Synergy of an Investigational Glycopeptide, LY333328, with Once-Daily Gentamicin against Vancomycin-Resistant Enterococcus faecium in a Multiple-Dose, In Vitro Pharmacodynamic Model

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The pharmacodynamics of an investigational glycopeptide, LY333328 (LY), alone and in combination with gentamicin, against one vancomycin-susceptible and two vancomycin-resistant Enterococcus faecium strains were studied with a multiple-dose, in vitro pharmacodynamic model (PDM). Dose-range data for the PDM studies were obtained from static time-kill curve studies. In PDM experiments conducted over 48 h, peak LY concentrations of 0.1× and 1× the MIC every 24 h and peak gentamicin concentrations of 18 µg/ml every 24 h (Gq24h) and 6 µg/ml every 8 h (Gq8h) were studied alone and in the four possible LY-gentamicin combinations. Compared to either antibiotic alone, LY-gentamicin combination regimens produced significantly higher apparent killing rates (KRs) calculated during the initial 2 h postdosing. The mean KRs for LY or gentamicin alone versus those for the LY-gentamicin combination regimens were 0.35 ± 0.55 log10 CFU/ml/h (95% confidence interval [CI95%], 0 to 0.70) and 1.46 ± 0.71 log10 CFU/ml/h (CI95%, 1.01 to 1.91), respectively (P < 0.0001). Bacterial killing at 48 h (BK48), which was calculated by subtracting the bacterial counts at 48 h from the initial inoculum, with a negative value indicating net growth, was also significantly greater. The mean BK48s were −0.69 ± 0.44 log10 CFU/ml (CI95% −0.41 to −0.97) and 3.72 ± 2.28 log10 CFU/ml (CI95%, 2.28 to 5.17) for LY or gentamicin alone versus LY-gentamicin combination regimens, respectively (P < 0.0001). None of the 12 regimens with LY or gentamicin alone but 75% (9 of 12) of the LY-gentamicin combination regimens were bactericidal. Eighty-three percent (10 of 12) of the LY-gentamicin combination regimens also demonstrated synergy. No significant differences between the pharmacodynamics of LY-gentamicin combination regimens containing Gq24h versus those containing Gq8h were detected.

Over the past decade, a significant increase in the prevalence of nosocomial enterococcal infections has been observed (11). Of greater concern has been the notable rise in multiple-drug-resistant strains and the difficulty encountered in the treatment of such pathogens (8, 10, 11, 19). In the United States, the prevalence of vancomycin-resistant enterococci (VRE), most commonly in Enterococcus faecium and frequently in association with multiple-drug resistance, increased 20-fold from 1989 to 1995 (7). VRE are often responsible for severe infections for which the antibiotic selection is limited for patients with significant comorbid diseases. These factors may explain the reported associations between vancomycin resistance and the increased mortality rates among patients with enterococcal bacteremia (4, 5, 18).

The treatment of systemic enterococcal infections is directed by antibiotic susceptibilities and may include single agents or combinations of penicillins, glycopeptides, aminoglycosides, quinolones, and other antibiotics (10). As levels of resistance continue to increase, antibiotic selection becomes more limited, and in some cases an appropriate antibiotic is unavailable. There is an urgent need for new antibiotics with activity against multiple-drug-resistant Enterococcus, specifically VRE. Preliminary susceptibility studies have demonstrated the promising activity of a class of glycopeptide derivatives which are related to vancomycin (9, 14, 15). Static time-kill curve studies of one compound, LY333328 (LY), have shown that it has dose-dependent, bactericidal activity against Enterococcus spp. including those with high-level resistance to vancomycin (16, 21). Only a few studies have investigated the potential for synergy between LY and gentamicin against aminoglycoside-sensitive, vancomycin-resistant strains (12, 20). Furthermore, there are no published data regarding the use of LY in combination with gentamicin administered once daily (Gq24h) versus the use of LY in combination with gentamicin administered thrice daily (Gq8h) against enterococcus.

The primary purpose of this research was to characterize the activity of LY against vancomycin-resistant E. faecium with a multiple-dose, in vitro pharmacodynamic model (PDM). The research included comparisons of (i) LY or gentamicin alone versus LY-gentamicin combination regimens (i.e., synergy) and (ii) LY-gentamicin combination regimens containing Gq24h versus the same combination regimens but with Gq8h against VRE in a PDM.

MATERIALS AND METHODS

Bacterial strains. Three E. faecium isolates obtained from cultures of blood were studied. The strains included a vancomycin-susceptible strain (strain 1338), PCR-positive vanB strain 563, and PCR-positive vanA strain 561. All strains had low-level aminoglycoside resistance.

