mutants and compensatory mutations**

Mutations in the RRDR prevent binding of RIF to RNAP and at the same time permit effective function of RNAP. When M. tuberculosis is grown in the presence of RIF, mutants with classic mutations in the RRDR are...
easily selected. Such laboratory-derived *M. tuberculosis* rpoB mutant strains show a variable reduction in fitness as measured by their *in vitro* growth rate. In contrast, some clinical isolates that are RIF resistant with the same RRDR mutation displayed fewer fitness costs (using *in vitro* growth rates) when compared with their wild-type counterparts, raising the possibility of compensatory mechanisms to correct for loss of fitness accrued by the original RIF resistance-conferring mutation (14).

Recent studies have compared laboratory-derived rpoB RIF-resistant strains, passaged *in vitro* to select for compensatory mutations, with collections of MDR clinical isolates. These studies have shown that RIF-resistant strains with classic mutations in the RRDR also contain other mutations that are associated with a reduction in the fitness cost of the RIF resistance-conferring mutation. These putative fitness-compensatory mutations localized to the rpoA gene encoding the α subunit of RNAP and to the rpoC gene encoding the β’ subunit of RNAP (15). Although functional analysis of these mutations has not been completed in *M. tuberculosis*, studies in other RIF-resistant organisms have confirmed that secondary mutations in rpoA and rpoC do act in a compensatory manner. Some also confer low-level resistance to RIF (16). Hence, it appears that mutations occurring in other subunits of the multi-subunit RNAP enzyme can compensate for the relative fitness loss of mutations in the rpoB RRDR that confer high-level RIF resistance.

**INH**

INH is a first-line drug for TB that has rapid bactericidal activity. During combination therapy of drug-susceptible TB infection, INH is the drug principally responsible for the 1 to 2 log decline of bacterial load in sputum seen within the first 14 days of treatment (17). INH is also widely used as a treatment for latent infection of *M. tuberculosis*. While its structure of a pyridine ring and hydrazide group is simple, the mechanism of action of INH is incredibly complex.

**Mechanism of action**

It has long been postulated that INH inhibits the biosynthesis of mycolic acids (18), resulting in accumulation of long-chain fatty acids (19) and cell death (20). However, the details of this complex process have only recently been elucidated.

INH is a prodrug that is activated by the mycobacterial catalase-peroxidase enzyme, KatG, encoded by katG (Rv1908c, MT1959) (21). Following activation by KatG, INH forms an adduct with NAD (22). This INH-NAD adduct then binds and inhibits a mycobacterial protein, InhA (23). *inhA* (Rv1484, MT1531) encodes InhA, an NADH-dependent enoyl-acyl carrier protein (ACP) reductase (24, 25), which is part of the fatty acid elongation system, fatty acid synthase type II (FASII), responsible for mycolic acid biosynthesis (26). Inhibition of InhA by the INH-NAD adduct results in intracellular accumulation of long-chain fatty acids, decreased mycolic acid biosynthesis, and eventual cell death.

**Mechanism of resistance: loss of activation: katG**

The two main molecular mechanisms of INH resistance are (i) loss of INH activation by katG (27) and (ii) increases in inhA expression or modification of the InhA target. Approximately 75 to 90% of INH resistance can be attributed to polymorphisms in the katG gene or the inhA promoter and gene (28).

The majority of clinical isolates that are resistant to INH harbor mutations in katG (29, 30). A broad range of polymorphisms has been reported worldwide including missense and nonsense mutations, insertions, deletions, truncation, and rarely, whole gene deletions (27, 31, 32). Mutations in katG result in a reduced ability to form the INH-NAD adduct (33) and a high level of INH resistance. The most common mutation occurring in clinical isolates in katG is S315T (30, 34–38).

Polymorphisms in katG may result in diminished or loss of catalase and peroxidase activity. Because these enzymatic activities are critical for *M. tuberculosis* defense against reactive oxygen species and virulence *in vivo* (39–41), mutations in katG can lead to a loss of fitness to the bacterium. However, unlike other polymorphisms, the S315T mutation renders katG unable to activate INH while still retaining some peroxidase and catalase activities, reducing the fitness cost to this particular mutation. It is likely that this retention of catalase-peroxidase activity and fitness with the S315T katG is responsible for the predominance of this mutation among drug-resistant isolates (41).

