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Pharmacodynamic Evaluation of a New Glycopeptide, LY333328, and In Vitro Activity against *Staphylococcus aureus* and *Enterococcus faecium*

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The objectives of the present study were to compare the in vitro activity of LY333328 (LY) to that of vancomycin (V) alone and in combination with gentamicin (G) and rifampin (R) against methicillin-resistant *Staphylococcus aureus* (MRSA) and V-resistant *Enterococcus faecium* (VREF), by using the killing curve methods. In addition, the effect of the inoculum size and protein on LY's activity was evaluated by using MICs and killing curves. MICs, MBCs, and killing curves were determined with supplemented Mueller-Hinton broth (B), B with albumin (4 g/dl) (A), and B with 50% pooled human serum (S). For MRSA, time to 99.9% killing after exposure to LY at four times the MIC (4× MIC) was achieved at 0.5 ± 0 h (mean ± standard deviation) and was significantly faster than that by V (8.54 ± 0.10 h; $P = 0.001$). Against VREF, LY decreased the inoculum by 2.2 $\log_{10}$ CFU/ml at 24 h ($P = 0.002$). With a large inoculum of MRSA, the activity of LY and V at 4× MIC was decreased compared to that with the standard inoculum ($P = 0.0003$) and regrowth occurred at 24 h. The reduction in the number of CFU per milliliter at 24 h to 2 $\log_{10}$ CFU/ml was restored by increasing the LY concentration to at least 16× MIC. At 24 h, the combinations of LY and G, LY and R, LY and V, and V and G were better than either LY or V alone against a large inoculum of MRSA ($P = 0.0002$). LY and G achieved 99.9% killing at 1.01 ± 0.03 h and was more rapid ($P < 0.007$) than all the other regimens studied except for V and G, which achieved 99.9% killing at 3.59 ± 0.01 h. Killing curves determined with different media against a standard inoculum of MRSA did not demonstrate a significant difference between LY and V at 24 h. Time to 99.9% killing was more rapid with LY than with V in B, A, and S ($P = 0.0002$). Times to 99.9% killing by LY in B, A, and S were not significantly different from each other. Against VREF, LY killed better than V in B, A, or S at 24 h ($P = 0.0002$). LY in B was more active than LY in A or S ($P = 0.0002$). LY is a new potent glycopeptide with a unique activity profile. It has a greater activity than that of V against MRSA and has activity against VREF. LY demonstrated synergism in combination with gentamicin against MRSA. LY was affected by large inoculum sizes and proteins in time-kill studies. However, the effect was compensated for by increasing the drug concentration to 16× MIC.

The development of antimicrobial resistance raises important concerns in the 1990s due to the prevalence of serious infections caused by drug-resistant bacteria (14, 18, 24). Enterococcus has been declared the pathogen of the 1990s (23, 30). It is the second leading cause of nosocomial infections, and an increase in the emergence of multidrug-resistant *Enterococcus faecium* has been described (32). Of particular concern are vancomycin-resistant *E. faecium* (VREF) isolates, since limited treatment options for these bacteria are available. The Centers for Disease Control and Prevention reported a 34-fold increase (0.3 to 7.9%) of VREF from 1989 to 1993 (4). At Detroit Receiving Hospital, in 1995, 50% of the *E. faecium* isolates cultured were resistant to vancomycin. This is in stark contrast to 1990, when none of the clinical isolates were reported to be vancomycin resistant. Unfortunately, effective therapy against VREF is presently lacking (32).

Another significant problem is the increase in methicillin-resistant *Staphylococcus aureus* (MRSA). As with multidrug-resistant *E. faecium*, effective drug treatments are limited. *S. aureus* has been well known for its ability to cause serious infections and it is the second-most-common pathogen found to infect patients with endocarditis (3). Vancomycin is the first choice of therapy for MRSA but has been associated with failure (13, 19, 28, 29). The future appearance of clinical MRSA isolates resistant to vancomycin is predictable. There is a definite need for new potent antimicrobial agents effective against resistant strains of enterococci and staphylococci.

