Selection of linezolid-resistant *Enterococcus faecium* in an *in vitro* dynamic model: protective effect of doxycycline

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Objectives: To relate the enrichment of linezolid-resistant *Enterococcus faecium* with linezolid pharmacokinetics, the pharmacodynamics of linezolid and its ability to prevent the selection of resistant mutants were studied in an *in vitro* model that simulates antibiotic concentrations in and out of the mutant selection window (MSW), i.e. the concentration range from the MIC to the mutant prevention concentration (MPC).

Methods: A clinical isolate of *E. faecium* (MIC 1.8 mg/L and MPC 7 mg/L) at a starting inoculum of 8 log cfu/mL was exposed to twice-daily linezolid, alone and in combination with once-daily doxycycline (MIC 0.2 mg/L and MPC 3.4 mg/L), for 3 consecutive days in a hollow-fibre two-compartment model.

Results: The ratios of 24 h area under the curve (AUC24) to MIC of linezolid were estimated at 70, 100 and 230 h and those of doxycycline were estimated at 230 and 720 h. At the two lower AUC24/MIC ratios of linezolid given alone, *E. faecium* resistant to 2 × MIC–16 × MIC and 2 × MIC–8 × MIC of linezolid, respectively, were selectively enriched with a concomitant slight loss in susceptibility. Neither growth on linezolid-containing media nor changes in susceptibility occurred at the high AUC24/MIC ratio. A similar protective effect was observed with the minimal AUC24/MIC ratio of linezolid (70 h) combined with doxycycline at an AUC24/MIC of 230 h.

Conclusions: This study suggests that selection of linezolid-resistant enterococci can be predicted from the MSW concept and can be prevented by linezolid given in combination with doxycycline, each at suboptimal AUC24/MIC ratios.

Keywords: pharmacodynamics, resistant *E. faecium*, oxazolidinones, enterococci

Introduction

Linezolid is a new oxazolidinone antibiotic active against Gram-positive cocci including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. However, despite its relatively recent clinical introduction, linezolid-resistant *S. aureus*¹ and *E. faecium*²–⁴ have been isolated from patients who received prolonged courses of the drug. Recent attempts in our laboratory to enrich resistant *S. aureus* exposed *in vitro* to fluctuating concentrations of linezolid in a dynamic model that simulates antibiotic pharmacokinetics were unsuccessful,⁵,⁶ possibly because of small numbers of resistant cells in the starting inoculum. The aim of this study was to use the same model to detect linezolid-resistant *E. faecium* at antibiotic concentrations in and out of the mutant selection window (MSW)⁷ and to evaluate the ability of doxycycline to prevent the enrichment of resistant enterococci.

Materials and methods

Antimicrobial agents, bacterial strain and susceptibility testing

Linezolid and doxycycline powders were provided by Pfizer, Inc. (Groton, CT, USA). A vancomycin-resistant clinical isolate of *E. faecium*, strain 392, was used for this study; vancomycin MIC was 80 mg/L, linezolid MIC was 1.8 mg/L, and doxycycline MIC

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was 0.20 mg/L. Susceptibility testing was performed by broth macrodilution techniques with the organism grown in brain–heart infusion (BHI) broth at an inoculum size of $5 \times 10^8$ cfu/mL.

The mutant prevention concentrations (MPCs) were determined as described elsewhere. Briefly, the tested microorganisms were cultured in fresh BHI broth and incubated overnight at 36°C. Then, two 50 mL aliquots were centrifuged at 2000 rpm for 30 min. The supernatant was discarded and the remaining pellets resuspended in 3 mL of fresh BHI. The 3 mL aliquots were thoroughly vortexed and combined in one tube to yield a concentration of $10^{10}$ cfu/mL. From the combined 6 mL suspension, one to eight dilution steps were made in sterile saline and 200 µL from each dilution step was plated on antibiotic-containing Trypticase soy agar (TSA) supplemented with 4% sheep blood (SB) plates that were prepared in our laboratory. Concentrations for the linezolid plates ranged from 1.80 to 46.15 mg/L, and from 0.20 to 5.12 mg/L for doxycycline.

