Time – Kill and Synergism Studies of Ceftobiprole against Enterococcus faecalis including β-Lactamase Producing and Vancomycin-Resistant Isolates

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ABSTRACT

Ceftobiprole (BAL9141) is an investigational cephalosporin with broad *in vitro* activity against Gram-positive cocci including enterococci. Ceftobiprole minimal inhibitory concentrations (MICs) were determined for 93 isolates of *E. faecalis* (including 16 β-lactamase (Bla) producers and 17 vancomycin-resistant isolates) using an agar dilution method following the CLSI recommendations. Ceftobiprole MICs were also determined using a high inoculum (10^7 CFU/ml) for a subset of five Bla+ producers belonging to different previously characterized clones using a broth dilution method. Time-kill and synergism studies (with either streptomycin or gentamicin) were performed using two β-lactamase producing isolates (TX0630 and TX5070) and two vancomycin-resistant isolates (TX2484, VanB and TX2784, VanA). The MIC_{50/90}'s of ceftobiprole were 0.25 µg/ml and 1 µg/ml, respectively. All Bla-producers and vancomycin-resistant isolates were inhibited by concentrations of ≤ 1 and ≤ 4 µg/ml, respectively, at standard inoculum. Ceftobiprole MICs at high inoculum for a subset of five Bla+ *E. faecalis* were ≤ 1 µg/ml. Bactericidal activity was observed for 4 isolates tested at concentrations as low as 1 µg/ml regardless of the production of β-lactamase or vancomycin resistance. The combination of ceftobiprole (0.5 µg/ml) and streptomycin (25 µg/ml) was synergistic against Bla+ TX0630 and TX5070. Ceftobiprole (0.5 µg/ml) plus gentamicin (10 µg/ml) was synergistic against the VanB isolate TX2484, and showed enhanced killing, but not synergism against TX2784 (VanA), despite absence of high level resistance to gentamicin. In conclusion, ceftobiprole exhibited good *in vitro* activity against *E. faecalis*, including Bla+ and vancomycin-resistant strains, and exhibited synergism with aminoglycosides for selected isolates.
INTRODUCTION

Enterococcal infections continue to be a challenge in clinical practice due to the fact that these organisms have the ability to quickly acquire and disseminate resistance genes. The introduction of new agents into clinical practice (e.g., linezolid and daptomycin, amongst others) has been shortly followed by the development of resistance (10). Furthermore, the treatment of certain enterococcal infections (e.g., endocarditis) requires the use of bactericidal (14) agents which decrease even further the choice of antimicrobials.

Ceftobiprole (BPR) is a novel broad-spectrum parenteral cephalosporin with high affinities for Gram-negative and Gram-positive penicillin-binding proteins (PBPs), including PBP 2a from methicillin-resistant *Staphylococcus aureus* (MRSA) (2, 6) and PBP 2X from resistant pneumococci (5, 11). The *in vitro* spectrum of activity includes both Gram-positive and Gram-negative organisms, including *S. aureus* (both MRSA and MSSA isolates) (2, 7), pneumococci (including penicillin and ceftriaxone resistant isolates) (5), *Streptococcus pyogenes* and other streptococci (11), *Haemophilus influenzae* and *Moraxella catarrhalis* (including β-lactamase producers) (1), *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Serratia marcescens, Citrobacter freundii, Pseudomonas aeruginosa* and *Proteus mirabilis* lacking extended spectrum β-lactamases (ESBLs) (11, 19). Against anaerobes, ceftobiprole had good *in vitro* activity against *Propionibacterium acnes, Peptostreptococcus anaerobius, Clostridium innocuum, Finegoldia magna, Porphyromonas asaccharolytica* (including a β-lactamase
producing isolate) and Porphyromonas somerae (9), although Bacteroides fragilis group isolates have been found to be resistant (9, 23).

Against enterococci, BPR was reported to have in vitro bactericidal activity against most strains of ampicillin and vancomycin-susceptible Enterococcus faecalis with MIC$_{50}$/MIC$_{90}$ values of 0.5 and 4 µg/ml for a small collection of E. faecalis isolates (6, 11). BPR exhibited bactericidal activity at concentrations of 4, 8 and 16 µg/ml using an ampicillin and vancomycin-susceptible E. faecalis isolate (E. faecalis ATCC 29212) (6). Time-kill studies failed to show synergism when ceftobiprole (8 µg/ml) was combined with gentamicin (at ¼ MIC) against two clinical isolates of E. faecalis (ampicillin and vancomycin-susceptible) (6). When tested against ampicillin-susceptible E. faecium, the BPR MIC$_{90}$ value was 8 µg/ml (11), but it lacked activity against ampicillin-resistant E. faecium (6, 11).

