Mechanisms of mycobacterial drug resistance: implications for treatment and prevention strategies

Erik C. Böttger, Zürich*

When addressing the problem of drug resistance in Mycobacterium tuberculosis, three issues need to be addressed: i) mechanisms and development of resistance, ii) therapeutic implications, iii) spread of resistance. During the past 15 years an enormous amount of knowledge has been gained concerning the mechanisms of mycobacterial drug resistance at a molecular level. In contrast to other bacterial pathogens, plasmid-mediated mechanisms of resistance are absent in mycobacteria; acquired drug resistance is exclusively due to chromosomal alterations, such as mutations or deletions. These chromosomal alterations either affect the drug target itself or bacterial enzymes activating the drug. Standardized short-course chemotherapy (SSC) regimes with first-line agents seem to be an adequate treatment for some patients with drug resistant tuberculosis, including multi-drug resistance. How can treatment of drug-resistant tuberculosis with SSC be successful? The hypothesis will be discussed that this paradox is a result of the methods used for in vitro drug susceptibility testing. In essence, this hypothesis implies that the seemingly paradoxical heterogeneity in treatment outcomes of multi-drug resistant tuberculosis may be due partly to limitations in the clinical predictive value of in vitro drug susceptibility testing, based on unique but mistakenly used techniques in diagnostic mycobacteriology. Experimental data and mathematical models indicate that the fitness cost (i.e., reduced transmission between hosts and reduced persistence and growth within hosts) conferred by a resistance determinant is the single most important parameter determining the spread of resistance. Most of the many chromosomal alterations that result in resistance to first line antituberculous drugs, e.g. isoniazid, rifampicin, streptomycin, may or may not be associated with a significant fitness cost. Based on work in experimental models and from observations in clinical drug resistant isolates a picture emerges in which, among the various resistance mutations that appear with similar rates, those associated with the least fitness cost are selected in the population. Thus, it can not be expected that drug resistance per se imposes a restriction on transmission. Keywords: Tuberculosis, resistance, treatment, prevention, fitness, susceptibility testing


In the early 90’s a long neglected disease, tuberculosis, made it into the head lines of such prestigious journals like Science, Nature and New England Journal of Medicine. The reason for this was a frank disaster – the development of multi-drug resistance in Mycobacterium tuberculosis, the agent responsible for the disease. Multi-drug resistance in M. tuberculosis has been and still is a frightening experience. For many patients suffering from this disease, there is very little, if any hope. MDR-TB is also an enormous financial burden. While treatment of drug-susceptible TB is one of the most cost-efficient therapies in clinical medicine – with drug costs of less than 100 Euro for a half year treatment –, the costs for treatment of a single case of MDR-TB may rise up to 100 000 Euro. World-wide drug resistant strains of M. tuberculosis have emerged as an increasing problem (fig. 1). In some areas, such as Estonia, Latvia, defined regions in Russia, China, Africa or Latin America, multi-drug resistant strains may represent up to 15% of the first-time TB cases. As well as TB is primarily a social disease affecting the poorest in the world, the disappointing lesson is: drug resistance in M. tuberculosis is entirely man-made – mainly due to non-compliance with treatment, may that be for individual, pharmacokinetic, social or even criminal reasons. In contrast to other bacterial pathogens, plasmid-mediated mechanisms of resistance are absent in mycobacteria. There is also no evidence that high-level MDR is conferred by transporters mediating this type of resistance. Rather, acquired drug resistance is exclusively due to chromosomal alterations, such as mutations or deletions. These chromosomal alterations affect either the drug target itself or bacterial enzymes activating the pro-drug. During the past 15 years, significant knowledge has been gained concerning the mechanisms of mycobacterial drug resistance at the molecular level [for review see 22]. Thus, resistance to isoniazid, pyrazinamide or ethionamide is mainly due to alterations in genes which metabolize the pro-drug into its active component, such as katG, pncA or ethA. In contrast, resistance to rifampicin, ethambutol, streptomycin or

Abbreviations:

MDR: multi-drug resistance
TB: tuberculosis
SSC: standard short-course

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quinolones is due to mutational alterations in genes encoding the drug target itself, such as \textit{rpoB}, \textit{embB}, \textit{rpsL}, \textit{rrs} or \textit{gyrA} (for a summary see \textbf{table 1}).