**Mechanism of resistance: overexpression or target modification: inhA**

While the majority of INH-resistant clinical strains contain katG mutations, a significant portion of INH-resistant clinical isolates have a wild-type katG, suggesting the existence of additional resistance mechanisms. Other such mechanisms involve changes in inhA expression and modification of the InhA target. Association with INH resistance has been well documented for
polymorphisms within the inhA promoter at the −15T and −8A loci. Mutations at this site result in overexpression of inhA, which confers low-level resistance to INH and cross-resistance to ethionamide (ETH) (42, 43). The prevalence of inhA promoter mutations varies geographically, but these mutations rarely account for greater than 20% of INH resistance (44, 45). While structural mutations in InhA are rare, the S94A mutation reduces affinity for the NADH cofactor and confers low-level resistance (25). Approximately 10% of INH resistance is unexplained by mutations in katG and inhA (28). Other genes including kasA, ahpC, ndh, and the ahpC-oxylR intragenic region have been associated with resistance to INH, but their impact on resistance among clinical isolates remains unclear (46–48).

Pyrazinamide

Pyrazinamide (PZA), a nicotinamide analogue, is a critically important first-line TB drug. The addition of PZA to the intensive phase of TB therapy has allowed treatment duration to be shortened from over 1 year to just 6 months of therapy (49). PZA has little bactericidal activity but has critical sterilizing activity during the later phases of treatment when relatively low numbers of persistor or semi-dormant organisms remain (50).

PZA is the least understood drug in the current TB drug arsenal. It has no activity against M. tuberculosis grown under normal in vitro culture conditions, but killing can be demonstrated in vitro at acidic pH, typically pH 5.5 (51). Hence, phenotypic testing for PZA susceptibility is complicated and has not been fully standardized; many clinical laboratories do not routinely test for PZA susceptibility. Another curiosity of PZA is that in contrast to M. tuberculosis, Mycobacterium bovis and M. bovis BCG are naturally resistant to the drug. Recently, as described below, the molecular basis for this difference in susceptibility has been determined.

Mechanism of action

PZA is a prodrug that is converted by the cytoplasmic mycobacterial enzyme, pyrazinamidase, into pyrazinoic acid (POA) (52). As described below, loss of the bacterial-encoded activating enzyme confers resistance to PZA. However, the molecular targets of POA remain uncertain. Several possible mechanisms have been postulated including inhibition of trans-translation, inhibition of fatty acid synthase I, and modulation of host targets.

Mechanism of resistance: loss of activation: pncA

In 1996, Zhang and colleagues identified mutations in the pncA gene (Rv2043c, MT2103), which encodes the mycobacterial pyrazinamidase, as the source of PZA resistance. The gene product is an amidase that hydrolyzes PZA to POA and ammonia. Mutations in pncA are the major mechanism for PZA resistance in M. tuberculosis (52–55). As might be expected, a plethora of mutations has been found in the pncA genes of PZA-resistant clinical isolates since virtually any mutation that confers loss of enzyme function is sufficient to confer PZA resistance. PZA resistance-conferring alterations are widely dispersed throughout the 561-amino-acid open reading frame (ORF), while resistance-conferring changes in the 82-base-pair region of the putative promoter are rare (56). M. bovis BCG, which is naturally resistant to PZA, has an H57D amino acid substitution in PncA, which leads to a lack of activation. Interestingly, up to 15 to 30% of PZA-resistant clinical isolates harbor an intact pncA gene (57–59).

Other factors in PZA resistance

In addition to the importance of pncA in activating PZA, three possible mechanisms and bacterial targets have been implicated in the drug’s action. The first is the weak acid activity of POA. Zhang and colleagues showed that following PZA entry into bacilli by passive diffusion and deamidation by cytoplasmic PncA, POA (pKₐ 2.9) is trapped in the bacterial cytoplasm because it is a carboxylate anion with only a small amount of elimination by bacterial efflux pumps. Because the protonated acid form of POA may diffuse back out as a neutral molecule, accumulation of protonated POA external to the cell leads to collapse of the bacterial membrane potential, killing the bacillus (60). A second postulated mechanism is an inhibitory effect of POA on the microbial fatty acid synthase type 1 (FAS-1) system. Zimhony and colleagues used 5-Cl-PZA and showed a potent inhibitory effect on Mycobacterium smegmatis that could be reduced by overexpression of the FAS-1 gene. They also demonstrated 5-Cl-PZA inhibitory activity against M. tuberculosis FAS-1 and that under acidic conditions, PZA itself inhibits the mycobacterial FAS-1, findings that suggest that FAS-1 is the target of activated PZA (61). Later studies, however, confirmed the activity of 5-Cl-PZA against FAS-1 but could not reproduce that the effects are not observed with PZA or POA themselves (62).

