

Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs

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Tuberculosis (TB) remains one of the leading public health problems worldwide. Declared as a global emergency in 1993 by the WHO, its control is hampered by the emergence of multidrug resistance (MDR), defined as resistance to at least rifampicin and isoniazid, two key drugs in the treatment of the disease. More recently, severe forms of drug resistance such as extensively drug-resistant (XDR) TB have been described. After the discovery of several drugs with anti-TB activity, multidrug therapy became fundamental for control of the disease.

Major advances in molecular biology and the availability of new information generated after sequencing the genome of *Mycobacterium tuberculosis* increased our knowledge of the mechanisms of resistance to the main anti-TB drugs. Better knowledge of the mechanisms of drug resistance in TB and the molecular mechanisms involved will help us to improve current techniques for rapid detection and will also stimulate the exploration of new targets for drug activity and drug development. This article presents an updated review of the mechanisms and molecular basis of drug resistance in *M. tuberculosis*. It also comments on the several gaps in our current knowledge of the molecular mechanisms of drug resistance to the main classical and new anti-TB drugs and briefly discusses some implications of the development of drug resistance and fitness, transmission and pathogenicity of *M. tuberculosis*.

Keywords: TB, MDR, XDR, quinolones, fitness

Introduction

The history of tuberculosis (TB) changed dramatically after the introduction of the first drugs with anti-mycobacterial activity. What was considered until then as a disease to be treated in sanatoria turned into a malady that could be managed with antibiotics.¹ However, not long after the first antibiotic was introduced in 1944, drug resistance emerged, mainly due to the use of streptomycin as monotherapy.² With the discovery of several other drugs with anti-TB activity, multidrug therapy became fundamental for the control of the disease by promoting the cure of the patients and interrupting the chain of transmission. More recently, new forms of antibiotic resistance have emerged. Multidrug-resistant TB (MDR-TB), caused by a strain of *Mycobacterium tuberculosis* resistant to at least rifampicin and isoniazid, and extensively drug-resistant TB (XDR-TB), caused by strains of *M. tuberculosis* that, in addition to being MDR, are also resistant to any fluoroquinolone and to at least one of the three injectable drugs kanamycin, capreomycin and amikacin, again threaten adequate control of the disease.^{3,4}

Major advances in molecular biology tools and the availability of new information generated after deciphering the complete genome sequence of *M. tuberculosis*^{5,6} increased our knowledge of the mechanisms of resistance to the main anti-TB drugs and

showed that specific gene mutations were associated with drug resistance.⁷ With almost 500 000 MDR-TB cases emerging each year worldwide and between 5% and 7% of them becoming XDR,^{3,8} a better knowledge of the mechanisms of drug resistance in TB and the molecular mechanisms involved will have an important impact on the improvement of currently available techniques for rapid detection and will also stimulate the exploration of new targets for drug activity and drug development. Several years have elapsed since two comprehensive reviews on the molecular mechanisms of drug resistance in *M. tuberculosis* were published.^{7,9} Here we present an updated review of the mechanisms and the molecular basis of drug resistance in *M. tuberculosis* to the main classical and new anti-TB drugs, and discuss some implications of the development of drug resistance and fitness, transmission and pathogenicity of the bacteria.

Intrinsic drug resistance

Intrinsic drug resistance of *M. tuberculosis* has traditionally been attributed to the unusual structure of its mycolic acid-containing cell wall that gives the bacteria a low permeability for many compounds such as antibiotics and other chemotherapeutic agents.¹⁰ More recently, the role of efflux mechanisms has also

been recognized as an important factor in the natural resistance of mycobacteria against antibiotics such as tetracycline, fluoroquinolones and aminoglycosides, among others.¹¹

In *Mycobacterium smegmatis*, it has been shown that mutants lacking the major porin MspA had an increased MIC of the β -lactam antibiotics ampicillin and cefaloridine. Deletion of the *mspA* gene also increased the MIC of vancomycin, erythromycin and rifampicin by 2- to 10-fold.¹² Using the same model organism, it was shown that deletion of the porins MspA and MspC increased the resistance to β -lactam antibiotics without affecting its β -lactamase activity, which remained unchanged. This study also showed that hydrophilic fluoroquinolones such as norfloxacin, but also chloramphenicol, diffuse through porins in mycobacteria.¹³ β -lactam antibiotics bind and inhibit the activities of penicillin-binding proteins involved in cell wall biosynthesis, but mycobacteria possess β -lactamase enzymes that degrade these drugs. This is the main mechanism conferring resistance to β -lactam antibiotics. In *M. tuberculosis*, β -lactamase activity is encoded by *blaC* and *blaS*. The use of β -lactamase-resistant β -lactam antibiotics or β -lactam in combination with β -lactamase inhibitors has been shown to be effective in killing *M. tuberculosis*.¹⁴ Recent studies have shown that in *M. tuberculosis*, Rv1698 can have the same function as MspA in contributing to intrinsic resistance to hydrophilic compounds.¹⁵ Moreover, bioinformatics analysis has identified both Rv1698 and Rv1973 as being mycobacterial outer membrane proteins (OMPs) with a possible role in the intrinsic resistance to some antibiotics.¹⁶

But not only permeability barriers or β -lactamases are responsible for the intrinsic resistance to antibiotics in mycobacteria. Physiological adaptations occurring within the host can also be responsible for antibiotic tolerance.¹⁷ Finally, in *M. tuberculosis*, *whiB7* is an MDR determinant whose deletion produces a multidrug-susceptible phenotype, suggesting its role as an ancestral MDR determinant.¹⁸

Intrinsic resistance is thus important since it limits the number of drugs available for treatment and favours the emergence of strains with a high level of drug resistance.

