Bactericidal Activity of Ramoplanin against Antibiotic-Resistant Enterococci

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Ramoplanin, a new lipoglycodepsipeptide antibiotic, was uniformly active against 65 strains of enterococci, including strains highly resistant to vancomycin, penicillin G, and gentamicin. MBCs were usually within a fourfold dilution of the MICs. In time-kill studies, ramoplanin alone demonstrated dose-dependent bactericidal activity against enterococcal strains that resisted killing by vancomycin or penicillin in combination with gentamicin.

Recently, the enterococcus has become a pathogen of great importance because of its ability to resist the activities of many antimicrobial agents and its potential for nosocomial transmission (3, 7, 9, 12, 16, 18, 21, 23, 24). Traditional therapy for serious enterococcal infections has relied on the use of a penicillin or a glycopeptide antibiotic in combination with an aminoglycoside (8, 10). Strains resistant to all of these agents are being reported in increasing frequency. Consequently, there is a need to expand the clinician's armamentarium with new antibiotics and antibiotic regimens that possess bactericidal activity against enterococci.

Ramoplanin (formerly A16686 and MDL 62198) is a novel lipoglycodepsipeptide with activity primarily against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, streptococci, *Clostridium difficile*, and *Listeria* spp. (1, 14). Generally, the spectrum of activity of ramoplanin is similar to that of vancomycin, but it has been reported to be four to eight times more active than vancomycin (6). Moreover, ramoplanin has greater bactericidal activity than the other glycopeptide antibiotics against strains of *S. aureus* and streptococci (11, 13). Ramoplanin also shows good activity against strains of *Enterococcus*, including those with moderate levels of resistance to ampicillin and vancomycin (22). The present study was undertaken to evaluate the activity of ramoplanin against a large number of strains of *Enterococcus*, including those that are highly resistant to vancomycin, penicillin G, and gentamicin. In addition, the bactericidal activity of the antibiotic was investigated in time-kill studies.

All enterococcal strains used in this study were unique clinical isolates recovered from patients located at our own and several regional medical centers between 1988 and 1991. Species identification was performed by using the automated MicroScan Walkaway System (Baxter Healthcare Corp., West Sacramento, Calif.) (20). Aliquots of each strain were frozen in cation-supplemented Mueller-Hinton broth (MHB) until required for study. All strains were clinically and epidemiologically unrelated. Vancomycin-resistant strains were confirmed as unique isolates by restriction endonuclease analysis of plasmid content.

The MICs of ramoplanin, vancomycin, teicoplanin, daptomycin, and penicillin (Sigma Chemical Co., St. Louis,

Mo.) were determined by the conventional agar dilution method (17). Antibiotics were prepared fresh on the day of the experiment and were diluted according to the manufacturers' recommendations. The following antibiotic powders were generously provided by the indicated manufacturers: ramoplanin and teicoplanin, Marion Merrell Dow Pharmaceuticals, Cincinnati, Ohio, and vancomycin and daptomycin, Eli Lilly & Co., Indianapolis, Ind.

A broth dilution method was also used to determine the MICs for selected enterococcal strains (n = 14) (17). All isolates were tested by using cation-supplemented MHB (20 to 25 mg of Ca^{2+} per liter) and a final inoculum of 5×10^5 to 1.0×10^6 CFU/ml. In experiments with daptomycin and ramoplanin, MICs were also determined in unsupplemented MHB to which calcium chloride was added to achieve final concentrations of 0, 25, 50, 100, and 200 mg of Ca^{2+} per liter. The MIC was read as the lowest concentration of each antimicrobial agent that prevented turbidity after 18 h of incubation at 37°C. MBCs, which were defined as the lowest concentration of drug that reduced the original inoculum by $\geq 99.9\%$ within 18 h, were determined as described by Pearson et al. (15). To test for the presence of high-level aminoglycoside resistance, 10^5 CFU of each strain per ml was incubated in the presence of 500 μg of gentamicin per ml in MHB. Resistance was defined as the presence of visible turbidity after 18 h of incubation.

Time-kill studies were performed on six enterococcal isolates that were resistant to vancomycin, penicillin G, high levels of gentamicin, or a combination of these. Survival of these strains was studied in flasks of MHB alone and in flasks containing MHB with vancomycin (50 μg/ml), penicillin G (50 μg/ml), and ramoplanin (at various concentrations), with and without gentamicin (3 μg/ml). A bacterial inoculum in the logarithmic phase of growth was added to each flask to achieve a final concentration of 5×10^5 CFU/ml. Time-kill studies with ramoplanin were also performed by using a final bacterial concentration of 10^7 CFU/ml. The flasks were then incubated in a shaking water bath at 37°C. Samples were removed at times of 0, 3, 6, and 24 h for enumeration by serial dilution and plating. Drug carryover effects were eliminated by dilution below the MIC for the organism. The lowest accurately countable number in the time-kill studies was 30 CFU/ml (1.5 in $\log_{10}$ units). All in vitro microbiologic studies were performed at least twice.