Recently, Shi and colleagues studied PZA-resistant M. tuberculosis strains that lack pncA mutations (63).
In three such strains they observed mutations in the \textit{rpsA} gene, which encodes the ribosomal protein S1; these mutations were ΔA438, T5S, and D123A. Overexpression of \textit{rpsA} increased \textit{M. tuberculosis} resistance to PZA in wild-type bacteria but not in the \textit{rpsA} ΔA438 mutant. RpsA in \textit{E. coli} has been shown to bind to transfer-messenger RNA (tmRNA) in a process called trans-translation that rescues stalled ribosomes during periods of slow growth or nutrient depletion. \textit{M. tuberculosis} RpsA was shown to have this tmRNA binding activity. This study suggests that PZA activity may interfere with \textit{M. tuberculosis} trans-translation, which may account for the drug’s unique activity against the persister forms.

**Host-directed activity of PZA**

In 2009, Mendez and colleagues (64) showed that PZA had an inhibitory effect on the parasitic organism \textit{Leishmania major} both in vitro and in a murine model. In addition to controlling the proliferation of \textit{L. major}, PZA treatment of macrophages in culture led to the upregulation of several cytokines including interleukin-12 (IL-12) in both the presence and absence of parasite infection. The authors concluded that PZA has a collateral host-directed effect that is beneficial in leishmaniasis. Recently, Manca and colleagues performed similar studies with \textit{M. tuberculosis} (65). They showed significant reductions in the secretion of the proinflammatory cytokines tumor necrosis factor alpha, IL-6, IL-1β, and monocyte chemotactic protein-1 when \textit{M. tuberculosis}-infected human monocytes were treated with PZA. The study also showed that on transcriptional analysis of the mouse lung transcriptome during \textit{M. tuberculosis} infection, treatment with PZA significantly upregulated the transcription of the adenylate cyclase and peroxisome-proliferator activated receptor (PPAR) genes that govern anti-inflammatory responses. The study suggests that PZA triggers an anti-inflammatory response in the host, which may play a role in the drug’s action during chemotherapy.

To evaluate the hypothesis that PZA may have a host-targeted mechanism of action, Almeida and colleagues tested PZA monotherapy against \textit{M. bovis} (resistant to PZA due to a \textit{pncA} mutation) in BALB/c mice and found no activity compared to untreated controls. They also evaluated the action of the drug in nude versus immunocompetent BALB/c mice (66). Over 8 weeks, BALB/c mice displayed a 4-log reduction on lung bacterial CFU counts with RIF-EMB-PZA and a 2-log reduction with RIF-EMB alone. Hence, PZA added 2 logs of killing in BALB/c mice. In contrast, nude mice showed only a 1.5-log reduction with both regimens, indicating that PZA did not add to the killing potency of the other two drugs in nude mice. These results suggest that in addition to the need for PncA-mediated drug activation, an intact host immune system is needed for bacterial killing by PZA.

**Ethambutol**

Ethambutol (EMB) is the least effective of the first-line antitubercular drugs and is considered bacteriostatic against metabolically active bacteria. It is incorporated in first-line therapy mainly to protect against the emergence of drug resistance.

**Mechanism of action**

While the mechanism of action and genetic basis of resistance to EMB have not been fully elucidated, the arabinosyltransferases have been identified as molecular targets for EMB (67, 68). These membrane-associated enzymes are involved in the polymerization of arabinan, a component of arabinogalactan, the major polysaccharide of the mycobacterial cell wall (69–71). Interruption of arabinan biosynthesis leads to intracellular accumulation of mycolic acids and eventual cell death. \textit{embCAB}, a three-gene operon encoding the arabinosyltransferases \textit{embC}, \textit{embA}, and \textit{embB}, has been identified in \textit{M. tuberculosis} (72). These enzymes share 65% homology with each other.

**Mechanism of resistance: target modification: \textit{embCAB} operon**

EMB resistance is most frequently associated with mutations in the \textit{embCAB} operon (Rv3793-5, MT3900-2). In particular, mutations in \textit{embC} and \textit{embB} have been associated with resistance, with codons 306, 406, and 497 in \textit{embB} being the most common (72). In some samples, 50 to 70% of EMB-resistant clinical isolates contain polymorphisms in codon 306 of \textit{embB}. While polymorphisms in codon 306 have also been identified among clinical isolates that are susceptible to EMB, this has previously been attributed to difficulties with phenotypic drug susceptibility testing for the low-level drug resistance conferred by \textit{embB} mutations (73, 74). Further evidence of the importance of codon 306 to EMB resistance is that the variant amino acid motifs at this locus in \textit{Mycobacterium leprae}, \textit{Mycobacterium abscessus}, and \textit{Mycobacterium chelonae} are thought to be responsible for the natural resistance of these organisms to EMB (68).

