Treatment of experimental endocarditis caused by multidrug resistant
*Enterococcus faecium* with ramoplanin and penicillin

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Antibiotic resistant strains of enterococci are being isolated with increasing frequency. Effective treatment of infections caused by *Enterococcus faecium* resistant to ampicillin, vancomycin and aminoglycosides has not been established. We studied the activity of ramoplanin, a new lipoglycopeptide antibiotic, against two strains of multidrug resistant *E. faecium*. In time kill studies, ramoplanin was bactericidal against both strains, but not in the presence of 50% serum. The combination of ramoplanin and penicillin was bactericidal even in the presence of serum. In rabbits with experimental endocarditis neither penicillin nor ramoplanin significantly reduced vegetation colony counts when given alone, although ramoplanin significantly reduced spleen and kidney bacterial counts of both strains. The combination of ramoplanin plus penicillin resulted in a significant reduction of vegetation bacterial counts (—3.2 and —3.7 log_{10} cfu/g for strains VA3 and MMC3, respectively, \( P < 0.01 \)). All spleen cultures and 9 out of 10 kidney cultures from each strain were sterile following combination therapy. While ramoplanin will not be available for parenteral therapy, further research into the development of other lipoglycopeptide antibiotics is warranted.

Introduction

Enterococcal infections are traditionally treated with the synergistic combination of a cell wall agent and an aminoglycoside. High-level aminoglycoside resistance results in loss of this synergy. The problem of high-level aminoglycoside resistance has been compounded recently by the emergence of vancomycin and penicillin resistance (Handwerger et al., 1992; Landman, Mobarakai & Quale, 1993). Resistance to cell wall agents also prevents synergy with aminoglycosides (Torres et al., 1993). Over the past 3 years, nearly 100 patients at our hospital have developed bacteraemic and non-bacteraemic infections with strains of *Enterococcus faecium* that are resistant to penicillin, vancomycin and aminoglycosides. No proven therapy is available for infections caused by these strains and so development of new antibiotic regimens is needed.

Ramoplanin is a lipoglycopeptide antibiotic with good activity *in vitro* against Gram-positive bacteria (Collins et al., 1993). In a preliminary investigation, we found that ramoplanin had excellent in-vitro activity against a collection of multidrug resistant
*E. faecium* when tested in broth, but not in the presence of serum (Mobarakai, Quale & Landman, 1994). The addition of ampicillin or penicillin resulted in enhanced activity even in the presence of serum (Mobarakai *et al.*, 1994). We studied the effectiveness of this regimen in a rabbit model of endocarditis caused by two of these strains.

**Materials and methods**

**In-vitro studies**

VA3 and MMC3 are clinical strains from patients with bacteraemia, isolated at two Brooklyn, NY hospitals. The organisms were identified as enterococci using standard microbiological techniques and identified as *E. faecium* according to established methods (Facklam & Collins, 1989). Genetic comparison between strains VA3 and MMC3 included plasmid profiles, pulsed-field gel electrophoresis, and Southern blot hybridization using a vanA gene probe (Murray *et al.*, 1990; Freiden *et al.*, 1993).

Tube MICs were determined using approximately $5 \times 10^5$ cfu/mL in cation-supplemented Mueller-Hinton broth (50 mg/L calcium and 25 mg/L magnesium). The following companies provided the antibiotics: Pfizer Inc., Groton, CT (penicillin and streptomycin), Eli Lilly Co., Indianapolis, IN (vancomycin), Marion Merrell Dow, Kansas City, MO, (ramoplanin and teicoplanin), and Schering-Plough Corp., Bloomfield, NJ (gentamicin). Penicillinase was detected by nitrocefin disc testing (Becton Dickinson, Cockeysville, MD). Time kill studies were performed using penicillin (40 mg/L) and/or ramoplanin (2 mg/L), as previously described (Mobarakai *et al.*, 1994). Bactericidal activity was defined as at least 99.9% killing of the inoculum after 24 h. Results are expressed as $\log_{10}$ cfu/mL.

The interaction between ramoplanin and penicillin was explored further by determining whether preincubation with ramoplanin resulted in increased susceptibility to penicillin. The *E. faecium* strains were grown to late logarithmic phase in supplemented Mueller-Hinton broth in the presence of ramoplanin at 0.2 x MIC. Tube MICs of penicillin were then performed in the continued presence of ramoplanin at 0.02 x MIC.

