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In Vitro Activities of an Investigational Quinolone, Glycylcycline, Glycopeptide, Streptogramin, and Oxazolidinone Tested Alone and in Combinations against Vancomycin-Resistant *Enterococcus faecium*

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We evaluated the in vitro activities of clinafloxacin, CL331,002, LY333328, quinupristin dalfopristin, and eperezolid (formerly known as U-100,592) against four strains of enterococci. All regimens tested resulted in the growth inhibition of each isolate. Against the three clinafloxacin-susceptible strains, clinafloxacin tested alone was the most active treatment, decreasing the bacterial inoculum by more than 3 $\log_{10}$ CFU/ml after 24 h in time-kill curve studies.

Enterococcus has emerged as a prominent nosocomial pathogen over the last decade. The prevalence of infections due to enterococcal species has been increasing, and enterococci are now classified as the fourth leading cause of nosocomial infections (8, 14). Enterococci have a low virulence potential, but problems arise from their ability to become resistant to multiple antibiotics. Combination therapy is required in order to obtain bactericidal activity against enterococci (4, 13). Typically, penicillin, ampicillin, or vancomycin in combination with an aminoglycoside is the treatment of choice for enterococcal endocarditis (10). However, high-level resistance to aminoglycosides and chromosomally mediated beta-lactam-resistance can prevent synergy from occurring with this combination (15). The emergence and proliferation of enterococcal strains resistant to glycopeptide antibiotics have now further limited therapeutic options (1).

VanA and VanB are common enterococcal phenotypes associated with glycopeptide resistance. VanA organisms are resistant to both vancomycin and teicoplanin, while VanB phenotypes typically remain susceptible to teicoplanin (1, 19, 20). Since VanA phenotypes are predominant and teicoplanin is investigational in the United States, combinations of currently available antibiotics have been evaluated to treat multidrug-resistant *E. faecium*, often with little success (19). Because of inadequate treatment options, we evaluated the activities of five investigational antibiotics alone and in combinations against multidrug-resistant enterococci in time-kill studies.

Four clinical isolates of vancomycin-resistant *Enterococcus faecium* (VREF) (SF10796, SF11346, SF12210, and SF12208) obtained from William Beaumont Hospital, Royal Oak, Mich., were tested. Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with calcium (25 mg/liter) and magnesium (12.5 mg/liter) (SMHB) was used for all susceptibility and time-kill curve experiments. Tryptic soy agar (TSA) (Difco) was used for performing colony counts. Antibiotics were obtained from their respective companies: CL331,002, a glycylcycline (Wyeth-Ayerst); clinafloxacin, a quinolone (Parke-Davis); LY333328, a glycopeptide (Eli Lilly & Co.); quinupristin-dalfopristin, a streptogramin (Rhone-Poulenc Rorer); and eperezolid (formerly known as U-100,592), an oxazolidinone (Pharmacia & Upjohn, Inc.). Antibiotic stock solutions were prepared immediately before use for all time-kill experiments.

The MIC and MBC of each antibiotic were determined by a broth microdilution technique following the National Committee for Clinical Laboratory Standards guidelines with a starting inoculum of 5×10^5 CFU/ml for all isolates (16). The E-test (AB Biodisk, Solna, Sweden) method was used to determine ciprofloxacin, amoxicillin, gentamicin, vancomycin, and teicoplanin MICs against all isolates. CL331,002, clinafloxacin, LY333328, quinupristin/dalfopristin, and eperezolid were tested alone and in combination against all strains of VREF by time-kill curve methods. All time-kill experiments were performed two to four times at a concentration equal to four times the MIC of each antibiotic, except for clinafloxacin against the SF12208 isolate. Due to the high clinafloxacin MIC and to be clinically relevant, a simulated peak concentration of 5 μg/ml was used for this isolate. An initial inoculum of 10^6 CFU/ml was obtained by diluting 1 ml of a 0.5 McFarland suspension obtained from a culture grown overnight in 9 ml of SMHB and then adding 0.8 ml of this solution to 7.2 ml of SMHB containing antibiotic(s). The test tubes were incubated at 37°C. Samples (0.1 ml) were taken in duplicate at 0, 8, and 24 h, serially diluted in normal saline, plated onto TSA, and incubated for 24 h at 37°C.

Synergy was defined as a ≥100-fold increase in killing at 24 h by a combination over that of the most active single drug. Antagonism was defined as a ≥100-fold decrease in killing at 24 h by a combination from that of the most active single drug (7). The changes in $\log_{10}$ CFU/milliliter at 24 h were compared by a two-way analysis of variance with Tukey's test for multiple comparison of significance. Time to achieve 99.9% killing was obtained by visual inspection of the log-linear phase of the log CFU/milliliter-versus-time plot. A P value of <0.05 was considered statistically significant.