Because a significant percentage of EMB-resistant isolates lack mutations in these genes, other yet unidentified
resistance mechanisms must be important (75). One of these mechanisms may involve the gene embR, which has been noted to modulate the level of arabinosyltransferase activity in vitro (76), but its role in clinical resistance is unknown. More recently, genomic characterization of in vitro-selected EMB-resistant strains identified some additional loci (77). For example, mutations in Rv3806c, encoding ubiA, an enzyme that synthesizes a donor substrate for arabinosyltransferases, were identified in EMB-resistant clinical isolates as well as selected for in vitro. In combination with embCAB mutations, ubiA mutations are associated with higher-level resistance.

**INJECTABLE AGENTS**

**Aminoglycosides**

Aminoglycosides have been used to treat TB since the introduction of the first antituberculous agent, streptomycin (STR). Currently, STR remains a first-line agent, although its use is largely restricted to retreatment cases because like other aminoglycosides it must be given parenterally. AMK and KAN are the other major aminoglycosides used for TB. These may be given intravenously or intramuscularly and are second-line agents used to treat MDR- or XDR-TB. Aminoglycosides are considered bactericidal drugs and achieve rapid bacterial killing during the initiation phase of treatment. They have poor sterilizing activity and must be combined with agents such as rifamycins and PZA in order to achieve durable cure.

**Mechanism of action**

Aminoglycosides inhibit protein synthesis by binding to the 30S subunit of the mycobacterial ribosome. The majority of mutations that confer aminoglycoside resistance lead directly or indirectly to alterations in the aminoglycoside binding pockets of the ribosome, which prevent drug binding but preserve ribosome function as occurs with mutations in the rpsL, rrs, and gidB genes. A minor mechanism of aminoglycoside resistance is drug modification like that which occurs with the Eis protein, an aminoglycoside acetyltransferase.

STR is a streptidine aminoglycoside. On binding to the 30S ribosomal subunit, STR inhibits translational initiation and may also cause misreading of mRNA (78). AMK and KAN are deoxystreptamine aminoglycosides, which bind to a different locus on the 30S ribosome. Because of these differences in ribosome binding site between STR and AMK/KAN, different drug-conferring mutations are associated with these two drug groups.

In contrast, owing to the structural and functional similarity between AMK and KAN, there is extensive cross-resistance between them.

**Mechanism of resistance:**

**target modification mutations**

**rpsL**

The rpsL gene (Rv0682, or MT0710) encodes the 12S protein, which is a structural component of the ribosome. The RpsL protein serves to stabilize the pseudoknot that is formed by the 16S rRNA component of the 30S ribosome. While rpsL is an essential gene, nonsynonymous mutations are tolerated for ribosomal function but result in reduced aminoglycoside binding. Common mutations in aminoglycoside resistance that confer resistance to STR are RpsL K43R and K88R (79, 80).

**rrs**

The rrs gene encodes the 16S rRNA itself. The 16S rRNA contains key structures such as the 330 stem loop and the 915 turn; both of these rRNA structures form contacts for the binding of aminoglycosides. Like rpsL, rrs is an essential gene. Nonsynonymous SNPs in the rrs gene result in reduced aminoglycoside binding while allowing for preserved ribosome function. Many bacteria harbor multiple copies of the rrs gene, and consequently single-allele rrs mutations confer low-level aminoglycoside resistance. In contrast, *M. tuberculosis* has only one rrs gene, and mutations in the *M. tuberculosis* rrs gene are usually associated with high-level aminoglycoside resistance.

While more than 20 rrs mutations have been associated with aminoglycoside resistance, the common polymorphisms are A1401G, A514C, and C517T. Some rrs mutations also confer resistance to the cyclic peptide antibiotic CAP (see below). The most common mutation for second-line aminoglycoside resistance is A1401G, which was found in 78%, 56%, and 76% of AMK-, KAN-, and CAP-resistant strains, respectively, in one large systematic review (81).

**gidB**

The gidB gene (Rv3919c, MT4038) encodes a 7-methylguanosine methyltransferase that specifically modifies residues on the 16S rRNA (rrs). gidB is a non-essential gene, and loss-of-function mutations in gidB result in failure to methylate G527 within the 530 loop of the 16S rRNA molecule (82). Reduced ribosomal methylation confers low-level aminoglycoside resistance by reducing the affinity of the drugs for the 16S rRNA.
binding site. Many different gidB mutations including deletions are associated with aminoglycoside resistance, suggesting that loss of function confers resistance. Polymorphisms in gidB have also been identified in drug-susceptible strains, indicating that as for other resistance-conferring loci, the presence of a mutation in a gene is not necessarily indicative of resistance.

