In Vitro Isolation and Characterization of Oxazolidinone-Resistant Mycobacterium tuberculosis

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ABSTRACT Oxazolidinones are promising candidates for the treatment of Mycobacterium tuberculosis infections. We isolated linezolid-resistant strains from H37Rv (Euro-American) and HN878 (East-Asian) strains; resistance frequencies were similar in the two strains. Mutations were identified in ribosomal protein L3 (RplC) and the 23S rRNA (rrl). All mutant strains were cross resistant to sutezolid; a subset was cross resistant to chloramphenicol. Mutations in rrl led to growth impairment and decreased fitness that may limit spread in clinical settings.

KEYWORDS tuberculosis, oxazolidinones, linezolid, resistance, fitness, ribosome, Mycobacterium, Mycobacterium tuberculosis, antibiotic resistance, mycobacteria

The oxazolidinone class of antibiotics inhibits the formation of protein synthesis initiation complexes by binding to domain V of the 23S rRNA (1). Linezolid (LZD), the first member of the oxazolidinones approved for clinical use, has recently been investigated as a potential treatment for drug-resistant strains of Mycobacterium tuberculosis (2, 3). LZD demonstrates time-dependent kill kinetics against replicating M. tuberculosis (4), bactericidal activity against nonre replicating bacilli (5), and good efficacy in mouse models (5). However, the long-term administration of LZD is limited due to side effects that include neuropathy and anemia (2, 6). Sutezolid (SZD; previously PNU-100480) is a next-generation oxazolidinone that has improved tolerance over long-term administration and improved efficacy against M. tuberculosis in a mouse model (7). Resistance to oxazolidinones has been studied in other bacteria and is mediated via mutations in domain V of the 23S rRNA (rrl), in the ribosomal protein L3 (rplC), or by the transporter OptrA (8). We sought to further characterize the mechanisms of oxazolidinone resistance in M. tuberculosis.

We isolated resistant mutant strains (RMs) against LZD by plating late-log-phase cultures of M. tuberculosis H37Rv (ATCC 25618) (Euro-American lineage) and HN878 (East-Asian lineage) on Middlebrook 7H10 agar with 10% (vol/vol) OADC (oleic acid-albumin-dextrose-catalase) supplement (Becton Dickinson) and 8 μg/ml of LZD (5 × MIC) (9). We confirmed resistance by determining MICs on solid medium or in liquid medium. Solid MICs were determined in 24-well plates on 7H10-OADC agar and were defined as the lowest concentrations that prevented growth. MIC90 was determined in Middlebrook 7H9 liquid medium with 10% (vol/vol) OADC and 0.05% (wt/vol) Tween 80 (Tw); bacterial growth was measured by the optical density at 590 nm (OD590) after 5 days, and the MIC90 was defined as the concentration at which 90% of growth was inhibited (10). Sixteen resistant strains were isolated in H37Rv at a frequency of 2.3 × 10−9 (Table 1); 12 resistant strains were isolated in HN878 at a similar frequency of 3.3 × 10−9 (Table 2). All strains were confirmed as resistant to LZD and were also cross resistant to sutezolid (SZD) (Tables 1 and 2).

We sequenced rrl and ribosomal protein L3 using primers TB-rrl-MMF1, CACACTGTGGGTCCTGA; TB-rrl-MMF2, TGGAATCCGCTGTGAA; TB-rrl-MMF3, CAGGAGGTTGGCTTAA; TB-rrl-MMF4, TCGTGAACACCCTTG; and TB-rrl-MMF1, CGCCGTAACTCTATGCA for...
**TABLE 1** Oxazolidinone-resistant mutant strains of *M. tuberculosis* H37Rv

| Strain          | MIC on solid medium (μM) | L3 mutation | 23S RNA mutation |
|-----------------|--------------------------|-------------|------------------|
|                 | Linezolid | Sutezolid |         |                     |
| H37Rv           | 3.1       | 1.6       | WT       | WT                  |
| LP-LZD-RM2      | 50        | 12.5      | WT       | G2814T              |
| LP-LZD-RM3      | 25        | 12.5      | WT       | G2814T              |
| LP-LZD-RM4      | 50        | 12.5      | WT       | G2814T              |
| LP-LZD-RM24     | 50        | 25        | WT       | G2814T              |
| LP-LZD-RM1      | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM11     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM12     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM13     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM14     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM15     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM16     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM17     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM21     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM22     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM23     | 50        | 12.5      | C154R    | WT                  |
| LP-LZD-RM25     | 50        | 12.5      | C154R    | WT                  |
| HN878 WT        | 1.6       | 0.4       | WT       | WT                  |

