In Vitro Resistance Studies with Bacteria that Exhibit Low Mutation Frequencies: the Prediction of “Antimutant” Linezolid Concentrations Using a Mixed Inoculum Containing both Susceptible and Resistant Staphylococcus aureus

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Bacterial resistance studies using *in vitro* dynamic models are highly dependent on the starting inoculum that might or might not contain spontaneously resistant mutants (RMs). To delineate concentration-resistance relationships with linezolid-exposed *Staphylococcus aureus*, a mixed inoculum containing both susceptible cells and RMs was used. An RM selected after the 9th passage of the parent strain (MIC 2 μg/ml) on antibiotic-containing media (RM9; MIC 8 μg/ml) was chosen for the pharmacodynamic studies because the MPC of linezolid against the parent strain in the presence of RM9 at 10^2 (but not at 10^3) CFU/ml did not differ from the MPC value determined in the absence of the RMs. Five-day treatments with twice daily linezolid were simulated at concentrations either between the MIC and MPC or above the MPC. *S. aureus* RMs (resistant to 2× and 4×MIC but not 8× and 16×MIC) were enriched at ratios of the 24-hour area under the concentration-time curve (AUC_{24}) to the MIC that provide linezolid concentrations between the MIC and MPC for 100% (AUC_{24}/MIC 60 h) and 86% of the dosing interval (AUC_{24}/MIC 120 h). No such enrichment occurred when linezolid concentrations were above the MIC and below the MPC for a shorter time (37% of the dosing interval; AUC_{24}/MIC 240 h) or when concentrations were consistently above the MPC (AUC_{24}/MIC 480 h). These findings obtained using linezolid-susceptible staphylococci supplemented with RMs support the mutant selection window hypothesis. This method provides an option to delineate antibiotic concentration-resistance relationships with bacteria that exhibit low mutation frequencies.

A specific mechanism of oxazolidinone action blocks functional initiation complexes in bacterial translation systems. Given the lack of analogues among available antimicrobials, a low probability of pre-existing, naturally occurring resistance mechanisms has been hypothesized (1, 2). For example, resistance of *Staphylococcus aureus* rarely occurred through spontaneous mutations at frequencies of only 10^{-9} - 10^{-11} (3-5). However, the first report on the detection of a clinical isolate of *S. aureus* with linezolid MIC >32 μg/ml was published as early as 2001 (6).
Analysis of 23S rRNA encoding DNA sequences showed that linezolid-resistant *S. aureus* had a G to T mutation at position 2576 (*Escherichia coli* numbering). The same point mutations also were observed in *S. aureus* isolated in other clinical studies (7-14). Later, amino acid substitutions in ribosomal protein L3 (50S large-subunit ribosomal protein) were associated with oxazolidinone resistance (linezolid MIC 8 μg/ml) in a clinical *S. aureus* strain (15). Thus, clinical data indicate that resistance to linezolid mediated by mutations in ribosomal genes may emerge more readily than was initially predicted by routine in vitro studies performed early in the development of oxazolidinones, because they were not specifically designed to simulate antibiotic exposures that would allow the enrichment of resistant mutants.

Resistance studies with linezolid cited above were not supported by concomitant pharmacokinetic studies to relate antibiotic concentrations to the selection of resistant mutants. To be sure, some in vitro model studies of the enrichment of resistant *S. aureus* simulated linezolid pharmacokinetics (16, 17). However, these attempts were unsuccessful because resistant mutants were not enriched, at least in simulations of oscillating antibiotic concentrations that mimic the usual linezolid dosing in humans. Linezolid-resistant staphylococci also were not enriched in other in vitro studies that were not designed to establish concentration-resistance relationships (18-20) probably because of the lack of spontaneous mutants in the starting inocula.

To more clearly delineate concentration-resistance relationships, the present study exposes linezolid to susceptible cells of *S. aureus* supplemented with their resistant mutants that had been selected by serial passaging on antibiotic-containing media. The combined inocula were examined with special emphasis on testing the mutant selection window (MSW) hypothesis (21).

