

Review

The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*

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Abstract

Multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* have emerged worldwide. In many countries and regions, these resistant strains constitute a serious threat to the efficacy of tuberculosis control programs. An important element in gaining control of this epidemic is developing an understanding of the molecular basis of resistance to the most important antituberculosis drugs: isoniazid, rifampin, and pyrazinamide. On the basis of this information, more exacting laboratory testing, and ultimately more appropriate and timely treatment regimens, can be developed.

Keywords: drug resistance, isoniazid, *Mycobacterium tuberculosis*, pyrazinamide, rifampin

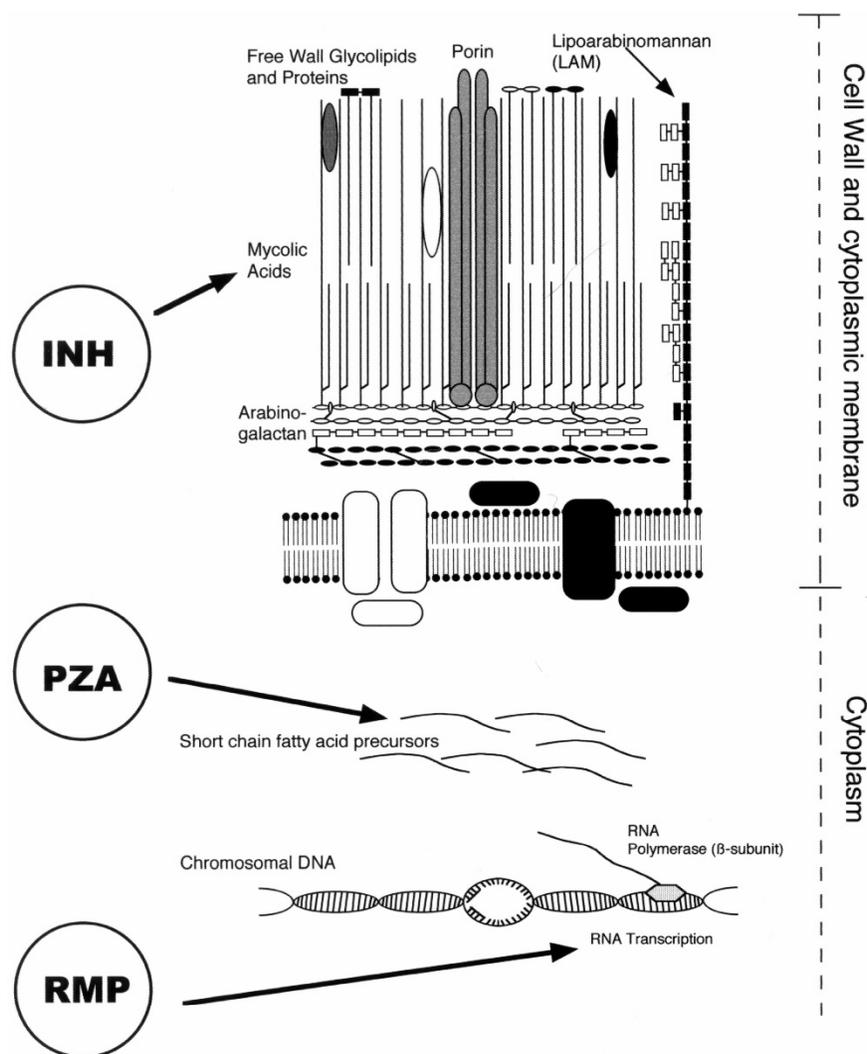
Introduction

Mycobacterium tuberculosis and other members of the *M tuberculosis* complex use several strategies to resist the action of antimicrobial agents. First, the mycobacterial cell is surrounded by a specialized, highly hydrophobic cell wall that results in decreased permeability to many compounds (Fig. 1) [1,2]. Active drug efflux systems and degrading or inactivating enzymes, and the genes that are associated with these functions, have been found in *M tuberculosis* [3,4]. However, genetic studies have shown that resistance of *M tuberculosis* to antimycobacterial drugs is the consequence of spontaneous mutations in genes that encode either the target of the drug, or enzymes that are involved in drug activation. Resistance-associated point mutations, deletions, or insertions have been described for all first-line drugs (isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin), and for several second-line and newer drugs

(ethionamide, fluoroquinolones, macrolides, nitroimidazopyrans) [5–8]. However, no single genetic alteration has yet been found that results in the MDR phenotype (defined as resistance at least to isoniazid and rifampin). Rather, MDR develops by sequential acquisition of mutations at different loci, usually because of inappropriate patient treatment. Because MDR strains are the result of cumulative mutations, growth of *M tuberculosis* can successfully be controlled in the host by concomitant treatment with more than one drug. Thus, treatment regimens that consist of three to four drugs are used routinely to treat patients with tuberculosis.