**In-vivo studies**

New Zealand White rabbits (weight, approximately 2 kg) were anaesthetised with ketamine (35 mg/kg) and xylazine (10 mg/kg) im. The right carotid artery was exposed and cannulated with a polyethylene catheter (PE90, Clay Adams, NJ). The catheter was advanced across the aortic valve, ligated, and left in place for the duration of the experiment. Twenty-four hours after catheter placement, rabbits were inoculated via the marginal ear vein with approximately $10^8$ cfu of one of the strains in 1 mL of saline. At 48 h following catheter placement, blood cultures were drawn to confirm the presence of bacteraemia; endocarditis was present in virtually all animals with a properly positioned catheter. Animals were then placed into one of four treatment groups: (1) untreated control, (2) penicillin ($2 \times 10^3$ units/kg aqueous penicillin plus $3 \times 10^5$ units/kg procaine penicillin im twice daily), (3) ramoplanin (10 mg/kg iv once daily), or (4) both penicillin plus ramoplanin. The dose of ramoplanin was chosen after
preliminary experiments revealed that this dose produced trough serum concentrations near the MIC of the study strains. After 3 days of therapy, animals were killed with sodium pentobarbital at the trough of their antibiotic regimen.

On the final day of therapy, serum was obtained 1, 6 and 12 h after the penicillin dose and 1, 12 and 24 h after the ramoplanin dose for the determination of antibiotic concentrations. Blood samples were also obtained for determination of white blood count, haemoglobin, alanine aminotransferase, and creatinine. Following death, 1 mL of blood was taken and cultured in 20 mL of trypticase soy agar. Cardiac vegetations were pooled, weighed (range 26.5–245 mg), and homogenised in 1 mL of normal saline. A 0.5 mL aliquot from each homogenate was cultured in 20 mL of trypticase soy agar. Serial ten-fold dilutions of the homogenates were subcultured on to trypticase soy agar with 5% sheep blood. Studies using similar concentrations of antibiotics and bacteria failed to demonstrate any antibiotic carry-over effect. Representative samples of spleen and kidney, including areas of infarction or abscess, were processed in a similar manner. Urine samples were aspirated from the bladder for measurement of antibiotic concentrations. Only animals that survived the duration of the experiment were included for bacteriological analysis.

The emergence of ramoplanin resistant enterococci was sought in vegetation homogenates of selected control, ramoplanin, and combined therapy animals. Tube ramoplanin MICs were performed on organisms isolated from the homogenates, as described above. In addition, samples of the homogenates were subcultured directly on to trypticase soy agar containing 5 × MIC of ramoplanin with an excess of penicillinase. Finally, cultures of the parent organisms were also subcultured on to plates containing 5 × MIC to determine the spontaneous rate of development of resistance. The plates were incubated at 37°C for 72 h.

Antibiotic concentrations were measured in serum, urine, and vegetation homogenates from selected animals using a well diffusion microbiological assay. A clinical isolate of Bacillus subtilis was used as the test organism for assaying ramoplanin. Antibiotic medium no. 5 containing 1% Tween 80 and an excess of penicillinase was used. Known ramoplanin concentrations were prepared in control rabbit serum for serum measurements, in control rabbit urine for urine measurements, and in control rabbit vegetation homogenate for the vegetations. Serum penicillin concentrations were also determined using a well diffusion assay using B. subtilis as the indicator organism. For both antibiotics, determinations were performed in duplicate on each assay plate and on duplicate plates. A minimum of four control concentrations were used for each assay. For the ramoplanin bioassay, the intraplate coefficient of variation was 10.3%, the interplate coefficient of variation was 9.3%, and the average linear correlation coefficient was 0.991.

Statistical analysis
Results of quantitative cultures of vegetation, kidney and spleen are expressed as log₁₀ cfu/g (mean ± s.d.). Specimens yielding no bacterial growth were considered sterile, and assigned the value of 2 cfu/w of the specimen, which was the lower limit of detection by our methods. The effectiveness of therapy and laboratory determinations were assessed by analysis of variance. The Bonferroni factor was used to correct for multiple comparisons. Proportional data were analysed by Fisher’s exact test. Significance was defined as a P value <0.05.
This study was approved by the animal studies subcommittee of the Institutional Review Board in our hospital.

Results

In-vitro studies

All three genetic approaches revealed the dissimilarity between VA3 and MMC3. VA3 lacks plasmid DNA whereas MMC3 has extrachromosomal DNA and the Smal pulsed-field gel electrophoresis restriction patterns were distinct. The HindIII hybridization patterns with the vanA probe demonstrated the presence of the gene in both strains; however, the banding patterns were different suggesting a different genetic organisation.