| Strain          | MIC90 in liquid medium (μM) | L3 mutation | 23S RNA mutation |
|-----------------|-----------------------------|-------------|------------------|
|                 | LZD | SZD | CM | GM | KM |         |                     |
| HN878 WT        | 3  | 2  | 7  | 3  | 1  | WT       | WT                  |
| HN-LZD-RM3      | 156 | 75 | 12 | 4  | 3  | WT       | G2299T              |
| HN-LZD-RM5      | 65  | 81 |     |     |     | WT       | G2299T              |
| HN-LZD-RM6      |     |     |     |     |     | WT       | G2299T              |
| HN-LZD-RM9      | 107 | 61 |     |     |     | WT       | G2299T              |
| HN-LZD-RM10     |     |     |     |     |     | WT       | G2299T              |
| HN-LZD-RM13     | 92  | 62 |     |     |     | WT       | G2299T              |
| HN-LZD-RM14     |     |     |     |     |     | WT       | G2299T              |
| HN-LZD-RM1      | 60  | 31 | 412 | 3  | 2  | WT       | A2689T              |
| HN-LZD-RM11     | 94  | 62 | 112 | 3  | 4  | WT       | G2814T              |
| HN-LZD-RM2      |     |     |     |     |     | WT       | G2814T              |
| HN-LZD-RM4      |     |     |     |     |     | WT       | G2814T              |
| HN-LZD-RM8      |     |     |     |     |     | WT       | G2814T              |
| H37Rv WT        | 3.1 | 1.6 |     |     |     | WT       | WT                  |

*a*LZD, linezolid; SZD, sutezolid; CM, chloramphenicol; GM, gentamicin; KM, kanamycin.

**TABLE 2** Oxazolidinone-resistant mutant strains of *M. tuberculosis* HN878

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| Strain          | MIC of in liquid medium (μM)* | L3 mutation | 23S RNA mutation |
|-----------------|-------------------------------|-------------|------------------|
|                 | LZD | SZD | CM | GM | KM |         |                     |
| HN878 WT        | 3  | 2  | 7  | 3  | 1  | WT       | WT                  |
| HN-LZD-RM3      | 156 | 75 | 12 | 4  | 3  | WT       | G2299T              |
| HN-LZD-RM5      | 65  | 81 |     |     |     | WT       | G2299T              |
| HN-LZD-RM6      |     |     |     |     |     | WT       | G2299T              |
| HN-LZD-RM9      | 107 | 61 |     |     |     | WT       | G2299T              |
| HN-LZD-RM10     |     |     |     |     |     | WT       | G2299T              |
| HN-LZD-RM13     | 92  | 62 |     |     |     | WT       | G2299T              |
| HN-LZD-RM14     |     |     |     |     |     | WT       | G2299T              |
| HN-LZD-RM1      | 60  | 31 | 412 | 3  | 2  | WT       | A2689T              |
| HN-LZD-RM11     | 94  | 62 | 112 | 3  | 4  | WT       | G2814T              |
| HN-LZD-RM2      |     |     |     |     |     | WT       | G2814T              |
| HN-LZD-RM4      |     |     |     |     |     | WT       | G2814T              |
| HN-LZD-RM8      |     |     |     |     |     | WT       | G2814T              |
| H37Rv WT        | 3.1 | 1.6 |     |     |     | WT       | WT                  |

*ZLD, linezolid; SZD, sutezolid; CM, chloramphenicol; GM, gentamicin; KM, kanamycin.
demonstrated bactericidal activity against WT H37Rv with a minimum bactericidal concentration (MBC) equivalent to the MIC (3 μM or 1 μg/ml) (Fig. 1A). The L3C154R and rrlG2814T mutant strains were resistant to LZD bactericidal activity up to 16 μM (5.4 μg/ml) (Fig. 1B and C). At 50 μM (17 μg/ml), LZD had activity against the L3C154R strains and was bacteriostatic against the rrlG2814T strain. Full kill was achieved against all strains at 125 μM (42 μg/ml). Thus, the increased MICs of L3 and rrl mutant strains were translated into proportional increases in MBCs.