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TABLE 1. Activities of selected antimicrobial agents against *Enterococcus* spp.

Enterococcal phenotype (no. of isolates)	Antibiotic	MIC (μg/ml)		% Susceptible to high levels of gentamicin
		Range	90%[a]	
Vancomycin resistant[b] (8)	Ramoplanin	0.39–1.6	1.6	38
	Vancomycin	200–800	800	
	Teicoplanin	50–200	200	
	Daptomycin	12.5–50	50	
	Penicillin	100–400	400	
Penicillin G resistant[c] (19)	Ramoplanin	0.39–1.6	1.6	63
	Vancomycin	1.6–3.1	3.1	
	Teicoplanin	0.78–3.1	1.6	
	Daptomycin	25–100	100	
	Penicillin	200–>400	>400	
Other[d] (38)	Ramoplanin	0.39–1.6	1.6	71
	Vancomycin	0.78–3.1	3.1	
	Teicoplanin	0.39–1.6	1.6	
	Daptomycin	12.5–100	25	
	Penicillin	1.6–100	50	

[a] 90%, MIC for 90% of isolates tested.
[b] Vancomycin MIC, ≥200 μg/ml.
[c] Penicillin MIC, ≥200 μg/ml.
[d] Includes one β-lactamase-producing strain.

The susceptibilities of 65 strains of enterococci (*E. faecium*, n = 32; *E. faecalis*, n = 30, and *E. durans*, n = 3) to various antimicrobial agents are reported in Table 1. Eight of these isolates were highly vancomycin resistant (MIC, ≥200 μg/ml) and 19 were highly penicillin G resistant (MIC, ≥200 μg/ml). Ramoplanin was uniformly active against all isolates, regardless of their susceptibilities to other antimicrobial agents. By broth dilution methods, ramoplanin MICs were essentially identical to those determined by agar dilution. Bactericidal activity was observed against all 14 tested strains. MBCs were within a twofold dilution of the MIC for six strains, within a fourfold dilution for six strains, and within an eightfold dilution for two strains. The activity of ramoplanin was neither increased nor decreased by variation of the calcium ion concentration of the medium, whereas the activity of daptomycin was markedly enhanced by an increase in the calcium ion concentration.

In time-kill studies with selected resistant isolates, vancomycin and penicillin, alone or in combination with gentamicin, failed to reduce colony counts at 24 h for five of the six strains (Table 2). However, ramoplanin demonstrated rapid, dose-dependent bactericidal activity against all isolates. Although killing occurred faster with the addition of gentamicin, synergy at 24 h was observed only for the combination

of gentamicin (3 μg/ml) with ramoplanin at a concentration of 0.5× the MIC (Fig. 1). Ramoplanin was equally bactericidal in time-kill studies when an initial high inoculum of 10⁷ CFU/ml was used.

Clinical observations and evidence from experimental models suggest that antibiotic treatment of serious enterococcal infections such as endocarditis requires bactericidal activity (8, 10, 12). This has usually been achieved by a combination of vancomycin or penicillin with gentamicin. When resistance to one or all of these agents occurs, no alternative treatment has been established as efficacious (5).

In this study, ramoplanin demonstrated excellent activity against all enterococci, including those resistant to other antimicrobial agents. Furthermore, ramoplanin had rapid, dose-dependent bactericidal activity that did not require the addition of an aminoglycoside. There was no cross-resistance between ramoplanin and glycopeptide (vancomycin, teicoplanin) or lipopeptide (daptomycin) antibiotics. This is consistent with observed differences in their structures and mechanisms of action (4). Ramoplanin inhibits cell wall synthesis by acting at the level of lipid-intermediate formation or utilization, whereas vancomycin and daptomycin interfere with peptidoglycan synthesis by preventing trans-glycosylation (19).