The World Health Organization [9] has recently recommended the following terminology changes for the different types of resistance to antituberculosis drugs. Isolation of drug-resistant *M tuberculosis* from patients without a history of previous treatment should be referred to as 'drug

Figure 1



Previously demonstrated and proposed sites of action of isoniazid (INH), pyrazinamide (PZA), and rifampin (RMP) on the *M. tuberculosis* cell. INH inhibits the synthesis of mycolic acids [19,20], PZA inhibits the synthesis of short-chain, fatty-acid precursors [32], and RMP inhibits transcription by binding to the β -subunit of RNA polymerase [28]. This figure is adapted in part from Parsons *et al* [40].

resistance among new cases' (instead of 'primary resistance'). Isolation of a drug-resistant strain from patients who have been treated for tuberculosis for at least 1 month should be referred to as 'drug resistance among previously treated patients' (instead of 'acquired resistance'). The more common of these occurrences is 'drug resistance among previously treated patients', in which inadequate treatment or lack of adherence by the patient results in the selection of naturally occurring resistant mutants [10]. Less common is cross-resistance, in which resistance occurs between drugs that are chemically related and/or have a similar target within the mycobacterial cell (ie rifampin and other rifamycin derivatives, or isoniazid and ethionamide) [11,12]. However, the *in vivo* relevance of the cross-resistance seen *in vitro* is not always clear.

The present review gives a concise summary of the mechanism of action and the molecular basis of resistance to isoniazid, rifampin, and pyrazinamide, the three most important antituberculosis drugs. In addition, the importance of using molecular assays for the rapid detection of drug-resistant strains of *M. tuberculosis* is emphasized.

Isoniazid

Isoniazid is a pro-drug that requires activation in isoniazid-susceptible mycobacterial species. Based on *in vitro* experiments, it has been proposed [13] that the activation of isoniazid results in a number of highly reactive species that are capable of either oxidizing or acylating groups in proteins. However, the actual form of isoniazid that is active *in vivo* is still unknown. It was observed soon after

isoniazid was introduced in the 1950s that isoniazid-resistant clinical isolates frequently lost catalase and peroxidase activity [14]. However, the association of this enzyme with isoniazid activation was not proven until the early 1990s, when the primary mycobacterial catalase-peroxidase gene (*katG*) was cloned and sequenced [15]. That study and others [8,16] revealed that mutations in this gene are found in 42–58% of isoniazid-resistant clinical isolates. A large number of different mutations have been described thus far; however, the Ser315Thr mutation is found most often, occurring in approximately 40% of all isoniazid-resistant strains [8,16,17]. The Ser315Thr mutation results in an enzyme without the ability to activate isoniazid, but retains approximately 50% of its catalase-peroxidase activity [18]. Thus, the altered catalase-peroxidase provides high-level resistance to isoniazid, while retaining a level of oxidative protection that is sufficient to enable the organism to maintain detoxifying activity against host antibacterial radicals. Isolates that carry other, less frequently occurring mutations in *katG* have been described as exhibiting varying levels of isoniazid resistance and catalase-peroxidase activity [8,16–18].

Significant evidence supports the concept that isoniazid blocks the synthesis of cell-wall mycolic acids, the major components of the envelope of *M tuberculosis* (Fig. 1). Two intracellular targets for the drug are currently being actively investigated [19,20]: the fatty-acid enoyl-acyl carrier protein reductase (*InhA*), and a complex of an acyl carrier protein (*AcpM*) and a β -ketoacyl-ACP synthase (*KasA*). These enzymes are involved in synthesis of mycolic acids, and mutations have been found in the promoter regions, or less commonly in the genes that encode these proteins (*inhA*, *acpM*, and *kasA*), in clinical isolates that exhibit low-level resistance to isoniazid (for review [8]). It is proposed that over-expression of one or more of these target proteins may be the reason for isoniazid resistance in these strains. However, the role of *kasA* mutations in isoniazid resistance is presently unclear, because similar mutations were also found in isoniazid-susceptible isolates, and, in cases of isoniazid resistance, mutations were also found in *katG* or *inhA* [21,22].

Mutations in the promoter region of a gene that encodes an alkyl hydroperoxidase reductase (*ahpC*) have been found in approximately 10% of isoniazid-resistant isolates, but mutations in *katG* were also found in these isolates [8,16,23]. The resulting over-expression of alkyl hydroperoxidase reductase may compensate for the loss of catalase-peroxidase activity in these mycobacteria [24].

Rifampin

One of the main reasons for treatment failure and fatal clinical outcome in tuberculosis patients is resistance to rifampin [25]. In addition to a significant early bactericidal effect on metabolically active *M tuberculosis*, rifampin also

exhibits excellent late sterilizing action on semidormant organisms undergoing short bursts of metabolic activity. The recognition of this late effect of rifampin, and the additional effectiveness of pyrazinamide, has allowed for the reduction of routine tuberculosis treatment from 1 year to 6 months [26,27]. Whereas monoresistance to isoniazid is quite common, monoresistance to rifampin is rare. Instead, rifampin resistance occurs most often in strains that are also resistant to isoniazid; thus, rifampin resistance can be used as a surrogate marker for MDR.

