Ceftobiprole Efficacy In Vitro against Panton-Valentine Leukocidin Production and In Vivo against Community-Associated Methicillin-Resistant Staphylococcus aureus Osteomyelitis in Rabbits

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Community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) can cause osteomyelitis with severe sepsis and/or local complications in which a Panton-Valentine leukocidin (PVL) role is suspected. In vitro sub-MIC antibiotic effects on growth and PVL production by 11 PVL+ MRSA strains, including the major CA-MRSA clones (USA300, including the LAC strain; USA400; and USA1000), and 11 PVL+ methicillin-susceptible S. aureus (MSSA) strains were tested in microplate culture. Time-kill analyses with ceftobiprole at its MIC were also run with LAC. Efficacies of ceftobiprole (40 mg/kg of body weight subcutaneously [s.c.] four times a day [q.i.d.] or vancomycin (60 mg/kg intramuscularly [i.m.] twice a day [b.i.d.]) alone or combined with rifampin (10 mg/kg b.i.d.) against rabbit CA-MRSA osteomyelitis, induced by tibial injection of 3.4 × 10^7 CFU of LAC, were compared. Treatment, started 14 days postinoculation, lasted 14 days. In vitro, 6/11 strains cultured with sub-MICs of ceftobiprole produced 1.6- to 4.8-fold more PVL than did the controls, with no link to specific clones. Rifampin decreased PVL production by all tested strains. In time-kill analyses at the LAC MIC (0.75 mg/liter), PVL production rose transiently at 6 and 8 h and then declined 2-fold at 16 h, concomitant with a 2-log_{10} CFU-count decrease. In vivo, the mean log_{10} CFU/g of bone for ceftobiprole (1.44 ± 0.40) was significantly lower than that for vancomycin (2.37 ± 1.22) (P = 0.034), with 7/10 versus 5/11 bones sterilized, respectively. Combination with rifampin enhanced ceftobiprole (1.16 ± 0.04 CFU/g of bone [P = 0.056], 11/11 sterile bones) and vancomycin (1.23 ± 0.06 CFU/g [P = 0.011], 11/11 sterile bones) efficacies. Ceftobiprole bactericidal activity and the rifampin anti-PVL effect could play a role in these findings, which should be of interest for treating CA-MRSA osteomyelitis.

Methicillin-resistant Staphylococcus aureus (MRSA) infections are increasingly being detected in the community worldwide (4). In the United States, the community-associated (CA) MRSA genotype USA300 has emerged as the major circulating strain type. CA-MRSA strains are generally more virulent than hospital-acquired (HA) MRSA, a finding consistent with the ability of CA-MRSA to cause disease in children and adults without predisposing factors (8). At present, skin and soft tissue infections represent the majority of the CA-MRSA disease burden, but severe acute diseases, such as necrotizing pneumonia, sepsis, and osteomyelitis, have also been described (3, 15, 16). Concomitantly with the recent worrying emergence of CA-MRSA strains, the S. aureus osteomyelitis incidence in hospitalized children doubled between 2002 and 2007 in the United States, and that increase was due exclusively to MRSA (14). Furthermore, pediatricians reported more unusual cases of staphylococcal osteomyelitis, characterized by severe sepsis (16) and/or local extension with concomitant myositis or pyomyositis (2).

Most CA-MRSA strains produce Panton-Valentine leukocidin (PVL). The PVL contribution to the course of osteomyelitis, suspected very early by Panton and Valentine themselves (31), was highlighted by the observation that S. aureus bone-and-joint infections were more severe and required prolonged treatment when the strain produced PVL (2, 9). In experimental Los Angeles County clone (LAC USA300) CA-MRSA rabbit osteomyelitis, PVL was shown to play a role in the local extension of the infection to muscle (6), a result consistent with the ability of PVL to cause muscle damage in a mouse model of necrotizing soft tissue infection (38).

At present, little is known about the therapeutic options for bone infections caused by PVL+ CA-MRSA. Therapeutic recommendations have been derived from those of HA-MRSA infections (25), and the optimal regimen remains to be defined. Unlike HA-MRSA, CA-MRSA strains are often susceptible in vitro to several oral antimicrobials (4). However, some of those compounds can be ineffective in vivo (32). In addition to the bacteriostatic/bactericidal activity, the effect of antibiotics on PVL release by the strains could also play a role in their in vivo efficacy. Indeed, it was shown in vitro that subinhibitory antibiotic concentrations influenced PVL release (10). In osteomyelitis, the bacteria may encounter such low concentrations because of poor antibiotic penetration into cortical bone (34).

Vancomycin alone or combined with rifampin remains the
first-line parenteral therapeutic option for MRSA osteomyelitis in the Infectious Diseases Society of America (IDSA) guidelines (25). However, vancomycin efficacy might not be optimal and some data suggested that it could be less effective than beta-lactams against methicillin-susceptible S. aureus (MSSA) bacteremia (36).