**Mechanism of resistance: inactivating mutations: eis**

The eis gene (Rv2416c, MT2489) encodes an aminoglycoside acetyltransferase, which has an affinity for KAN. KAN acetylation inactivates the drug by preventing it from binding to the 30S ribosome. Promoter upregulating mutations in the 5′ untranslated region of eis are associated with clinically relevant M. tuberculosis resistance to KAN. While the Eis protein is capable of acetylating AMK, its affinity for AMK is low, and studies of clinical isolates with eis promoter-up mutations reveal selective KAN resistance with relative preservation of AMK susceptibility (83). Nevertheless, clinical isolates with eis mutations that are KAN- and AMK-resistant have been described (79), so the specificity of eis mutations for KAN resistance remains uncertain.

**Mechanism of resistance: upregulation of drug inactivator and drug efflux: whiB7**

Recently, mutations in the promoter region of the transcriptional activator whiB7 have been identified in clinical isolates. These promoter mutations result in upregulation of the whiB7 regulon, leading to an increase in eis expression and resulting KAN resistance (84). The whiB7 regulon also includes Rv1258c, which encodes an efflux pump, tap, that is upregulated in tandem with eis, resulting in STR resistance. whiB7 promoter mutations therefore can result in both a target modification and efflux mechanism of resistance, leading to cross-resistance to two aminoglycoside drug groups (84).

**STR resistance-conferring mutations:**

**rpsL, rrs, gidB**

High-level STR resistance is achieved predominantly by mutations in rpsL (∼50% of STR-resistant strains) and rrs (∼15% of STR-resistant strains). Important rrs mutations associated with STR resistance are rrs A514C and A908C. While the rrs A514C mutation is associated with high-level resistance to STR, the most common rrs mutation conferring AMK and KAN resistance (rrs A1401G) does not confer STR cross-resistance. gidB mutations are less frequent among STR-resistant strains (∼20% of strains) and are associated with low-level STR resistance (79).

**Mechanisms of low-level resistance have not been well characterized but probably involve drug efflux.**

**AMK- and KAN-resistance-conferring mutations: rrs, eis**

The rrs A1401G is the most frequent mutation conferring AMK and KAN resistance, occurring in ∼85% or more of strains that are AMK or KAN resistant. The presence of A1401G appears to be 100% specific for coresistance to AMK and KAN. Of AMK- and KAN-resistant strains, 10 to 15% harbor eis mutations, indicating that eis is a minor determinant of AMK and KAN resistance (45, 79).

**Capreomycin**

CAP and viomycin (VIO) are cyclic peptide antibiotics with structural similarity. The drugs have uniform cross-resistance in M. tuberculosis and appear to have similar mechanisms of action. While VIO is rarely used due to high toxicity, CAP is an injectable drug commonly used as a second-line agent in the management of MDR- and XDR-TB that is resistant to aminoglycosides.

Like the structurally unrelated aminoglycosides, CAP and VIO are bactericidal drugs that inhibit protein synthesis. VIO has been shown to bind both the 30S and 50S ribosome subunits and to inhibit ribosomal transllocation by interference with the peptidyl tRNA acceptor site (85, 86). Due to overlap in the binding region of CAP and the aminoglycosides, certain mutations confer cross-resistance to CAP and AMK/KAN. In contrast, cross-resistance between CAP and STR is rare. The major mechanisms of CAP resistance are mutations that result in ribosome modification, particularly rrs and tlyA. Interestingly, tlyA mutations uniquely affect CAP resistance and do not appear to play a role in resistance to aminoglycosides.

**Mechanism of resistance: target modification mutations**

**rrs**

The rrs A1401G mutation is found in ∼85% of CAP-resistant XDR-TB strains. Other rrs mutations including C1402T and G1484T are also associated with CAP resistance (79).

**tlyA**

The tlyA gene (Rv1694, MT1733) is a nonessential gene found in many bacteria. Transposon mutants of tlyA as well as spontaneous point mutants display significant CAP and VIO resistance. Biochemical, genetic, and comparative genomics suggest that the tlyA gene is an
rRNA methyltransferase and that loss of methyltransferase activity yields an unmethylated ribosome that is resistant to CAP inhibition (87). This mechanism is similar to that of the eis gene that confers resistance to KAN. Numerous thyA mutations have been reported including L180R, S265T, S64W, frameshift at 218L, N236K, and L150P (79, 87, 88).