Previously described dynamic model

A previously described dynamic model\(^7\) was used in the study. Briefly, this two-compartment model consists of a central compartment and three bioreactors, artificial chambers (Fibercell Systems Inc., Frederick, MD, USA) connected in series, which represent the peripheral compartments. For all experiments, the bacterial inoculum was prepared from previously frozen inocula by thawing, diluting with an equal part of BHI and incubated for 90 min at 36°C to bring the organisms into growth phase.

This mixture was then inoculated into each peripheral compartment, which also contained BHI, via an entry port and incubated until a density of $\sim 10^{10}$ cfu/mL was achieved, at which time the antibiotic was introduced into the central compartment (time zero). Given a 20 mL volume of the peripheral compartment, the total number of organisms in the starting inoculum reached $\sim 2 \times 10^9$ cfu. Control experiments without antimicrobial agent were performed to characterize growth kinetics. All dynamic model experiments were performed in triplicate.

A series of monoexponential profiles that mimic twice-daily administration (60 min infusion) of linezolid (half-life 6 h)\(^9\) and once-daily doxycycline (half-life 15 h)\(^10\) alone and in combination (1:3 ratio based on 24 h AUC/MICs) were simulated for 3 consecutive days. Overall, six dosing regimens were simulated: linezolid AUC\(_{24}\)/MIC 60 h; linezolid AUC\(_{24}\)/MIC 120 h; linezolid AUC\(_{24}\)/MIC 240 h; doxycycline AUC\(_{24}\)/MIC 240 h; doxycycline AUC\(_{24}\)/MIC 720 h; linezolid AUC\(_{24}\)/MIC 60 h + doxycycline AUC\(_{24}\)/MIC 240 h.

To provide simultaneous monoexponential elimination of linezolid and doxycycline, a previously described dynamic model was modified according to Zinner et al.\(^11\)

As the antimicrobial effect depends on antibiotic concentration in the peripheral compartments (where the organisms come into contact with antibiotic), peripheral compartments were sampled to determine linezolid concentrations by bioassay using well plates of TSA seeded with a clinical strain of coagulase-negative Staphylococcus and doxycycline with bioassay well plates of Antibiotic Medium #8 seeded with Bacillus cereus ATCC 11778.

Quantification of the antimicrobial effect and susceptibility changes

In each experiment, the peripheral compartments were sampled to determine bacterial concentrations. The numbers of surviving organisms were determined by serial dilution of samples in cold sterile saline and inoculating 20 µL in triplicate onto commercially available Mueller–Hinton agar supplemented with 5% SB. After overnight incubation at 36°C, the resulting bacterial colonies were counted and the numbers of cfu/mL were calculated. The detection limit was 10 cfu/mL. The duration of the experiments was defined as the time (after the last dose) until antibiotic-exposed bacteria reached the same maximum numbers as observed in the absence of antibiotic ($>10^8$ cfu/mL).

Changes in susceptibility of E. faecium 392 were examined by repeated MIC determinations and by plating a standard loopful of each 24 h specimen on TSA-SB plates containing no antibiotic, 2×MIC, 4×MIC, 8×MIC, and 16×MIC of linezolid or doxycycline. The stability of observed resistance was determined daily by consecutive passaging E. faecium on antibiotic-free agar plates for 5 consecutive days.

