Antimicrobial Resistance to Linezolid

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Acquired resistance to linezolid, the first approved oxazolidinone, has been selected in laboratory experiments and has been observed in clinical isolates of gram-positive cocci. This resistance has typically been associated with single-nucleotide changes in varying numbers of copies of the genes encoding 23S ribosomal RNA. In the current environment of increasingly prevalent resistance to standard antibiotics, linezolid is an important drug because of its activity against a number of clinically significant gram-positive cocci, including multidrug-resistant staphylococci and enterococci. Although resistance to linezolid remains uncommon, the development of resistance by clinical isolates should prompt increased attention to susceptibility testing for this agent and should be taken into account in consideration of the therapeutic use of this drug.

Linezolid (Zyvox; Pfizer) is the first member of the oxazolidinone class of antimicrobials approved for use in the United States, and oxazolidinones are the first new class of antibacterial agents that have been introduced since 1980 [1]. After a priority review, the US Food and Drug Administration (FDA) approved the use of linezolid, in April 2000, for vancomycin-resistant Enterococcus faecium (VREF) infection, nosocomial pneumonia, and complicated skin and skin-structure infections. Linezolid is the first agent approved to treat infections caused by methicillin-resistant Staphylococcus aureus (MRSA) in >40 years [2] and the second agent (and first oral agent) approved to treat infections caused by vancomycin-resistant enterococci (VRE).

Early studies showed that linezolid was active against a number of clinically important gram-positive cocci, including S. aureus, Staphylococcus epidermidis, Enterococcus species, and streptococci [3]. In the results of time-kill assays, linezolid demonstrated bacteriostatic activity against staphylococci and enterococci (when the conventional definition of bactericidal activity was used—namely, the killing of ≥3.0 log cfu/mL during a 24-h incubation), and it demonstrated bactericidal activity against some streptococci [3].

Linezolid also showed activity against aerobic and anaerobic gram-positive bacilli, anaerobic gram-positive cocci, some gram-negative anaerobes, Nocardia species, and mycobacteria species [1–3]. However, oxazolidinones generally are not active in clinically relevant concentrations against aerobic gram-negative organisms, such as Escherichia coli. With regard to this organism, resistance is most likely due to drug efflux by AcrAB, a proton–motive force efflux pump [4].

Linezolid has a number of characteristics that were thought to be likely to mitigate against the development of drug resistance. First, linezolid is a totally synthetic agent; thus, there is a low probability of pre-existing, naturally occurring resistance mechanisms, such as those that might exist in antibiotic-producing organisms or in organisms that share the same environment with an antibiotic producer [1]. Second, although the precise mechanism of action has not been determined conclusively, numerous studies have established that oxazolidinones inhibit bacterial ribosomal protein synthesis [5–8], and existing mechanisms of resistance to other ribosomal agents do not confer cross-resistance to linezolid [9]. Third, linezolid binds to rRNA [7], specifically to domain V of the 23S rRNA of the 50S ribosomal subunit, which is encoded by genes (rDNA) present in multiple copies in clinically relevant species. For example, 4 copies are present in Enterococcus faecalis, and 5–6 copies are present in E. faecium and S. aureus [10]. Selection for mutational resistance was predicted to be difficult, since resistance would likely require mutations in multiple copies of 23S rDNA [11]. Finally, in vitro studies have demonstrated that selection for linezolid-resistant mutants of various species is difficult [3].

IN VITRO STUDIES OF LINEZOLID RESISTANCE

Perhaps because of the synthetic nature of linezolid, susceptibility to this agent was remarkably uniform in large surveys of naive
populations of target organisms [12]. As noted above, several studies have established that oxazolidinones inhibit bacterial ribosomal protein synthesis [5–8]. Fines and Leclercq [9] looked for cross-resistance to linezolid by looking for mechanisms of resistance to other inhibitors of protein synthesis (such as chloramphenicol, macrolides, lincosamides, streptogramins, aminoglycosides, and tetracyclines). The in vitro activity of linezolid was determined by use of isogenic pairs of isolates harboring genes encoding resistance mechanisms—such as modifying enzymes, efflux pumps, and modification or protection of the bacterial ribosome—known to confer resistance to these other antibiotics. Fines and Leclercq determined that none of these mechanisms conferred cross-resistance to linezolid.

