First Linezolid-Resistant Clinical Isolates of *Mycobacterium tuberculosis*

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Linezolid resistance was found in 4 (1.9%) of 210 multidrug-resistant *Mycobacterium tuberculosis* strains. The MICs of linezolid were 4 μg/ml (one strain) and 8 μg/ml (three strains). Since no mutations were detected in potential target genes, the mechanism of resistance remains unclear.

The ongoing global burden of tuberculosis (TB) and the increasing problem of multidrug-resistant (MDR) TB (resistant to at least isoniazid and rifampin) have led to the increased use of second-line anti-TB drugs. Linezolid is a recently approved antituberculosis drug belonging to a new class of antibiotics, the oxazolidinones (1). Early studies have shown that linezolid is a protein synthesis inhibitor that interacts with domain V of the 23S rRNA (9). This domain is also the binding site for chloramphenicol, macrolides, and lincosamides, but the lack of cross-resistance between oxazolidinones and other antibiotics supports evidence for a novel mechanism of action (5, 10–12).

To date, linezolid-resistant *Mycobacterium tuberculosis* clinical strains seem to be rare. In this study, 210 M. *tuberculosis* strains sent to the German National Reference Laboratory for Mycobacteria from 2003 to 2005 were examined for linezolid resistance, using the BACTEC 460 and BACTEC MGIT 960 systems (Becton Dickinson Diagnostic Systems, Sparks, MD). Linezolid susceptibility testing was performed at 1 μg/ml, as this was the critical concentration determined in a previous investigation (7). Of the 210 strains tested, 4 (1.9%) exhibited linezolid resistance, defined as an MIC of >1 μg/ml.

The four patients harboring these strains were known to be infected with strains resistant to at least isoniazid, rifampin, streptomycin, and ethambutol (Fig. 1). The nonrelatedness of the strains was ascertained by the different origins of the patients, diverse susceptibility patterns of the strains, and different IS6110 patterns of the strains, which remained identical within each patient over the years. The reasons for the occurrence of linezolid resistance were determined by referring to the patients’ treatment histories shortly before resistance was observed. In that period, patient 1 was treated with pyrazinamide (to which the strain was already resistant), cycloserine, capreomycin, (three times weekly), and linezolid. Capreomycin was stopped after 2 months. Patient 2 was treated with a six-drug combination (rifabutin, ofloxacin, pyrazinamide, cycloserine, lamprrene, and linezolid), although the strain was resistant to rifabutin and ofloxacin. Despite controlled treatment in a hospital, linezolid resistance evolved. Few data were available for patient 3. Within 2 years, the patient was admitted to several hospitals. During that time, linezolid resistance developed. Approximately one-half to 1 year before linezolid resistance was detected, patient 4 was treated as an outpatient with cycloserine, capreomycin (three times weekly), and linezolid. The possible causes for the development of resistance in these cases might be lack of compliance, intermittent treatment, and/or weaker tuberculostatic activities of second-line drugs.

To determine the levels of linezolid resistance, the MICs of all linezolid-resistant strains and their susceptible parent strains were determined. For the four resistant strains, the MICs were estimated to be 4 (patient 3) and 8 μg/ml (patients 1, 2, and 4). The MIC of the susceptible parent strains from patients 1, 2, and 4 and of an MDR control strain was estimated to be 0.5 μg/ml, while that of the susceptible parent strain from patient 3 and of *M. tuberculosis* H37r was 1 μg/ml. MICs were identical irrespective of the method used (BACTEC MGIT 960 or BACTEC 460).

Two relevant parameters for the efficiency of a drug are its concentration and duration at the site of action. In a pharmacokinetic study, the mean 12-h concentration of linezolid in the inflammatory fluid was 4.9 μg/ml, and peak levels were between 6 and 21 μg/ml in plasma and inflammatory fluid (3). The MICs estimated in that study shifted from 0.5 and 1 to 4 and 8 μg/ml, respectively, which may point out the clinical significance of these findings.

There were no obvious differences in the growth characteristics of linezolid-resistant and -susceptible strains after growth on Löwenstein-Jenssen medium, with (linezolid-resistant strains) or without linezolid.

To investigate whether the linezolid-resistant strains belonged to a certain genotype, real-time PCRs with all four linezolid-resistant strains and their respective susceptible parents were performed. Real-time PCR is able to distinguish between Beijing and non-Beijing genotype strains by targeting a specific deletion uniquely present in Beijing strains (4). Since two isolates and their respective susceptible parents were identified as Beijing genotypes (from patients 1 and 3) and the other two were identified as non-Beijing genotype *M. tuberculosis* strains, it can be stated that linezolid resistance is not restricted to one of the two groups.