SECOND-LINE AGENTS

Quinolones

Fluoroquinolones are second-line antitubercular agents, and they currently form the backbone of MDR-TB therapy. In addition to use in drug-resistant TB, quinolones are indicated for the treatment of drug-susceptible TB among patients intolerant to components of first-line regimens.

Mechanism of action

Quinolones inhibit bacterial topoisomerases, enzymes that regulate the supercoiling of DNA and are thus essential for DNA replication, transcription, and recombination (89). While many bacterial species contain both topoisomerase II (also known as DNA gyrase) and topoisomerase IV, M. tuberculosis lacks an analogue of topoisomerase IV (90). Thus, DNA gyrase is the sole target for quinolone activity in M. tuberculosis. Clinically observed differences in efficacy among quinolones may be explained by specificity for different topoisomerases; ciprofloxacin, which is less effective in M. tuberculosis, primarily targets topoisomerase IV, which is lacking, whereas newer-generation fluoroquinolones that are also known to have improved efficacy, including moxifloxacin and levofloxacin, preferentially target DNA gyrase (91).

DNA gyrase is an ATP-dependent enzyme that cleaves and religates double-stranded DNA, which allows for the introduction of negative supercoils into DNA. DNA gyrase is a tetrameric protein, comprised of two α and two β subunits, encoded by the genes gyrA (Rv0006, MT0006) and gyrB (Rv0005, MT0005), respectively (92).

Mechanism of resistance: target modification: gyrA and gyrB

A highly conserved area within gyrA, known as the quinolone-resistance-determining region (QRDR), contains mutations that have been shown to confer fluoroquinolone resistance in many bacterial species, including M. tuberculosis (92). In addition, gyrB mutations can also confer fluoroquinolone resistance. More than 90% of quinolone-resistant M. tuberculosis strains have mutations in gyrA or gyrB (93, 94). Classically, mutations in codons 90 and 94 of gyrA are most commonly associated with drug resistance, with A90V, D94G, and D94H frequently noted among clinical isolates (45, 92, 94–97). Among quinolone-resistant isolates, double mutants in gyrA or additionally in gyrB have been noted to have higher MICs (98, 99).

Two additional potential mechanisms of quinolone resistance are efflux and DNA mimicry (100–103). However, to date these resistance mechanisms have been identified only in laboratory M. tuberculosis strains, so the clinical relevance and epidemiological impact of this resistance mechanism are unknown.

Ethionamide/Prothionamide

ETH is a commonly used oral, second-line agent used for the treatment of MDR- and XDR-TB. It is occasionally used in the treatment of drug-susceptible TB, primarily when patients cannot tolerate INH. Prothionamide is also in the thionamide drug class, and it contains the same active moiety as ETH. Because there is complete cross-resistance between ETH and prothionamide, these drugs are used interchangeably and can be considered functionally identical (104, 105).

Mechanism of action

ETH is a structural analog of INH, and like INH it inhibits mycolic acid biosynthesis. It has long been known that strains displaying low-level resistance to INH are also ETH-resistant but that strains with high-level INH resistance can remain ETH-susceptible (106). Like INH, ETH is a prodrug and requires activation, but in contrast to INH, which requires KatG activation, ETH is activated by a mono-oxygenase encoded by ethA. The EthA mono-oxygenase is unique for ETH and does not activate INH. Following EthA activation, ETH forms an adduct with NAD, and this adduct subsequently inhibits the NADH-dependent enoyl-ACP reductase, InhA, in the same manner as the INH-NAD adduct.

Hence, ETH and INH have a common mechanism of action but different bacterial activating enzymes. Thus, katG mutations confer exclusively high-level INH resistance, whereas mutants in the common target of INH and ETH (inhA) lead to low-level cross-resistance to INH and ETH. Resistance to ETH arises by two major mechanisms: (i) loss of activating enzyme activity (ethA) and (ii) target amplification (inhA promoter-up mutations).
Mechanism of resistance: loss of activation: \textit{ethA}

The \textit{ethA} gene (Rv3854c, MT3969) encodes a nonessential mono-oxygenase. Its association with ETH resistance was identified in 2000 \cite{107, 108}. Expression of \textit{ethA} is under the control of a TetR-like repressor known as \textit{ethR} (Rv3855, MT3970), and overexpression of \textit{ethR} leads to ETH resistance. Consistent with the fact that loss of function confers resistance, over 25 mutations in \textit{ethA} have been associated with ETH resistance. In contrast to KatG, where the dominant INH-resistance-conferring mutation is S315T, a mutation that preserves catalase-peroxidase activity while failing to activate INH, EthA mutations are widely dispersed throughout the \textit{ethA} gene. This suggests that there is little fitness cost for loss of EthA mono-oxygenase activity, possibly because there are over 30 other mono-oxygenases in the \textit{M. tuberculosis} genome that may play compensatory roles \cite{109}.