In vitro studies of candidate oxazolidinone compounds (linezolid and eperezolid) showed that resistance occurred rarely through spontaneous mutation in S. aureus (at a frequency of <1 linezolid-resistant mutant per 8 × 10^{11} cfu tested) and only with some difficulty (in 1 isolate of E. faecalis) or not at all (in 3 S. aureus isolates and 1 E. faecalis isolate) under selective antimicrobial pressure, using a spiral gradient protocol [3]. In the first description of a mechanism for acquired resistance to linezolid, Swaney et al. [13] showed that laboratory-derived linezolid- or eperezolid-resistant E. faecalis and S. aureus isolates had a single G→U mutation at position 2447 or 2576 (E. coli numbering system) of the central loop of domain V of 23S rRNA. Prystowsky et al. [14] also studied laboratory-derived linezolid-resistant enterococci and identified mutations that mapped to domain V, including C2512U, G2513U, C2610G, and G2505A. The finding that 23S rRNA was the target for linezolid was significant, since nearly all bacteria have multiple copies of the gene encoding 23S rRNA [10]. In studies with E. coli, Xiong et al. [11] found that, because of the presence of 7 rRNA gene copies, the preponderance of wild-type rRNA masked spontaneous mutations arising in only one copy. Other researchers have studied linezolid resistance by using organisms with single copies of 23S rRNA. Kloss et al. [15] conducted experiments with Halobacterium halobium, a halophilic organism that contains only one copy of the 23S rRNA gene. In both of these studies, all laboratory-derived linezolid-resistant isolates contained nucleotide mutations that mapped to the domain V region of 23S rRNA.

Sander et al. [16] conducted experiments with a laboratory strain of Mycobacterium smegmatis, which had only 1 functional rRNA gene (vs. 2 copies found in wild-type isolates). This group identified 2 classes of linezolid-resistant mutants. Class I mutants had higher levels of resistance (MIC for linezolid, 64–128 μg/mL), and ribosomes from these mutants were resistant to oxazolidinones in assays in vitro, which indicated that resistance was associated with changes in the ribosomes. Class II mutants, on the other hand, had lower levels of resistance (MIC for linezolid, 4–8 μg/mL), and ribosomes from these mutants showed wild-type susceptibility in assays in vitro. This suggested a nonribosomal mechanism of resistance in these class II mutants, but the specific nature of this mechanism was not determined.

Howe et al. [17] reported results of another in vitro study that selected for linezolid resistance in clinical MRSA isolates, by means of daily passages in liquid media containing increasing concentrations of linezolid. In some strains, emergence of linezolid resistance was accompanied by the loss of macrolide resistance. Sequencing of domain V of 23S rRNA in these strains identified a single mutation, either G2447U or C2192U. However, a linezolid-resistant strain with the G2576T mutation remained resistant to macrolides.

**LINEZOLID RESISTANCE AMONG CLINICAL ISOLATES**

*Enterococci.* The results of a number of these early laboratory investigations suggested that resistance to linezolid might be slow to emerge; in fact, resistance was not seen in staphylococci or streptococci during the initial clinical trials [18]. However, 2 of 169 patients with VREF infection treated with linezolid, as part of the Linezolid Compassionate Use Program (1999), developed resistant infection [18]. Of note, both patients had complicated clinical courses, bacteremia, and indwelling intravascular devices. Zurenko et al. [18] reported that multiple copies of the 23S rRNA gene were present in these isolates and that a G2576T mutation was present in the linezolid-resistant isolates; they also showed that the level of linezolid resistance correlated with an increasing number of copies of the mutant gene. Additional reports of clinical linezolid-resistant E. faecium and E. faecalis have been published subsequently [19–21].