Since LZD and chloramphenicol share a binding site in the 23S rRNA (14), we looked at cross-resistance. Strains carrying rrlA2689T or rrlG2814T were cross resistant to chlor-
amphenicol, while rrlG2299T did not confer resistance (Table 2). As expected, rrl mutant strains remained susceptible to kanamycin and gentamicin, both of which bind to the 16S rRNA (Table 2). Cross-resistance to chloramphenicol is consistent with data from Staphylococcus aureus (15, 19) and Mycobacterium smegmatis (20). However, the lack of cross-resistance for rrlG2299T strains is surprising since the equivalent nucleotide in E. coli (G2061) interacts with the hydroxyl group of chloramphenicol (21). Furthermore, mutations of G2061 and A2062 are associated with chloramphenicol resistance in Thermus thermophilus (22). These results suggest that there are sufficient structural differences in the 23S rRNA of M. tuberculosis that cause chloramphenicol to bind in a different manner.

Mutations in 23S rRNA are commonly associated with growth defects in a diverse range of bacterial species, including M. smegmatis and M. tuberculosis (17, 19, 23). We conducted growth curves for representative strains in liquid medium; cultures were grown in 16-mm borosilicate tubes containing 5 ml of 7H9-OADC-Tw and incubated at 37°C with stirring at 250 rpm using an 8-mm stirrer bar. All strains with rrl mutations demonstrated impaired growth compared to WT strains (Fig. 1D and E), while strains with mutations in L3 were unimpaired (Fig. 1D).

The fitness cost of resistance mutations is an important contributor to the emergence and expansion of drug-resistant strains (24, 25). To investigate the fitness cost of LZD resistance, we conducted in vitro competition experiments as described previously (25). Briefly, 100 ml of 7H9-OADC was inoculated with ~10^6 CFU of the WT and mutant strains in a 450-cm² roller bottle. Coculture experiments were grown at 37°C until stationary phase (OD590 ~1). Serial dilutions were plated onto 7H10-OADC with and without 5 μM (1.7 μg/ml) LZD at day 0 and at stationary phase. CFUs were counted after 4 to 5 weeks of incubation at 37°C. The relative fitness (W) of resistant (R) compared to that of susceptible (S) strains was calculated by W = ln(RF/RI)/ln(SF/SI) (25), where RI and SI are the number of resistant and susceptible cells at day 0, and RF and SF are the number of resistant and susceptible cells at stationary phase. Experiments were performed in biological triplicate. The H37Rv rrlG2814T strain had a fitness cost compared to that of the susceptible parental strain (Fig. 1F). In contrast, the L3C154R mutant strain had no fitness cost relative to that of the parent (Fig. 1F). Relative fitness costs have been previously shown to influence the spread of resistance, with low-cost resistance phenotypes being the most prevalent within clinical populations (25). From a limited number of clinical studies, the L3C154R single nucleotide polymorphism (SNP) is more prevalent than rrl SNPs within LZD-resistant strains (6, 11, 13). Whether or not this is because of the associated fitness cost requires further investigation. Resistance defects can be overcome by compensatory mechanisms as, for example, in S. aureus where changes in the copy number of 23S rRNA can achieve a balance between fitness and resistance (19). M. tuberculosis is unique in that it contains only a single copy of 23S rRNA, so it may not have access to the same compensatory mutations. Identifying compensatory mechanisms that overcome the fitness defects associated with rrl SNPs would provide further insights.

In conclusion, we demonstrate that mutations in the 23S rRNA (rrl) and the ribosomal protein L3 (RplC) are associated with resistance to the oxazolidinones LZD and SZD. Resistance led to decreased bactericidal activity from LZD. Mutations in rrl, but not L3, had a competitive fitness cost in vitro, suggesting that their appearance may be limited in clinical settings.

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