LY333328 is a new investigational glycopeptide antibiotic. The mechanism of action is still unknown but is thought to be similar to that of vancomycin. The primary mechanism appears to be the inhibition of the cell wall synthesis and assembly by complexing with the D-alanyl-D-alanine precursor. It might also impair RNA synthesis (12).

LY333328 has in vitro bactericidal activity against enterococci, including VREF, and staphylococci. The reported MICs at which 90% of isolates are inhibited for different strains of MSSA, MRSA, and vancomycin-resistant enterococci (VRE) (*vanA* and *vanB* strains) are ≤1.0 μg/ml. Overall, LY333328 has been shown to be 10 times more active against MRSA than vancomycin, exhibiting a higher rate of killing and a concentration-dependent killing effect against both staphylococci and enterococci (2, 9, 34, 35). The pharmacokinetic data available so far for rats have shown that the drug is 77% bound to protein, has a slow clearance (0.04 liter/hr/kg of body weight), a volume of distribution of 0.23 liter/kg, and a terminal half-life

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TABLE 1. Summary of MICs and MBCs

Strain	SMHB								Albumin				PHS			
	SI				LI											
	LY		V		LY		V		LY		V		LY		V	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
MRSA-494	1	2	0.5	1	2	8	0.5	8	2	8	1	8	*[b]	*	0.5	1
VREF-7303	1	4	512	1,024	2	8	1,024	1,024	8	8	>1,024	>1,024	2	4	1,024	>1,024

[a] Abbreviations used: SMHB, SMHB alone; albumin, SMHB with albumin (4 g/dl); PHS, SMHB with 50% PHS; SI, standard inoculum (5×10^5 CFU/ml); LI, large inoculum (10^7 and 10^8 CFU/ml for microtiter and macrodilution techniques, respectively); LY, LY333328; V, vancomycin.

[b] *, results inconsistent.

of 9.77 h. The slow elimination in rats suggests that a therapeutic level can be achieved for a long period of time with a reduced dosage. The relation between dose and concentration achieved in plasma is not a resolved issue, but with a 5-mg/kg dose to rats, the levels obtained were effective against a strain of VRE (20).

The objectives of this investigation were (i) to evaluate the effects of protein, inoculum, and concentration on LY333328's activity, (ii) to compare the activity of LY333328 alone and in combination with other antimicrobial agents, namely, gentamicin, rifampin, and vancomycin, and (iii) to determine the postantibiotic effect (PAE) of LY333328 against MRSA and VREF.

MATERIALS AND METHODS

Bacterial strains. The following clinical strains were tested: *S. aureus* MRSA-494 and *E. faecium* VREF-7303 (multidrug resistant). The bloodstream isolates were obtained from the University of Michigan Medical Center, Ann Arbor, Mich., and William-Beaumont Hospital, Royal Oak, Mich., respectively.

Media and antibiotics. 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 killing curve experiments. Tryptic soy agar (TSA) (Difco) and Mueller-Hinton agar (Difco) were used for performing colony counts and detecting antibiotic resistance, respectively. LY333328 was obtained from Eli Lilly (lot no. X03059 S105109); gentamicin (lot no. 14H06088), rifampin (lot no. 81H3317), and vancomycin (lot no. 112H075025) were all supplied by Sigma Chemical Company (St. Louis, Mo.).

In vitro antibiotic susceptibility tests. MICs and MBCs were determined for each drug (LY333328, vancomycin, gentamicin, and rifampin) by broth microdilution following the technique of the National Committee for Clinical Laboratory Standards, with a starting inoculum of 5×10^5 CFU/ml for both isolates (27). The inoculum effect was determined by microdilution and macrodilution MIC methods and killing curve experiments using two different bacterial densities, i.e., 5×10^5 and 1×10^7 to 1×10^8 CFU/ml, respectively, for both the *S. aureus* and *E. faecium* strains.