The objective of this work was to evaluate the in vitro activity of BPR against a larger collection of E. faecalis isolates and use time-kill curves and synergism studies (with aminoglycosides) to specifically assess ceftobiprole bactericidal activity against β-lactamase producing (Bla+) and vancomycin-resistant isolates.
MATERIALS AND METHODS

**Bacterial strains.** A total of 93 isolates of *E. faecalis* were included in the study. They were isolated from five different countries (USA, Chile, Argentina, Lebanon and Thailand) and the majority of the isolates were obtained from patients’ clinical samples (mainly blood from endocarditis and non-endocarditis patients and urine). The collection included 17 vancomycin resistant isolates and 15 β-lactamase producing *E. faecalis*. The Bla+ isolates had previously been characterized by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (17) and confirmation of β-lactamase activity was performed with nitrocefin disks following the Clinical Laboratory Standards Institute (CLSI) recommendations (3). Isolates obtained from fecal samples of healthy volunteers (2 isolates), animals and animal feed (7 isolates) and laboratory strains (*E. faecalis* JH2-2 (12) and OG1RF (16) were also included in the study. All isolates were kept at -80°C and recovered from frozen stocks.

**MIC determinations.** MICs of vancomycin, ampicillin, BPR (BAL9141), gentamicin and streptomycin were determined by an agar dilution method on Mueller-Hinton agar II (Becton Dickinson and Company, Cockeysville, Md) as recommended by the CLSI (3). BPR was diluted in 9.9% glacial acetic acid and 1% high quality dimethy sulfoxide (DMSO) as recommended by the manufacturer (Johnson & Johnson, Raritan NJ). Susceptibilities of isolate TX5070 (a Bla+ transconjugant of *E. faecalis* JH2-7 (Table 1), which is a thymine auxotroph and cannot grow on Mueller-Hinton) (12), were performed on brain heart infusion (BHI) agar with a starting inoculum of $10^4$ CFU/spot. For selected
Bla+ *E. faecalis* isolates, BPR and ampicillin MIC determinations were performed at high inocula (10^7 CFU/ml) in Mueller-Hinton broth (Becton Dickinson and Company, Cockeysville, Md). Control strains included *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213 and *E. coli* 25922.

**Time-kill and synergism studies.** The bactericidal activity of BPR was evaluated by time-kill curves. Synergism studies were performed using sub-inhibitory concentrations of either gentamicin (10 µg/ml) or streptomycin (25 µg/ml) (14). The following *E. faecalis* isolates were chosen for time-kill and synergism studies (Table 1): The Bla+ strains *E. faecalis* TX0630 (a clinical blood isolate originally recovered in Argentina (4)) and TX5070 (a laboratory strain obtained in a mating experiment between the first ever discovered Bla+ enterococcal isolate (*E. faecalis* HH22, used as donor) (15) and *E. faecalis* JH2-7 (a thymine auxotroph) (12)) and the vancomycin-resistant, ampicillin-susceptible isolates TX2484 and TX2784. Bacteria were grown in flasks at a final volume of 20 ml in BHI broth with a starting inoculum of 10^7 CFU/ml from an overnight culture. Ceftobiprole was added at concentrations of 1 and 2 µg/ml for time-kill curves and 0.5 µg/ml for synergism studies which are below the therapeutic levels achieved in humans (22). The concentrations of aminoglycosides used in the synergy studies were below the MIC of the organisms and produced no significant growth inhibition in the absence of ceftobiprole. Concentrations of gentamicin 10 µg/ml and streptomycin 25 µg/ml were found to yield the best killing activity. Viable counts were determined at times 0, 4 and 24 h by plating appropriate dilutions of the cultures on BHI agar plates. Antibiotic carryover was eliminated by centrifuging 1 ml samples of the culture and resuspending
the pelleted bacteria in 0.9% saline before plating. Time-kill and synergism studies were performed from 2 to 4 times per strain. The level of detection was 10 CFU/ml assuming maximum plating efficiency. Bactericidal activity was defined as \( \geq 3\log_{10} \) decrease in CFU/ml between counts at time 0 h and those observed at time 24 h. Synergism was defined as a \( \geq 2\log_{10} \)- decrease in CFU/ml between the combination of ceftobiprole plus aminoglycosides (gentamicin or streptomycin) and ceftobiprole alone at 24 h, with a concentration of the aminoglycoside that did not affect the growth curve of the test organism when used alone.