Antibiotics and in vitro susceptibility testing. LY (Eli Lilly & Co., Indianapolis, Ind.), vancomycin (Eli Lilly & Co.), teicoplanin (Merrell Dow, Laval, Quebec, Canada), and gentamicin (Schering Corp. Ltd., Pointe-Claire, Quebec, Canada) MICs were determined in Mueller-Hinton broth (MHB; Difco Labo-
ratories, Detroit, Mich.) supplemented with 25 μg of calcium per ml and 12.5 μg of magnesium per ml by the broth macrodilution method described by the National Committee for Clinical Laboratory Standards (13). Vancomycin and teicoplanin MICs were determined to confirm the phenotypic designations of the isolates. Gentamicin MICs were determined to select isolates with low-level aminoglycoside resistance. MICs were verified in triplicate on separate occasions.

**Static time-kill curve studies.** Dose-range data for the PDM studies were obtained from static time-kill curve experiments. Single-dose, static time-kill curve experiments were conducted over 24 h for LY alone and for LY in combination with gentamicin against all strains (20, 21). Static time-kill curve studies were performed in flasks containing total volumes of 10 ml. The volume of water used from drug stock solutions was limited to 0.1 ml. The bacteria were grown to the logarithmic phase by inoculating cation-supplemented MHB (CSMHB) which was incubated in a shaking water bath at 37°C for 1.5 h. One-milliliter aliquots containing 10⁶ CFU/ml were added to 9 ml of CSMHB to yield an initial inoculum of approximately 10⁸ CFU/ml. This initial inoculum was selected for all static time-kill curve and PDM experiments to enable the detection of synergy between LY and gentamicin. LY concentrations of 1×, 10×, 20×, 50×, and 100× the MIC were studied alone and in combination with gentamicin concentrations of 0.6 μg/ml. LY concentrations were empirically selected, whereas the gentamicin concentration was based on the levels achieved in blood with traditional dosing regimens (i.e., 1 to 1.5 mg/kg of body weight given every 8 h). Samples (0.1 ml) were collected at 0, 1, 2, 4, 8, and 24 h. Samples were serially diluted in normal saline at 4°C; 10⁶- and 10⁷-μl aliquots were plated in duplicate onto blood agar, and the plates were incubated at 35°C for 24 h. Viable colonies present at between 10 and 100 per plate were counted, and a lower limit of detection of 10³ CFU/ml was used. All experiments were performed in triplicate on separate occasions.

Residual antibiotic effects were assessed during initial experiments by running concurrent samples which were washed twice to remove antibiotic. Washing was performed by centrifugation at 4,000 × g for 10 min, decanting of the supernatant, and resuspension of the pellet in CSMHB. The colony counts from unwashed and washed samples were compared.

**PDM studies.** A multiple-dose, one-compartment PDM was used to simulate the treatment of bacteremic infections over 48 h (6). The central chambers (690 ml) were washed before each experiment to remove the previous inocula and for another hour during equilibration of the PDM. Antibiotic doses were injected into the central chambers at appropriate intervals to produce the desired peak and trough concentrations. All antibiotic concentrations in the PDM represented steady-state, free levels which simulate the active antibiotic component. Free antibiotic concentrations in vivo, which are dependent on protein binding and which are proportionally related to the total concentrations in vitro, can be calculated for antibiotics with known levels of protein binding (i.e., 77% for LY on the basis of data from studies with animals).

Because the primary purpose of the PDM studies was to investigate the potential for synergy between LY and gentamicin, low concentrations of the invesigational compound, LY, were used. The static time-kill curve experiments demonstrated maximal activity (i.e., less than the lower limit of detectable colonies at 24 h) for all G/LY-gentamicin combination regimens containing LY at ≤10× the MIC, and therefore, only the lowest LY concentration of 1× the MIC was studied for the PDM studies (20, 21). For LY alone, the MIC was also chosen to study potential synergy between LY and gentamicin. On the other hand, gentamicin is an established antibiotic which would be used only in combination for the treatment of enterococcal infections. As a result, clinically relevant gentamicin concentrations were used including peak levels of 12.5 μg/ml in regimens containing Gq24h and 10 μg/ml in regimens containing Gq48h. All LY and gentamicin dosage regimens were studied alone and in the four possible LY-gentamicin combinations.

