Alpe Adria Microbiology Journal (1998) 7(4):275-283

REVIEW ARTICLE

MOLECULAR MECHANISMS OF DRUG RESISTANCE IN *Mycobacterium tuberculosis*

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Received 4 December 1998

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Key words: *Mycobacterium tuberculosis*, drug resistance, molecular mechanisms

The burden of tuberculosis worldwide

Mycobacterium tuberculosis, and the less frequent species *Mycobacterium bovis* and *Mycobacterium africanum*, are the causative agents of tuberculous disease. Humans are the only reservoir of *M. tuberculosis* which is now estimated to involve one third of the world's population and which was first documented in 3,000 BC [1]. Although only a low proportion of infected individuals develop the disease, the World Health Organization has estimated that eight million new cases and three million deaths occur each year [2], making tuberculosis the leading single infective cause of death worldwide. Ninety five per cent of the cases of tuberculosis have been reported by non-industrialized countries, yet in industrialized countries, where the disease was considered to be under control and on the way to eradication, an alarming increase in incidence has been observed since the 1980s [3]; this increasse has been made even more alarming by the emergence of multidrug resistance [4].

The mycobacterial envelope

M. tuberculosis is intrinsically resistant to most antibiotics. This resistance seems to be

mainly due to the organism's envelope which is mostly made up of lipids (up to 60% of its weight). The asymmetric lipid bilayer, with an inner leaflet containing mycolic acids and an outer leaflet of extractable lipids, acts as a permeability barrier to hydrophylic solutes, whose influx is limited by the low number of porins present in the mycobacterial envelope [5]. The diffusion of lipophilic solutes, such as rifamycins, is slowed down by the low fluidity of mycolic acids and by the thickness of the lipid bilayer.

Metabolic conditions of *M. tuberculosis*

M. tuberculosis may be present in tissues under different metabolic conditions: it replicates actively in the aerobic and neutral-pH pulmonary environment, yet its growth is extremely slow in caseous lesions and macrophages, where pH and oxygenation are lower, and is completely blocked in dormant bacilli surviving under anaerobic conditions.

Replicating conditions of *M. tuberculosis* largely influences the activity of single drugs., Among the first line drugs, only rifampicin is active against both extra- and intra-macrophagic bacilli, and none of these drugs is active against dormant bacilli [6].

Antimicrobial resistance

Molecules active against *M. tuberculosis* are divided into first-line and second-line drugs; the first-line drugs, which are the drugs of choice for treating disease due to susceptible strains, include isoniazid, streptomycin, ethambutol, rifampicin and pyrazinamide.

M. tuberculosis acquires antibiotic resistance through mutations occurring within its chromosome, given that no plasmid resistance has been detected to date. Drug resistant mutants arise spontaneously, independently from the exposure to drugs; their frequency varies with the specific drug and has been estimated to be 10^{-6} for isoniazid, streptomycin and ethambutol and 10^{-8} for rifampicin [7].

While therapeutic regimens using a single drug can rapidly select for the rare resistant mutants, double mutations affording resistance to two antibiotics are rare in the population of bacilli typically present in tuberculous lesions [8].

The definition of resistance of *M. tuberculosis* is based on a single empirically determined criterion: the detection of resistance in vitro involving at least 1% of the mycobacterial population [9]. The molecular basis of *M. tuberculosis* resistance is complex and remains to be fully elucidated.

Isoniazid

The isonicotinic acid hydrazide (isoniazid) was discovered in 1912 but its use in tuberculosis only started in 1952. The drug has strong bactericidal activity against *M. tuberculosis* complex, but its activity is scanty or absent against other mycobacterial species. The efficacy of isoniazid is limited to actively growing organisms under aerobic conditions. The mechanism of action is unknown, but it appears to be mediated by the enzyme catalase-peroxidase, the enzyme eliminating reactive oxygen resulting from cell metabolism, which likely catalyses a peroxidative transformation of the drug into a bactericidal derivative [10]. The main target of its action appears to be the mycolic acid biosynthesis.