Gonzales et al. [19] described infections due to linezolid-resistant E. faecium and VREF in 5 patients at the researchers’ medical centers. Each patient was treated with linezolid for VRE infection. The 4 isolates available for testing were genetically unrelated, as determined by PFGE. These data, combined with the clinical descriptions, suggest that these cases of infection represented independent events of de novo selection of resistant mutants in the presence of linezolid. Pai et al. [22] performed a follow-up, retrospective, case-control study of linezolid-treated patients, in which they compared case patients with linezolid-resistant VREF infection with control subjects with VREF infection. In general, the case patients received longer courses of linezolid treatment (median of 38 days vs. 11 days for the control subjects), and multivariable logistic regression identified that, among patients infected with VREF, receipt of linezolid prior to hospital admission was independently associated with the development of resistance to linezolid.

A report by Herrero et al. [21] highlighted the potential for nosocomial spread of linezolid-resistant VREF. A liver transplant recipient who received linezolid treatment for an abdom-
inal infection caused by VRE became the apparent index case for a cluster of cases caused by linezolid-resistant VREF (all containing the G2576T mutation in 23S rRNA). Five patients in a liver, kidney, and pancreas transplantation unit who had not received linezolid treatment acquired an *E. faecium* strain that was indistinguishable (by PFGE) from the linezolid-resistant strain. None of these patients had overt evidence of infection by the outbreak strain, and this nosocomial acquisition occurred despite appropriate infection-control measures.

Laboratory studies by Marshall et al. [23] corroborated the earlier work of Zurenko et al. [18], by providing evidence of a gene-dosage effect for mutant alleles of rDNA in a study of 9 *E. faecium* isolates and 1 *E. faecalis* isolate. Specifically, Marshall et al. found that the G2576U mutation was associated with resistance to linezolid in these clinical enterococcal isolates and that the level of resistance correlated with the proportion of mutant 23S rRNA genes. An *E. faecium* isolate with a mutation in 1 of 6 23S rRNA genes had an MIC of 8 μg/mL for linezolid, whereas a second *E. faecium* isolate with mutations in 5 of 6 23S rRNA genes had an MIC of 64 μg/mL for linezolid.

Marshall et al. [23] also suggested that the presence of clinical isolates containing multiple mutated copies of the 23S rRNA gene might indicate homologous recombination between mutant and wild-type copies, under antibiotic selective pressure [23]. Lobritz et al. [24] studied this phenomenon, referred to as gene conversion, in experiments with laboratory strains of *E. faecalis*. These authors found that a resistant mutant with an MIC of 4 μg/mL for linezolid (vs. a baseline MIC of 2 μg/mL) had 2 of 4 copies of 23S rDNA bearing the G2576T mutation; however, in a recombination-deficient *E. faecalis* strain, a different mutation, G2505A, was selected. Apparently, although it may theoretically have been able to mutate a single copy to G2576T at the same rate as the wild-type strain, the recombination-deficient strain could not perform the subsequent recombination events to acquire a sufficient percentage of mutated copies to show detectable resistance.

Mazur et al. [25] studied another potential impact of linezolid resistance—namely, the relative fitness of clinical isolates of linezolid-resistant *E. faecalis* with the G2576U mutation. Growth rates were determined in brain-heart infusion broth and in Mueller-Hinton broth, with and without linezolid supplementation. They found that the presence of 6 copies of the G2576U mutation led to decreased fitness but that the presence of 4 copies did not, as determined by in vitro growth in the absence of antibiotic pressure. The nosocomial spread of linezolid-resistant enterococci to untreated patients, as documented by Herrero et al. [21], supports the concept that linezolid-resistant enterococci may remain relatively fit [21].