When LY or gentamicin was tested alone, fresh CSMHB was pumped through the central chambers at flow rates producing half-lives of either 24 h for LY or 2 h for gentamicin. During studies with the drug combinations, fresh broth or LY-supplemented broth was delivered by separate computerized pumps at flow rates that concurrently produced the LY and gentamicin half-lives (1). Samples (0.1 ml) were collected at 0, 1, 2, 5 or 6, 24, 25, 26, 29 or 30, and 48 h. During experiments with Gq48h only, samples were also collected at 8, 10, 16, 18, 32, 34, 40, and 42 h. The samples were then processed as described above for the time-kill curve experiments. The pharmacokinetic profiles obtained in the PDM were verified by determining gentamicin concentrations by immunosay (TDx; Abbott, Chicago, Ill.) in the samples obtained at 2, 6, and 24 h. All experiments were performed in triplicate on separate occasions.

Results from the PDM studies were used to compare the activity of LY or gentamicin alone to the activities of the LY-gentamicin combination regimens. Bactericidal activity was defined as a BK24 > 1.4 log₉₂₀ CFU/ml at 24 or 48 h. Synergy was determined from PDM experiments and was defined as a ≥2 log₁₀ decrease in the numbers of CFU per milliliter between the combination and its most active constituent at 24 and 48 h when at least one of the agents was present at a concentration that did not affect the growth curve of the test organism when the agent was used alone (17). Finally, the activities of Gq24h- versus Gq48h-containing combination regimens were compared. Statistical comparisons were performed by a t test (α = 0.05).

**RESULTS**

**Susceptibility testing.** Vancomycin, teicoplanin, LY, and gentamicin MICs were 0.5, 0.25, 0.06, and 16 μg/ml, respectively for strain 1338 (vancomycin sensitive), 16, 0.5, 0.03, and 32 μg/ml, respectively, for strain 563 (vanB, 512, 32, 0.25, and 16 μg/ml, respectively, for strain 561 (vanA). Isolate 1338 was vancomycin and teicoplanin sensitive. Strain 563 demonstrated low-level vancomycin resistance and teicoplanin sensitivity, whereas strain 561 had high-level vancomycin resistance and teicoplanin resistance. The LY MICs were similar for strain 1338 and strain 563, but the LY MIC for strain 561 was higher. All strains demonstrated low-level aminoglycoside resistance, with MICs of either 16 or 32 μg/ml.

**Static time-kill curve study results.** The colony count variation at each time point within and between experiments was less than 10%. In addition, no antibiotic carryover was observed, as detected from the colony counts for unwashed samples. KR and BK24 results from the static time-kill curve studies are presented in Tables 1 and 2, respectively. For LY alone, the KR and BK24 were dose dependent over the concentration range studies for all strains. Variability in parameters was demonstrated between strains. For example, although the KR against 561 (vanA) was less than those against the other strains at 1×, 10×, and 20× the MIC, it was higher than those against the other strains at 50× and 100× the MIC. Furthermore, LY at 20× the MIC was bactericidal against 1338 (vancomycin-resistant strain 1338), whereas it was only bacteriostatic against 561 and 563 (vanA).

**TABLE 1. Time-kill curve KRs for LY alone and in combination with gentamicin**

<table>
<thead>
<tr>
<th>LY conc (× MIC)</th>
<th>KR (log₉₂₀ CFU/ml)</th>
<th>LY + G</th>
<th>LY + G</th>
<th>LY + G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×</td>
<td>0.73</td>
<td>3.53</td>
<td>0.69</td>
<td>1.27</td>
</tr>
<tr>
<td>10×</td>
<td>2.40</td>
<td>3.69</td>
<td>1.90</td>
<td>2.98</td>
</tr>
<tr>
<td>20×</td>
<td>2.30</td>
<td>4.10</td>
<td>1.90</td>
<td>3.55</td>
</tr>
<tr>
<td>50×</td>
<td>2.95</td>
<td>3.80</td>
<td>1.93</td>
<td>3.01</td>
</tr>
<tr>
<td>100×</td>
<td>3.00</td>
<td>5.00</td>
<td>3.48</td>
<td>5.00</td>
</tr>
</tbody>
</table>

* The KR was calculated during the initial 2 h postdosing. G, gentamicin.

**TABLE 2. BK24 time-kill curves for LY alone and in combination with gentamicin**

<table>
<thead>
<tr>
<th>BK24 (log₉₂₀ CFU/ml)</th>
<th>LY + G</th>
<th>LY + G</th>
<th>LY + G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×</td>
<td>0.03</td>
<td>5.00</td>
<td>-1.44</td>
</tr>
<tr>
<td>10×</td>
<td>2.64</td>
<td>5.00</td>
<td>1.03</td>
</tr>
<tr>
<td>20×</td>
<td>3.72</td>
<td>5.00</td>
<td>1.12</td>
</tr>
<tr>
<td>50×</td>
<td>5.00</td>
<td>5.00</td>
<td>1.64</td>
</tr>
<tr>
<td>100×</td>
<td>5.00</td>
<td>5.00</td>
<td>3.88</td>
</tr>
</tbody>
</table>

* BK24 was calculated by subtracting the bacterial count at 24 h from the initial inoculum, with a negative value indicating net growth. G, gentamicin.
susceptible) and 561 (vanA) but was not bactericidal against 563 (vanB) until the concentration was 100× the MIC.