**MATERIALS AND METHODS**

**Antimicrobial agents, bacterial strains, and susceptibility testing.** Linezolid powder was kindly provided by Pfizer Corp. (Kalamazoo, MI). *S. aureus* 10, a clinical isolate, with linezolid
MIC of 2 μg/ml and its resistant mutants (RMs, see below) were used in the study. Susceptibility testing was performed in triplicate using broth microdilution techniques after a 24-hour post exposure with the organism grown in Ca²⁺- and Mg²⁺-supplemented Mueller–Hinton broth II (MHB) at an inoculum size of 5×10⁵ colony-forming units (CFU)/ml.

**Resistance selection studies at static conditions.** To select linezolid RMs, serial passages of *S. aureus* 10 were performed daily with MHB containing consecutively increasing concentrations of linezolid (from 1 to 512 μg/ml). For each subsequent daily passage, an inoculum was taken from the tube with the maximal linezolid concentration that showed visual growth that matched the turbidity of a growth control tube. Then, a sample was plated on Mueller-Hinton II agar (MHA) with the same linezolid concentration, and the cycle was repeated. Daily passages and susceptibility testing were performed over 15 days. Stability of resistance was determined by MIC determinations after 15 passages of RM colonies on antibiotic-free MHA.

**Mutant prevention concentration (MPC) determinations.** To optimize the relative concentrations of the parent strain and its RMs in the starting inoculum, MPCs were determined with and without RMs. MPCs were determined as described elsewhere (22). Briefly, the tested microorganism (*S. aureus* 10) was cultured in MHB and incubated for 24 h. The suspension was then centrifuged (4,000×g for 10 min) and resuspended in MHB to yield a concentration of ~10¹⁰ CFU/ml. A mixture of *S. aureus* 10 and its RM was prepared by inoculating the final suspension of the parent strain (10¹⁰ CFU/ml) with RM cells (10² or 10⁴ CFU/ml) to achieve a mutation frequency of 10⁻⁸ or 10⁻⁶. A series of MHA agar plates containing known linezolid concentrations was then inoculated with ~10¹⁰ CFU of *S. aureus* alone or supplemented with RMs. The inoculated plates were incubated for 48 h at 37°C and screened visually for growth. To estimate the MPC, logarithms of bacterial numbers were plotted against antibiotic concentrations. The MPC was taken as the point at which the plot intersected the lower limit of detection (log CFU/ml = 1).
In vitro dynamic model and simulated pharmacokinetic profiles. A previously described dynamic model (23) was used in the study. Briefly, the model consisted of two connected flasks, one containing fresh MHB and the other with a magnetic stirrer, the central unit, with the same broth containing either a bacterial culture alone (control growth experiment) or a bacterial culture plus antibiotic (killing/regrowth experiments). Peristaltic pumps circulated fresh nutrient medium to the flasks and from the central 100-ml unit at a flow rate of 11.6 ml/h.

The system was filled with sterile MHB and placed in an incubator at 37°C. The central unit was inoculated with an 18-h culture of S. aureus. After a 2-hour incubation the resulting exponentially growing cultures reached $\sim 10^8$ CFU/ml ($10^{10}$ CFU per 100 ml central unit), and 1 ml of a bacterial suspension with $10^2$ CFU/ml of RM cells was added to the central unit. Then, a mixture of S. aureus and RMs (one linezolid-resistant cell per $10^8$ CFUs of susceptible cells) was exposed to twice-daily linezolid over 5 days. The duration of the experiments was defined in each case as the time after the last dose when linezolid-exposed bacteria reached numbers observed at the beginning of the experiment ($\geq 10^8$ CFU/ml). To compare initial growth rates of the parent strain and its RMs in antibiotic-free media, the starting inoculum in the central unit was $10^6$ CFU/ml. Each experiment was performed at least in duplicate. Antibiotic dosing and sampling the central unit of the dynamic model were processed automatically, using computer-assisted systems.