TABLE 2. Bactericidal action of selected antimicrobial agents against *Enterococcus* spp. in time-kill studies

Strain (resistance[a])	Cumulative change ($\log_{10}$ CFU/ml) 24 h after the growth in:			
	Control	Ramoplanin[b]	Vancomycin (50 μg/ml) and gentamicin (3 μg/ml)	Penicillin G (50 μg/ml) and gentamicin (3 μg/ml)
620 (BLA⁺, Gent)	2.2	−3.8	0.1	0.2
294 (Gent)	3.7	−3.2	−0.3	3.0
298 (Gent)	3.0	−3.4	−2.7	3.0
401 (Van)	3.2	−3.1	3.1	3.2
418 (Van)	2.7	−3.2	3.3	1.8
410 (Van, Gent)	3.2	−3.2	3.0	3.1

[a] BLA⁺, β-Lactamase positive; Gent, gentamicin MIC, >500 μg/ml; Van, vancomycin MIC, ≥200 μg/ml; penicillin MICs (in micrograms per milliliter) for the following strains: 620, 6.2; 294, 200; 298, 200; 401, 200; 418, 100; 410, 200.
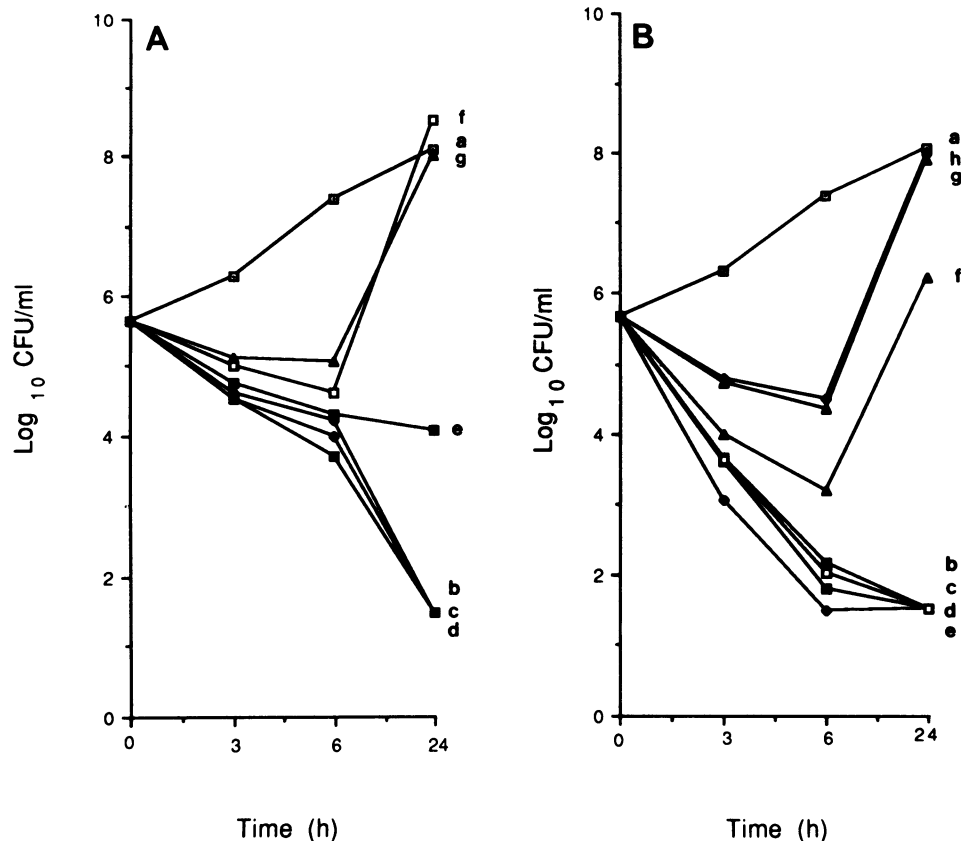[b] Ramoplanin kill curve was determined at the MIC for the isolate.

FIG. 1. Time-kill study of ramoplanin at various concentrations alone (A) and in combination with gentamicin (3 μg/ml) (B) against vancomycin-resistant *E. faecium* 401. a, control; b, ramoplanin, 1.6 μg/ml (4× the MIC); c, ramoplanin, 0.8 μg/ml (2× the MIC; d, ramoplanin, 0.4 μg/ml (MIC); e, ramoplanin, 0.2 μg/ml (0.5× the MIC); f, ramoplanin, 0.1 μg/ml (0.25× the MIC); g, ramoplanin, 0.05 μg/ml (0.12× the MIC); h, gentamicin, 3 μg/ml.

In preliminary studies, ramoplanin has been poorly tolerated following intravenous or intramuscular injection. However, it has been considered promising as a topical agent because of its spectrum of activity, lack of systemic absorption, and good local tolerability (2). Since nosocomial transmission of antibiotic-resistant strains of enterococci has been associated with fecal carriage, ramoplanin may have a role in eradicating colonization from the gastrointestinal tract (16, 23). Furthermore, its excellent bactericidal activity against multiply antibiotic-resistant strains suggests that efforts be made to develop this or a related lipoglycodepsipeptide antibiotic for systemic use.

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