One of the most important questions in the epidemiology of drug resistance concerns the underlying mechanisms which determine resistance spread. A common concept in drug resistance implies that drug resistance has a cost: in essence this means that while a drug resistance determinant provides an advantage in the presence of the drug, in the absence of the drug the resistance determinant is associated with a fitness burden [5, 6, 26, for review 1]. Thus, the probability of having a fitness lowering mutation accompanied by a second-site compensatory mutation is \(10^{-12}\) to \(10^{-16}\). These numbers can only be met by the assumption that following acquisition of the first costly mutation there must be a positive selection for organisms carrying the fitness lowering mutation in the patient, in most instances there will be competition between different resistance mutations occurring simultaneously in a strictly stochastic fashion. Thus, the resistant population initially is quite heterogeneous. This principle of initial competition between different resistance mutations has mostly not been properly addressed by the in vitro laboratory conditions, chosen to study these phenomena.

It was demonstrated some 50 years ago that resistance to isoniazid – as conferred by deletion of \textit{katG}, i.e. complete loss of katalase-peroxidase activity – is

The simple equation of fitness cost and restricted drug use resulting in elimination of resistance is complicated by the existence of so-called compensatory mutations. Experimentally, in the laboratory, it has been demonstrated that compensatory mutations may occur which counteract the fitness burden associated with a resistance determinant [3, 5–7, 18, 20]. These compensatory mutations have been suggested to maintain the spread of resistance even in the absence of antibiotics [27]. In case a fitness lowering chromosomal alteration occurs, two possibilities thus exist:

- the mutant carrying the chromosomal alteration becomes extinct, or
- the chromosomal alteration is fixed in the population by means of a compensatory second-site mutation.

In general, mutations occur in a strictly stochastic fashion. Given known mutation frequencies, the statistical likelihood of obtaining a defined mutation, such as a chromosomal resistance mutation, is in the range of \(10^{-6}\) to \(10^{-8}\). In case of a fitness lowering mutation, the probability of a compensatory mutation to occur is in approximately the same range. Thus, the probability of having a fitness lowering mutation accompanied by a second-site compensatory mutation is \(10^{-12}\) to \(10^{-16}\). These numbers can only be met by the assumption that following acquisition of the first costly mutation there must be a positive selection for organisms carrying the fitness lowering mutation – in order to increase population numbers, the substrate for the compensatory second mutation to occur.

Another important issue is the structure of the population studied, i.e. whether a homogeneous population versus a population of genetically diverse organisms is present. In case of a homogeneous population of mutants all having the same fitness lowering mutation there will be a high probability that ultimately an off-spring carrying a compensatory second-site mutation will be selected and will become the dominant part of the population. However, under conditions of selecting for drug resistance in the patient, in most instances there will be competition between different resistance mutations occurring simultaneously in a strictly stochastic fashion. Thus, the resistant population initially is quite heterogeneous. This principle of initial competition between different resistance mutations has mostly not been properly addressed by the in vitro laboratory conditions, chosen to study these phenomena.

It was demonstrated some 50 years ago that resistance to isoniazid – as conferred by deletion of \textit{katG}, i.e. complete loss of katalase-peroxidase activity – is