**RESULTS**

**Ceftobiprole MICs.** The BPR MIC distribution of all isolates including the subgroups of vancomycin-resistant and Bla+ isolates is shown in Figure 1. The BPR MIC\textsubscript{90} and MIC\textsubscript{50} for all isolates were 1 µg/ml and 0.25 µg/ml, respectively, ranging from <0.015 to 4 µg/ml. The presence of vancomycin resistance did not influence BPR susceptibility: 94% of vancomycin resistant isolates had MICs of ceftobiprole \( \leq 1 \) µg/ml. Among the 16 \( \beta \)-lactamase producers, the BPR MIC\textsubscript{100} was 1 µg/ml. Table 2 shows the BPR MICs for a subset of five \( \beta \)-lactamase producers from different clonal origins at two inocula. The use of a high inoculum (10\(^7\) CFU/ml) for MIC determination in broth for these isolates resulted in an increase of 2 to 8 fold in the MIC but all MIC’s were of \( \leq 2 \) µg/ml (Table 2). A similar increase occurred in isolates TX2484 and TX2784 lacking the \( \beta \)-lactamase enzyme (2 and 4 fold increase in MIC at high inoculum).
Bactericidal activity of ceftobiprole. The bactericidal activity of BPR was assessed by time-kill assays against four different isolates of *E. faecalis* (Table 1). Fig. 2 shows the *in vitro* activity of ceftobiprole against two Bla+ strains (TX0630 panel A, and TX5070, panel B). Both isolates were highly resistant to gentamicin but lacked high level resistance (HLR) to streptomycin (Table 1). Concentrations of BPR as low as 1 and 2 µg/ml were bactericidal against both Bla+ strains, decreasing the viable bacterial count (CFU/ml) ca. 4 log₁₀ from the starting inoculum (time 0) (Fig. 2). Fig. 3 shows that ceftobiprole was also bactericidal against vancomycin resistant isolates. For both TX2484 (a VanB isolate) and TX2784 (a VanA isolate), BPR decreased the viable counts > 3 log₁₀ CFU/ml at 24 h from the starting inoculum.

Synergism between ceftobiprole and aminoglycosides. The synergistic activity of aminoglycosides (either gentamicin or streptomycin) was evaluated for two Bla+ and two vancomycin resistant *E. faecalis* isolates. For the Bla+ strains (which exhibit high level resistance to gentamicin but not streptomycin), the addition of streptomycin (25 µg/ml) to ceftobiprole was synergistic (Fig. 2). The decrease in viable counts (CFU) at 24 h was > 2 log₁₀ (ca. 3 log₁₀ for TX0630 and ca. 4 log₁₀ for TX5070 when compared with ceftobiprole alone) (Fig. 2A and 2B). Similarly, the combination of ceftobiprole and gentamicin (10 µg/ml) was synergistic against the vancomycin-resistant isolate TX2484 (Fig. 3A) (reduction of ca. 2.5 log₁₀ CFU/ml when gentamicin was added compared to ceftobiprole alone). The addition of gentamicin (10 µg/ml) for isolate TX2784 decreased counts by less than 2 log₁₀ CFU/ml at 24 h (Fig. 3B). The lack of synergism in strain
TX2784 was observed at concentrations of 0.25, and 1 µg/ml of ceftobiprole and using concentrations of gentamicin of 5 and 8 µg/ml (data not shown).

**DISCUSSION**

Ceftobiprole is a novel cephalosporin that has been shown to be active against Gram-positive organisms (including MRSA) and also maintains the spectrum of extended spectrum cephalosporins against Gram-negative bacteria (2, 11). The basis for ceftobiprole’s potent activity against many organisms is its high affinity for penicillin binding proteins (including PBP2a of MRSA) and stability against hydrolysis by β-lactamases (11). Against enterococci, ceftobiprole displays unique properties amongst the cephalosporins, since it has good activity against isolates of *E. faecalis* (6). A previous study (11) showed that the MIC<sub>90</sub> for a collection of 14 clinical isolates of ampicillin-susceptible *E. faecalis* was 4 µg/ml. The results of our work support the potent in vitro activity of ceftobiprole against *E. faecalis* from different geographical, clinical and host origins (MIC<sub>90</sub> of 1 µg/ml for our isolates). Furthermore, our findings confirm that susceptibility to ceftobiprole in *E. faecalis* is not affected by the presence of vancomycin resistance or by β-lactamase production in enterococci. Although a modest inoculum effect was seen with ceftobiprole for both Bla<sup>+</sup> and Bla<sup>−</sup> isolates, the MIC remained \( \leq 2 \) µg/ml.

