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# Comparative Antimycobacterial Activities of Rifampin, Rifapentine, and KRM-1648 against a Collection of Rifampin-Resistant *Mycobacterium tuberculosis* Isolates with Known *rpoB* Mutations

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**A collection of 24 rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* with characterized RNA polymerase β-subunit (*rpoB*) gene mutations was tested against the antimycobacterial agents rifampin, rifapentine, and KRM-1648 to correlate levels of resistance with specific *rpoB* genotypes. The results indicate that KRM-1648 is more active in vitro than rifampin and rifapentine, and its ability to overcome rifampin resistance in strains with four different genetic alterations may prove to be useful in understanding structure-function relationships.**

Rifampin resistance in *Mycobacterium tuberculosis* correlates closely with a cluster of genetic alterations in a very limited region of the RNA polymerase β-subunit gene (*rpoB*). Collectively, DNA sequence studies demonstrated that 96% of resistant isolates have missense mutations, deletions, or insertions within an 81-bp region, in contrast to susceptible isolates, which revealed no nucleotide changes (6, 7, 13, 18, 19). The correlation was further supported by genetic complementation experiments that demonstrated that the wild-type *rpoB* allele restored susceptibility to rifampin when introduced into a resistant isolate (12).

We have previously reported on our collection of genetically characterized *M. tuberculosis* strains that were genotyped with IS*6110* (10). DNA isolated from both mono-rifampin-resistant and multidrug-resistant subtypes was further catalogued on the basis of their *rpoB* sequence changes (6, 7, 11). As a result, we have been able to define a test panel of 25 *M. tuberculosis* strains that reflect the genetic diversity in both the clonal types and in the *rpoB* genetic alterations. In this study, each strain was tested against three rifamycin derivatives, rifampin, rifapentine, and KRM-1648, to correlate levels of resistance with specific *rpoB* genotypes.

Twenty-four rifampin-resistant clinical isolates of *M. tuberculosis* and one pansusceptible strain, isolated predominantly from New York City tuberculosis patients from 1992 to 1995, were selected from the strain database. This panel includes 22 different IS*6110* subtypes and 17 allelic differences in the *rpoB* gene (Table 1).

Rifampin was purchased from Sigma Chemical Co., St. Louis, Mo., rifapentine was obtained from Marion Merrell Dow Inc., Cincinnati, Ohio, and KRM-1648 was obtained from PathoGenesis Corporation, Seattle, Wash., and Kaneka Corporation, Osaka, Japan. Stock solutions were made at a concentration of 10 mg/ml in dimethylsulfoxide.

Frozen cultures of *M. tuberculosis*, stored at −70°C in 7H9 broth in 15% glycerol, were recovered on Lowenstein-Jensen slants. Strain identity was confirmed by DNA fingerprinting of each recovered culture and matching of the new IS*6110* pattern to the archived pattern. Once identity was confirmed, the cultures on Lowenstein-Jensen slants were used for susceptibility testing.

Susceptibility testing was performed by agar diffusion as described previously (15). Serial twofold dilutions of three compounds were incorporated into 7H10 agar (Difco, Detroit, Mich.) at concentrations that ranged from 0.25 to 32 μg/ml.

Each *M. tuberculosis* isolate was subcultured on 7H10 agar, suspended in 7H9/ADC broth (Difco) in the presence of glass beads to a concentration of $10^7$ CFU/ml, diluted 1:100, and plated (0.1 ml per quadrant). Plates, tested in duplicate, were incubated at 35°C in the presence of 5% $CO_2$. Each plate was checked weekly, and results were recorded after weeks 3 and 4.

A summary of the MICs of all three rifamycin derivatives for the 25 genetically characterized *M. tuberculosis* test isolates is listed in Table 1. Among the 25 isolates there were 22 different genetic subtypes as defined by IS*6110* genotyping; 5 had unique fingerprint patterns, and 17 had DNA fingerprint patterns that were identified previously in the Public Health Research Institute Tuberculosis Center database and were assigned 17 strain cluster types.

Clustered isolates with the same *rpoB* mutations, such as the two C strains (TN1916 and TN987) and the closely related W and W1 strains (TN565, TN635, and TN3806), behave as clones, and the MIC profiles of the three rifamycin derivatives are the same for isolates within a cluster.