Compared to LY alone, LY-gentamicin combination regimens produced higher KRs; however, the difference was not statistically significant. The mean KRs for LY alone and the LY-gentamicin combination regimens were 2.1 log₁₀ CFU/ml/h (95% confidence interval [CI₉₅%], 1.3 to 2.8) and 3.3 log₁₀ CFU/ml/h (CI₉₅%, 2.6 to 4.1), respectively (P > 0.05). Compared to LY alone, LY-gentamicin combination regimens increased the BK₂₄ significantly, with mean values of 2.4 log₁₀ CFU/ml (CI₉₅%, 1.2 to 3.6) and 4.8 log₁₀ CFU/ml (CI₉₅%, 4.5 to 5.1), respectively (P < 0.0001). The addition of gentamicin lowered the bactericidal concentrations of LY (i.e., the LY concentration required to kill ≥ 3 log₁₀ CFU/ml) significantly to 1× the MIC against all strains.

PDM study results. The pharmacokinetic profiles in the central chambers of the PDM were verified by gentamicin concentration determinations. Extrapolated peak levels and calculated elimination rates (i.e., half-lives) were within 10% of the expected values (i.e., peak level = 18 or 6 mg/liter and half-life = 2 h). The colony count variation at each time point within and between experiments was less than 10%. In addition, no antibiotic carryover was observed, as detected from the colony counts for unwashed samples. PDM killing curves for LY alone at 0.1× and 1× the MIC are depicted in Fig. 1A. LY at 0.1× the MIC against strain 1338 (vancomycin sensitive); +, LY at 1× the MIC against strain 1338 (vancomycin sensitive); □, LY at 0.1× the MIC against strain 563 (vanB); ●, LY at 0.1× the MIC against strain 561 (vanA); ○, LY at 1× the MIC against strain 561 (vanA). (B) BK curves for regimens with Gq₂₄h and Gq₈₄h against E. faecium in a multiple-dose, in vitro PDM. –, growth control; ○, Gq₂₄h against strain 1338 (vancomycin sensitive); +, Gq₂₄h against strain 563 (vanB); □, Gq₂₄h against strain 561 (vanA); #, Gq₈₄h against strain 1338 (vancomycin susceptible); ●, Gq₈₄h against strain 563 (vanB); ○, Gq₈₄h against strain 561 (vanA).
of LY had minimal KRṣ against all strains tested. PDM killing curves for Gq24h and Gq8h alone are presented in Fig. 1B. The Gq8h regimen had no activity, whereas the first doses of the Gq24h regimen produced notable killing of all strains. Again, regrowth to the initial inoculum occurred at 24 h.

PDM killing curves for LY-Gq24h and LY-Gq8h combination regimens are depicted in Fig. 2A and B, respectively. Compared to either drug alone, the LY-gentamicin combination regimens produced significantly higher KRṣ, with mean values of 0.35 ± 0.55 log₁₀ CFU/ml/h (CI₉⁵, 0 to 0.70) and 1.46 ± 0.71 log₁₀ CFU/ml/h (CI₉⁵, 1.01 to 1.91), respectively (P < 0.0001). BK₄₈ was also significantly greater, with mean values of −0.69 ± 0.44 log₁₀ CFU/ml (CI₉⁵, −0.41 to −0.97) and 3.72 ± 2.28 log₁₀ CFU/ml (CI₉⁵, 2.28 to 5.17) for LY or gentamicin alone versus the combination regimens, respectively (P < 0.0001). None of the regimens with LY or gentamicin alone were bactericidal, whereas 75% (9 of 12) of the LY-gentamicin combination regimens were bactericidal at both 24 and 48 h. The exceptions were regimens containing LY at 0.1 × the MIC against strain 563 (vanB) and the regimen with LY at 0.1 × the MIC against strain 1338 (vancomycin sensitive), which produced BKs of 2.41 and 2.13 log₁₀ CFU/ml at 24 and 48 h, respectively. Eighty-three percent (10 of 12) of the LY-gentamicin combination regimens were synergistic at both 24 and 48 h. The exceptions again included the regimen containing LY at 0.1 × the MIC against strain 563 (vanB).
There was no statistically significant difference in the pharmacodynamic parameters of LY-gentamicin combination regimens containing Gq24h versus those containing Gq8h. The mean KRs were 1.92 ± 0.51 log₁₀ CFU/ml/h (CI₉₅%; 1.38 to 2.46) and 1.01 ± 0.59 log₁₀ CFU/ml/h (CI₉₅%; 0.39 to 1.62) for the regimens containing Gq24h and Gq8h, respectively (P > 0.05). Mean BK₄₈ were also similar, with values of 3.91 ± 2.31 log₁₀ CFU/ml (CI₉₅%; 1.48 to 6.33) and 3.54 ± 2.45 log₁₀ CFU/ml (CI₉₅%; 0.97 to 6.11) for the regimens containing Gq24h and Gq8h, respectively (P > 0.05).