Synergy testing. LY333328 and vancomycin were tested in combination with gentamicin and rifampin by the killing curve method to determine the potential for synergism. Synergy was defined as a ≥100-fold increase in killing at 24 h (as measured by colony counts) with the combination, in comparison with the killing by the most active single drug (10). All killing curves were performed at four times the MIC (4× MIC) of each antibiotic except for vancomycin against *E. faecium*, which was performed at a standard concentration of 30 μg/ml. An initial inoculum of 10^6 CFU/ml was obtained by diluting 1 ml of a 0.5 McFarland suspension, which was prepared with two to three colonies from an overnight TSA plate, in 9 ml of SMHB and then by adding 0.8 ml of the diluted 0.5 McFarland suspension to 7.2 ml of SMHB containing antibiotics. Samples (0.1 ml) were taken at 0 (inoculum control), 0.5, 1, 2, 4, 8, and 24 h for each organism.

Effect of concentration. The effect of concentration on the bactericidal activity of LY333328 and vancomycin was compared by killing curve experiments at various multiples of the MIC for both organisms. Increments of 1/4, 1/2, 1, 4, 8, 16, and 32× MIC were tested and compared (17).

Effect of proteins. The effect of proteins on the activities of LY333328 and vancomycin was compared by using microdilution MIC and killing curve methods in the presence and absence of albumin (4 g/dl) (Baxter) and 50% pooled healthy volunteer human serum (50% PHS) (1, 8, 16, 17, 31).

PAE. The PAE method used was as described by Craig and Gudmundsson (7). LY333328 and vancomycin were tested at different multiples of the MIC. SMHB was used in the test tubes, and a 1/1,000 dilution removed the antibiotics after the bacteria were exposed to them for 1 h. The PAE was calculated by using the following equation: $PAE = T - C$, where T is the time required for the count in the test culture to increase 1 $\log_{10}$ above the count observed immediately after drug removal and C is the time required for the count of the untreated control tube to increase by 1 $\log_{10}$.

Statistical analysis. The number of $\log_{10}$ CFU per milliliter at 24 h was compared between regimens by an analysis of variance with Tukey's test for multiple comparisons of significance. The time required to achieve 99.9% killing was determined by linear regression. A P of <0.05 was considered statistically significant.

RESULTS

Susceptibility testing. The MIC and MBC results were similar for LY333328 and vancomycin performed with standard or large inoculum in SMHB alone, SMHB with albumin (4 g/dl), and 50% SMHB plus 50% PHS (Table 1). The only difference in MICs was seen when LY333328 was tested in albumin; there was a four- to eightfold increase in this MIC compared to those in SMHB with 50% PHS or SMHB alone, respectively. Moreover, there was no significant difference found when we compared the MIC and MBC results obtained by microtiter dilution or macrodilution technique. The MBC/MIC ratio doubled for LY333328 at a large inoculum and with albumin against MRSA compared to LY333328 tested in SMHB alone with a standard inoculum of MRSA. However, for the VREF isolate, the effect of inoculum and protein on the MBC/MIC ratio was inconsistent. In the presence of a large inoculum, the MBC/MIC ratio remained the same as the ratio obtained with a standard inoculum, while with protein, we observed a decrease in the ratio.

Killing curve results. Time to 99.9% killing after exposure to 4× MIC of LY333328 was achieved at 0.5 ± 0 h (mean ± standard deviation), versus 8.54 ± 0.1 h for vancomycin in SMHB alone ($P = 0.001$). At 24 h there was no significant difference between the killing by LY333328 and that by vancomycin in the three different media used: SMHB alone, 50% SMHB with 50% PHS, and SMHB with albumin (4 g/dl). The time required to achieve 99.9% killing and the colony counts at 24 h when LY333328 was used in SMHB alone were not statistically different from those in SMHB with 50% PHS and SMHB with albumin (4 g/dl). The effect of proteins, if any, on LY333328's killing activity against this strain of MRSA was impossible to detect by killing curve methods since killing in all media tested was achieved in the same brief time (Fig. 1).