Reduction or lack of catalase activity in isoniazid-resistant *M. tuberculosis* was first detected many years ago [11] and has been reported to be associated with a reduced virulence in the guinea pig model. It is now known that these changes result from mutations or deletions of the gene *katG*, which encodes for the catalase-peroxidase enzyme [12, 13]. In fact, almost 60% of isoniazid-resistant *M. tuberculosis* strains present mutations in the *katG* locus [14]; such mutations probably imply the impossibility of converting the drug into its active moiety, as apparently confirmed by the restoration of susceptibility to isoniazid following the genetic transfer of an effective *katG* locus [13].

In a limited number of isoniazid-resistant-*katG*-defective strains, a further mutation in the promoter of *ahpC*, the gene encoding for alkyl hydroperoxiyde reductase C, has been detected [15]. This mutation, which leads to overexpression of *ahpC*, seems, however, to be only indirectly associated with isoniazid-resistance and is most likely acquired to compensate for the loss of catalase-peroxidase activity [15].

A further resistance to isoniazid has been found to be associated with resistance to ethionamide and with a mutation in the *inhA* gene [16], whose product, *inhA* protein, is probably involved in fatty acid biosynthesis [17] and could be the target of activated isoniazid.

At least one other mechanism of resistance to isoniazid may exist, given that a significant proportion (15-25%) of isoniazid-resistant *M. tuberculosis* isolates have wild type genes within both the *katG* and *inhA* regions. A very recent study suggests that the target of isoniazid may be, instead of *inhA*, the *kasA* protein, which is involved in fatty acid elongation; mutations of *kasA* locus have been found in about 15% of isoniazid-resistant strains [18].

Streptomycin

Streptomycin was discovered in 1943 and was the first available scientifically proven agent for treating tuberculosis. Unlike other first-line anti-tuberculosis drugs, it has a broad spectrum of antibacterial activity. The specific tuberculocidal activity of streptomycin is limited to continuously growing extracellular mycobacteria.

The mechanism of action of streptomycin, and of other aminoglycosides as well, is characterized by binding to 16S ribosomal RNA, thus inhibiting the binding of aminoacyl-tRNA and consequently perturbing the synthesis of proteins [19].

Resistance to streptomycin in *M. tuberculosis* is associated with mutations in the rrs gene encoding for 16S rRNA and the *rpsL* gene [20, 21] encoding for the S12 ribosomal protein, which interacts and stabilizes the higher order structure of 16S rRNA. Aminoglycoside-modifying enzymes, very frequent mechanism of resistance in many eubacteria, are not present in *M. tuberculosis* [22]. Almost 30% of streptomycin-resistant strains do not present mutations in the *rrs* or *rpsL* gene [23]. The mechanism of resistance in such organisms is unknown; however, since they usually display lower levels of resistance, alterations of the permeability barrier may be hypothesized.

The development of streptomycin resistance is more efficient in *M. tuberculosis*, in which mutations of *rrs* gene directly confer resistance, given that such species, like all slowly growing mycobacteria, possess only one copy of the 16S rRNA gene [24], differently from most eubacteria, which have multiple copies of the locus.

Ethambutol

This drug, first synthesized in 1961, is bacteriostatic and is active only against multiplying mycobacteria.

The molecular mechanism of action is unknown, though several targets of its inhibitory action have been hypothesized: the rRNA metabolism [25], the phospholipid synthesis [26], the transfer of mycolic acids to the cell wall-linked arabinogalactan [27], and the glucose conversion into the precursors used for the synthesis of cell wall polysaccharides [28]. The most probable target is currently considered to be the polymerization of arabinan in the arabinogalactan and lipoarabinomannan of the mycobacterial cell wall [29]. In fact, drug resistance has been recently found to be associated with a single codon mutation of *emb*B, a gene of *emb* locus, which encodes for arabinosyltransferases, the enzymes that are probably the target of ethambutol [30].