Consequently, drugs inhibiting these mechanisms would enable potential new use against *M. tuberculosis* of many antibiotics that are already available but have not been used before in the treatment of TB.^{19,20}

Acquired drug resistance

Unlike the situation in other bacteria where acquired drug resistance is generally mediated through horizontal transfer by mobile genetic elements, such as plasmids, transposons or integrons, in *M. tuberculosis*, acquired drug resistance is caused mainly by spontaneous mutations in chromosomal genes, producing the selection of resistant strains during sub-optimal drug therapy.²¹

Although no single pleiotropic mutation has been found to cause an MDR phenotype in *M. tuberculosis*, a possible complex association between classical mutations associated with resistance to one drug could be related to initial steps in the resistance to other drugs.²²

In prokaryotes, spontaneous mutations occur at a low rate of 0.0033 per replication. The mutation rate per bp is inversely proportional to the genome size. Previous studies have shown that the rate of mutation depends on the nature of the drug selection, but for most of the main anti-TB drugs, this occurs at a rate of 10^{-9} mutations per cell division. This is the main reason why anti-TB drugs are given as a combination, as the risk of a mutant containing two resistance mutations is $<10^{-18}$.²³

In the following sections we will present an updated review on individual anti-TB drugs, including new proposed anti-TB antibiotics, and their molecular mechanisms of drug resistance. In addition, see Table 1. It is noteworthy that most of the drugs currently in use to treat TB are specific for *M. tuberculosis*.

Isoniazid

Isoniazid is one of the main drugs for the treatment of TB. It has a simple structure containing a pyridine ring and a hydrazide group, with both components being essential for the high activity

Table 1. Drugs, MICs and mechanisms of drug resistance

| Drug | MIC (mg/L) | Gene | Role of gene product | Reference |
|--------------------------------|---------------------|------------------|-------------------------------------|-----------|
| Isoniazid | 0.02–0.2 (7H9/7H10) | <i>katG</i> | catalase/oxidase | 26 |
| | | <i>inhA</i> | enoyl reductase | 35 |
| | | <i>ahpC</i> | alkyl hydroperoxide reductase | 38 |
| Rifampicin | 0.05–0.1 (7H9/7H10) | <i>rpoB</i> | β -subunit of RNA polymerase | 54 |
| Pyrazinimide | 16–50 (LJ) | <i>pncA</i> | PZase | 66 |
| Streptomycin | 2–8 (7H9/7H10) | <i>rpsL</i> | S12 ribosomal protein | 78 |
| | | <i>rrs</i> | 16S rRNA | 78 |
| | | <i>gidB</i> | 7-methylguanosine methyltransferase | 81 |
| Ethambutol | 1–5 (7H9/7H10) | <i>embB</i> | arabinoxyl transferase | 84 |
| Fluoroquinolones | 0.5–2.0 (7H9/7H10) | <i>gyrA/gyrB</i> | DNA gyrase | 99 |
| Kanamycin/amikacin | 2–4 (7H9/7H10) | <i>rrs</i> | 16S rRNA | 112 |
| Capreomycin/viomycin | 2–4 | <i>tlyA</i> | rRNA methyltransferase | 116 |
| Ethionamide | 10 (7H11) | <i>inhA</i> | enoyl reductase | 30 |
| <i>p</i> -amino salicylic acid | 0.5 (LJ) | <i>thyA</i> | thymidylate synthase A | 127 |
| PA-824 and OPC-67683 | 0.03 (7H9/7H10) | Rv3547 | hypothetical 16.4 kDa | 147 |
| TMC207 | 0.03 (7H9/7H10) | <i>atpE</i> | ATP synthase | 151 |

against *M. tuberculosis*. Despite this simple structure, the mode of action of isoniazid is more complex and isoniazid-resistant strains had already been isolated as soon as its anti-TB activity was recognized. Very early on, it was also shown that *Mycobacterium bovis* and *M. tuberculosis* isoniazid-resistant strains lacking catalase activity were highly attenuated in guinea pigs.^{24,25}

Resistance to isoniazid is a complex process. Mutations in several genes, including *katG*, *ahpC*, *inhA*, *kasA* and *ndh*, have all been associated with isoniazid resistance. Isoniazid is a pro-drug requiring activation by the catalase/oxidase enzyme encoded by *katG*.²⁶ Activated isoniazid interferes with the synthesis of essential mycolic acids by inhibiting NADH-dependent enoyl-ACP reductase, which is encoded by *inhA*.²⁷ Two molecular mechanisms have been shown to be the main cause for isoniazid resistance: mutations in *katG* and mutations in *inhA*, or more frequently in its promoter region.^{28,29}

A study by Hazbón *et al.*³⁰ analysed 240 alleles previously described in association with isoniazid resistance and found that mutations in *katG*, *inhA* and *ahpC* were most strongly associated with isoniazid resistance, while mutations in *kasA* were not associated with resistance. Similar results were reported in another previous study by Larsen *et al.*³¹

A decrease in or total loss of catalase/oxidase activity as a result of *katG* mutations are the most common genetic alterations associated with isoniazid resistance.²⁶ So far, more than a hundred mutations in *katG* have been reported, with MICs ranging from 0.2 to 256 mg/L. Missense and nonsense mutations, insertions, deletions, truncation and, more rarely, full gene deletion have been observed. The most common mutation is S315T, which results in an isoniazid product that is highly deficient in forming the isoniazid-NAD adduct related to isoniazid antimicrobial activity.³²

Interestingly, it has been shown that the mutation S315T in *katG* occurs more frequently in MDR than in isoniazid mono-resistant strains, and it has been postulated that this alteration does not produce a fitness cost, while *inhA* overexpression would produce a fitness cost.^{30,33} Also, isoniazid-resistant strains with the *katG* mutation S315T were found to be in clusters almost as frequently as the isoniazid-susceptible strains showing no transmissibility difference with the latter.³⁴ It has been proposed that the combination of isoniazid resistance with retention of virulence presumably offers the basis for persistence and the possibility to gain additional resistance to other drugs. This could be partially explained by the hypothesis that *katG* S315T mutants are the result of a second-step mutation, occurring after a long period of inappropriate chemotherapy.³⁰

Mutations in *inhA* cause not only resistance to isoniazid, but also resistance to the structurally related second-line drug ethionamide.³⁵ The most common *inhA* mutation occurs in its promoter region (-15C→T) and it has been found more frequently associated with mono-resistant strains.³⁶