In some surveys of clinical isolates, mutation in \textit{ethA} occurs in 50% of strains with or without other ETH-resistance-associated mutations, while \textit{inhA} mutations account for the remaining 50% \cite{109}. Other surveys have found that up to 50% of ETH resistance is unexplained by \textit{inhA} or \textit{ethA} mutations \cite{48}.

Mechanism of resistance: target modification: \textit{inhA}

\textit{inhA} encoding an NADH-dependent enoyl-ACP reductase, which is required for mycolic acid biosynthesis, is the target of both the ETH-NAD adduct and the INH-NAD adduct. Among strains showing ETH resistance without mutations in \textit{ethA}, \textit{inhA} promoter mutations account for about two-thirds of resistance, while promoters in combination with ORF mutations account for one-third. A small number of ETH-resistant strains have mutations only in the \textit{inhA} ORF, usually with the S94A, S94W, or L11V alterations \cite{48, 109}.

\textit{mshA} (Rv0486, MT0504), which encodes for a glycosyl-transferase involved in mycothiol biosynthesis, may also be associated with drug resistance to ETH in certain isolates \cite{110}.

**Cycloserine**

\(d\)-Cycloserine (CYS) is an analogue of the amino acid \(d\)-alanine, which competitively inhibits two essential enzymes required for peptidoglycan formation and thus cell wall biosynthesis. Terizidone (TER) combines two molecules of cycloserine and is thought to act similarly. Alanine racemase (\textit{alr}, Rv3423c, MT3532) converts \(l\)-alanine to \(d\)-alanine, which is a substrate for the second enzyme, \(d\)-alanine-\(d\)-alanine ligase (\textit{ddlA}, Rv2981c, MT3059), which incorporates \(d\)-alanine into the elongating pentapeptide necessary for peptidoglycan synthesis \cite{111, 112}. In \textit{M. smegmatis}, overexpression of \textit{alr} and \textit{ddlA} results in resistance to CYS \cite{113, 114}.

\textit{CycA} (Rv1704c, MT1744), a bacterial \(d\)-serine/\(l\)- and \(d\)-alanine/glycine/\(d\)-cycloserine proton symporter, has also been implicated in CYS susceptibility. A nonsynonymous SNP in \textit{cycA}, G122S, has recently been identified in BCG, which has now been shown to be partially responsible for BCG’s innate resistance to CYS \cite{115}.

**Para-Aminosalicylic Acid**

Para-aminosalicylic acid (PAS) is a second-line oral drug for TB. It was introduced in 1948 shortly after STR, and its early use without partner drugs was associated with the rapid emergence of resistance. PAS is an analogue of para-amino benzoic acid (PABA) and has long been known to interfere with folic acid biosynthesis. \textit{thyA} (Rv2764c, MT2834) encoding thymidylate synthase was thought to be a resistance gene; however, large surveys have shown only a weak association between \textit{thyA} polymorphisms and PAS resistance among clinical isolates \cite{116}. Recent studies have shown that the enzyme dihydropteroate synthase misincorporates PAS as if it were PABA at the dihydropteroate stage of the enzymatic pathway \cite{117}. This altered substrate serves to block the biosynthetic pathway downstream of dihydropteroate synthase including dihydrofolate reductase. \textit{RibD}, a bifunctional enzyme involved in riboflavin biosynthesis (\textit{ribD}, Rv2671, MT2745), was found to have dihydrofolate reductase activity, and clinical isolates that overexpress \textit{RibD} due to mutation are PAS-resistant \cite{118}. To date, specific mutations in additional pathway enzymes that confer PAS resistance remain poorly defined in clinical isolates.

**Oxazolidinones**

Linezolid (LIN), an oxazolidinone, has shown promise among limited numbers of patients in treatment for drug-resistant TB \cite{119, 120}. LIN binds the bacterial 23S portion of the 50S subunit of rRNA, thereby inhibiting formation of the initiation complex. Mutations in the
23S rRNA gene have been documented with in vitro selection (121). More recently, mutations in the 50S ribosomal protein L3, rplC, at position T460C have been noted among in vitro-selected LIN-resistant isolates and also among several LIN-resistant clinical isolates (122).

**β-Lactams**

β-Lactam antibiotics bind transpeptidases that cross-link peptidoglycans and thus inhibit cell wall synthesis. M. tuberculosis possesses a highly active β-lactamase, BlaC, which hydrolyzes β-lactams, rendering them ineffective against M. tuberculosis except in the presence of a β-lactamase inhibitor (123). A β-lactam-β-lactamase inhibitor combination, meropenem-clavulanate, has been associated with encouraging clinical outcomes in a limited number of patients with XDR-TB (124). The genomic markers of β-lactam drug resistance in M. tuberculosis have yet to be elucidated.