A series of monoexponential profiles that mimic twice-daily dosing of linezolid with a half-life of 6 h in accordance with values reported in humans (24) was simulated for 5 consecutive days. The profiles were designed to provide 24-h ratios of area under the curve (AUC$_{24}$) to the MIC from 60 to 480 h. According to the AUCs reported in human pharmacokinetic studies with linezolid (the 12-h AUC of 99.5 mg×h/l (25) and AUC$_{24}$ of 228 mg×h/l (26)), AUC$_{24} \approx 200$ mg×h/l can be taken as a clinically achievable figure. Given this estimate, the simulated AUC$_{24}$/MIC ranges covered the clinically attainable AUC$_{24}$/MIC ratio ($200/2 = 100$ h). The reliability of antibiotic pharmacokinetic simulations and the high...
reproducibility of the time–kill curves provided by the model have been reported elsewhere (16). The determined linezolid concentrations were in concordance with the target values. The estimated half-life of linezolid (6.2 h) was close to the target value (6 h).

Quantitation of the antimicrobial effect, population analysis and susceptibility changes. In each experiment, bacteria-containing medium from the central unit of the model was sampled to determine bacterial concentrations throughout the observation period. Samples (100 µl) were serially diluted as appropriate and 100 µl was plated onto MHA plates, which were placed in an incubator at 37°C for 24 h. The lower limit of accurate detection was $2 \times 10^2$ CFU/ml (equivalent to 20 colonies per plate).

To reveal resistant mutants, each sample was serially diluted if necessary and plated manually onto agar plates containing 2×, 4×, 8×, and 16×MIC of linezolid. The lower limit of detection was 10 CFU/ml (equivalent to at least one colony per plate). Based on time-kill data, area under the bacterial concentration-time curves with mutants (AUBC$_{M5}$ (22)) was determined from the beginning of treatment to 144 h and corrected for the area under the lower limit of detection over the same time interval.

To reveal changes in susceptibility of linezolid-exposed bacterial cultures, MICs were determined prior to and after 5-day simulated linezolid treatments.

Mechanisms of resistance. Nucleotide sequences of the domain V region of 23S rRNA genes of the parental strain, S. aureus 10, and its mutant derivative, RM9, were analysed by PCR amplification and direct sequencing of individual copies of rRNA operons essentially as described elsewhere (11). Since no prior information about the copy number of rRNA genes carried in the genome of S. aureus 10 was available, all published combinations of primers corresponding to the reference genomes of S. aureus N315 (GenBank accession no. NC_002745) and S. aureus MW2 (GenBank accession no. NC_003923) were used: rrn1(1)-rrn1(2), rrn2(1)-rrn2(2), rrn3(1)-rrn3(2), rrn4(1)-rrn4(2), rrn5(1)-rrn5(2) (11, 27), rrn6(1)-rrn2(2), and rrn6(1)-rrn6(2) (11, 28). Bacterial genomic DNA was extracted using the InstaGene Matrix Kit (Bio-Rad...
Laboratories, Richmond, CA, USA). Encyclo Plus PCR kit (Evrogen, Moscow, Russia) was used for “long-range” PCR. The amplification was carried out in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) under the previously described conditions (11). PCR fragments were analyzed by agarose (1%) gel electrophoresis and were used as matrix for sequencing reactions after purification by exonuclease I/shrimp alkaline phosphatase (ExoSAP-IT) treatment (GE Healthcare Ltd., Buckinghamshire, UK). Sequencing of the of the domain V region was performed using the primers described by Meka et al (11), the BigDye terminator v3.1 cycle sequencing kit and ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

RESULTS

Serial passaging cell suspensions of *S. aureus* cultures on linezolid-containing MHB led to a systematic loss in susceptibility beginning from the sixth passage (2-fold MIC elevation). With further passages, the MIC of linezolid increased gradually: 4-fold elevation after the 9th passage and 64-fold elevation after the 15th passage (Fig. 1). Elevated linezolid MICs with mutants selected after the 9th (RM9) and 15th (RM15) passages were stable after 15 passages on antibiotic-free plates. As expected, both mutants showed slower growth in MBH compared to the parent strain, with a more prolonged lag-time (upper plots on Fig. 1). As seen in the figure, growth of RM9 and RM15 started one and two hours later than the parent strain.