In *M. tuberculosis*, *ahpC* codes for an alkyl hydroperoxidase reductase that is implicated in resistance to reactive oxygen and reactive nitrogen intermediates. It was initially proposed that mutations in the promoter of *ahpC* could be used as surrogate markers for the detection of isoniazid resistance.³⁷ However, several other studies have found that an increase in the expression of *ahpC* seems to be more a compensatory mutation for the loss of catalase/oxidase activity rather than the basis for isoniazid resistance.³⁸

Described for the first time in *M. smegmatis*, mutations in *ndh* reduce the activity of NADH dehydrogenase and produce resistance to isoniazid and ethionamide.³⁹ In *M. tuberculosis*, mutations in *ndh* have been associated with isoniazid resistance alone or in combination with other gene mutations such as *inhA* and *katG*. Although the mutations A13C and V18A in *ndh* were reported in two isoniazid-resistant *M. tuberculosis* strains, both also had mutations in *katG*. Moreover, mutation V18A was previously described in an isoniazid-susceptible strain.^{29,30,40}

Arylamine *N*-acetyltransferases are cytosolic conjugating enzymes that transfer an acetyl group from acetyl coenzyme A to an acceptor substrate. Mammalian arylamine *N*-acetyltransferases are involved in drug detoxification, showing an important pharmacogenetic role.⁴¹ In humans, there are two isoenzymes encoded by polymorphic genes that lead to different rates of inactivation of drugs, including isoniazid.⁴² Genomic and experimental studies have identified *N*-acetyltransferase homologues in bacteria, including *M. tuberculosis*, that inactivate isoniazid.⁴³ It would be expected that mutations resulting in the loss of *N*-acetyltransferase activity would improve susceptibility to isoniazid; however, no clear correlation between *nat* mutations and isoniazid susceptibility has been found in studies with clinical strains, although this does not permit the conclusion that arylamine *N*-acetyltransferase is directly related to isoniazid resistance.⁴⁴ Interestingly, the arylamine *N*-acetyltransferase protein appears to play an important role in the synthesis of the mycobacterial cell wall in slow-growing mycobacteria and has been suggested to be a target for anti-mycobacterial therapy.⁴⁵

Down-regulation of *katG* expression has also been recently shown to be associated with resistance to isoniazid.⁴⁶ Three novel mutations in the *furA-katG* intergenic region were identified in 4% of 108 isoniazid-resistant strains studied; none of these was present in 51 isoniazid-susceptible strains. Reconstructing these mutations in the *furA-katG* intergenic region of isogenic strains decreased the expression of *katG* and conferred resistance to isoniazid.

Similarly, mutations in the intergenic region *oxyR-ahpC* can reduce the level of expression of *inhA* and have been associated with resistance to isoniazid. A study by Dalla Costa *et al.*⁴⁷ found mutations in the intergenic region *oxyR-ahpC* in 8.9% of 224 isoniazid-resistant strains studied, confirming its less frequent involvement as a cause of resistance to isoniazid. The role of some of these genes in isoniazid resistance, however, has not been completely elucidated.

Isoniazid and tolerance

Antibiotic tolerance can be defined as the ability of bacteria to stop growing in the presence of an antibiotic, while still surviving to resume growth once the antibiotic has been removed. In *M. tuberculosis*, tolerance or phenotypic resistance occurs when changes in the metabolism or physiological status of the bacteria make them temporarily resistant to a certain drug. It has been shown that isoniazid induces alterations in the expression of several genes in both drug-resistant and drug-tolerant *M. tuberculosis* strains. Some of these genes fall into the functional class of lipid metabolism, while others belong to the class of cell wall and cell processes or transporters.⁴⁸

It has also been described that *M. tuberculosis iniA* gene (Rv0342), part of the three-gene operon (Rv0341, Rv0342,

Rv0343) induced in the presence of isoniazid, participates in the development of tolerance to both isoniazid and ethambutol. The same study also suggested that *iniA* functions through an MDR-pump-like mechanism, although *IniA* does not appear to directly transport isoniazid from the cell.⁴⁹

Isoniazid also induces several other genes, including an operon cluster of five genes that code type II fatty acid synthase enzymes and *fbpC*, which encodes trehalose dimycolyl transferase. Other genes also induced are *efpA*, *fadE23*, *fadE24* and *ahpC*, which mediate processes linked to the toxic activity of the drug and efflux mechanisms.⁵⁰

Rifampicin

Rifampicin is a lipophilic ansamycin introduced in 1972. Due to its efficient antimicrobial action, it is considered, together with isoniazid, to be the basis of the short-course treatment regimen for TB.⁵¹

The target of rifampicin in *M. tuberculosis* is the β -subunit of RNA polymerase, where it binds and inhibits the elongation of messenger RNA.⁵² An important characteristic of rifampicin is that it is active against actively growing and slowly metabolizing (non-growing) bacilli.⁵³

The great majority of *M. tuberculosis* clinical isolates resistant to rifampicin show mutations in the gene *rpoB* that encodes the β -subunit of RNA polymerase. This results in conformational changes that determine a low affinity for the drug and consequently the development of resistance.⁵⁴

Mutations in a 'hot-spot' region of 81 bp of *rpoB* have been found in about 96% of rifampicin-resistant *M. tuberculosis* isolates. This region, spanning codons 507–533, is also known as the rifampicin resistance-determining region (RRDR).⁷ Mutations in codons 531 and 526 are the most frequently reported mutations in most of the studies.^{55,56} Some studies have also reported mutations outside of the hot-spot region of *rpoB* in rifampicin-resistant *M. tuberculosis* isolates.⁵⁷

The mutation A191C in Rv2629 was initially described by Wang *et al.*⁵⁸ as being associated with resistance to rifampicin. However, other studies have found that this allele is related to the W-Beijing genotype but not to resistance to rifampicin.⁵⁹

Cross-resistance between rifampicin and other rifamycins do exist; some mutations in codons 516, 518, 522, 529 and 533 have been associated with low-level resistance to rifampicin but susceptibility to rifabutin and rifalazil.^{60,61} Rifabutin can continue to be used in TB patients receiving antiretroviral treatment since it induces the cytochrome P450 CYP3A oxidative enzymes at a lower level compared with rifampicin and rifapentine.⁶²

An important finding related to resistance to rifampicin is that almost all rifampicin-resistant strains also show resistance to other drugs, particularly to isoniazid. For this reason, rifampicin-resistance detection has been proposed as a surrogate molecular marker for MDR.⁶³

Pyrazinamide

Pyrazinamide was discovered in 1952 and introduced into TB chemotherapy in the early 1950s. Its use allowed the length of treatment to be reduced from 9 to 6 months. One key

characteristic of pyrazinamide is its ability to inhibit semi-dormant bacilli residing in acidic environments.⁶⁴

Pyrazinamide is a structural analogue of nicotinamide and is a pro-drug that needs to be converted into its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase (PZase).⁶⁵ PZase is encoded in *M. tuberculosis* by the gene *pncA*.⁶⁶ It has been postulated that the mechanism of action of pyrazinamide is through pyrazinoic acid, its active moiety, by disrupting bacterial membrane energetics and inhibiting membrane transport. This correlates with the increased activity of pyrazinamide against non-replicating bacilli with lower membrane potential and its disruption by pyrazinoic acid in acid environments.⁶⁷ Previous studies have shown that pyrazinamide enters *M. tuberculosis* by passive diffusion, is converted into pyrazinoic acid by PZase, and is excreted by a weak efflux pump. Under acid conditions, the protonated pyrazinoic acid is reabsorbed and accumulates inside the cell due to an inefficient efflux pump, resulting in cellular damage.⁶⁸ Another proposed theory states that pyrazinoic acid and its *n*-propyl ester inhibit the fatty acid synthase type I in replicating bacilli.⁶⁹

Mutations in *pncA* are the main mechanisms for pyrazinamide resistance in *M. tuberculosis*. Most alterations occur in a 561 bp region of the open reading frame or in an 82 bp region of its putative promoter.^{70,71} There is a high degree of diversity of *pncA* gene mutations among pyrazinamide-resistant strains; however, some pyrazinamide-resistant strains do not show mutations in *pncA* or its promoter region. In this case, it has been postulated that resistance to pyrazinamide could be due to mutations occurring in an unknown *pncA* regulatory gene.⁷² An alternative explanation could be the difficulty in performing drug susceptibility testing for pyrazinamide and that these strains are falsely resistant by the phenotypic test. Furthermore, a small proportion of pyrazinamide-resistant strains that have low-level resistance and retain PZase activity are considered to have another alternative mechanism of resistance.⁷³

The highly specific activity of pyrazinamide for *M. tuberculosis*, with little or no activity against other mycobacteria, can be explained by the fact that *pncA* is altered in many species of mycobacteria;⁷⁴ e.g. in *M. bovis* subsp. *bovis*, the natural substitution H57A produces a non-effective PZase determining intrinsic resistance to pyrazinamide.⁶⁶

Streptomycin

Streptomycin is an aminocyclitol glycoside antibiotic that was the first antibiotic used in the treatment of TB. Streptomycin was first isolated from the soil microorganism *Streptomyces griseus*. In prokaryotes, its mechanism of action is to inhibit the initiation of translation by binding to the 16S rRNA.⁷⁵ Initially it was observed that strains of *M. tuberculosis* isolated from patients before treatment were remarkably uniform in their susceptibility to streptomycin. Unfortunately, as it was used as mono-therapy, resistance to streptomycin emerged quite rapidly.⁷⁶

Even though in other mycobacteria such as *Mycobacterium fortuitum* it has been shown that an aminoglycoside 3''-O-phosphotransferase is involved with resistance to streptomycin,⁷⁷ in *M. tuberculosis* the genetic basis of resistance to streptomycin is mostly due to mutations in *rrs* or *rpsL*, which

produce alterations in the streptomycin binding site. However, such mutations are identified in slightly more than 50% of the strains studied to date.⁷⁸

The majority of point mutations resulting in streptomycin resistance occur in *rpsL*, with the most common mutation being K43R. Some clinical isolates showing low-level resistance to streptomycin and no mutation in *rpsL* or *rrs* have also been found,⁷⁹ and have consequently been theorized as an alternative mechanism for streptomycin resistance.⁸⁰ More recently it has been shown that mutations in *gidB*, which encodes a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, can confer a low level of streptomycin resistance.^{81,82}

Ethambutol

Ethambutol, 2,2'-(1,2-ethanediyldiimino)bis-1-butanol, was first used in 1966 against TB and constitutes, together with isoniazid, rifampicin and pyrazinamide, the first-line drugs currently in use for treatment of the disease. It is active against multiplying bacilli, where it interferes in the biosynthesis of cell wall arabinogalactan.⁸³

Some years ago, it was shown that in *M. tuberculosis* the genes *embCAB* were organized as a 10 kbp operon encoding for mycobacterial arabinosyl transferase. Using a panel of *M. tuberculosis* comprising ethambutol-resistant isolates, it was found that close to 50% of them had mutations in codon 306 of *embB*.⁸⁴ Further investigation into the association of *embB* and resistance to ethambutol was conducted by Sreevatsan *et al.*,⁸⁵ who showed that in 50% of ethambutol-resistant isolates there were mutations in *embB*. In 69 ethambutol-resistant and 30 ethambutol-susceptible additionally evaluated isolates from different geographical origins, 69% of ethambutol-resistant isolates had amino acid substitutions in *embB* that were not present in any of the ethambutol-susceptible isolates. Furthermore, some mutations were related to a high level of resistance, with MIC values ≥ 40 mg/L.⁸⁵

Other studies have found that there were mutations present in *embB* codon 306 in up to 20% of ethambutol-susceptible isolates evaluated.^{86,87} Furthermore, studies performed with MDR strains susceptible to ethambutol by phenotypic methods found an association between mutations in *embB306* and broad drug resistance rather than with resistance to ethambutol.⁸⁸ Another recent study, performed with panels of *M. tuberculosis* from different geographical origins and comprising ethambutol-susceptible and ethambutol-resistant isolates with or without MDR, found, after re-testing for discordant results, *embB306* mutations only in ethambutol-resistant isolates. They concluded that the association of *embB306* mutations found in ethambutol-susceptible strains could be related to problems with the phenotypic susceptibility testing performed for ethambutol.⁸⁹

More recently, it has been postulated that mutations in *embB306* may be related to variable degrees of ethambutol resistance and that this molecular alteration could be necessary, but not sufficient, for high-level ethambutol resistance. Allelic exchange studies showed that individual mutations producing certain amino acid substitutions caused ethambutol resistance while other amino acid substitutions had little or no effect on ethambutol resistance.²² As for pyrazinamide, it is well known

that ethambutol is also a difficult antibiotic to test and discordant results are frequently found.⁹⁰ There is, nonetheless, an important percentage of ethambutol-resistant isolates that do not have mutations in *embB*, stressing the fact that there must be another mechanism for ethambutol resistance that has not yet been described.⁹¹

Fluoroquinolones

Fluoroquinolones are bactericidal antibiotics currently in use as second-line drugs in the treatment of TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of nalidixic acid, the parent compound discovered in 1965 as a by-product in the purification of the antimalarial drug chloroquine.⁹² Currently a new generation of fluoroquinolones, such as moxifloxacin and gatifloxacin, are under clinical evaluation and are being proposed as first-line antibiotics with the goal of shortening the duration of TB treatment.⁹³⁻⁹⁵ In *M. tuberculosis*, only type II topoisomerase (DNA gyrase) is present and thus is the only target for fluoroquinolone activity.⁹⁶ Type II topoisomerase is a tetramer composed of two A and B subunits encoded by the genes *gyrA* and *gyrB*, respectively, that catalyses the supercoiling of DNA.⁹⁷⁻⁹⁹

Initial studies performed in laboratory strains of *M. tuberculosis* and *M. smegmatis* showed that resistance to fluoroquinolones was the result of amino acid substitutions in the putative fluoroquinolone binding region in *gyrA* or *gyrB*.⁹⁹ This association of mutations in the so-called quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB* and resistance to fluoroquinolones has been confirmed now in multiple studies. Fluoroquinolone-resistant strains of *M. tuberculosis* show mutations in a conserved region of *gyrA*, with Ala-90 and Asp-94 as the most frequently mutated positions; nevertheless, mutations at Ala-74, Gly-88 and Ser-91 have also been reported.^{100,101} Codon 95 contains a naturally occurring polymorphism (Ser or Thr) that is not related to fluoroquinolone resistance, as it occurs in both fluoroquinolone-susceptible and fluoroquinolone-resistant strains.⁹ Interestingly, strains with mutations at position 80 of *gyrA* have been reported to cause hypersusceptibility, especially if present together with other resistance mutations.¹⁰²

Other studies that looked in more detail into the mechanisms of fluoroquinolone resistance in laboratory-selected mutants of *M. tuberculosis* compared with clinical isolates found that the same *gyrA* mutations gave different MICs of ofloxacin for laboratory-selected or clinical isolates.¹⁰¹ Moreover, double point mutations seem to be more frequently found in clinical isolates, although they correlated with higher MIC values in both groups of strains. Also, for the same mutation in *gyrA*, the MIC value was higher in the laboratory-selected strains. These findings indicated that other factors are also involved in resistance, especially in laboratory-selected strains, such as alterations in membrane permeability or increased expression of efflux mechanisms.

More recent studies have already reported resistance to moxifloxacin and gatifloxacin, two third-generation fluoroquinolones that are currently undergoing clinical testing.^{103,104} Although full cross-resistance is commonly assumed between fluoroquinolones, von Groll *et al.*¹⁰³ recently reported a strain with an

Asn-533-Thr mutation in *gyrB* that was resistant to moxifloxacin and gatifloxacin but susceptible to ofloxacin.

The involvement of efflux pumps in resistance to fluoroquinolones has been shown in other bacterial species.¹⁰⁵ In *M. tuberculosis*, several studies have already shown the presumptive participation of efflux mechanisms in resistance to fluoroquinolones.¹⁰⁶ ABC-type efflux pumps have also been described in *M. tuberculosis*.^{107,108} In *M. tuberculosis*, around 2.5% of the genome includes genes coding predicted ABC transporters; however, most of them have not yet been fully characterized.¹⁰⁹

An interesting observation was reported by Hegde *et al.*,¹¹⁰ who described a fluoroquinolone-resistance protein from *M. tuberculosis* that mimics DNA. Expression of MfpA, a member of the pentapeptide repeat family of proteins in *M. tuberculosis* caused resistance to ciprofloxacin and sparfloxacin. By binding to DNA gyrase, MfpA inhibits its activity. Determination of its three-dimensional structure revealed a resemblance in terms of size, shape and electrostatic similarity to B-form DNA. This DNA mimicry would explain the inhibitory effect on gyrase and fluoroquinolone resistance.¹¹⁰ However, the significance of the MfpA protein to fluoroquinolone resistance in clinical isolates of *M. tuberculosis* has not been evaluated.

Kanamycin, amikacin, capreomycin and viomycin

Kanamycin and amikacin are aminoglycoside antibiotics, while capreomycin and viomycin are cyclic peptide antibiotics. All four are used as second-line drugs in the treatment of MDR-TB. Although belonging to two different antibiotic families, all exert their activity at the level of protein translation. Cross-resistance among kanamycin, capreomycin and viomycin have been reported since the studies performed by Tsukamura and Mizuno.¹¹¹ Several other studies have also reported cross-resistance between kanamycin and amikacin or between kanamycin and capreomycin or viomycin to variable degrees.^{112,113} The most common molecular mechanism of drug resistance has been associated with an A1401G mutation in the *rrs* gene coding for 16S rRNA. This mutation occurs more frequently in strains with high-level resistance to kanamycin and amikacin.¹¹⁴

Capreomycin and viomycin are structurally similar antibiotics with full cross-resistance shown in previous studies.¹¹⁵ Mutations in the gene *tlyA* have been implicated in resistance to capreomycin and viomycin. This gene codes an rRNA methyltransferase specific for 2'-O-methylation of ribose in rRNA. When mutated, it determines an absence of methylation activity.¹¹⁶ Interestingly, resistance to ribosome-targeting drugs is generally associated with the addition of methyl groups to rRNA rather than their loss.¹¹⁷ However, other more recent studies have not found any mutations in *tlyA* in CAP-resistant strains.¹¹⁴