In general, the MICs of rifampin and rifapentine for the panel of isolates were indistinguishable, and nearly all revealed high-level resistance (>32 μg/ml). The strain with two missense mutations in adjacent codons 511 and 512 (TN801) was reported as resistant to rifampin at 2 μg/ml but susceptible to rifapentine, which had an MIC of 1 μg/ml. KRM-1648, which is a derivative of 3′-hydroxy-5′-aminobenzoxazinorifamycin characterized by an isobutyl-1-piperazinyl group at the 5′ position (20, 21), was more active, with an MIC of <1 μg/ml, and in general its MICs were lower for each strain tested. Among the nine isolates with four different point mutations in codon 526, two strains had a His-to-Leu substitution; the MICs of rifampin and rifapentine for these strains were 8 μg/ml, but for

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TABLE 1. Clinical isolates of *M. tuberculosis* with *rpoB* gene mutations tested against rifampin, rifapentine, and KRM-1648

| Strain | FP[a] | No. of bands | Culture date[b] | Amino acid position | Base change(s) | Amino acid change(s)[c] | MIC (μg/ml) of[d]: | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | RMP | RPE | KRM |
| TN801 | 001 | 14 | 8/93 | 511, 512 | CTG to CGG, AGC to ACC | Leu to Arg, Ser to Thr | 2 | 1 | <1 |
| TN794 | AH | 4 | 8/93 | 511, 516 | CTG to CGG, GAC to TAC | Leu to Arg, Asp to Tyr | >32 | >32 | <1 |
| TN715 | AB | 11 | 4/93 | 513 | CAA to AAA | Gln to Lys | >32 | >32 | 16 |
| TN808 | CP | 7 | 7/93 | 513 | CAA to CTA | Gln to Leu | >32 | >32 | 16 |
| TN1733 | AF3 | 12 | 5/93 | 513, 514 | Deletion of CAA, TTC | Deletion of Gln, Phe | >32 | >32 | 8 |
| TN728 | 001 | 14 | 4/93 | 513–516 | Deletion of AA-TTC-ATG-G | Gln-Phe-Met-Asp to His | >32 | >32 | <1 |
| TN804 | AR1 | 12 | 8/93 | 516, 517 | Deletion of GAC, CAG | Deletion of Asp, Gln | >32 | >32 | 8 |
| TN1916 | C | 3 | 8/92 | 517, 518 | Deletion of CAG, AAC | Deletion of Gln, Asn | >32 | >32 | 16 |
| TN987 | C | 3 | 8/92 | 517, 518 | Deletion of CAG, AAC | Deletion of Gln, Asn | >32 | >32 | 16 |
| TN565 | W | 16 | 7/92 | 526 | CAC to TAC | His to Tyr | >32 | >32 | 32 |
| TN635 | W1 | 17 | 7/93 | 526 | CAC to TAC | His to Tyr | >32 | >32 | >32 |
| TN3806 | W1 | 17 | 8/94 | 526 | CAC to TAC | His to Tyr | >32 | >32 | >32 |
| TN800 | L | 3 | 8/93 | 526 | CAC to TAC | His to Tyr | >32 | >32 | 16 |
| TN994 | T | 12 | 9/92 | 526 | CAC to AAC | His to Asn | 16 | 16 | 4 |
| TN644 | A | 8 | 1/93 | 526 | CAC to GAC | His to Asp | >32 | >32 | 16 |
| TN981 | AC | 7 | 7/92 | 526 | CAC to GAC | His to Asp | >32 | >32 | 16 |
| TN659 | CM | 19 | 12/92 | 526 | CAC to CTC | His to Leu | 8 | 8 | 8 |
| TN792 | 001 | 12 | 8/93 | 526 | CAC to CTC | His to Leu | 8 | 8 | <1 |
| TN640 | AT | 7 | 1/93 | 531 | TCG to TTG | Ser to Leu | >32 | >32 | >32 |
| TN1811 | W12 | 16 | 2/94 | 531 | TCG to TTG | Ser to Leu | >32 | >32 | >32 |
| TN807 | 001 | 14 | 7/93 | 531 | TCG to TGG | Ser to Trp | >32 | >32 | 2 |
| TN798 | 001 | 11 | 8/93 | 531, 514 | TCG to TTG, TTC to TTT | Ser to Leu, Phe to Phe | >32 | >32 | 16 |
| TN805 | H | 2 | 8/93 | 531, 528 | TCG to TTG, CGC to CGT | Ser to Leu, Arg to Arg | >32 | >32 | >32 |
| TN1595 | N2 | 15 | 10/93 | 533 | CTG to CCG | Leu to Pro | >32 | >32 | 4 |
| TN657 | H | 2 | 2/93 | | Wild type | Wild type | 1 | 1 | 1 |

[a] FP, IS*6110* DNA fingerprint code (letter assignment described previously [10]). Unique (not previously identified) fingerprints were assigned code 001.
[b] Month/year.
[c] Amino acid abbreviations: Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; His, histidine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine.
[d] RMP, rifampin; RPE, rifapentine; KRM, KRM-1648.

one of these strains (TN792), the MIC of KRM-1648 was <1 μg/ml. The findings that two strains with identical missense mutations respond differently to KRM-1648 and that 4% of rifampin-resistant isolates have the wild-type *rpoB* nucleotide sequence suggest that mutations may be present in other regions of the *rpoB* gene or that there is another rifamycin target in *M. tuberculosis*. The ability of KRM-1648 to overcome rifampin and rifapentine resistance was observed in two additional strains, one with a 9-bp deletion that replaced four consecutive amino acids (codons 513 to 516) with a single histidine (TN728) and one with missense mutations in codons 511 and 516 (TN794). In both cases these strains grew in the presence of 32 μg of rifampin and rifapentine per ml, but strikingly, the MIC of KRM-1648 for both was <1 μg/ml.