Ceftobiprole is the first broad-spectrum cephalosporin with bactericidal activity against MRSA, including CA-MRSA isolates (23, 43). It was shown to be as effective as vancomycin against MRSA, including CA-MRSA isolates (25, 42). However, its in vivo efficacy against PVL-producing CA-MRSA osteomyelitis models has already been demonstrated (37, 42). However, its in vivo efficacy against PVL-producing CA-MRSA osteomyelitis has never been tested.

The goals of this study were (i) to evaluate in vitro ceftobiprole activity against the isolates belonging to major PVL⁺ CA-MRSA clones circulating worldwide and its impact on their capacity to produce PVL and (ii) to compare the in vivo efficacies of ceftobiprole and vancomycin alone or combined with rifampin against experimental CA-MRSA osteomyelitis in rabbits.

(MThis study was presented, in part, at the 21st European Congress of Chemotherapy, ECCMID-ICC, Milan, Italy, 7 to 10 May 2011 [35a].)

**MATERIALS AND METHODS**

### In vitro study design.

**i) Bacterial strains.** The PVL⁺ S. aureus LUG855 strain obtained by lysogenization of the reference strain RN6390 with phage phISLT as control (28); 11 S. aureus isolates, including the major PVL-producing CA-MRSA clones; and 10 PVL⁻ MSSA strains were used (Table 1). Strains were characterized by multilocus sequence typing (MLST), with spa typing and mecA and pvl gene detection performed as previously described (12, 17, 20). Six mecA-negative strains showed agr-1 and ST8 by MLST and t008, t024, or t1911 (spa type) and belonged to the USA300 lineage. The mecA⁺ strains belonged to the major CA-MRSA clones spreading throughout the United States: 5 strains showed agr-1 and ST8 (MLST) and t008 (spa type) and belonged to the USA300 major lineage, including the LAC strain (kindly provided by Franck DeLeo, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT) (41); the 5 agr-3 and ST1 (MLST) and t125, t128, t175 spa-type strains belonged to the USA400 lineage; the agr-1, ST39, and t216 strain belonged to the USA1000 lineage.

(ii) Antibiotics. Antibiotic MICs were determined for each isolate using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) in Mueller-Hinton medium (bioMérieux, Marcy l’Etoile, France) (5) and adapted to CCY medium containing casein hydrolysate and yeast extract (bioMérieux) because optimal PVL yield is obtained in this medium. Ceftobiprole was provided by Basilea Pharmaceutica (Basel, Switzerland), whereas oxacillin was purchased from Biochemika Fluka (Buchs, Switzerland), and rifampin and vancomycin were purchased from Sigma-Aldrich (L’Isle d’Abeau, France). S. aureus strain ATCC 29213 served as the control for MIC determinations.

(iii) Culture conditions for PVL production in the presence of antibiotics. Isolates were preincubated aerobically on blood agar at 37°C overnight before being tested for PVL production using the broth microdilution method in modified CCY medium, without or with antibiotics (at 0.5×, 0.25×, and 0.12× MIC) according to the CLSI standard procedure. After 24 h at 37°C without shaking, samples were taken for counting of bacterial colonies in diluted broth and PVL quantification by enzyme-linked immunosorbent assay (ELISA), as previously described (1, 10). Experiments were run in triplicate. Results are expressed as the mean ratios of μg of PVL/log₁₀ CFU of bacteria grown with each antibiotic concentration to that of bacteria grown without antibiotic (control).

Time-kill experiments cultured the LAC strain in CCY medium without or with ceftobiprole at its MIC starting with an inoculum of 10⁷ cfu/mL.

### Table 1. Characterization of clinical strains and MICs of selected antibiotics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence type</th>
<th>spa type</th>
<th>mecA status</th>
<th>agr allele</th>
<th>lukSF-PV</th>
<th>Source</th>
<th>MIC (mg/liter) in CCY medium</th>
</tr>
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<tbody>
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<td>8</td>
<td>t1911</td>
<td>−</td>
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<td>0.25 0.007 0.5 1</td>
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<td>−</td>
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<td>+</td>
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</tr>
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<td>t008</td>
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<td>t528</td>
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<td>This study</td>
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<td>LAC USA300</td>
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<td>ND</td>
<td>ATCC</td>
<td>0.12 0.007 0.25 0.5</td>
<td></td>
</tr>
</tbody>
</table>

**a** Abbreviations: ND, not determined; OXA, oxacillin; CEF, ceftobiprole; RIF, rifampin; VAN, vancomycin.

**b** MICs were determined according to CLSI recommendations for 22 strains cultured in CCY broth.
Given for 7 days following surgery. A 3.4 CFU/g of bone and as the percentage of animals with sterile bone. