Rifampicin-resistant and rifabutin-susceptible Mycobacterium tuberculosis strains: a breakpoint artefact?

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Objectives: It has long been assumed that some rifampicin-resistant Mycobacterium tuberculosis strains are susceptible to, and thus treatable with, rifabutin. However, clinical breakpoints for susceptibility testing of rifabutin as well as the evidence for a clinical effect of rifabutin in rifampicin-resistant strains remains poorly defined. The objective of this study was to re-evaluate the breakpoint for rifabutin in relation to its MIC wild-type distribution and the presence of mutations in rpoB.

Methods: The MIC in 7H10 Middlebrook medium was determined for clinical isolates of M. tuberculosis (n=95), where a majority were multidrug resistant. Additionally, all strains were screened for rpoB mutations by sequencing and the GenoType MTBDRplus assay.

Results: Rifampicin resistance was confirmed by genotypical and/or phenotypical tests in 73 isolates (76.8%). Nineteen isolates, defined as rifampicin resistant and rifabutin susceptible according to the present breakpoint, exhibited significantly higher MICs of rifabutin (0.064–0.5 mg/L) than rifabutin-susceptible isolates without any detectable mutations in rpoB (P<0.001). These 19 isolates were clearly resistant to rifampicin (MIC 2–256 mg/L) and all but one had mutations in rpoB, with 9 (47.4%) specifically in Asp516Val.

Conclusions: Our results indicate that rifampicin-resistant but rifabutin-susceptible isolates according to the present breakpoints harbour rpoB mutations and have a rifabutin MIC significantly higher than strains without any detectable mutations in rpoB. So far there are no clinical, pharmacological or microbiological data to confirm that such isolates can be treated with rifabutin and we suggest a revision of the current breakpoints.

Keywords: epidemiological cut-offs, ECOFFs, minimal inhibitory concentrations, MICs, multidrug-resistant tuberculosis, MDR, HIV, rpoB

Introduction

As a result of increasing drug resistance in Mycobacterium tuberculosis, it is of importance to optimize the use of the current antimycobacterial drugs. An important step is the adaptation of clinical breakpoints for antimicrobial susceptibility testing to modern principles used for other clinically important bacterial pathogens.1 It is currently believed in clinical practice that rifampicin-resistant but rifabutin-susceptible isolates may be treatable with rifabutin.2 However, these data have not been substantiated in prospective clinical trials as reviewed in a meta-analysis.3 Rifampicin and rifabutin belong to the rifamycin group of antibiotics together with rifapentine. The pharmacological properties show differences in that the free Cmax is lower for rifabutin (0.12 mg/L after a 300 mg dose) than for rifampicin (8 mg/L after a 600 mg dose).4 However, initial studies in mouse models indicated that rifabutin was more effective than rifampicin at reducing the bacterial load, which was ascribed to an increased intracellular accumulation of rifabutin.5 Compared with rifampicin, rifabutin is a minimal inducer of CYP3A4/5, which is of relevance for HIV/tuberculosis (TB) coinfecion, in which it causes fewer interactions with antiretroviral drugs such as the protease inhibitors.6 The CLSI recommends 0.5 mg/L as a critical concentration for rifabutin in Middlebrook 7H10 medium and others have suggested similar breakpoints for liquid-based methods.5,7 However,
full-range MIC testing has shown an MIC distribution of rifabutin-susceptible isolates at $\leq 0.1$ mg/L.\textsuperscript{9,10} Thus the suggested critical concentration for rifabutin (0.5 mg/L) is significantly higher than both the epidemiological wild-type cut-off ($\leq 0.1$ mg/L) and the free \( C_{\text{max}} \) (0.12 mg/L).

Our aim was to re-evaluate the breakpoint for rifabutin in relation to mutations in the \( rpoB \) gene and the MIC wild-type distribution.