\begin{table}
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Drug} & \textbf{Resistance Mechanism} & \textbf{Mechanism} \\
\hline
Isoniazid & katG & \textit{inhA} (target) \\
\hline
Rifampicin & \textit{rpoB} & (target) \\
\hline
Ethambutol & \textit{embB} & (target) \\
\hline
Pyrinamide & \textit{pncA} & \\
\hline
Streptomycin & \textit{rpsL} & \textit{rrs} (target) \\
\hline
Aminoglycosides & \textit{rrs} & (target) \\
\hline
Macrolides & \textit{rif} & (target) \\
\hline
Quinolones & \textit{gyrA} & (target) \\
\hline
Ethionamide & \textit{ethA} & \textit{inhA} (target) \\
\hline
\end{tabular}
\caption{Mycobacterial drug resistance mechanisms}
\end{table}
associated with a significant fitness cost, at least as determined in the guinea pig animal model [14]. More recently it was shown that complete loss of KatG activity in clinical isoniazid resistant strains is associated with a secondary mutation resulting in over-expression of the alkyl-hydroperoxidase AhpC [28]. It was hypothesized, that isoniazid resistance carries a fitness cost and compensatory mutations in ahpC will develop over time, ultimately facilitating transmission and spread of resistant microorganisms. However, all attempts to demonstrate a direct role for AhpC in virulence failed [16, 29]. Thus, the reason for the surprising association of loss of KatG activity and AhpC upregulation is somewhat unclear – as yet there is no causal link for this association. In addition, complete loss of KatG activity represents only a minor fraction of isoniazid resistance in clinical isolates.

A different and particular instructive picture emerged when looking at the mechanisms mediating streptomycin resistance in M. tuberculosis. Here, in vitro experimental data on the genetics and costs associated with a resistance determinant were used for interpretation of resistant clinical isolates in vivo. The procedure involved two steps:

i) to determine the frequency of resistance mutations in clinical drug resistant isolates versus in in vitro selected drug resistant mutants,

ii) to introduce resistance determinants by means of genetic techniques to obtain isogenic mutants, which are subsequently investigated in an in vitro competition assay.

Plotted in figure 2 is the relative frequency of a given resistance mutation in clinical isolates versus the relative fitness as determined in the in vitro competition growth assay. A variety of mutations in either ribosomal protein S12 or the small subunit rRNA may result in resistance to streptomycin. However, there is a strict correlation between the frequency of a given resistance mutation in clinical isolates and its fitness cost as determined in vitro. The no-cost Lys → Arg alteration at position 42 of rpsL is by far the most frequent streptomycin resistance mutation in clinical isolates [11, 23]. An important control was to determine the stochastic probability of the different resistance mutations in M. tuberculosis (see table 2). Most importantly, and this makes his study so particularly interesting and insightful, Canetti categorized streptomycin resistance into low-level (≥4 µg/ml), intermediate-level (≥10 µg/ml) and high-level (≥100 µg/ml) drug resistance. At this gross view there was no significant difference between primary and acquired drug resistance with respect to the relative proportions of the different streptomycin resistance levels. In other words, within streptomycin resistant mutants high-level drug resistance mutations must exist which do not impede transmission. Available evidence suggests that within a spectrum of possible mutational resistance alterations each being associated with a distinct fitness cost, a selection for those resistance mutations with the least resistance-associated cost seems to exist in vivo [4, 10, 11, 21, 30, 31]. This selection is best explained by fluctuating environments, i.e. expansion of mutants experiencing a low cost of resistance in the absence of antibiotics during periods in which selection for antibiotic resistance is removed. The rare existence of high cost resistance mutations can best be explained

1. by the stochastic probability of a resistance mutation in a size limited bacterial population and

<table>
<thead>
<tr>
<th>Tab. 2. Primary resistance and acquired resistance to streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cases</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Primary resistance</td>
</tr>
<tr>
<td>Acquired resistance</td>
</tr>
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</table>

**Fig. 2. Relationship between frequency of streptomycin resistance mutation and relative fitness in clinical M. tuberculosis isolates (n = 79)**
2. by bottleneck phenomena which take place in transmission. Our studies in M. tuberculosis revealed that there is a priori no need for compensatory evolution in maintaining persistence of drug resistance, as resistance mutations exist which carry little or no fitness cost at all. As long as for a given drug different resistance mutations exist with only one of these being a no cost resistance mutation, the stochastic probability of selection for this particular mutation in a given population is much higher than the probability of two mutations occurring either simultaneously or successively with one compensating for disadvantages conferred by the other. The finding that no or low cost resistance mutations are common in clinical isolates has important implications for measures to combat the spread of resistance. It indicates that drug resistance per se can not be expected to restrict transmission, but that active infection disease control measures are necessary to counteract the spread of resistance.