**Clofazimine**

Clofazimine (CFZ) is a riminophenazine dye that has recently shown promise for use in MDR-TB and may allow for treatment regimen shortening (125). Though the precise mechanism of action of CFZ has remained elusive, the drug may target NDH-2, the primary NADH dehydrogenase involved in the respiratory chain, resulting in production of bacterial reactive oxygen species (126).

**NOVEL AGENTS**

**Bedaquiline**

Bedaquiline (BDQ, formerly known as TMC207) is a diarylquinoline drug approved for use against MDR-TB in 2012. It is the first new drug class to be introduced in the treatment of TB since the rifamycins in the 1960s. BDQ binds to and inhibits the M. tuberculosis ATP synthase gene encoded by the essential gene atpE (Rv1305, MT1345) (127). AtpE forms a part of the F1F0 proton ATP synthase, a transmembrane protein complex that generates ATP from proton translocation, and inhibition of ATP biosynthesis has been shown to kill both actively dividing and nondividing bacteria (128).

In vitro resistance to BDQ in mycobacteria has been shown to involve mutations in atpE (129). However, resistant mutants have been identified that do not have any mutations in atpE or in the other genes encoding components of ATP synthase, suggesting alternative mechanisms of resistance or perhaps other targets for BDQ (130).

Verapamil, an efflux pump inhibitor, was recently found to decrease profoundly the MIC of BDQ and CFZ to M. tuberculosis by 8- to 16-fold (131). Thus, efflux inhibition is an important sensitizer of BDQ and CFZ, and efflux may emerge as a resistance mechanism to these drugs.

**DIAGNOSTIC TOOLS FOR DETECTING DRUG-RESISTANT M. TUBERCULOSIS**

As discussed above, for each first-line anti-TB drug, there are several known M. tuberculosis genetic mutations commonly associated with bacterial drug resistance. Capitalizing on these genetic associations has allowed for the development of molecular diagnostics, including the GeneXpert and the Hain Line Probe Assay MTBDRplus, which are able to accelerate the identification of drug resistance to two of the most important TB drugs, RIF and INH.

GeneXpert is a PCR-based, point-of-care system that is able to quickly and accurately analyze a sample for the presence of the M. tuberculosis complex as well as assay for RIF resistance (132). Building upon the assumption that approximately 95% of RIF resistance is conferred by mutations in the rpoB gene, GeneXpert utilizes a molecular beacon to detect the presence of a mutated rpoB gene as an indicator of drug-resistant TB. Similarly, MTBDRplus uses M. tuberculosis complex-specific probes to assess for wild-type or mutant-specific probes to identify mutations conferring resistance in the RRDR of rpoB, as well as katG and inhA mutations that are known to confer INH drug resistance (133). A similar test that includes mutations to select second-line agents is also available.

However, the genetic mutations included in these assays do not account for all known drug resistance to their cognate anti-TB drugs. This genomic ambiguity translates into a relatively high false-negative rate of the above-mentioned genetics-based assays, which is a major limitation to their clinical utility. Indeed, MTBDRplus fails to identify 15 to 30% of INH resistance and 5% of RIF resistance. Improving the performance of these diagnostic tests will require identification of the additional molecular mechanisms of resistance and inclusion of additional polymorphisms that can be used to identify a minority of resistant phenotypes.

Genome sequencing shows promise as a reliable modality for the rapid identification of resistance. One recent study showed that by subjecting the primary growth of an isolate to genomic sequencing, XDR-TB
could be detected weeks before the culture-based diagnosis was made (134).

GENOMICS AND DRUG RESISTANCE

While significant progress has been made in identifying independent genes in which mutations confer resistance, some resistance cannot be accounted for by the current models. A possible explanation is that multiple incremental mutations combine to produce phenotypic resistance. Recently, there have been several genomic surveys seeking resistance “signatures” that may correlate with either individual resistance phenotypes or certain multidrug-resistant phenotypes. Several recent whole-genome sequencing projects involving resistant M. tuberculosis strains have led to the identification of over 100 genetic loci that are associated with resistance including genes involved in the synthesis or regulation of surface exposed lipids (135, 136). As sequencing of M. tuberculosis strains becomes more common, these signatures will have better definition and will have the potential to identify additional resistance mechanisms as well as form the basis of algorithmic prediction tools for diagnosing all drug-resistant TB.

REFERENCES

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