Against VREF when tested in SMHB, LY333328 reduced the number of CFU by 2.2 $\log_{10}$ CFU/ml at 24 h. LY333328 in SMHB alone was significantly more effective than LY333328 in 50% PHS or in albumin (4 g/dl) ($P \leq 0.0002$). Although LY333328 was affected by the presence of proteins, an increase in the drug concentration to at least 16× MIC compensated for the effect of proteins (Fig. 2). LY333328 was significantly

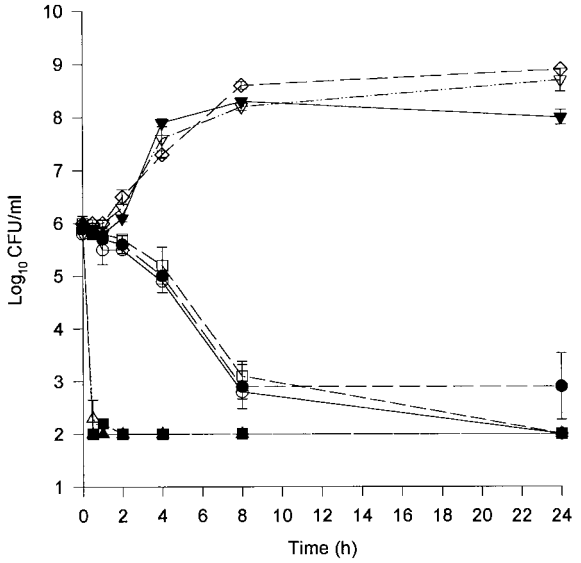

FIG. 1. LY333328 (LY), vancomycin (V), and growth control (GC) in SMHB (B), 50% PHS (S), and albumin (A) against MRSA. Symbols: ○, V in B; ●, V in S; □, V in A; ■, LY in B; △, LY in S; ▲, LY in A; ▽, GC in B; ▼, GC in S; and ◇, GC in A. Error bars, standard deviations.

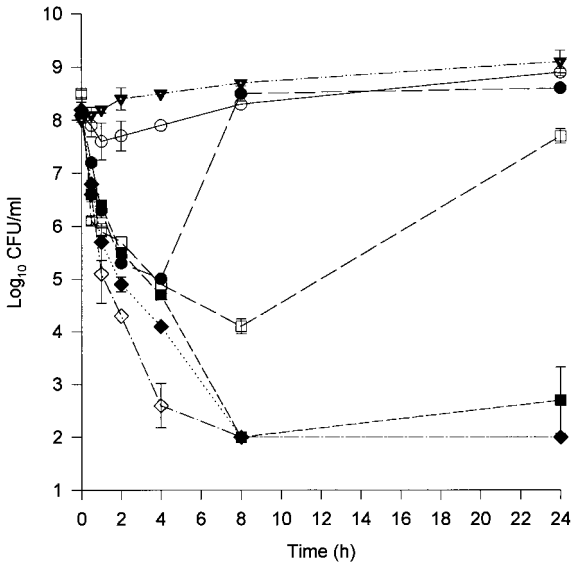

FIG. 3. LY333328 against large inoculum of MRSA. Symbols: ○, 1/2× MIC; ●, 1× MIC; □, 4× MIC; ■, 8× MIC; ◆, 16× MIC; ◇, 32× MIC; and ▼, growth control. Error bars, standard deviations.

more effective than vancomycin against VREF in all media tested ($P \leq 0.0002$).

Figure 3 represents the inoculum effect on LY333328's activity against MRSA-494. At a large inoculum (10^8 CFU/ml), the activity of LY333328 at 4× MIC was decreased compared to that at the standard inoculum (10^6 CFU/ml) and regrowth occurred at 24 h. The reduction in bacterial density to our limit of detection of 2 $\log_{10}$ CFU/ml at 24 h was restored when the LY333328 concentration was increased to at least 16× MIC.

Against VREF, a concentration-dependent effect was observed with LY333328 at concentrations varying from 1/4 to 32× MIC in SMHB. LY333328 achieved 99.9% killing at 0.5 h at 32× MIC, at 1.16 h at 16× MIC, at 3.09 h at 8× MIC, and at >24 h at 4× MIC. Concentrations lower than 4× MIC did not significantly reduce the bacterial density at 24 h (Fig. 4). For MRSA, LY333328 at 4× MIC decreased the inoculum size to our limit of detection in ≤30 min. Using killing curve methods, we were unable to detect a concentration-dependent effect against the MRSA strain. Concentrations lower than 4× MIC reduced the colony counts to 2 $\log_{10}$ CFU/ml at 0.5 to 1 h after exposure, but regrowth occurred at 24 h (1/4× MIC, 8.2 $\log_{10}$ CFU/ml; 1/2× MIC, 6.9 $\log_{10}$ CFU/ml; 1× MIC, 5.4 $\log_{10}$ CFU/ml).