All isolates were susceptible to all the antibiotics tested, with

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TABLE 1. Susceptibility results

Antibiotic	Susceptibility[a] of *E. faecium* isolate:							
	SF10796		SF11346		SF12210		SF12208	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Clinafloxacin	0.5	2.0	0.5	2.0	2.0	4.0	4.0	16.0
CL331,002	0.06	>4.0	0.06	>4.0	0.03	>4.0	0.03	4.0
LY333328	0.5	32.0	0.5	32.0	1.0	4.0	1.0	16.0
Q/D[b]	0.5	32.0	0.5	>32.0	0.25	>32.0	0.5	8.0
Eperezolid	2.0	32.0	2.0	8.0	2.0	32.0	2.0	64.0
Ciprofloxacin	8	ND	4	ND	4	ND	>32	ND
Amoxicillin	64	ND	96	ND	48	ND	96	ND
Gentamicin	>256	ND	>256	ND	>256	ND	>256	ND
Vancomycin	>256	ND	>256	ND	>256	ND	>256	ND
Teicoplanin	64	ND	96	ND	64	ND	48	ND

[a] Measured by MICs and MBCs (units for both MICs and MBCs are micrograms per milliliter). ND, not determined.
[b] Q/D, quinupristin/dalfopristin.

the exception of SF12208, which was resistant to clinafloxacin. MIC and MBC results are listed in Table 1.

In time-kill experiments, all regimens tested were significantly better than the growth control ($P < 0.0002$) (Fig. 1). Clinafloxacin was significantly more potent than all other antibiotics tested alone or in combination, except for clinafloxacin given in combination with CL331,002 or LY333328 and LY333328 plus quinupristin/dalfopristin where no significant differences were found. Furthermore, clinafloxacin was the only agent that achieved 99.9% killing at 24 h. When organisms were examined individually, quinupristin/dalfopristin alone and in combination with CL331,002 and LY333328 decreased the bacterial density by >3 $\log_{10}$ CFU/ml at 24 h in two of the four strains studied (data not shown). None of the combinations studied were synergistic when the organisms were evaluated separately or pooled together (using the mean CFU/milliliter of all isolates for each regimen). However, the combination of clinafloxacin plus eperezolid was antagonistic. CL331,002 was the least effective agent with all regimens, except for eperezolid alone and combined with CL331,002, which achieved a greater kill at 24 h.

There was no significant correlation found between the MIC or MBC of the organisms and the bacterial density at 24 h for all antibiotics tested. However, there was good correlation between the MBC/MIC ratio in the SF12208 and SF12210 isolates and the inoculum at 24 h ($r \geq 0.73, P < 0.002$). For the other two isolates, a correlation existed between MBC/MIC ratio and $\log_{10}$ CFU/milliliter at 24 h, but it was not statistically significant ($r = 0.4, P = 0.3$).

With the increasing problem of multidrug-resistant *E. faecium*, recent attention has focused on new antibiotics with in vitro activity against VREF. Our study suggests that quinupristin/dalfopristin, LY333328, clinafloxacin, CL331,002, and eperezolid may all have a role in treating VREF. Clinafloxacin tested alone had good killing activity against susceptible strains of *E. faecium*. The previously reported clinafloxacin MICs for *E. faecium* have ranged from 0.12 to 4.0 μg/ml (17, 18). All the enterococcal isolates studied were resistant to ciprofloxacin with MICs of ≥4 μg/ml. Ciprofloxacin MICs did not correlate with the clinafloxacin MICs for three of the strains, which is consistent with what has been reported for other organisms, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia* (11, 21). Clinafloxacin is active against many ciprofloxacin-resistant strains; therefore, susceptibility to ciprofloxacin cannot be used to determine susceptibility to clinafloxacin.

The 3 $\log_{10}$ CFU/ml decrease in inoculum caused by clinafloxacin at 24 h was similar to that observed by Burney et al. (3), who reported a 2.9 $\log_{10}$ CFU/ml decrease in the inoculum at 24 h with clinafloxacin (1 μg/ml) against 12 *E. faecium* strains, which required MICs of <1.0 μg/ml. For all the organisms tested, the MBC/MIC ratio for clinafloxacin remained low (between 2 and 4), which might explain the superiority of this agent in decreasing the bacterial inoculum. Since antagonism was seen with the combination of clinafloxacin plus eperezolid against all four strains of enterococcus, fluoroquinolones in combination with an oxazolidinone may not warrant further study.