**DISCUSSION**

The methodology, antibiotic concentrations, bacterial inoculum, and measured endpoints can all influence the results of synergy testing (2). Traditional techniques (i.e., checkerboards and static time-kill curve studies) and the extrapolation of their results to the in vivo situation have obvious limitations. In comparison, PDM more closely simulates the antibiotic concentrations observed in vivo and allows the study of multiple-dose regimens over prolonged periods. PDM offers an alternative method for synergy testing and warrants further investigation (2). An important consideration, however, is the variation in PDM studies (i.e., design and analysis), with our infection model, for example, most closely simulating the activities of free antibiotic concentrations obtained from multiple-dose regimens against bacteria in the blood of immunocompromised patients. As a result, methodological standards for the use of synergy testing with the PDM should be developed.

As was consistent with our static time-kill curve data, LY alone at the low doses used in the PDM studies demonstrated little activity (20, 21). In humans, LY dosage regimens and their resulting concentrations are still being studied. In addition, there is controversy regarding the pharmacokinetics of LY, with recent data suggesting that it underestimates the activity of LY.

The significant activities of some regimens with gentamicin alone may have been related to the peak concentrations achieved in the PDM (Fig. 1B). The regimens with Gq8h alone produced peak concentrations (i.e., 6 µg/ml) well below the MICs for the isolates (i.e., 16 to 32 µg/ml) and were relatively inactive. In comparison, the regimens with Gq24h alone had peak levels which approached the MICs (i.e., approximately 0.5× to 1× the MIC) and reduced bacterial counts by at least 2 log₁₀ CFU/ml at 4 h for all strains. Despite this initial activity, however, there was rapid regrowth which began at 5 h and which reached or exceeded the initial inoculum at 24 h. This may have been the result of declining gentamicin concentrations in the PDM, which were approximately 3 µg/ml at 5 h and <0.3 µg/ml at 12 h. Lastly, a phenomenon more difficult to explain was the lack of bacterial killing following administration of the second doses for the regimen with Gq24h.

The detection of synergy between LY and gentamicin against VRE in our study is consistent with previous results (12). Mercier et al. (12) showed in static time-kill curve experiments that LY plus gentamicin was significantly more potent than LY alone against a VRE strain with resistance to multiple drugs. In our study, LY-gentamicin combination regimens produced statistically significant increases in all pharmacodynamic parameters including KR, BK₄₈, and BK₄₈. Eighty-three percent (10 of 12) of the LY-gentamicin combination regimens were synergistic (i.e., ≥2 log₁₀ decrease in the numbers of CFU per milliliter between the combination and its most active constituent) at both 24 and 48 h. Strain variability was demonstrated by the lack of synergy with the regimens containing LY at 0.1× the MIC against strain 563 (vanB). The concentration dependence of synergy testing was also shown by the presence of synergy with the regimens containing LY at 1× the MIC against the same strain. Finally, the regimens with Gq24h alone were more active than those with Gq8h alone; however, no statistically significant differences between the LY-gentamicin combination regimens containing Gq24h versus those containing Gq8h were detected.

**Conclusion.** LY is a new glycopeptide with activity against vancomycin-susceptible and vancomycin-resistant *E. faecium*. Synergy between LY and gentamicin against vancomycin-susceptible *E. faecium* and VRE was demonstrated by PDM experiments. The activities of combination regimens containing Gq24h were no different from the traditional regimens containing Gq8h. Strain variability in synergy testing was demonstrated by the consistent lack of synergy with one LY concentration-strain combination. Since our data represent those for only three isolates, all of which demonstrated low-level resistance to gentamicin, studies that include more strains with different susceptibilities to LY and gentamicin are needed. The urgent need for new treatments for VRE further warrants pharmacodynamic investigations of LY alone and in combination with other antibiotics.

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**REFERENCES**