In order to be able to detect synergism with LY333328

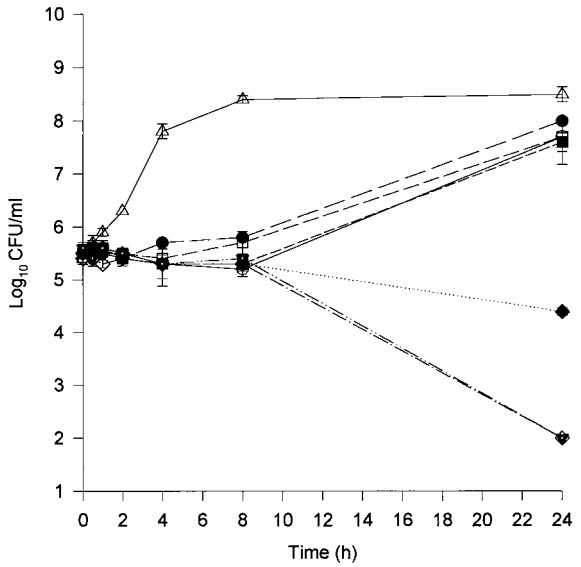

FIG. 2. LY333328 in 50% PHS against VREF. Symbols: ○, 1/4× MIC; ●, 1/2× MIC; □, 1× MIC; ■, 4× MIC; ◆, 8× MIC; ◇, 16× MIC; ▼, 32× MIC; and △, growth control. Error bars, standard deviations.

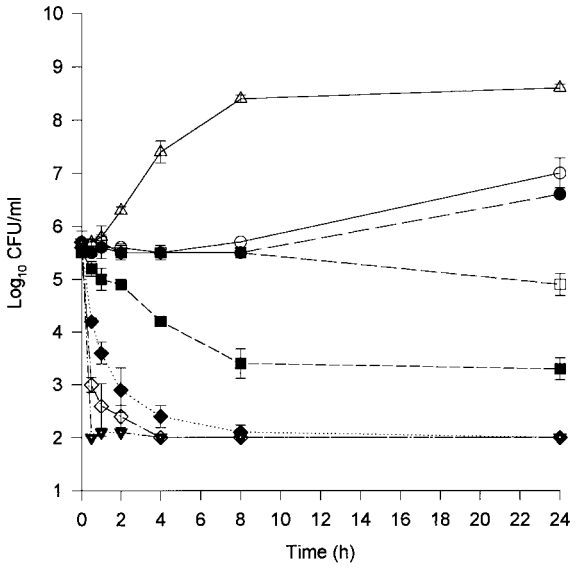

FIG. 4. Concentration-dependent effect of LY333328 against VREF-7303 in SMHB. Symbols: ○, 1/4× MIC; ●, 1/2× MIC; □, 1× MIC; ■, 4× MIC; ◆, 8× MIC; ◇, 16× MIC; ▼, 32× MIC; and △, growth control. Error bars, standard deviations.