Although full cross-resistance between kanamycin and amikacin was previously assumed, other studies have shown that kanamycin and amikacin cross-resistance is not absolute, demonstrating highly variable patterns and levels of resistance that allow inference of the existence of other molecular changes related to resistance.¹¹⁸ A recent study found that mutations in the promoter region of the *eis* gene in *M. tuberculosis*, which encodes an aminoglycoside acetyltransferase, produced an overexpression of the protein and conferred low-level

resistance to kanamycin but not amikacin. The same study found that 80% of clinical isolates evaluated and having low-level resistance to kanamycin had mutations in the *eis* promoter.¹¹⁹ Similar results have been obtained in two recent studies that assessed low-level kanamycin-resistant clinical isolates.^{120,121}

Ethionamide

Similar to isoniazid, ethionamide is a pro-drug requiring activation to form an adduct with NAD that subsequently inhibits the NADH-dependent enoyl-ACP reductase InhA. Activation of ethionamide occurs via *ethA*-encoded mono-oxygenase, yielding the ethionamide-NAD adduct.¹²² Mutations in *ethA* and *inhA* confer resistance to ethionamide.³⁰ Furthermore, co-resistance to isoniazid and ethionamide can be mediated by mutations that alter the InhA target or cause their overexpression, or by mutations in *ndh* that increase the intracellular concentration of NADH.^{123,124} More recently, *mshA*, a gene encoding a glycosyltransferase involved in mycothiol biosynthesis, has also been suggested as a possible target for ethionamide.¹²⁵

p-Amino salicylic acid

p-Amino salicylic acid was one of the first antibiotics to show anti-TB activity and was used in the treatment of the disease in combination with isoniazid and streptomycin.¹²⁶ However, its mechanism of action was never clearly elucidated. It was suggested that it may compete with *para*-amino benzoic acid for dihydropteroate synthase, an enzyme needed in folate biosynthesis. More recently, using transposon mutagenesis in *M. bovis* BCG, an association of *p*-amino salicylic acid resistance with mutations in the *thyA* gene that encodes thymidylate synthase A for thymine biosynthesis was shown.¹²⁷ This same study found that *p*-amino salicylic acid-resistant clinical isolates of *M. tuberculosis* harboured mutations in *thyA* resulting in decreased enzyme activity. Other studies assessing the role of enzymes in the folate pathway determined that *p*-amino salicylic acid was a pro-drug whose activation required a viable ThyA.¹²⁸ However, only 37% of the evaluated clinical isolates of *M. tuberculosis* and spontaneous mutants had mutations in *thyA*, suggesting the existence of additional mechanisms for *p*-amino salicylic acid resistance. These studies reported Thr202Ala as the most common mutation associated with *p*-amino salicylic acid resistance, although a few susceptible isolates have been found to contain this same mutation.¹²⁹ These findings have been more recently challenged by a study that found the Thr202Ala mutation to be a marker for the Latin American Mediterranean (LAM) lineage of *M. tuberculosis* rather than for resistance to *p*-amino salicylic acid,¹³⁰ indicating the need for additional studies to further elucidate the mechanism of action and resistance to *p*-amino salicylic acid.

Macrolides

Macrolide antibiotics are commonly used to treat infections caused by the *Mycobacterium avium* complex and other non-tuberculous mycobacteria (NTM). However, they have little or no effect on mycobacteria belonging to the *M. tuberculosis*

complex.²⁰ This intrinsic resistance to macrolides such as clarithromycin has been associated with low cell wall permeability and expression of the *erm37* gene, which encodes an enzyme that methylates a specific site in the 23S rRNA, preventing antibiotic binding. Moreover, in studies performed with *M. tuberculosis* and *Mycobacterium microti*, it was found that intrinsic resistance was inducible with subinhibitory concentrations of clarithromycin, causing a 4- to 8-fold increase in MIC values. The same study also found an increase in the levels of *erm37* mRNA.¹³¹ On the other hand, it has been found that subinhibitory concentrations of ethambutol reversed resistance to clarithromycin in clinical isolates of *M. tuberculosis*, supporting the concept that macrolide intrinsic resistance could be related to a permeability barrier.¹³²

The possible role of macrolides in the treatment of TB is still an open question because recent studies have found the occurrence of synergy when combined with subinhibitory concentrations of other antibiotics or anti-TB drugs.^{133,134}

Linezolid

Linezolid is the first compound belonging to the oxazolidinones approved for clinical use. Its mode of action is by inhibiting an early step in the protein synthesis by binding to the ribosomal 50S subunit.¹³⁵ Linezolid has shown significant *in vitro* and *in vivo* activity in animal models against *M. tuberculosis*.^{136,137} PNU100480, a structure analogue of linezolid, showed activity against *M. tuberculosis* similar to that of isoniazid and rifampicin in a murine model.¹³⁷ Recent *in vitro* studies have also shown activity of PNU100480 against drug-resistant *M. tuberculosis* isolates.¹³⁸

Resistance to linezolid in *M. tuberculosis* has been reported as rare. A study by Richter *et al.*¹³⁹ found 1.9% resistance among 210 MDR strains. When looking at *in vitro*-selected linezolid-resistant mutants, the mutations G2061T and G2576T in the 23S rRNA gene were found in strains with MICs of 16–32 mg/L. Mutants with MIC values of 4–8 mg/L and the parental susceptible strains had no alterations in the 23S rRNA gene.¹⁴⁰ This allows us to infer other possible mechanisms of linezolid resistance, such as the possible involvement of efflux pumps¹⁰⁶ or other non-ribosomal alterations, as has been shown in *M. smegmatis* mutants.¹⁴¹

Cycloserine

D-cycloserine is an analogue of D-alanine that inhibits the synthesis of peptidoglycan by blocking the action of D-alanine:D-alanine ligase (Ddl). It also inhibits D-alanine racemase (Alr) involved in the interconversion of L-alanine and D-alanine, which then serves as a substrate for Ddl.¹³⁵ In studies with *M. smegmatis* it was shown that overexpression of *alrA* in a multicopy vector was responsible for resistance to D-cycloserine in the recombinant clone.¹⁴² Although the cellular target(s) of D-cycloserine in *M. tuberculosis* is still unknown, the studies performed in other mycobacteria suggest a similar mechanism in the tubercle bacillus.