The mechanism of action of rifampin is to inhibit mycobacterial transcription by targeting DNA-dependent RNA polymerase (Fig. 1). The development of resistance to rifampin is due to mutations in a well-defined, 81 base pair (bp) (27 codons) central region of the gene that encodes the β -subunit of RNA polymerase (*rpoB*) [28]. More than 96% of the rifampin-resistant strains contain a mutation in this 81 bp region of *rpoB*, thus facilitating a straightforward approach to detecting rifampin resistance and/or MDR rapidly [8,16].

The most common mutations (65–86%) alter either codon 526 or codon 531, and result in high-level resistance to rifampin (minimal inhibitory concentration [MIC] >32 μ g/ml). However, not all mutations within the 81 bp region exhibit the same level of resistance. For example, alterations in codons 511, 516, 518, and 522 result in organisms that have low-level resistance to rifampin and another rifamycin derivative (rifapentin), but remain susceptible to two other rifamycins (rifabutin and rifalazyn) [29,30] (Parsons LM, unpublished data). Rare mutations associated with rifampin resistance have also been found in the amino-terminal region of *rpoB* [8,31]. Most reference laboratories that use molecular methods examine only the 81 bp region. However, it is advisable to screen for amino-terminal mutations in cases in which rifampin resistance is suspected, but no mutation is found in the 81 bp region.

Pyrazinamide

Pyrazinamide has an excellent sterilizing effect on semidormant tubercle bacilli and, when used in combination with rifampin, shortens the duration of treatment of tuberculosis patients from 1 year to 6 months [26]. Pyrazinamide, a nicotinamide analog that is believed to target an enzyme involved in fatty-acid synthesis [32], is a pro-drug that is converted to its active form (pyrazinoic acid [POA]) by the mycobacterial enzyme pyrazinamidase. In *M tuberculosis*, an accumulation of intracellular POA occurs when the extracellular pH is acidic. Experimental evidence suggests that pyrazinamide diffuses into *M tuberculosis* in a passive manner, is converted into POA by pyrazinamidase, and, because of an inefficient efflux system, accumulates in huge amounts in the bacterial cytoplasm [33,34]. The accumulation of POA lowers the intracellular pH to a sub-optimal level that is likely to inactivate a vital target enzyme such as fatty acid synthase I [32].

It has been observed that the pyrazinamide-resistant *M tuberculosis* isolates usually lose their pyrazinamidase activity [35]. After cloning and sequencing the gene that encodes pyrazinamidase (*pncA*), it was found that 72–97% of all pyrazinamide-resistant clinical isolates tested carry a mutation in the structural gene or in the putative promoter region of the gene [8,36]. However, the involvement of other mechanisms (ie those that involve pyrazinamide uptake, *pncA* regulation, or POA efflux) is indicated by the existence of isolates that exhibit a high level of pyrazinamide resistance without mutations in the *pncA* gene [37]. Two other members of the *M tuberculosis* complex, *M bovis* and *M bovis* BCG, are naturally resistant to pyrazinamide. In these organisms, pyrazinamide resistance is due to a unique C to G point mutation in codon 169 of *pncA*. In contrast, mutations in pyrazinamide-resistant *M tuberculosis* have been found scattered throughout *pncA* [36].

Conclusion

The action and mechanism of resistance to the three most important antituberculosis drugs are still not fully understood. However, current molecular evidence indicates that routine application of rapid molecular tests in the clinical management of drug-resistant tuberculosis is essential. Many approaches have been used successfully to detect and identify the most common mutations associated with drug resistance [8,16,38]. In many instances, the knowledge gained from determining the particular mutation can provide significant information on the following: drug resistance, the level of resistance, cross-resistance to similar drugs, relatedness of strains, and virulence.

Total dependence on results that are provided 2–4 weeks (or even months) later by the conventional 'gold standard' susceptibility methods might not be sufficient for optimal patient outcome. The tuberculosis laboratory should no longer allow its pace to be dictated by the slow growth of the pathogenic mycobacteria. Rather, the laboratory is a vital part of a renewed global commitment that is aimed at the elimination of tuberculosis [39]. The ultimate goals are provision of timely, appropriate, and adequate services. These must be provided, and continually evaluated and updated. A highly infectious tuberculosis patient must have access to state-of-the-art laboratory services, even if the patient resides in an area where a local laboratory is not capable of providing those services. Innovative ideas, such as centralized testing by a large public health laboratory, may be required to achieve this goal. District, state, or national boundaries should not limit access to these laboratory services. The benefits of providing new and more clinically relevant assays to a larger population, especially in high-incidence regions of the world, would be of great public health significance to all populations.

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