Results

Pharmacodynamics and resistance in single-drug treatments with linezolid

Simulated pharmacokinetics and killing kinetics of linezolid-exposed E. faecium are presented in Figure 1. As seen in the top panel of the figure, the estimated AUC\(_{24}\)/MIC ratios were close to the target values: 70 h versus 60 h, 100 h versus 120 h, and 230 h versus 240 h. At the lowest AUC\(_{24}\)/MIC, linezolid concentrations fell in the MSW for most of the dosing interval: after the first dose, the time within the MSW ($T_{MSW}$) was 100% of the interval. At the intermediate AUC\(_{24}\)/MIC, linezolid concentrations were within the MSW for a shorter period: $T_{MSW}$ of 70%, whereas at the highest AUC\(_{24}\)/MIC, the simulated concentrations were above the MPC throughout the dosing interval (out of the MSW): $T_{MSW}$ approached zero. The bottom panel of Figure 1 shows similar patterns of the time–kill curves: a gradual decrease in viable counts over 80 h (AUC\(_{24}\)/MIC 70 h), 90 h (AUC\(_{24}\)/MIC 100 h) and 100 h (AUC\(_{24}\)/MIC 230 h) was followed by bacterial regrowth. The respective $I_E$ were AUC\(_{24}\)/MIC-dependent: the higher the AUC\(_{24}\)/MIC, the greater the $I_E$ (see diagram in the right corner of the figure). The AUC\(_{24}\)/MIC-response relationship was log-linear (data not shown).

Population analysis of specimens sampled from peripheral units of the dynamic model demonstrates a clear relationship between the enrichment of resistant mutants and simulated pharmacokinetics of linezolid (Figure 2). Treatments of E. faecium at AUC\(_{24}\)/MIC of 70 and 100 h that provide antibiotic concentrations within the MSW over at least a part of the first dosing interval ($T_{MSW}$ 100% and 70%, respectively) were accompanied by bacterial growth on the antibiotic-containing plates. This growth in the presence of 2×, 4× and 16×MIC, but not 8×MIC of linezolid, began earlier at AUC\(_{24}\)/MIC of 70 rather...
than 100 h. The concomitant loss in susceptibility observed by 72–96 h after the start of treatment was more pronounced at AUC\textsubscript{24}/MIC = 70 h (post-exposure MIC 2.2-fold higher than the pre-exposure MIC) than at AUC\textsubscript{24}/MIC = 100 h (1.5-fold difference between pre- and post-exposure MICs). This loss at both simulated AUC\textsubscript{24}/MICs was unstable: the differences between pre- and post-exposure MICs disappeared after six passages (data not shown). Unlike the lower AUC\textsubscript{24}/MICs, at the highest AUC\textsubscript{24}/MIC ratio (230 h) when antibiotic concentrations were out of the MSW, the starting population was not enriched by resistant mutants and the susceptibility of \textit{E. faecium} was not changed.

**Pharmacodynamics and resistance in combined treatments with linezolid and doxycycline**

Comparative killing kinetics of \textit{E. faecium} in treatments with linezolid and doxycycline given alone and in combination are shown in Figure 3. As seen in the figure, despite the rapid initial killing of \textit{E. faecium} exposed to doxycycline, its effect was

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**Figure 1.** \textit{In vitro} simulated pharmacokinetics of linezolid (upper panel) and time–kill curves of \textit{E. faecium} exposed to linezolid (bottom panel). Arrows indicate linezolid administration. The respective AUC\textsubscript{24}/MIC ratio (h) is indicated for each curve. Diagram in the right corner of the bottom panel shows AUC/MIC-dependent antimicrobial effects of linezolid (see text for a description of the determination of $I_E$).

**Figure 2.** Effect of AUC\textsubscript{24}/MIC on the selection of linezolid-resistant \textit{E. faecium} after exposure to linezolid: population analysis. Simulated AUC\textsubscript{24}/MIC ratios (in hours) are indicated for the curves.
transient; bacterial regrowth was observed after the first dose. Much more stable effects were seen with linezolid (regrowth only after the end of treatment), although the simulated AUC_{24}/MIC of linezolid was 3.4 times lower than that of doxycycline (70 h versus 230 h). Both in terms of the minimal number of surviving organisms and the time to regrowth, even more pronounced killing was observed in the combined treatment of *E. faecium* with linezolid and doxycycline. On the basis of the comparison of the respective *I_{ES}* (see the diagram in the right corner of Figure 3), the combination of linezolid with doxycycline was synergistic: the *I_{ES}* that reflects the effect of combined treatment was 4.5 times greater than the sum of linezolid and doxycycline *I_{ES}* in the single-drug treatments.