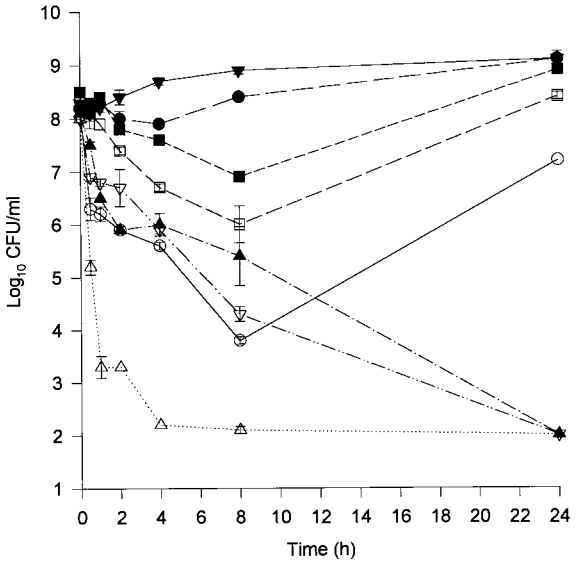

FIG. 5. Combination therapy killing curves of MRSA-494 at large inoculum. Symbols: ○, LY333328; ●, vancomycin; □, gentamicin; ■, rifampin; △, ▲, and ▽, LY333328 plus gentamicin, rifampin, and vancomycin, respectively; and ▼, growth control. Error bars, standard errors of the means.

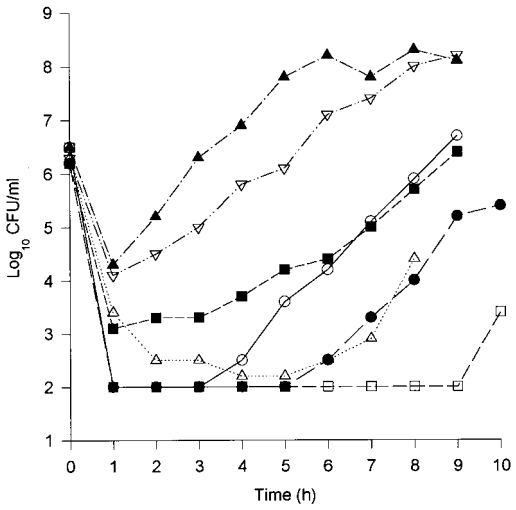

FIG. 6. PAE of LY333328 against MRSA and VREF. Symbols: ○, MRSA, 1× MIC; ●, MRSA, 2× MIC; □, MRSA, 4× MIC; ■, VREF, 1× MIC; △, VREF, 4× MIC; ▲, MRSA, growth control; and ▽, VREF, growth control.

against MRSA, an inoculum of 10^8 CFU/ml was used. LY333328 in combination with gentamicin and rifampin and vancomycin plus gentamicin were significantly more effective than LY333328 and vancomycin alone. LY333328 combined with gentamicin achieved 99.9% killing at 1.01 ± 0.03 h and was more rapid than LY333328 alone, LY333328 plus rifampin, and LY333328 plus vancomycin. LY333328 in combination with gentamicin was not significantly different from vancomycin plus gentamicin at 24 h (Fig. 5).

Against VREF, LY333328 plus gentamicin was significantly more potent than LY333328 alone at 24 h, even though the isolate was highly resistant (MIC > 1,024 μg/ml) to aminoglycosides ($P = 0.001$). LY333328 alone and LY333328 plus vancomycin were not statistically different from each other, but both reduced the inoculum size better than LY333328 plus rifampin ($P = 0.01$).

The PAE of LY333328 against MRSA and VREF was found to be concentration dependent. At 1× MIC of LY333328 against MRSA, the PAE was 2.35 h; at 2× MIC, it was 4.62 h; and at 4× MIC, it increased to 7.68 h. For VREF, the PAE of LY333328 lasted 1.85 and 4.25 h at 1× MIC 4× MIC, respectively (Fig. 6).

DISCUSSION

In previous studies, LY333328 has been shown to have potent bactericidal activity against gram-positive bacteria (2, 9, 20, 35). Similar to those investigations, we have found that LY333328 was active against a strain of MRSA and a strain of VREF, although at a concentration equal to 4× MIC, LY333328 was only bactericidal against the MRSA strain. Our investigations verified the concentration-dependent killing effect of LY333328 against VREF. This is a unique finding, since concentration-dependent killing has not been previously reported for the glycopeptides (1, 5, 12). LY333328, LY191145, and vancomycin are structurally related glycopeptides derived from the same fungus, *Amycolatopsis orientalis* (26). LY333328 is an *N*-alkyl derivative of the glycopeptide antibiotic LY264826 (Fig. 7). We have reported a trend towards a concentration-dependent effect with LY19145 (15). This finding suggests that slight changes in the active chemical side chains may contribute to this effect. It has been previously suggested that concentration-dependent killing is a function of the antibiotic's mechanism of action. Antibiotics which interact with protein synthesis, such as the aminoglycosides, or with the DNA gyrase, such as the quinolones, exhibit concentration-dependent killing. Antibiotics which disrupt cell wall integrity, such as the beta-lactams and vancomycin, are concentration independent (6). Although the mechanism of action of LY333328 is thought to be similar to that of vancomycin, its action against VREF and its concentration-dependent killing effect suggest that LY333328 may have specificity for the peptidyl termini of the peptidoglycan precursors of VREF or possesses an alternative mechanism(s) of action. The concentration-dependent effect is significant, because LY333328 is affected by proteins and inoculum, and a slight increase in drug concentration can easily compensate for these effects. Studies with daptomycin have demonstrated that increases in dapto-