**S. aureus.** In July 2001, Tsiodras et al. [26] reported the first clinical staphylococcal isolate with resistance to linezolid, which was recovered from a patient receiving oral linezolid for treatment of peritoneal dialysis–associated peritonitis. The linezolid-resistant isolate appeared after the drug was used to treat a genetically distinct MRSA isolate (as determined by PFGE). Analysis of the 23S rRNA–encoding sequences of the resistant isolate revealed a G2576T mutation.

In a follow-up study of this strain, Pillai et al. [27] performed PCR amplification of individual copies of 23S rDNA, followed by PCR amplification of a portion of the domain V region in each copy. The results of this method, corroborated by detection of a restriction fragment–length polymorphism by Southern blot analysis, confirmed that all 5 copies contained the G2576T mutation. The linezolid-resistant isolate maintained its phenotype despite serial passages in antibiotic-free media and did not show any significant decrease in growth in vitro, compared with genetically unrelated linezolid-susceptible MRSA isolate recovered from the same patient.

Subsequently, additional reports of clinical *S. aureus* isolates with resistance to linezolid emerged. Wilson et al. [28] provided the second report of a linezolid-resistant MRSA isolate from a patient in England who received linezolid therapy (intravenously and then orally) for 21 days for treatment of an empyema. A pretreatment isolate had an MIC of 2 μg/mL for linezolid. Linezolid-resistant isolates (MIC, 8–32 μg/mL) were detected 20 days after the completion of linezolid therapy and were admixed with linezolid-susceptible strains that were indistinguishable by PFGE. Sequencing of domain V of 23S rRNA revealed the G2576T mutation and was consistent with a gene-dosage effect. An isolate with an MIC for linezolid of 8 μg/mL had mutations in 2 of 6 23S rRNA copies, whereas an isolate with an MIC of 32 μg/mL for linezolid had mutations in 5 of 6 23S rRNA copies. Also, the linezolid-resistant isolates had become susceptible to erythromycin.

At the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAc) in 2003, additional reports of clinical linezolid-resistant *S. aureus* were presented. Machado et al. [29] identified a linezolid-resistant MRSA isolate from a patient with cystic fibrosis. This patient had received multiple courses of linezolid therapy to treat *S. aureus* pulmonary infections, and sequencing of domain V of the 23S rRNA gene identified a G2576T mutation. At the ICAAC (and in a subsequent letter to the editor), Paterson et al. [30, 31] described a patient with an MRSA isolate found to have decreased linezolid susceptibility. After the patient had completed 10 days of linezolid and rifampin therapy for ventilator-associated MRSA pneumonia, an MRSA isolate obtained from blood cultures had an MIC of 8 μg/mL for linezolid. Sequencing of domain V revealed that 2 of 5 copies of the 23S rRNA gene had the G2576T mutation. Also of note, the concomitant use of rifampin did not prevent the development of resistance.

We recently reported the characterization of a novel 23S rRNA mutation in sequential MRSA isolates from the blood-
stream of a single patient [32]. The patient was suspected of having an endovascular focus of infection as the source for recurrent cases of MRSA bacteremia, but this site of infection could not be approached surgically. The patient was placed on oral linezolid therapy for suppression, during which linezolid resistance emerged. Analysis of this series of isolates detected the presence of a T2500A mutation in domain V of the 23S rRNA gene, and 2 of the resistant isolates also had lost a single copy of the 23S rRNA gene (a wild-type copy in other isolates in the series). This series of MRSA isolates also demonstrated a gene-dosage effect. The final isolate, recovered 7 months after discontinuation of linezolid, had reverted to a susceptible phenotype, with the loss of the T2500A mutation. This suggests that gene conversion resulted in replacement of mutant with wild-type 23S rDNA copies and in reversion to the wild-type phenotype, in the absence of antibiotic pressure.