The studied combination also appeared to be efficient in preventing the selection of linezolid-resistant *E. faecium*. As seen in Figure 4 (left-hand panel), organisms resistant to 2×MIC were enriched much later in the combined treatment than with linezolid monotherapy at the same AUC_{24}/MIC. Moreover, enterococci resistant to the higher linezolid concentrations (4×, 8× and 16×MIC) were not enriched at all in the presence of doxycycline, with no loss in susceptibility of antibiotic-exposed *E. faecium*. In contrast, the selection of doxycycline-resistant organisms also began later in combinations with linezolid, but it occurred at all doxycycline concentrations (from 2×MIC to 16×MIC) (Figure 4, right-hand panel).

The described effects of linezolid on doxycycline pharmacodynamics and the emergence of enterococcal resistance to doxycycline could not be achieved by using a greater AUC_{24}/MIC ratio of doxycycline given alone. As seen in Figure 5, an almost 4-fold increase in the simulated AUC_{24}/MIC (from 230 to 720 h) with *T_{MSW}* of 100% and 35% of the first dosing interval, respectively (upper panel), provided only a 1 day delay in regrowth of the doxycycline-resistant subpopulation with similar minimal numbers of surviving organisms (upper middle panel). In contrast to these minor differences between the time–kill curves, the combination of doxycycline (AUC_{24}/MIC 230 h) with linezolid (AUC_{24}/MIC 70 h) delayed regrowth for 4 days and yielded a much smaller minimal number of survivors.

Similarly, at the increased AUC_{24}/MIC (720 h), the amplification of *E. faecium* resistant to 2×–16×MIC of doxycycline occurred at the same time as at the lower AUC_{24}/MIC (230 h)—both much earlier than with the combined treatment—see the lower middle panel in Figure 5 showing the respective data on plates with 4×MIC of doxycycline. Similar data were obtained on antibiotic-containing plates at 2×, 8× and 16×MIC (data not shown). Minimal differences in the amplification of resistant *E. faecium* exposed to the smaller and larger AUC_{24}/MICs of doxycycline are in concordance with similar concomitant changes in susceptibility of doxycycline- and doxycycline plus linezolid-exposed organisms (Figure 5, bottom panel). At both AUC_{24}/MICs, the MICs of doxycycline increased 3 days earlier with doxycycline monotherapy than with the doxycycline/linezolid combination, although a similar loss in doxycycline susceptibility (30- to 35-fold difference between pre-exposure and post-exposure MICs) occurred in all treatments.

**Discussion**

This *in vitro* study demonstrates concentration-dependent pharmacodynamics of linezolid and selection of linezolid-resistant mutants, in this case with *E. faecium*. A log-linear relationship was established between the intensity of the antimicrobial effect and simulated AUC_{24}/MIC ratios in 3 day treatments with linezolid. In concordance with the MSW hypothesis, *E. faecium* mutants resistant to 2–16×MIC of linezolid were enriched when the antibiotic concentrations were within the MSW (AUC_{24}/MIC 70 and 100 h), but not when concentrations were out of the MSW (AUC_{24}/MIC of 230 h). The estimated ‘anti-MSW AUC_{24}/MIC ratio’ is comparable to those reported in our studies with fluoroquinolone- and glycopeptide-exposed *S. aureus* (201–244 h^{14} and 200 h^{15}) and with moxiﬁxacin-exposed *Streptococcus pneumoniae* (>100 h)^{16} as well as in a similar *in vitro* study with garenxacin-exposed *S. aureus* (190 h)^{17} Given these consistent findings to other antibiotic-pathogen pair organisms, it is unclear why linezolid-resistant *S. aureus* did not emerge resistance in a similar experimental setting.5,6