We also evaluated the bactericidal activity of ceftobiprole against four strains of *E. faecalis* exhibiting either vancomycin resistance (VanA and VanB phenotypes, Table 1)
or ampicillin resistance due to the production of the β-lactamase enzyme. Ceftobiprole was bactericidal in time-kill studies against all strains at concentrations as low as 1 µg/ml. Pharmacokinetic studies (21, 22) have shown that single infusions of 750 mg of ceftobiprole medocaril (a BAL9141 prodrug) led to mean plasma concentrations above 4 µg/ml for approximately 7 h (22) (the MIC at which 100% of our E. faecalis were inhibited, including Bla+ isolates at high inoculum). Our results support the fact that ceftobiprole would likely exhibit bactericidal activity against E. faecalis at the dose proposed. Our findings are also in agreement with those of Despande et al (6), who showed that ceftobiprole (at concentration of 4, 8 and 16 µg/ml) was bactericidal against E. faecalis ATCC 29212 and two additional E. faecalis clinical isolates (tested at 8 µg/ml). The bactericidal activity against E. faecalis is a unique characteristic of ceftobiprole amongst the cephalosporins and is likely to be due to the high affinity for the enterococcal penicillin binding proteins (PBPs). Ceftobiprole has been shown to exhibit increased affinity for PBPs of several Gram-positive organisms (particularly PBP2a of MRSA and S. epidermidis) (11) when compared with other β-lactams. Moreover, it has been shown that ceftobiprole acylates PBP 2a more rapidly than other β-lactam antibiotics and forms a more stable acyl-enzyme complex through a unique mode of interaction with the protein (11).

Another important feature of ceftobiprole is its β-lactamase stability. The production of this enzyme is rare among clinical isolates of E. faecalis but its presence compromises the use of the most effective anti-enterococcal β-lactams (e.g., ampicillin). The enterococcal β-lactamase is identical to the staphylococcal class A enzyme encoded by
the blaZ gene (15), and ceftobiprole is a poor substrate of these enzymes which explains its excellent activity against β-lactamase producing *E. faecalis*.

The combination of β-lactams and aminoglycosides has been widely used in the treatment of enterococcal infections that require bactericidal therapy for optimal cure rates (e.g., endocarditis) (14). Previously, no synergistic activity was observed for two strains of *E. faecalis* when using concentrations of ceftobiprole of 8 µg/ml and gentamicin at ¼ of the MIC of the strains (6). In contrast with these data, we were able to demonstrate synergism in 3 out of 4 isolates of *E. faecalis* when using concentrations of ceftobiprole of 0.5 µg/ml (which is at MIC value or slightly higher). An explanation for this discrepancy is that, at concentrations of ceftobiprole as high as 8 µg/ml (as used by Deshpande et al. (6)), the killing effect of ceftobiprole is so marked that it may mask the effect of the aminoglycoside. Consistent with this hypothesis is the fact that we were unable to show any synergism when using concentrations of 1 µg/ml, 2 µg/ml and 4 µg/ml of ceftobiprole (data not shown).

Synergistic activity was evident in the presence of β-lactamase in different host backgrounds. Both TX0630 (a Bla+ clinical strain) and TX5070 (a Bla+ laboratory strain) exhibited synergism when ceftobiprole was combined with streptomycin at concentrations of 25 µg/ml. Both isolates have HLR to gentamicin but not to streptomycin. Similarly, the combination of ceftobiprole (0.5 µg/ml) and gentamicin (10 µg/ml) was synergistic against one vancomycin-resistant (VanB) isolate of *E. faecalis*. However, although a decrease in viable counts by the combination of ceftobiprole and
gentamicin compared to ceftobiprole alone was observed at 24 h in isolate TX2484 (a VanA human fecal isolate from Spain), the reduction in CFU/ml did not reach the cut-off for synergism (> 1 log_{10} but ≤ 2 log_{10}). These results indicate that, with selected isolates of Bla+ or vancomycin-resistant \textit{E. faecalis}, the combination of ceftobiprole and aminoglycosides could be potentially useful in clinical settings where bactericidal therapy is important. \textit{In vivo} studies will be of paramount importance to clarify this issue.