The MICs of penicillin, vancomycin, and teicoplanin for strain VA3 were 128, 256, and 4 mg/L, respectively, and for strain MMC3 were 256, 512, and 64 mg/L, respectively. The MIC of ramoplanin for both strains was 0.5 mg/L; in the presence of 10% serum, the MICs rose four-fold. The MICs of gentamicin and streptomycin were >2000 mg/L for both strains. Neither strain produced β-lactamase. Growth in the presence of subinhibitory concentrations of ramoplanin did not change the MIC of penicillin. Time kill studies revealed that both ramoplanin and ramoplanin plus penicillin were bactericidal when tested in broth (change in log$_{10}$ cfu/mL at 24 h for strain VA3 of $-3.0$ and $-3.5$, respectively and for strain MMC3 of $-4.0$ and $-3.2$, respectively). However, when tested in the presence of 50% serum, only the combination of ramoplanin plus penicillin remained bactericidal ($-3.5$ for strain VA3 and $-3.2$ for strain MMC3).

In-vivo studies

Ramoplanin serum concentrations were measured in 15 animals. The peak, 12 h and trough concentrations (mean ± s.d.) were $28.2 ± 11.5$, $6.4 ± 2.4$ and $1.3 ± 0.5$ mg/L, respectively. Serum ramoplanin concentrations in the combination treatment group were not different from those in the ramoplanin treatment group. Urine ramoplanin concentrations measured at autopsy in six animals were $1.25 ± 0.7$ mg/L. The ramoplanin concentrations in cardiac vegetations measured in nine animals were $20.1 ± 7.6$ μg/g vegetation. Penicillin serum concentrations were measured in four animals. The peak, 6 h and trough concentrations (mean ± s.d.) were $110 ± 65$, $33.6 ± 7.7$ and $6.5 ± 1.0$ mg/L, respectively. White blood count, alanine aminotransferase, and creatinine reassessed on the third day of therapy were unchanged from pre-treatment values for both the ramoplanin and control groups. Haemoglobin concentrations on the final day were 10.5 ± 1.1 and 10.7 ± 0.6 g/dL in the control and ramoplanin groups, respectively, and were both significantly lower than pre-treatment values (13.7 ± 1.7 g/dL, $P < 0.05$).

In the endocarditis study with strain VA3, 25% of controls died, compared with 10% in the penicillin group, 17% in the ramoplanin group, and 0% in the combined therapy group ($P = NS$). Among surviving animals, blood cultures remained positive in 100% of controls, 89% in the penicillin group, 80% in the ramoplanin group, and 30% in the combined group ($P ≤ 0.01$, combination group vs control). For strain MMC3, 40% of controls died, compared with 11% in the penicillin group and 0% in the ramoplanin
Table. Results of quantitative cultures of the two E. faecium strains, from vegetations, kidneys, and spleens

<table>
<thead>
<tr>
<th>Treatment group (n)</th>
<th>Vegetation mean log_{10} cfu/g ± S.D. (no. sterile/n)</th>
<th>Kidney mean log_{10} cfu/g ± S.D. (no. sterile/n)</th>
<th>Spleen mean log_{10} cfu/g ± S.D. (no. sterile/n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain VA3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (12)</td>
<td>9.0 ± 0.6 (0/12)</td>
<td>6.0 ± 1.5 (0/12)</td>
<td>5.8 ± 1.6 (0/12)</td>
</tr>
<tr>
<td>penicillin (9)</td>
<td>9.0 ± 1.3 (0/9)</td>
<td>5.0 ± 2.1 (0/9)</td>
<td>4.3 ± 1.2 (1/9)</td>
</tr>
<tr>
<td>ramoplanin (10)</td>
<td>9.5 ± 0.6 (0/10)</td>
<td>3.2 ± 2.1 (3/10)*</td>
<td>3.5 ± 1.5 (3/10)*</td>
</tr>
<tr>
<td>ramoplanin + penicillin (10)</td>
<td>6.7 ± 1.5 (0/10)*</td>
<td>1.4 ± 0.2 (10/10)*</td>
<td>1.5 ± 0.3 (10/10)*</td>
</tr>
<tr>
<td>Strain MMC3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (10)</td>
<td>9.4 ± 0.5 (0/10)</td>
<td>5.2 ± 1.9 (0/10)</td>
<td>5.0 ± 1.8 (0/10)</td>
</tr>
<tr>
<td>penicillin (8)</td>
<td>8.6 ± 0.9 (0/8)</td>
<td>3.8 ± 1.6 (0/8)</td>
<td>3.6 ± 1.3 (0/8)</td>
</tr>
<tr>
<td>ramoplanin (10)</td>
<td>8.2 ± 0.4 (0/10)</td>
<td>2.8 ± 2.3 (6/10)*</td>
<td>1.2 ± 0.2 (9/10)*</td>
</tr>
<tr>
<td>ramoplanin + penicillin (10)</td>
<td>5.7 ± 1.9 (1/10)*</td>
<td>1.8 ± 1.8 (9/10)*</td>
<td>1.3 ± 0.2 (10/10)*</td>
</tr>
</tbody>
</table>

*P ≤ 0.01 compared with other three groups
*P ≤ 0.01 compared with control group.
*P ≤ 0.05 compared with control group.

and combination groups (P ≤ 0.01, ramoplanin and combination groups vs control). Among surviving animals, blood cultures remained positive in 100% of controls, 88% in the penicillin group, 20% in the ramoplanin group, and 10% in the combination group (P ≤ 0.01, ramoplanin and combination groups vs control).