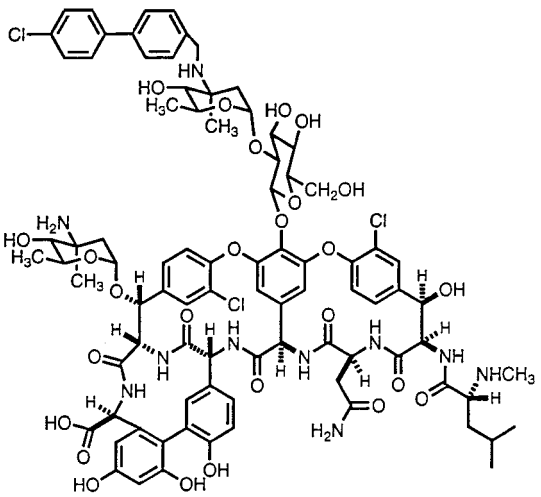

FIG. 7. Structure of LY333328.

mycin concentrations also overcome the inoculum and protein binding effects that hamper its killing activity (16, 17). The lack of data regarding pharmacokinetics in humans and toxicity with LY333328 creates uncertainties as to whether it is possible to achieve the desired concentrations in humans that would compensate for the inoculum effect and protein binding without causing serious side effects. Such a problem has been described in the past with the use of daptomycin (34).

As demonstrated by killing curve methods, LY333328 in combination with gentamicin resulted in synergistic activity at 24 h against a large inoculum of MRSA. Even though this subject is still controversial, some of the earlier investigations have shown that glycopeptides such as vancomycin demonstrated synergism against MRSA when combined with an aminoglycoside (22, 25, 33). Since our VREF isolate displayed high-level aminoglycoside resistance, we were unable to detect synergism, although there was a beneficial effect found from the combination of LY333328 with gentamicin. We expect that against an aminoglycoside-sensitive enterococcus strain, the combination of LY333328 with an aminoglycoside would be synergistic, but further studies are necessary to prove our hypothesis.

The combination of LY333328 with rifampin was synergistic at 24 h, but as seen with vancomycin plus rifampin, this may be a strain-dependent effect, as rifampin has been shown to have synergism, antagonism, or indifference when combined with vancomycin against *S. aureus* (11, 21). More strains need to be tested to evaluate the overall interaction between LY333328 and rifampin. Our VREF isolate was resistant to rifampin (MIC = 8 μg/ml), and no benefits from the combination of LY333328 and rifampin were found.

Conclusion. LY333328 is a new glycopeptide with bactericidal activity against resistant strains of *S. aureus* and *E. faecium*. LY333328 is affected by a large inoculum, but an increase in drug concentration can minimize this effect. As with other glycopeptides, LY333328 is synergistic in combination with gentamicin against MRSA. The presence of albumin and serum significantly reduced the killing activity of LY333328 against our strain of VREF. We were unable to detect the effect of proteins with a standard inoculum of MRSA due to the high potency of the antimicrobial agent against this isolate. An increase in drug concentration may compensate for the protein effect, as shown with VREF and a large inoculum of MRSA. Also, LY333328 possesses a prolonged concentration-dependent PAE. In conclusion, LY333328 is an interesting new compound that deserves further investigations for its use in animals as well as in humans, in order to better establish its pharmacokinetic and pharmacodynamic profiles as well as its toxic effects.

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