To determine the maximal burden of resistant mutants that when added to linezolid-susceptible cells will not shift the MPC estimate using a mutant-free inoculum of *S. aureus*, MPCs were assessed with and without RM9 or RM15. As seen in Fig. 2, curves plotting the numbers of surviving colonies against linezolid concentrations exhibited the same shape with susceptible cells ($10^{10}$ CFU/ml) enriched with RM9 ($10^{2}$ CFU/ml) compared to the mutant-free inoculum. As the viable count - antibiotic concentration plots were virtually superimposed, the MPC of linezolid was the same in both cases (16 µg/ml). However, at the greater RM9 burden ($10^{3}$ CFU/ml), the shape of the concentration relationship with viable counts changed – a distinct
plateau was inherent in the plot, and the estimated MPC was higher than the parent strain (32 µg/ml). Similar plateaus were observed with RM15 both at the minimal (10^2 CFU/ml) and the maximal load (10^4 CFU/ml), and the estimated MPCs were much greater (128-256 µg/ml) than without the mutant (data not shown). Therefore, only the less resistant mutant, i.e., RM9 (10^2 CFU/ml), was used in further simulations using the dynamic model.

To determine the mechanism of decreased linezolid susceptibility in the RM9 mutant, nucleotide sequences of individual copies of domain V region of 23S rRNA genes were analysed and compared to those of the parental strain, *S. aureus* 10. Five copies of the *rrn* locus were detected by PCR, one of which (*rrn3*), in both strains, contained a deletion in 23 rRNA gene as evidenced by the smaller-than-expected size of the PCR product (ca. 2400 bp instead of 5577-5655 bp) and by the lack of sequencing products with primers for domain V region. In addition, RM9 contained a ca. 500-bp deletion in *rrn4* outside the domain V region as compared to the parental strain. Nevertheless, the sequences of the domain V region of all *rrn* loci of the parental strain and RM9 were identical and contained no mutations in comparison to the reference sequence of *S. aureus* MW2 (GenBank accession no. NC_003923).

Simulated pharmacokinetics of linezolid given twice daily for five days and the respective time courses of susceptible and resistant subpopulations of *S. aureus* using mixed inocula (one linezolid-resistant cell per 10^8 CFUs of susceptible cells) are shown in Fig. 3. At the relatively low AUC24/MIC ratios (60 and 120 h), when linezolid concentrations were inside the mutant selection window (*T* _{MSW}) for most of the dosing interval (100% and 86%, respectively), mutants resistant to 2× and 4×MIC (but not 8× and 16×MIC) of antibiotic were enriched, starting from the 2nd-3rd day of treatment. This enrichment continued almost up to complete replacement of susceptible organisms by resistant mutants and was accompanied by 4-fold elevations of the culture MIC. As expected, the amplification of resistant mutants in the total bacterial population kept the linezolid antibacterial effect low. A slight reduction in the initial inoculum was followed by bacterial regrowth, so that bacterial counts reached the initial
inoculum size at 96 h (AUC<sub>24</sub>/MIC 60 h) and 120 h (AUC<sub>24</sub>/MIC 120 h) after the start of simulated treatments.

In contrast, at the higher AUC<sub>24</sub>/MIC ratios, when simulated antibiotic concentrations either fell into the mutant selection window for 37% of the dosing interval (AUC<sub>24</sub>/MIC 240 h) or were consistently above the MPC (AUC<sub>24</sub>/MIC 480 h), the enrichment of resistant mutants did not occur (two right columns in Fig. 3), and there was no loss in susceptibility of linezolid-exposed *S. aureus*. Unlike AUC<sub>24</sub>/MIC of 60 and 120 h, a pronounced reduction in numbers of survivors was observed at AUC<sub>24</sub>/MIC of 240 and 480 h during treatment. Moreover, at AUC<sub>24</sub>/MIC of 480 h, there was no bacterial regrowth even after stopping treatment.

Concentration-dependent differences in the time courses of resistant mutants were consistent with results obtained using the AUBCM parameter, an integral index of time-kill curves observed with resistant mutants (22). Based on population analysis data obtained with RMs resistant to 2×MIC of linezolid, the AUBCMs estimated at the AUC<sub>24</sub>/MIC of 60 and 120 h were as high as 525 and 477 (log CFU/ml)×h, respectively, and with RMs resistant to 4×MIC - 507 and 436 (log CFU/ml)×h, respectively. In contrast, at the AUC<sub>24</sub>/MIC of 240 and 480 h the AUBCMs were equal to zero.