Resistance to rifampin among *M. tuberculosis* strains closely correlates in 96% of the tested isolates to a cluster of genetic alterations in an 81-bp region of the RNA polymerase β-subunit gene. This finding is greatly strengthened by the fact that rifampin-susceptible isolates show no mutations in this region, and together the data suggest that the only mutations seen in *M. tuberculosis* are those under selective pressure (8).

Proper antimycobacterial therapy, which requires treating the patient with two agents to which the strain is susceptible, eliminates the selective advantage of any one random mutational event (5). Therefore, it is consistent that the development or acquisition of drug resistance in *M. tuberculosis* is the result of inadequate therapy; this is commonly the result of patient noncompliance, but other factors such as doctors not prescribing adequate drug regimens or patients suffering from chronic diarrhea may also contribute to the selection of drug-resistant mutants (2, 16). In addition, human immunodeficiency virus infection also appears to have an impact on the selective development of rifampin monoresistance, although the mechanism has not been elucidated (11).

Shortening the length of therapy and developing compounds that require less dosing have been two effective methods of improving patient compliance and limiting the acquisition of resistance (1, 4). It has been suggested that the promising in vitro and in vivo activity of KRM-1648 could lead to a significant reduction in the duration of treatment. This suggestion was partially based on the KRM-1648 antitubercular activity in a murine model compared with rifampin and isoniazid activities (9). That study revealed that 6-week treatment with KRM-1648 resulted in the lack of detection of culturable mycobacteria in both spleens and lungs whereas rifampin or isoniazid never reduced cell counts below detectable levels, even after 12 weeks of treatment. In addition, a KRM-1648–isoniazid combination therapy maintained sterilization 6 months after the cessation of treatment, whereas all other drug treatments failed to prevent growth 2 weeks after the regimen was stopped (9).

In this study, the in vitro activity of KRM-1648 was further analyzed by testing against a panel of rifampin-resistant *M. tuberculosis* strains with characterized *rpoB* mutations. Several of the MICs of KRM-1648 were lower than those of both rifampin and rifapentine, and a significant difference in activity (±1 dilution) against strains with five different mutant alleles was observed. A survey of the data in Table 1 reveals that both the amino acid position and the substitution are clearly important. Among the nine isolates with a total of four different missense mutations at codon 526, the strains with CAC-to-CTC (His-to-Leu) genetic alteration required lowers MICs of rifampin and rifapentine (8 μg/ml) and isolate TN792 was susceptible to 1 μg of KRM-1648 per ml.

In a related study, Bodmer and coworkers (3) compared the relative activities of rifampin, rifapentine, and rifabutin against a panel of *rpoB*-typed isolates. In accord with the findings in this study, all mutants resistant to rifampin were also resistant to rifapentine, but rifabutin had lower MICs, and in four instances the drug overcame the mutant phenotype. In both studies, rifabutin and KRM-1648 were active against mutants which had alterations of the leucine at position 511 and aspartic acid at position 516. It is interesting that the strain with the missense mutation at codon 531 that changes a serine to tryptophan showed high-level resistance to rifampin and rifapentine (MIC, >32 μg/ml), and MICs of rifabutin and KRM-1648 were 8 and 2 μg/ml, respectively.

The discrepancies in MICs for *M. tuberculosis* isolates with the same *rpoB* mutations, but from different studies, were observed with isolates with a leucine-to-proline change at codon 533. Although we and others have previously reported isolates with this mutation resistant, and in the present study this mutation is associated with an MIC of rifampin of >32 μg/ml, Ohno and coworkers (17) identified two susceptible isolates (not genetically compared) for which MICs were <1.0 μg/ml.

The protein structure of the RNA polymerase β-subunit is not resolved to the atomic level and little is known of its interaction with rifamycin derivatives. It has been proposed that rifampin acts as a plug that appears to inhibit the extrusion of the nascent RNA transcript and that mutations in the *rpoB* gene likely affect this protein-drug pocket and provide a space for the RNA to escape (14). In the absence of a protein structure for rational drug design, the results with both KRM-1648 and rifabutin revealed that a robust collection of genetically characterized rifampin-resistant strains with known *rpoB* mutations provides an excellent screen panel to evaluate and catalogue rifamycin derivatives. This approach might lead to the identification of a "cocktail" of compounds that together overcome most rifampin-resistant genotypes.

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