Rifampicin

Rifampicin has been used for treating tuberculosis since the early 1970s; the drug binds to the prokaryotic RNA polymerase and thus interferes with transcription and elongation of rRNA [31]. Resistance to rifampicin is associated with mutations clustering into a "hot spot" (81 bp) of the *rpo*B region encoding for the β-subunit of RNA-polymerase [32], with more than three quarters of mutations affecting specific positions and usually resulting in a single amino-acid change.

In *M. tuberculosis* only, resistance to this drug appears to be imputable to mutations in a single gene (*e.g.*, mutated *rpo*B gene). In fact, this gene has been observed in over 97%, of the rifampicin-resistant isolates investigated to date, [32]. The possibility of conferring rifampicin-resistance using a mutant *rpo*B allele [31] and of restoring susceptibility with a plasmid carrying the wild type *rpo*B are strong evidence that specific mutations directly confer rifampicin-resistance [14].

Rifabutin, although sharing the same mechanism of action as rifampicin, is active *in vitro* against more than 10% of rifampicin-resistant *M. tuberculosis* [33]; such strains are characterized by specific *rpo*B mutations, but they present minimal inhibitory concentrations within the range of drug concentration effective *in vivo* [34].

Pyrazinamide

Pyrazinamide, a derivative of nicotinamide first synthesized in 1952, is inactive against replicating *M. tuberculosis*. With respect to bacilli surviving within macrophages, pyrazinamide is bacteriostatic when used alone, yet it is rapidly bactericidal who used in association with isoniazid. Pyrazinamide, which, *in vitro*, is only active at an acid pH, is converted, *in vivo*, to pyrazinoic acid, its active moiety, by pyrazinaminidase [35]. The lowering of pH brought about by pyrazinoic acid is thought to contribute to the drug's damaging effect of *M. tuberculosis* [35].

The lack, or the unusually low levels, of pyrazinamidase in most pyrazinamide-resistant strains of *M. tuberculosis* [36] suggest that this enzyme is involved in the mechanism of resistance; this hypothesis seems to be supported by the recent detection, in over 80% of pyrazinamide-resistant strains, of mutations arrayed along the whole locus encoding for pyrazinamidase (*pncA* gene) [37, 38].

Quinolones

Several quinolones, such as ciprofloxacin, ofloxacin and sparfloxacin, are bactericidal against *M.tuberculosis* at concentrations easily achievable *in vivo*. Although they are not considered first-line antimycobacterial drugs, their role is becoming increasingly important, particularly for the treatment of multiresistant strains.

The target of quinolones is the DNA-gyrase, the enzyme that relaxes and recoils the DNA during transcription and replication. The binding of the drug to the gyrase leads to the inhibition of DNA-synthesis. Acquired high-level resistance in mycobacteria, which arises at a frequency of 10^{-7}-10^{-8}, is associated with point mutations in the *gyrA* locus, one of the two genes encoding for subunits of the DNA-gyrase [39].

Mechanism of multidrug resistance

Investigations concerning *M. tuberculosis* multidrug resistance have not been able to detect any generalized mechanism restricting drug access or activating drug efflux; thus, the only currently excepted explanation of this phenomenon is the accumulation of acquired mutations. Resistance to isoniazid or rifampicin, the multidrug-resistance-defining molecules, has been proposed as a surrogate for the lacking marker of multidrug-resistance. Rifampicin is at present the most important candidate, since resistance to this drug is rarely encountered alone and is often present with resistance to isoniazid.

Genotypic methods for detection of drug resistance

The detection of mutations associated with drug resistance has in recent years led to the development of a number of techniques for assessing drug susceptibility at the genotype level. In theory, these techniques reduce to several hours the time needed to culture and to perform conventional susceptibility testing, which otherwise takes at least three weeks even with the radiometric method.