Other gram-positive organisms. Reports of linezolid resistance among other gram-positive organisms have been unusual. Data from the SENTRY Antimicrobial Surveillance Program, which screened 9833 gram-positive isolates from 1 January 2001 to 30 June 2002, revealed that 8 linezolid-resistant strains had been detected in 8 different patients in 7 different participating institutions (from 6 states) [33]. In addition to 6 linezolid-resistant enterococci, 1 S. epidermidis strain and 1 Streptococcus oralis strain were found. Although linezolid resistance remains rare (8 [0.08%] of 9833 isolates), this report highlighted the fact that resistance was no longer limited to enterococci and S. aureus. No information was provided on the suspected mechanism of resistance.

Similar results also were described in the recent report from a large survey of oxazolidinone resistance across 4 continents (North America, South America, Europe, and Asia): Jones et al. [34] analyzed a total of 200 gram-positive organisms collected in 2002 from 55 different laboratories. They found no significant variation in the distribution of MICs for linezolid between geographic regions, but they did identify 4 resistant isolates (all from the United States), including 1 isolate each of S. aureus, coagulase-negative staphylococci, viridans group streptococci, and E. faecium. All 4 isolates had the G2576U mutation.

No reports of clinical Streptococcus pneumoniae isolates with resistance to linezolid have yet been published; however, several groups used in vitro models to study this possibility [35, 36]. In each report, resistance was due to mutations that mapped to domain V of 23S rRNA (4 copies in S. pneumoniae). Carsenti et al. [35] also showed that 2 of 28 strains (selected for linezolid resistance by means of daily passages in subinhibitory concentrations of linezolid) had reverted to erythromycin susceptibility. In addition, these 2 strains had lost expression of the erm gene.

**CLINICAL RELEVANCE OF LINEZOLID RESISTANCE**

Overall, linezolid resistance remains uncommon, although some centers have reported notable rates of resistance among VRE isolates: for example, 4% of 98 VRE isolates from sterile sites were found to be nonsusceptible in a recent study [37]. Clinical microbiology laboratories should test appropriate isolates for susceptibility to linezolid and should perform surveillance to track resistance, in an effort to detect outbreaks of resistant organisms. Certain mutations of a limited number of the multiple copies of 23S rDNA, which confer resistance to linezolid, do not seem likely to be particularly deleterious to bacterial fitness. To date, all of the published reports describing genetically characterized linezolid-resistant clinical isolates have described mutations of the 23S rRNA genes; thus, the likelihood of transferable resistance conferred by this mechanism seems fairly remote, although not impossible. Resistance among gram-positive cocci will most likely continue to arise in individual patients exposed to the drug, and, as has been reported for linezolid-resistant enterococci, transmission of resistant clones from patient to patient will occur, even in the absence of selective pressure from linezolid.

On the basis of case reports of linezolid-resistant clinical isolates, a number of fairly predictable caveats emerge that pertain to the therapeutic use of this drug. Linezolid should be used with caution in cases involving infected sites with poor penetration of the drug, infected foreign bodies, lengthy and/or repeated courses, long-term suppression, oral treatment, and patients undergoing hemodialysis (which may reduce serum levels of linezolid [38], increasing the risk of selection for resistance).

Characterization of resistance mutations in clinical isolates may be helpful in efforts to design the next generation of oxazolidinones. To our knowledge, linezolid-resistant isolates appear to be cross-resistant to the currently reported investigational oxazolidinones, although some of these drugs are incrementally more active than linezolid. Despite some tantalizing data demonstrating the loss of erythromycin resistance that accompanies the emergence of resistance to linezolid [17, 28, 35], no convincing evidence to date indicates that the use of other drugs in combination with linezolid will prevent the development of resistance. In view of the growing prevalence of multiresistant gram-positive cocci and the relatively limited treatment options for many of the infections caused by these organisms, oxazolidinones are likely to remain an important part of our antimicrobial formulary.

**Acknowledgments**

The authors thank Robert C. Moeller, Jr., for critical review of the manuscript.

**Conflict of interest.** V.G.M. and H.S.G. are recipients of an Independent Medical Grant from Pfizer.
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