**Methods**

**Clinical isolates and control strains**

Clinical isolates were selected from the collection at the Swedish Institute for Communicable Disease Control (SMI) and included 95 unique isolates of \( M. \) tuberculosis confirmed by molecular typing (restriction fragment length polymorphism analysis) and the reference strain H37Rv (ATCC 25618). Among the clinical isolates, 73 isolates (76.8%) were multidrug-resistant (MDR) or extensively drug-resistant (XDR) TB strains. All strains were stored at $-70^\circ$C from primary cultures on standard Lowenstein–Jensen medium in the biosafety level 3 laboratory at SMI.

**Determination of rifabutin and rifampicin MICs**

Stock solutions of rifampicin and rifabutin (30.7 g/L) were prepared in DMSO. All drugs and solvents were from Sigma (Stockholm, Sweden). The methodology has been previously described in detail.\textsuperscript{11} Briefly, bacterial suspensions were transferred by using a 96-stick replicator to Middlebrook 7H10 agar plates with serial 2-fold dilutions from 0.002 to 512 mg/L for the drugs. The MIC was defined as the first antibiotic concentration where there was less growth compared with the 1:100 diluted control of the corresponding strain.

**Detection of mutations in \( rpoB \)**

DNA was extracted from the \( M. \) tuberculosis strains as previously described.\textsuperscript{12} A 382 bp fragment containing the \( rpoB \) hot spot region (ATCC 25618). Among the clinical isolates, 73 isolates (76.8%) were wild-type H37Rv's \( rpoB \) gene (Rv0667). The nucleotides and codons were designated based on \( Escherichia coli \) codon numbering. Additionally, isolates were tested using the MTBDRplus assay (Hain Life-science, GMBH, Nehren, Germany), as previously described.\textsuperscript{7,13}

**Results and discussion**

Rifampicin resistance according to the critical concentration (1 mg/L) was confirmed by genotypical and/or phenotypical testing in 73 isolates (76.8%). Nineteen of these rifampicin-resistant strains would be classified as susceptible to rifabutin according to the CLSI-derived critical concentration (0.5 mg/L). It should be noted that these strains, all but one having \( rpoB \) mutations, had a significantly higher rifabutin MIC (0.064–0.5 mg/L, \( P \leq 0.001 \)) than the rifabutin-susceptible strains without any detectable mutations in \( rpoB \) (Figure 1). Consequently, these strains exhibit resistance mechanisms not only to rifampicin, but most likely also to rifabutin, which indicates that a critical concentration of 0.5 mg/L would fail to correctly classify such isolates. Additionally, there is very limited clinical, pharmacological or MIC data to support the current breakpoint. Of the 19 rifampicin-resistant strains that would be classified as rifabutin susceptible, 9 (47%) had an \( rpoB \) mutation in Asp516Val (\( rpoB \) mutation one (MUT1) according to Hain.

**Figure 1.** MIC distribution for rifabutin in relation to resistance mutations in \( rpoB \). Rifabutin- and rifampicin-susceptible isolates without any resistance mutations in \( rpoB \) (\( n = 19 \)) are distributed between MICs of 0.004 and 0.032 mg/L. Three isolates without resistance mutations in \( rpoB \) that had rifampicin MICs $\geq 16$ mg/L had rifabutin MICs of 0.25, 2 and 16 mg/L, respectively. Miscellaneous mutations are described in detail in Table 1 (\( n = 18 \)). The most frequent substitution (Ser531Leu, \( n = 46 \)) is shown in black and shows a Gaussian distribution. Nine isolates that are classified as susceptible according to the current critical concentration (CC; 0.5 mg/L) and show a significantly increased MIC distribution (0.064–0.25 mg/L) compared with the rifabutin-susceptible population at MICs of 0.004–0.032 mg/L exhibit an \( rpoB \) substitution in Asp516Val (\( P < 0.0001 \), Mann–Whitney \( U \)-test).
MTBDRplus] and the others except for one isolate had miscellaneous mutations (Table 1). In one of the two isolates with both rifabutin and rifampicin resistance both lacking mutations in \( rpoB \), the HAIN MTBDRplus was interpreted as resistant by two independent evaluators. Although this seems to be a very rare event even in this collection of MDR and XDR strains, one plausible explanation may be a mixed bacterial population where phenotypical resistance is not detectable by the \( rpoB \) assay.