As opposed to \textit{E. faecalis} isolates, \textit{E. faecium} have developed different strategies for β-lactam resistance which include the hyperproduction of PBP5 which has low affinity for β-lactams and is capable of substituting for the functions of β-lactam susceptible PBPs (8) and by introducing amino acid substitutions in the penicillin-binding domain of PBP5 (13, 20, 24). Previous studies (6) have shown that ceftobiprole had no activity against ampicillin-resistant \textit{E. faecium} indicating that it is likely that the ceftobiprole affinity for the PBP5 of \textit{E. faecium} is low and therefore not clinically useful for the treatment of ampicillin-resistant \textit{E. faecium} infections.

In conclusion, we demonstrated that ceftobiprole has potent \textit{in vitro} activity against the largest collection of \textit{E. faecalis} tested to date. The activity was not affected by vancomycin-resistance or production of β-lactamase and synergism with aminoglycosides can be achieved in selected strains. Therefore, ceftobiprole emerges as a promising agent with potential for future use to treat Bla+ and vancomycin-resistant \textit{E. faecalis} infections.
ACKNOWLEDGEMENTS

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Legends to Figures

**Figure 1.** MIC distributions for ceftobiprole (BPR9141) against clinical isolates of *E. faecalis* including vancomycin-resistant isolates; VanR, vancomycin resistant

**Figure 2.** Time-kill and synergism studies of ceftobiprole and streptomycin against two Bla+ isolates of *E. faecalis*. Panel A, TX0630; panel B, TX5070. Detection limit 10 CFU/ml. BPR, ceftobiprole; STR, streptomycin.

**Figure 3.** Time-kill and synergism studies of ceftobiprole and gentamicin against two vancomycin-resistant isolates of *E. faecalis*. Panel A, TX2484 (VanB); panel B, TX2784 (VanA). Detection limit 10 CFU/ml. BPR, ceftobiprole; GEN, gentamicin.
### Table 1. *E. faecalis* strains used in time-kill and synergism studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
<th>Bla+</th>
<th>MIC (µg/ml)</th>
<th>HLR* to Aminoglycosides</th>
<th>Synergism&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX0630</td>
<td>Blood isolate from Argentina recovered in 1990</td>
<td>(4)</td>
<td>YES</td>
<td>0.25</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>TX5070</td>
<td>JH2-7 transconjugant resulting from mating</td>
<td>(12)</td>
<td>YES</td>
<td>0.125&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>with a bla+ strain (HH-22). Thymine auxotroph</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX2484</td>
<td>Houston blood isolate, 1994, VanB</td>
<td>(4)</td>
<td>NO</td>
<td>0.5</td>
<td>0.5</td>
<td>&gt;256</td>
</tr>
<tr>
<td>TX2784</td>
<td>Human fecal isolate from Spain, 1998. VanA</td>
<td>(18)</td>
<td>NO</td>
<td>0.25</td>
<td>0.5</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

<sup>a</sup>HLR, high level resistance

<sup>b</sup>Performed in brain heart infusion (BHI) agar since TX5070 does not grow on Mueller-Hinton agar. *E. faecalis* ATCC 29212 used as control.

<sup>c</sup>Using either gentamicin 10 µg/ml or streptomycin 25 µg/ml when appropriate

BPR, ceftobiprole; AMP, ampicillin; VAN, vancomycin; GEN, gentamicin; STR, streptomycin

<sup>d</sup> > 1 log<sub>10</sub> but < 2 log<sub>10</sub> decrease in CFU/ml vs BPR at 24 h.
Table 2. Ceftobiprole (BAL9141) MICs at different inocula against β-lactamase (Bla) producing *E. faecalis* from different clonal origins

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clonal origin (MLST type)</th>
<th>BPR MIC</th>
<th>Aminoglycoside HLR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inoculum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TX0921</td>
<td>BVE&lt;sup&gt;a&lt;/sup&gt; (ST-7)</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>TX0614</td>
<td>BVE (ST-6)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TX0638</td>
<td>BVE (ST-7)</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>TX0630</td>
<td>ACB&lt;sup&gt;b&lt;/sup&gt; (ST-9)</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td>TX0645</td>
<td>Unrelated</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> BVE, Bla-vancomycin-resistant-endocarditis clone

<sup>b</sup> ACB, Argentina-Connecticut-Bla clone

<sup>c</sup> Standard inoculum was 10⁴ CFU/spot in agar and high inoculum was 10⁷ CFU/ml in broth; HLR, High level resistance; GEN, gentamicin; STR, streptomycin