To identify the mechanism of resistance, DNA sequencing of putative target genes was performed for all the linezolid-resistant strains and their respective susceptible parents. The sequences of the complete 23S rRNA gene, the rplV and rplD genes, coding for ribosomal proteins L4 and L22, the
erm-37 gene, coding for a 23S rRNA methyltransferase, and a putative regulator, the \textit{whiB7} gene, were determined. The primers listed in Table 1 were used for amplification and sequencing. The alignment of all sequences revealed no alterations between susceptible or resistant strains or \textit{M. tuberculosis} H37.

Possible effects of efflux pump inhibitors could give the first hints of a resistance mechanism. Therefore, linezolid MICs of resistant strains were determined in the presence of the inhibitor reserpine (12\,\mu g/ml) (6). Linezolid MICs were reduced only slightly, from 8 to 4\,\mu g/ml and from 4 to 2\,\mu g/ml. Reserpine alone showed no inhibitory effect on growth. Thus, aside from the first hint that efflux pumps could be involved in linezolid resistance, the mechanism remains unexplained. Sander and coworkers isolated \textit{Myco-}bacterium \textit{smegmatis} class I and II linezolid-resistant mutants, suggesting different mechanisms of resistance (8). Class I mutants, with alterations in 23S rRNA, had high MICs of \(>64\,\mu g/ml\). Class II mutants had wild-type growth characteristics, unaltered peptidyl transferase activity, and MICs of 4 to 8\,\mu g/ml (8). If similar mechanisms for the emergence of resistance are assumed for the isolated \textit{M. tuberculosis} strains, then they could be classified as class II mutants (lacking mutations and having similar growth characteristics and MICs). Sander and coworkers proposed a nonribosomal resistance mechanism for class II mutants (8). Further studies on the functionality of the ribosomes of the linezolid-resistant \textit{M. tuberculosis} strains, the investigation

![FIG. 1. Diagrammatic representation of the time-dependent development of drug resistance in the four linezolid-resistant strains. The drugs for which resistance was determined over time are indicated in boxes. INH, isoniazid; RMP, rifampin; SM, streptomycin; PZA, pyrazinamide; EMB, ethambutol; OFX, ofloxacin; LEV, levofloxacin; LAM, lamprene; LIN, linezolid; PTH, protoniamide; AK, amikacin; CM, capreomycin; RBT, rifabutin; MOX, moxifloxacin; CS, cycloserine. Arrows indicate drug susceptibility tests with a result of linezolid susceptibility, and asterisks specify susceptible parent strains that were chosen for MIC determinations.](http://aac.asm.org/)

### TABLE 1. Primers used in the present study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name (sequence [5’-3’])&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>23S rRNA</td>
<td>23SF500* (5’CCTTGGTGTGGGTTGGTGTG) 23SR1268 (5’TCAAAAGGCAGCCATCAGCA)</td>
</tr>
<tr>
<td>23SF1001 (5’GGTTAGAAGCTGAGGAGG)</td>
<td>23SF2030* (5’AGAACCTTGCCCGCCGAAAGA) 23SR3910* (5’CATGCGGCCTGGCAAGCTTAG)</td>
</tr>
<tr>
<td>23SF1488 (5’GGTTGAAGACTGAGGGGATGAG)</td>
<td>23SF2454 (5’GCCAGATTTACGGGGGACATT) 23SR3307 (5’AAGACCCACAGGCGGCGCTT)</td>
</tr>
<tr>
<td>23SF2030* (5’AGAACCTTGCCCGCCGAAAGA)</td>
<td>23SF3075 (5’GGGACAGTCCGCGGGGCTG) 23SR3588 (5’AGATGCTTTCAGCGGTTATTC)</td>
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<td>23SF2454 (5’GCCAGATTTACGGGGGACATT)</td>
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<sup>a</sup> The primers were designed to correspond to sequences with GenBank accession numbers BX842576.1 (23S RNA; H37Rv region 90499 to 93929), BX842574.1 (rplD [Rv0702] and rplV [Rv0706]), BX842578.1 (erm-37; Rv1988), and BX842582.1 (whiB7; Rv3197A). Primers with asterisks were used for amplification. With the exception of primers EMT1 and EMT2 (2), all primers were newly designed for this study.
of potential reserpine-resistant efflux pumps, or a reduced input rate of the drug could elucidate the mechanism of linezolid resistance in *M. tuberculosis*.

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REFERENCES