The rifabutin MIC distribution (0.004–0.032 mg/L) for strains without any detectable mutations in \( rpoB \) in this study was similar to our previous investigation of consecutive susceptible isolates, where the putative wild-type MIC distribution ranged from 0.008–0.064 mg/L.\(^9\) The intra- and inter-assay quality controls with both resistant and susceptible isolates for rifabutin and rifampicin showed a variation less than or equal to one MIC dilution step.

In another study of 41 isolates with known \( rpoB \) mutations,\(^{14} \) 35 were rifabutin resistant using the indirect proportion method on Middlebrook 7H10. Six isolates with rifabutin MICs of 2–16 mg/L were ‘susceptible’ to rifabutin at the critical concentration of 0.5 mg/L, but without any exact MIC determination. In other reports, mutations in the 531 and 526 codons correlated with resistance to both rifabutin and rifampicin, whereas Asp516Val, Asp516Tyr, Met515Leu and Leu533Pro mutations correlated with resistance to rifampicin, but with MICs below the CLSI-derived critical concentration of 0.5 mg/L for rifabutin.\(^2,14\)

Until more pharmacodynamic and clinical outcome data are available, we suggest that an intermediate (I) category is warranted for strains with a rifabutin MIC of 0.064–0.25, as the currently used breakpoint at 0.5 mg/L is high in relation to the epidemiological wild-type cut-off (≤0.1 mg/L) and the free \( C_{\text{max}} \) obtained (0.12 mg/L), even if a potential increased intracellular accumulation is taken into account. This is supported by previous data where rifabutin MIC values of 0.125–0.25 mg/L were obtained for 5 of 21 rifampicin-resistant isolates where 0.125 mg/L has been considered as a breakpoint.\(^{10} \)

As pointed out previously, there are very limited published data supporting the successful treatment of MDR TB with rifabutin,\(^3,15–18 \) and even less so for rifabutin-‘susceptible’ strains with \( rpoB \) mutations and with resistance to rifampicin. A recent study showed that in the case of rifampicin, \( rpoB \) mutations were associated with treatment failures even if the strains had been determined as susceptible by phenotypic testing.\(^{19} \)

In a retrospective study, rifabutin was suggested to show an increased treatment success rate in MDR patients defined as susceptible to rifabutin (20 mg/L on Lowenstein–Jensen medium), although the sample size was limited (14 patients), did not include \( rpoB \) analysis and the group receiving rifabutin were treated with significantly more drugs.\(^{18} \) The use of other drugs together with rifabutin will also introduce a significant bias in the interpretation of the few available prospective studies, suggesting an effect of rifabutin in sporadic MDR patients. The increased effect from rifabutin (free \( C_{\text{max}} \) 0.6 mg/L) in a case report where amikacin was introduced at the same time in a rifampicin-resistant but rifabutin-susceptible MDR TB strain was

### Table 1. Distribution of rifabutin MIC in relation to rifampicin MIC, phenotypic susceptibility interpretation, \( rpoB \) sequence and Hain MTBDRplus genotypic interpretation