Treatment of multi-drug resistance in M. tuberculosis is associated with an apparent paradox. By definition MDR implies resistance to at least isoniazid and rifampicin. These two drugs are the cornerstone of short-course therapy. However, it has been observed in some studies that for more than one third of MDR-TB SSC therapy is an effective treatment [e.g. 15]. How to explain effective chemotherapy despite drug resistance [24, 25]?

Drug susceptibility testing of mycobacteria in the clinical laboratory is characterized by peculiarities which most people are not aware of. The definition of resistance in the mycobacteriology laboratory dates back to Mitchinson in 1962: “Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug” [13]. Thus, until today, drug susceptibility testing in the mycobacteriology laboratory is not related to treatment outcome – the ultimate and only parameter validating in vitro drug susceptibility testing. Interestingly, it has been observed back to 1969 that the prognostic significance of in vitro determined drug resistance may be limited. “There is evidence that the presence of resistance to a single drug has little or no effect on the outcome of treatment with the three drugs isoniazid, streptomycin and para-aminosalicylic acid (International Union against Tuberculosis, 1964). Furthermore, even in the presence of primary resistance to two first-line drugs, a bacteriological response is not infrequently obtained with the three drug regimen” [13].

When we studied the mechanisms of streptomycin resistance in M. tuberculosis [19], we made the following observations (see table 3):

1. There is a strict correlation between the molecular mechanism of resistance and the phenotypic resistance level conferred. Thus, mutations in rpsL mediate high-level drug resistance and those in ribosomal RNA intermediate levels of resistance.

2. A significant part of drug resistant clinical strains exhibits a low-level resistance phenotype. Here, no mutual target alteration in either rpsL or rrs is present. Further experimental evidence suggests that this low-level drug resistance phenotype is due to alterations in cell wall permeability. Similar low-level drug resistant phenotypes have been described for other aminoglycosides, isoniazid and rifampicin [e.g. 8, 32].

In the diagnostic laboratory, drug susceptibility testing for mycobacteria is very different compared to other bacterial microorganisms. Rather than to determine minimal inhibitory concentrations, a single drug concentration, termed critical concentration, is used to categorize a clinical isolate as susceptible or as resistant [17]. A most important issue to be noted here is first that this so-called critical concentration bears no relationship to the drug concentrations, which are present in vivo in the patient (see table 4). Second, regardless of whether high-level, intermediate-level or low-level drug resistance is present, the corresponding isolate is categorized as resistant in the diagnostic laboratory. However, it is clear that the biological implications of low-level versus high-level drug resistance must be different for a number of reasons.

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### Tab. 3. Comparison of genotype and antimicrobial susceptibility test results

<table>
<thead>
<tr>
<th>Strain</th>
<th>Drug susceptibility</th>
<th>Genotype</th>
<th>Mutations</th>
<th>Substitutions</th>
<th>Phenotype (streptomycin MIC [mg/l])</th>
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</thead>
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<td>0.5–1.0</td>
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<td>Wild type</td>
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<td>0.5–1.0</td>
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<tr>
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<td>Wild type</td>
<td>1.0–2.0</td>
<td>0.5–1.0</td>
</tr>
<tr>
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<td>Wild type</td>
<td>1.0–2.0</td>
<td>0.5–1.0</td>
</tr>
<tr>
<td>4649/83</td>
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<td>rpsL</td>
<td>43-Lys→Arg</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>4513/83</td>
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<td>rpsL</td>
<td>43-Lys→Arg</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
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<td>43-Lys→Arg</td>
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<td>11966/89</td>
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<tr>
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<td>88-Lys→Arg</td>
<td>500–1,000</td>
<td>250–500</td>
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<tr>
<td>K8/94</td>
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<td>25–50</td>
</tr>
<tr>
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<td>526-C→T&lt;sup&gt;b&lt;/sup&gt;</td>
<td>250–500</td>
<td>25–50</td>
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<td>25–50</td>
<td>2.0–6.0</td>
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</tbody>
</table>

<sup>a</sup> Sm<sup>+</sup>: streptomycin susceptible; Sm<sup>−</sup>: streptomycin resistant

<sup>b</sup> Corresponding E. coli position
1. The relationship between phenotypic resistance level in vitro and drug concentration in vivo is of clinical relevance and is commonly addressed in bacteriology by the definition of break points. Here, the resistance phenotype determined in vitro is related to the drug levels, which can be achieved in vivo.