The first steps of all currently used genotype analyses consist of the destruction of the mycobacterial envelope and the polymerase chain reaction amplification of the genetic regions containing the known molecular targets at the basis of drug resistance.

Genetic sequencing of the previously amplified target gene [40] currently represents the gold standard in genotype-based techniques for susceptibility assessment. Such technology is in fact suitable for unambiguously detecting mutations known to be associated with phenotypic resistance. Recent advances in automated sequencing have largely contributed to the standardization of this technique and to bringing its cost within the reach of major laboratories.

Alternative and less expensive techniques have also been recently developed.

Single strand conformation polymorphism (SSCP) is based on the heat-separation of a double stranded amplified target gene; the conformation spontaneously adopted by cooled single strands which fold intramolecularly is highly specific and can be easily detected with simple electrophoresis in non-denaturing gel, revealing the electrophoretic motility related to the specific molecular conformation [32, 39, 41-44].

The sensitivity of SSCP is enhanced when using dideoxy-fingerprinting, a technique employing a single dideoxy-nucleotide to produce variable-length single-strand termination products to be subjected to the electrophoretic conformation analysis [45].

A technique that is conceptually similar to SSCP is heteroduplex formation, in which single strands of the amplified target are blended with strands from a susceptible *M. tuberculosis* control strain. Because of their different motility, heteroduplex resulting from the combination of a susceptible and a resistant strand are easily differentiated from homogeneous strands by means of gel electrophoresis [46].

Restriction fragment length polymorphism (RFLP), the most widely used technique for epidemiological investigations, has also been used for the genotypic investigation of drug resistance. Knowledge on site-specific mutations associated with *M. tuberculosis* resistance and the use of properly selected restriction enzymes allows the loss or gain of a restriction site related to the mutation to be easily detected [47].

Sequencing by hybridization is the only technique available as a commercial kit (Inno LiPA Rif TB, Innogenetic, Belgium). In this approach, the nucleotide sequence of the resistance marker is determined resorting to multiple short DNA probes specific for mutant and wild type "hot spots" of the target molecule. The resistance is detected by hybridization with one or more of the mutation-containing probes and the simultaneous absence of base pairing with the wild type ones. In the commercially available kit, developed for genetic detection of rifampicin-resistant *M. tuberculosis*, various probes are attached, in lines, to a nitrocellulose surface, and hybridization is revealed using the biotin-peroxidase reaction [48-51].

Genotypic assessment of drug susceptibility is extremely suitable for *M. tuberculosis* because of the long time required by conventional susceptibility testing; however, despite the enormous progress made in recent years, a number of problems still limit this genotypic approach. Specifically, all current methods require a large amount of the amplification product, which is hardly feasible with clinical specimens, which often have a small number of mycobacterial genomes or present some inhibitors. Unambiguous results are obtained with cultures, but this approach reduces only the time needed for phenotypic determination of susceptibility.

The single criterion adopted for the definition of *M. tuberculosis* resistance, (*e.g.*, the presence of as few as 1% of resistant mutants within a mycobacterial population) does not always allow resistance at the genetic level to be unambiguously recognised, particularly when the resistant cells represent less than 15% of the entire population.

For most antitubercular drugs affected by mutations conferring resistance, susceptibility testing is hindered by the absence of a single mechanism valid for a substantial proportion of strains. Thus multiple tests would be necessary for each drug; yet the sensitivity of tests is often insufficient, owing to the existence of resistance whose genetic markers are unknown.

Furthermore, polymorphism-based techniques may lead to conserved mutations (*e.g.*, mutations without amino-acid changes) being misdefined as mutations producing resistance.

Despite these problems, and the added considerations of cost and the need for skilled personnel, the results of current studies suggest that the role of susceptibility testing at the genotypic level will become increasingly prominent in the near future.

Acknowledgement

We thank Pasquale Urbano (Istituto di Microbiologia, Università di Firenze, Firenze, Italy) for reading the manuscript.

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