<table>
<thead>
<tr>
<th>n</th>
<th>MIC (7H10) RIB</th>
<th>CLSI S/R (RIB) 0.5 mg/L</th>
<th>MIC (7H10) RIF</th>
<th>WHO S/R (RIF) 1 mg/L</th>
<th>( rpoB )</th>
<th>Hain MTBDRplus</th>
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<tbody>
<tr>
<td>1</td>
<td>0.004</td>
<td>S</td>
<td>0.064</td>
<td>S</td>
<td>wt</td>
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<td>7</td>
<td>0.008</td>
<td>S</td>
<td>0.016–0.25</td>
<td>S</td>
<td>wt</td>
<td>S</td>
</tr>
<tr>
<td>9</td>
<td>0.016</td>
<td>S</td>
<td>0.064–0.25</td>
<td>S</td>
<td>wt</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>0.016</td>
<td>S</td>
<td>0.125–1</td>
<td>S</td>
<td>Leu511Pro(^2), Leu511Pro(^2)/Met515Val</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>0.032</td>
<td>S</td>
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<td>S</td>
<td>wt</td>
<td>S</td>
</tr>
<tr>
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<td>S</td>
<td>0.5</td>
<td>S</td>
<td>Asp516Tyr</td>
<td>R</td>
</tr>
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<td>S</td>
<td>2–32</td>
<td>R</td>
<td>Asp516Val (2), Asp516Tyr, Leu533Pro, Ser531Leu</td>
<td>R</td>
</tr>
<tr>
<td>5</td>
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<td>S</td>
<td>4–32</td>
<td>R</td>
<td>Asp516Val (4), His526Leu</td>
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</tr>
<tr>
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<td>S</td>
<td>32</td>
<td>R</td>
<td>wt</td>
<td>S</td>
</tr>
<tr>
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<td>2–64</td>
<td>R</td>
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</tr>
<tr>
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<td>256</td>
<td>R</td>
<td>Ser531Tyr</td>
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<tr>
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<td>8–256</td>
<td>R</td>
<td>Ser531Leu (9), Gin490Arg</td>
<td>R</td>
</tr>
<tr>
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<td>2</td>
<td>R</td>
<td>16</td>
<td>R</td>
<td>wt</td>
<td>S</td>
</tr>
<tr>
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<td>4</td>
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<tr>
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<td>R</td>
<td>wt</td>
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<tr>
<td>4</td>
<td>16</td>
<td>R</td>
<td>128–256</td>
<td>R</td>
<td>Ser531Leu, His526Asp, His526Tyr (2)</td>
<td>R</td>
</tr>
</tbody>
</table>

ND, not determined; RIB, rifabutin; RIF, rifampicin; wt, wild-type; R, resistant; S, susceptible.

\(^{a}\)Leu511Pro is considered as an allelic variation rather than a resistance mutation.
difficult to separate from amikacin and other drugs to which the bacillus was susceptible. In a trial from Hong Kong, 22 MDR patients were treated with rifampicin or rifabutin without any clinical effect. A temporary effect on bacteriological results was observed, which was most pronounced in two patients with rifabutin-susceptible isolates (MIC < 0.2 mg/L). However, rifabutin resistance emerged at 2 and 14 weeks of treatment for these patients. Early bactericidal activity studies using 300 and 600 mg of rifabutin showed a considerably lower effect of rifabutin than rifampicin at similar doses. In a French trial, 23 MDR TB patients with bilateral cavitary disease were treated with a rifabutin-based regimen for at least 1 year. In this trial, one rifampicin-resistant strain with a low MIC of rifabutin (0.125 mg/L) was included, but there is no information about the outcome in this patient. It was difficult to assess the respective role of rifabutin and companion drugs in cases of successful treatment (spu
tum conversion at 12 months in 14 of 39). In the trials including MDR patients, high doses of rifabutin at 450–600 mg were given, which have been reported to be associated with a higher rate of side effects. Thus there are no convincing clinical data confirming that rifabutin-susceptible but rifampicin-resistant strains with mutations in rpoB may be treated with rifabutin at normal dosages.

We conclude that the presence of rifabutin-susceptible but rifampicin-resistant isolates is probably caused by a breakpoint artefact. Until firm evidence exists for a clinical effect in strains with a rifabutin MIC of 0.064–0.5 mg/L and a resistance mutation in rpoB, they should not be classified as susceptible to avoid giving MDR patients an ineffective treatment regimen.

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**Transparency declarations**

None to declare.

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