**DISCUSSION**

Using a mixed inoculum of linezolid-susceptible and –resistant *S. aureus* mutants, this *in vitro* study has demonstrated a means to establish concentration-dependent resistance relationships with bacteria that exhibit low mutation frequencies. In five-day simulations of twice-daily linezolid pharmacokinetics, *S. aureus* mutants resistant to 2× and 4×MIC were enriched at lower AUC<sub>24</sub>/MIC ratios (60 and 120 h) but not at higher AUC<sub>24</sub>/MICs (240 and 480 h). The amplification of resistant mutants occurred when linezolid concentrations were inside the MSW for most of the dosing interval (*T<sub>MSW</sub>* 100% and 86% at the AUC<sub>24</sub>/MIC ratios of 60 and 120 h, respectively). When antibiotic concentrations were inside the MSW for only 37% of the dosing
interval (AUC$_{24}$/MIC 240 h) or when they were consistently above the MPC (AUC$_{24}$/MIC 480 h), resistant mutants were not enriched.

The critical importance of achieving antibiotic concentrations above the MPC to restrict bacterial resistance was noted earlier in studies with fluoroquinolones (29-31) and carbapenems (32). These studies led to an awareness of the heterogeneity of the MSW (29) and an understanding of why the maximal time inside the MSW at which resistant mutants are still not enriched is shorter at antibiotic concentrations below the MPC than above MPC (30). As seen in Fig. 3, there was no amplification of resistant mutants only when linezolid concentrations were above the MPC for most or entire dosing interval. Interestingly, these results also can be explained by considering the time above the MIC for RM9 (MIC$_{RM9}$ = 8 μg/ml). Indeed, resistant mutants were not enriched only when linezolid concentrations were above the MIC$_{RM9}$ (two right columns in Fig. 3). Thus, in this specific case, when S. aureus mutants more resistant than RM9 were not selected, the cross section of the concentration – time plots at the level of the MIC$_{RM9}$ appears to be even more discriminative than the MPC. However, consideration of the MICs of resistant mutants may be useful to explain but not predict resistance, because these MICs cannot be predicted before treatment. For example, unlike this linezolid study, the post-exposure culture MICs of ciprofloxacin determined in our study with Escherichia coli (33) were greater but not less than the MPCs. Moreover, these MICs were dependent on antibiotic exposure: the appearance of more resistant mutants was associated with the maximal mutant enrichment. Therefore, currently there are no alternatives to these MPC-based predictions of resistance.