LY264826 is a naturally occurring glycopeptide with four to eight times more activity against gram-positive organisms than vancomycin. Derivatives of this compound, such as LY333328, have potent activity against vancomycin- and teicoplanin-resistant enterococci, demonstrating MICs of ≤2.0 μg/ml (22). Synergy against enterococci has been demonstrated by fractional inhibitory concentration index for vancomycin plus quinupristin/dalfopristin. Since LY333328 is also a glycopep-

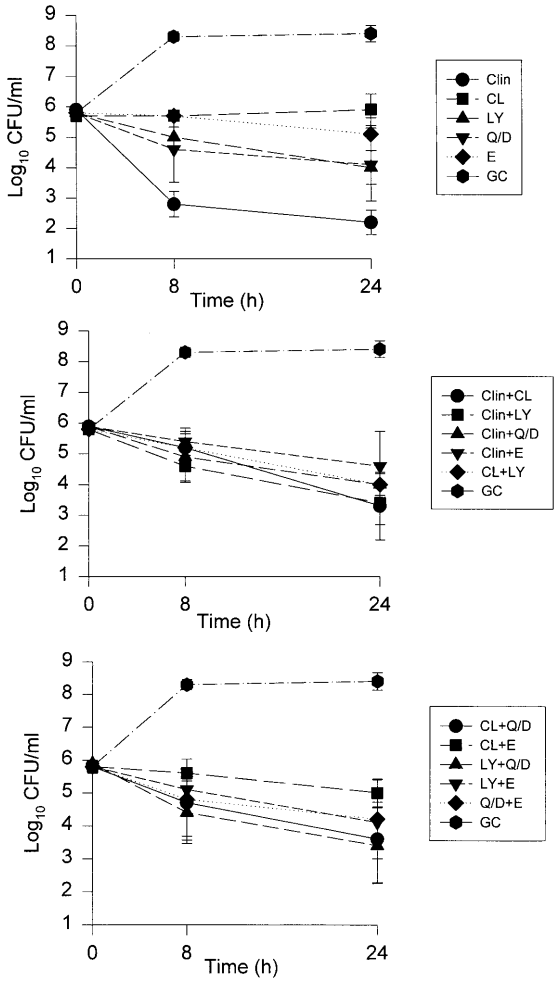

FIG. 1. Investigational antibiotics alone and in combination against vancomycin-resistant *E. faecium*. Each time-kill curve shows the means of the four isolates tested (except for clinafloxacin for which only three strains are averaged). Abbreviations: Clin, clinafloxacin; CL, CL331,002; LY, LY333328; Q/D, quinupristin/dalfopristin; E, eperezolid; GC, growth control.

tide, as is vancomycin, synergism might be expected with the combination of LY333328 plus quinupristin/dalfopristin (12). This was not the case in our isolates tested. Additional studies of LY333328 in combination with currently available antibiotics, such as gentamicin and beta-lactams, are needed. Also, triple-antibiotic combinations of a glycopeptide, gentamicin, and ceftriaxone, such as those carried out by Caron et al. (6) in which LY333328 could replace either vancomycin or teicoplanin may be of interest. Quinupristin/dalfopristin, a streptogramin-type antibiotic, is further along in its development than the other agents we tested, so any data obtained from time-kill studies with quinupristin/dalfopristin may hint at clinical relevance in a shorter period of time.

Another class of investigational agents, the glycylcyclines, have also been shown to have potent in vitro activity against resistant strains of enterococci (9). As monotherapy, CL331,002 and eperezolid inhibited the growth of enterococci over a 24-h period. Combinations with other agents did not improve the activity of either of these compounds. Bostic et al. (2) did not find synergy when eperezolid was combined with gentamicin, ampicillin, or streptomycin. We have now shown that many additional classes of drugs have now failed to interact synergistically with eperezolid. Cappelletty et al. (5) did not find synergy between CL331,002 with either ofloxacin or rifampin against *E. faecium*. CL331,002 also failed to demonstrate synergism when combined with the investigational agents we tested.

In conclusion, most antimicrobial agents are bacteriostatic when tested separately against enterococci. The search to find antimicrobial combinations that are consistently bactericidal against multidrug-resistant *E. faecium* is of prime importance. Our investigation did not find any superior combinations but did demonstrate that in vitro, clinafloxacin appears to be the best agent tested alone. Four strains of VREF were utilized in this in vitro study, which limits our ability to generalize to all VREF isolates. However, our results support what others have found on the properties of these new compounds. Additional combinations involving these new antibiotics should continue to be pursued.

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