The results of quantitative cultures of vegetations, kidneys and spleens are shown in the Table. Penicillin produced no significant reduction in colony counts from any site for either strain. When used alone, ramoplanin did not produce significant reduction in vegetation bacterial counts, but did reduce kidney and spleen bacterial concentrations. This was particularly true for strain MMC3 for which 6/10 kidneys and 9/10 spleens were sterilised. In contrast, combination therapy with ramoplanin and penicillin resulted in a reduction in vegetation bacterial counts of 3.2 log_{10} cfu/g for strain VA3 and 3.7 log_{10} cfu/g for strain MMC3 (P ≤ 0.01). For both strains, combination therapy resulted in sterilisation of 9/10 kidney cultures and 10/10 spleen cultures.

At concentrations of 10^9 cfu/mL of the parent organism, no ramoplanin resistant isolates could be recovered on plates containing the antibiotic at 5 × MIC. Similarly, ramoplanin resistant organisms could not be recovered from vegetations of treated animals. The MICs of ramoplanin for the organisms isolated from the vegetations in animals treated with ramoplanin were similar to the parent organism. Therefore, development of resistance could not be detected in animals following treatment with ramoplanin.

Discussion

Antibiotic resistant enterococci have become a widespread problem. The treatment of enterococcal endocarditis is difficult even when caused by susceptible strains. The emergence and spread of multidrug resistant enterococci has left clinicians with few
therapeutic options. The combination of penicillin, vancomycin and gentamicin has demonstrated good activity against a vancomycin and penicillin resistant strain (Caron, Carbon & Gutmann, 1991). This combination was also effective against a strain with a higher level of penicillin resistance (Caron et al., 1993). For both of these strains (Caron et al., 1991, 1993), penicillin/vancomycin synergy was present and high-level aminoglycoside resistance was absent. Many vancomycin resistant enterococci do not possess these characteristics (Cercenado et al., 1992; Handwerger et al., 1992). The combination of ciprofloxacin, rifampin and gentamicin was active against a strain that was susceptible to rifampin and low concentrations of aminoglycosides (Whitman et al., 1993). Neither of these regimens is likely to be effective against the multidrug resistant strains of *E. faecium* that have plagued New York City (like VA3 and MMC3) and have concomitant high-level aminoglycoside and rifampin resistance (Freiden et al., 1993; Landman et al., 1993). In previous studies of the treatment of experimental endocarditis caused by VA3 and MMC3, we found modest activity with the combination of ciprofloxacin and novobiocin (Quale, Landman & Mobarakai, 1993) and disappointing activity with the combination of ampicillin and ciprofloxacin (Landman et al., 1995), despite promising activity in vitro with these combinations. There is a clear need for the development of new agents against these pathogens.

Ramoplanin is a lipoglycopeptide antibiotic which interferes with peptidoglycan synthesis in Gram-positive bacteria (Somner & Reynolds, 1990). The inhibition precedes transpeptidation; ramoplanin presumably interferes with the transfer of N-acetylmuramylpentapeptide to lipid intermediate I, resulting in depletion of lipid intermediate II (Somner & Reynolds, 1990). This mechanism is different from that of β-lactams which inhibit penicillin-binding proteins that serve as transpeptidases for peptidoglycan synthesis. Our experiments suggest that the combination of diminished pentapeptide transport with some inhibition of transpeptidation led to enhanced killing of resistant enterococci. We were unable to detect the development of ramoplanin resistance in vitro or in vivo; therefore, the mechanism of enhanced activity of the combination does not appear to be due to the prevention of resistant mutants. Preincubation with subinhibitory concentrations of ramoplanin did not alter the MIC of penicillin. It is possible that higher concentrations of ramoplanin may affect penicillin-binding proteins. Further studies are necessary to determine the mechanism of interaction of these two antibiotics.

In the present study, ramoplanin alone was ineffective in reducing vegetation bacterial counts. This finding is not surprising in view of the in-vitro studies showing that the activity of ramoplanin was markedly diminished by the addition of protein. The combination of ramoplanin and penicillin, however, was able to reduce vegetation colony counts significantly after only 3 days of therapy, and sterilised nearly all cultures of kidney, spleen and blood. These results concur with the in-vitro finding of enhanced activity with the combination of ramoplanin plus penicillin. Although ramoplanin has been developed for topical use only, further study of other lipoglycopeptide antibiotics is warranted.

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References


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