Concentration-dependent differences in the time courses of linezolid-resistant mutants of S. aureus are consistent with the MSW hypothesis, although a relatively narrow, i.e., an eight-fold range of the simulated AUC$_{24}$/MIC ratio made delineation of the full AUC$_{24}$/MIC relationships with AUBCM impossible. In fact, AUC$_{24}$/MIC-dependent changes in the AUBCM can be considered as a fragment of a bell-shaped curve that might be observed over a wide range
of linezolid concentrations. To date, such AUC\textsubscript{24}/MIC-resistance relationships have been established with \textit{S. aureus} exposed to fluoroquinolones (34-35) and glycopeptides (36-37). It is very likely that similar bell-shaped AUC\textsubscript{24}/MIC relationships will be observed with the enrichment of linezolid-resistant staphylococci. The resistant mutant of \textit{S. aureus} 10 (RM9) with a linezolid MIC of 8 µg/ml used in this pharmacodynamic study was selected by passaging the parent strain on antibiotic-containing media. Earlier, the same method had been used in other static resistance studies (4, 5, 38-40) that allowed selection of \textit{S. aureus} mutants with the same linezolid resistance mechanisms as found in clinical mutants. The results of PCR amplification and sequencing of 23S rRNA genes revealed a deletion in one of the copies of \textit{rrn} locus (\textit{rrn4}) but did not reveal specific mutations in domain V region in RM9 as compared to the parental strain. It is possible that decreased linezolid susceptibility in RM9 was due to a combination of deletion in \textit{rrn4} and mutation outside domain V, e.g. in the genes for L3 or L4 proteins, which has been described in both laboratory mutants and clinical isolates of \textit{S. aureus} exhibiting the same low-level resistance as RM9 (5, 15). It should be noted that the RM used to supplement susceptible subpopulations of \textit{S. aureus} 10 in the pharmacodynamic studies and the ratio of RM to the parent strain in the starting inoculum were chosen to avoid RM-induced shifting of the MPC. In the presence of RM9 (at 10\textsuperscript{2} but not 10\textsuperscript{4} CFU/ml) supplemented to susceptible \textit{S. aureus} 10 (10\textsuperscript{10} CFU/ml) the MPC of linezolid did not differ from value determined in the absence of the RMs. Unlike RM9, a more resistant RM15 (MIC 128 µg/ml) did shift the MPC of linezolid towards greater values. The pronounced enrichment of RMs was observed in our simulations with linezolid-exposed \textit{S. aureus} at AUC\textsubscript{24}/MICs of 60 and 120 h (Fig. 3). The latter exposure is similar to the clinically attainable AUC\textsubscript{24}/MIC ratio (100 h – see Materials and Methods) that is two-fold lower than needed to prevent the amplification and/or to suppress the growth of RMs. This shows that dosing decisions based on the overly optimistic expectations that multiple mutations would be
required for the development of resistance to linezolid may be flawed. It appears that the only way to ensure “antimutant” linezolid dosing might be to combine it with other anti-staphylococcal agents. Indeed, both rifampin and gentamicin given in combination with linezolid protected against the enrichment of linezolid-resistant mutants of *S. aureus* in an *in vitro* dynamic model (41, 42). Similarly, combined use of linezolid and doxycycline in subtherapeutic doses blocked the selection of *Enterococcus faecium* resistant to both antibiotics (43).

Overall, this study suggests a methodological option to delineate concentration-resistance relationships with bacteria that exhibit low mutation frequencies. Using a susceptible subpopulation of *S. aureus* supplemented by RMs, a reasonable AUC24/MIC relationship with resistance was established. Hopefully, the potential clinical application of these findings might limit resistance selection with optimal dosing strategies. Also, the use of the mixed inoculum containing susceptible and resistant cells allows better standardization of the experimental conditions compared to the use of conventional inocula that might or might not contain spontaneous mutants.

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REFERENCES


FIGURE LEGENDS

FIG 1 Loss in susceptibility of *S. aureus* 10 passaged on linezolid-containing MHB. Upper plot: *S. aureus* growth in antibiotic-free MHB. Symbols: ○, *S. aureus* 10; △, RM9; □, RM15.

Linezolid MIC for parent strain is indicated by dotted line.

FIG 2 Linezolid MPC determination with *S. aureus* 10 alone and supplemented by RM.

Symbols: ○, parent strain; △, parent strain (10^{10} CFU/ml) + RM9 (10^4 CFU/ml); ▲, parent strain (10^{10} CFU/ml) + RM9 (10^8 CFU/ml).

FIG 3 Simulated pharmacokinetics of linezolid and time courses of susceptible (0× the MIC) and resistant (2×, 4× and 8× the MIC) subpopulations of antibiotic-exposed *S. aureus* supplemented with its RM. Arrows reflects antibiotic dosing.
Viable count (log CFU/ml) | Concentration (µg/ml)
---|---
0 | 0
2 | 2
4 | 4
6 | 6
8 | 8
10 | 10
24 | 24
48 | 48
72 | 72
96 | 96
120 | 120
144 | 144

AUC24/MIC | TMSW %
---|---
60 h | 100%
120 h | 86%
240 h | 37%
480 h | 0%

MIC | MPC
---|---
0×MIC | 0×MIC
2×MIC | 2×MIC
4×MIC | 4×MIC
8×MIC | 8×MIC

2×, 4×, 8×MIC | 2×, 4×, 8×MIC

AUC24/MIC 48h | TMSW 0%