New drugs, new targets and new resistance mechanisms

Several new drugs are being proposed as candidates for the treatment of TB. They exert their activity by interacting with different targets, which are in many cases different from the classical targets of other anti-TB drugs. Surprisingly, and even before these drugs have been put into clinical use, new mechanisms of resistance have already been identified.

Nitroimidazoles

The compounds PA-824, a nitroimidazo-oxazine, and OPC-67683, a nitroimidazo-oxazole, have shown activity against *M. tuberculosis* strains susceptible and resistant to classical anti-TB drugs.^{143,144} MIC values reported for PA-824 are in the range of 0.015–0.25 mg/L for drug-susceptible strains and 0.03–0.53 mg/L for drug-resistant strains.¹⁴⁵ PA-824 has also shown activity against anaerobic non-replicating bacilli.¹⁴⁶ PA-824 is a pro-drug that needs to be metabolized by *M. tuberculosis* in order to be activated, involving a bioreduction of the aromatic nitro group to a reactive nitro radical anion intermediate within the cell. The mechanism of action has been found to be inhibition of cell wall lipid and protein synthesis,¹⁴³ although its activity against non-replicating bacteria shows that cell wall biosynthesis inhibition is probably not the only mode of action. When studying drug-resistant strains, it was found that resistance was mediated by the loss of a specific glucose-6-phosphate dehydrogenase or its deazaflavin cofactor F₄₂₀, which could provide electrons for reduction.¹⁴³ Rv3547, a protein with high structural specificity for binding to PA-824, is also involved in the activation process.¹⁴⁷

OPC-67683, on the other hand, has shown MIC values of 0.006–0.024 mg/L with no cross-resistance with first-line drugs. The mode of action of OPC-67683 is by inhibition of the synthesis of methoxy- and keto-mycolic acids, but not alpha-mycolic acid. It is not known if, like PA-824, it has some effect on protein synthesis, or has activity on other targets. OPC-67683 is also a pro-drug that needs to be activated by *M. tuberculosis*. Drug-resistant strains do not metabolize the drug and contain a mutation in the Rv3547 gene, suggesting its involvement in drug activation.¹⁴⁸

SQ109

SQ109 is a 1,2-diamine analogue of ethambutol obtained after analysis of a library of 63,238 compounds. It has shown good activity, with MIC values ranging from 0.16 to 0.63 mg/L, and intermediate cytotoxicity assessed in cell viability assays.¹⁴⁹ The mode of action of SQ109 is not well known, but it is believed that it affects mycobacterial cell wall synthesis in a different manner to that exerted by ethambutol. It has been found that in strains resistant to isoniazid, ethambutol and SQ109, there is up-regulation of *ahpC*, suggesting a possible role in the development of resistance to this drug.¹⁵⁰

TMC207

TMC207, formerly known as R207910, is a diarylquinoline with inhibitory activity against both drug-susceptible and drug-resistant *M. tuberculosis* and other mycobacteria.¹⁵¹

TMC207 has been shown to be bactericidal in patients with drug-susceptible pulmonary TB⁹⁵ and also, when added to standard therapy for MDR-TB, to reduce the time and increase the proportion of patients with sputum conversion.¹⁵²

The mechanism of action of TMC207 was described as specific inhibition of mycobacterial ATP synthase.^{151,153} *In vitro*-generated mutants resistant to TMC207 showed an A63P mutation in the *atpE* gene, suggesting that the drug acts on the proton pump of ATP synthase. Furthermore, in a separate study with *in vitro*-generated mutants, Petrella *et al.*¹⁵⁴ found the same mutation, A63P, and a new mutation, I66M, in *atpE*. They also analysed the genetic diversity of *atpE* in 13 other mycobacterial species and found that this region is highly conserved, except in *Mycobacterium xenopi*, in which residue Ala63 is replaced by Met, which could explain the natural resistance of *M. xenopi* to TMC207.

The exceptional specificity of TMC207 is probably a consequence of the limited sequence similarity among bacterial AtpE proteins. As with other specific anti-TB compounds, such as isoniazid and pyrazinamide, that require enzymatic activation by the mycobacteria, it would be worthwhile to investigate if TMC207 is a pro-drug also requiring initial activation by the bacteria.¹⁵⁵

More recent studies with *in vitro*-generated mutants resistant to TMC207 suggest that alternative mechanisms for drug resistance might exist, since in 38 out of 53 mutants evaluated no mutation in *atpE* could be observed; the same was true in three strains where the complete F0 and F1 operons were sequenced.¹⁵⁶

NAS-21 and NAS-91

The anti-malarial agents NAS-21 and NAS-91 have recently been shown to have potent antimycobacterial activity, inhibiting mycolic acid biosynthesis and profoundly altering the production of oleic acids.¹⁵⁷ In studies with *M. bovis* BCG, it has been suggested that the main target could be the FAS-II dehydratase coded by Rv0636. It has been shown that strains resistant to these compounds overexpress Rv0636 gene analogues.¹⁵⁸

Phenothiazines

Phenothiazines such as thioridazine and chlorpromazine can be considered as calmodulin antagonists and their anti-TB activity has been related to the presence of a calmodulin-like protein in the bacilli.¹³⁵ Thioridazine has shown *in vitro* activity against drug-susceptible and drug-resistant strains of *M. tuberculosis*. More importantly, they have shown activity against bacilli inside macrophages.¹⁵⁹

The mechanism of action of thioridazine as an anti-TB compound has not been completely elucidated. Related to its inhibition of calcium transport and enzymes dependent on calcium, it would also inhibit the generation of cellular energy and ATP hydrolysis. Among these are efflux pumps, and for this reason phenothiazines are considered as putative efflux pump inhibitors.¹⁶⁰ More recently thioridazine has been shown to have an effect on the sigma factor network in *M. tuberculosis* that would play a role in the bacteria's defence against cell damage.¹⁶¹