2. From experience in the treatment of streptococcal endocarditis, the problem of low-level resistance is known. Streptococci naturally exhibit a low-level resistance phenotype to aminoglycosides. This is due to impermeability of their cell wall; the drug target itself — the ribosome — is susceptible. In the presence of drugs which target cell wall synthesis, such as beta-lactams, aminoglycosides now become bactericidal. The current perception is that the beta-lactams interfere with the integrity of the cell wall allowing aminoglycosides to penetrate and to reach their target. Combination therapy of tuberculosis involves drugs which target cell wall synthesis, such as isoniazid and ethambutol. We can safely assume that the integrity of the cell wall will be affected here.

The hypothesis can be put forward that a successful treatment outcome despite a resistant phenotype — as determined by routine drug susceptibility testing — reflects limitations of the procedures used to determine drug susceptibility in the mycobacteriology laboratory and that low-level drug resistance does not correspond to clinical resistance [9]. This hypothesis can be tested, namely by determination of minimal inhibitory concentrations for those isolates resistant to the „critical concentration” combined with prospective studies which correlate the treatment response with the results of quantitative drug susceptibility testing. The view is emerging that the term “resistance” biologically is a heterogeneous mixture. Some patients suffering from drug-resistant tuberculosis may benefit from standard therapy — for reasons outlined above. For other patients, however, those who are suffering from a high-level drug resistant phenotype the mere continuation of standard therapy is likely to contribute to the build-up of further resistance. I think it is time to change the procedures used for drug susceptibility testing in diagnostic mycobacteriology, i.e. to implement the determination of minimal inhibitory concentrations for clinical strains which have been categorized as resistant. It is gratifying to note that — although not as a radical — the NCCLS subcommittee has at least incorporated parts of this hypothesis in its guidelines edited 2003. „In the case of isoniazid, if an isolate is resistant to the critical concentration of 0.2 µg/ml but susceptible to the higher concentration of 1.0 µg/ml the following comment should be given — the test results indicate low-level resistance to isoniazid; some evidence suggests that patients infected with corresponding strains may benefit from continuing therapy with isoniazid.”

In the treatment of MDR-TB a single drug should never be added to a failing regimen, as this involves the risk of development of resistance to the new drug. For treating MDR there are basically two options: i) use a standardized regimen, such as a combination of a chinolone, amikacin or streptomycin, pyrazinamide, ethambutol and ethionamide, or ii) individualize treatment according to the drug susceptibility pattern of the isolate, a strategy referred to as DOTS-PLUS.

There is still a debate going on which of these two strategies is the more appropriate for controlling MDR-TB in developing countries given their limited resources. However, there is consensus that for developed countries the individualized approach is preferable. Rather than treating empirically, the best here is to treat according to the results of drug susceptibility testing.

There is a word of caution necessary: the widespread use of chinolones for long term treatment of MDR-TB in developing countries may lead to building up resistance to these drugs in other pathogens of major significance, such as Salmonella or E. coli. This is a lesson to be learnt from the past when these drugs were used for long-term treatment of patients with kidney problems – not only did chinolone resistance in E. coli, which was once thought to be impossible, emerge under these circumstances, but corresponding isolates are now present in the community. The cost-benefit ratio of strategies for treatment of multidrug resistant tuberculosis should carefully be weighed so as not to run the risk that focusing too much on one aspect may lead to blindness for others.

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References


<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC [µg/l] of susceptible M. tuberculosis (average)</th>
<th>Concentration [µg/l] in serum used for testing</th>
<th>Concentration [µg/l] used for testing</th>
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</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>0.05–0.2</td>
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<td>Streptomycin</td>
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