In the present study with a specific strain of *E. faecium*, an AUC_{24}/MIC ratio >200 h was estimated to protect against the selection of linezolid-resistant enterococci. This estimated value is two times larger than the AUC_{24}/MIC of 100 h (186 mg×h/L^{3}/1.8 mg/L) that can be provided by a 600 mg clinical dose of twice-daily linezolid. One approach for restricting resistance is the use of linezolid in combination with other antibiotics. The combination of linezolid plus doxycycline (both at AUC_{24}/MICs at which amplification of resistant *E. faecium* occurred in monotherapy) appears promising. Doxycycline was shown to strengthen the effects of linezolid on both susceptible and resistant *E. faecium* subpopulations. Unlike monotherapy, organisms resistant to 2×MIC of linezolid were enriched only after the end of the combined treatment, and those resistant to 4–16×MIC were not enriched at all. In contrast to linezolid-resistant staphylococci, the combined use of linezolid with doxycycline was able to delay the selection of enterococcal mutants resistant to 4–16×MIC of doxycycline, but did not prevent it. This can be explained by the fact that the simulated concentrations of linezolid (AUC_{24}/MIC = 70 h) were within its MSW over only part of the dosing interval, whereas concomitant concentrations of doxycycline (AUC_{24}/MIC = 240 h) were
Figure 4. Selection of linezolid (LZD)- and doxycycline (DOX)-resistant *E. faecium* after exposure to LZD alone and in combination with DOX: population analysis.
within its MSW over the entire dosing interval. Although linezolid influenced the pharmacodynamics of doxycycline to a lesser extent than doxycycline did with linezolid, the linezolid-induced delay in the selection of doxycycline-resistant enterococci was much more pronounced than what could be provided by a 4-fold greater doxycycline AUC$_{24}$/MIC ratio given alone. Earlier, a similar combination of suboptimal AUC$_{24}$/MICS of moxifloxacin and doxycycline, i.e. AUC$_{24}$/MICS smaller than the anti-mutant values, was shown to prevent the enrichment of resistant staphylococci.\(^{18}\)

On the basis of the comparison of \(I_{E8}\) determined in simulations of the pharmacokinetics of linezolid and doxycycline given alone and in combination, the combination’s effect on the susceptible subpopulation of \(E.\ faecium\) was synergistic. Recently, similar synergism was reported in our pharmacodynamic studies with the same combination against \(S.\ aureus\).\(^5\,^6\) These findings are consistent with a clinical report on bacteriologic cure of a neutropenic patient with fever caused by VRE with the addition of doxycycline to linezolid after unsuccessful monotherapy with daptomycin and linezolid.\(^2\) These data are not consistent with an in vitro study with \(E.\ faecium\)\(^{19}\) that demonstrated only minimal if any advantages of the linezolid/doxycycline combination in a relatively short 48 h simulation.

Overall, these data suggest that linezolid-resistant enterococci are enriched when drug concentrations fall into the MSW and that the amplification of resistant mutants may be restricted by using linezolid in combination with doxycycline, even if both agents are given at suboptimal individual doses.

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**Transparency declarations**

None to declare.

**References**


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**Figure 5.** \textit{In vitro} simulated pharmacokinetics of doxycycline (DOX)—upper panel; time–kill curves of \textit{E. faecium} exposed to DOX alone and in combination with linezolid (LZD)—upper middle panel; bacterial survival on plates with \(4 \times\) MIC of DOX—lower middle panel; and time courses of changes in susceptibility of \textit{E. faecium} to DOX—bottom panel. Arrows indicate DOX administration. Simulated AUC$_{24}$/MIC ratios (in hours) are indicated in parentheses.