Benzothiazinones

A new class of antimycobacterial agents, 1,3-benzothiazin-4-ones, has been recently synthesized and characterized. 1,3-benzothiazin-4-one was found to kill *M. tuberculosis in vitro*, *ex vivo* and in a mouse model of TB infection.¹⁶² The DprE1 subunit of the enzyme decaprenylphosphoryl- β -D-ribose 2'-epimerase has been identified as the major target. When this enzyme activity is inhibited, it aborts the formation of decaprenylphosphoryl arabinose, a precursor in the synthesis of cell wall arabinan. When spontaneous 1,3-benzothiazin-4-one-resistant laboratory mutants were generated, codon Cys387 of *dprE1* was replaced by an Ser or Gly. On the other hand, in *M. avium*, which is naturally resistant to 1,3-benzothiazin-4-ones, codon Cys387 is replaced by an Ala codon.

In clinical isolates of *M. tuberculosis*, no resistance has been found yet. A recent evaluation performed in four hospitals in Europe found uniform susceptibility to 1,3-benzothiazin-4-ones among the 240 isolates evaluated, with susceptibility in the range of 0.75–30 ng/mL. None of them showed any mutation in *dprE1*.¹⁶³ An alternative mechanism for resistance to 1,3-benzothiazin-4-ones has been recently described in *M. smegmatis*. Overexpression of nitroreductase NfnB inactivated the drug by reducing a critical nitro group to an amino group. This is due to a common amino acid stretch between NfnB and DprE1 that facilitates interaction with 1,3-benzothiazin-4-ones.¹⁶⁴ *M. tuberculosis*, however, seems to lack nitroreductases able to inactivate these drugs.

Resistance and biological fitness

It is commonly accepted that microorganisms pay a physiological cost for the acquisition of drug resistance. This fitness cost has been demonstrated in several bacteria and is expressed as a reduction in growth, virulence or transmission.¹⁶⁵ In *M. tuberculosis*, it has been shown that *rpoB* mutations responsible for resistance to rifampicin found in clinical isolates were the same as those having less fitness cost *in vitro*.¹⁶⁶

Although rifampicin resistance mutations in *rpoB* have been found to clearly cause a fitness cost *in vitro*, some mutations caused only a minor cost, suggesting there is a small possibility for reversion to susceptibility.¹⁶⁷ The severity of this fitness cost is also dependent on the specific resistance mutation and the genetic background of the strain.¹⁶⁸ This phenomenon is apparently more commonly found in mutants selected *in vitro*. When rifampicin-resistant clinical isolates obtained from patients receiving anti-TB treatment were compared with their parental susceptible strain, four out of five strains with the mutation S531L had no fitness cost.¹⁶⁹ This could mean that this particular mutation had a low fitness cost or that a compensatory mutation offset the initial fitness cost associated with this mutation. Similar findings were obtained when looking into *katG* mutations associated with resistance to isoniazid. The mutation S315T in *katG* is associated with high levels of resistance to isoniazid, but retaining catalase/peroxidase activity was associated with higher rates of transmission in a population-based study conducted in San Francisco.¹⁷⁰

There are limited data available on the effect of resistance to other drugs and the fitness cost in *M. tuberculosis*. The fitness cost of various chromosomal mutations in streptomycin-resistant

strains was found to be dependent on the gene mutation mediating the resistance.¹⁷¹ Different mutations have been found in *in vitro*-selected mutants. However, in clinical isolates of *M. tuberculosis* found to be resistant to streptomycin, the lysine to arginine alteration at position 42 of *rpsL* is most commonly seen.¹⁷² Interestingly, this mutation has been found not to carry a fitness cost when tested experimentally *in vitro*.¹⁷¹

Recently Motiwala *et al.*¹⁷³ analysed MDR- and XDR-TB strains from an outbreak in KwaZulu Natal in South Africa and compared them with susceptible strains or with resistant strains found in other settings. They identified 12 new mutations, which were not located in highly repetitive genes, and which were apparently unique to that outbreak. Furthermore, they found that those virulent XDR strains in HIV-infected patients evolved without any change in their fitness.¹⁷³ Just recently, von Groll *et al.*¹⁷⁴ confirmed *in vitro* a fitness advantage of strains belonging to the LAM family when compared with other genotypes.

There are limitations, however, in some of these studies: the main one being the use of *in vitro* models or non-isogenic strains. Host and environmental factors, as well as the genetic background of the strain, can influence the transmission dynamics of drug-resistant bacteria, while the virulence of strains may be a reflection of other genomic differences not related to drug resistance. Nevertheless, these studies are important since the effects of resistance mutations on the fitness of *M. tuberculosis* can be related to epidemiological findings that could explain the spread of drug-resistant TB in several settings.¹⁷⁵

Concluding remarks

Even though mutations in several genes are evidently related to drug resistance in *M. tuberculosis*, there are numerous resistant strains that do not present these classic mutations. From the clinical point of view, it is probably more important to have diagnostic tools that are easy to use, inexpensive and provide rapid results of drug susceptibility or resistance of a strain. However, regarding the dynamics of TB transmission, and also in view of the need to develop new anti-TB drugs, it is extremely important to further our knowledge on the molecular basis of drug resistance and all its complexity. It is necessary, for instance, to clarify the association between specific mutations and the development of MDR-TB or the association between drug resistance and fitness. This would allow better evaluation of the transmission dynamics of resistant strains and more accurate prediction of a future disease scenario. Furthermore, knowledge of the molecular basis of drug resistance will allow more rational development of new drugs; something that is urgently needed, when taking into account the increasing rates of MDR- and XDR-TB around the world.¹⁷⁶ In this framework it is useful to have additional sources of information, such as the recently described Tuberculosis Drug Resistance Mutation Database: a web site compiling this type of information.¹⁷⁷ Ideally, new drugs should bypass the molecular mechanisms of resistance in currently available drugs and also offset intrinsic resistance, as, for example, that provided by efflux mechanisms.

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None to declare.

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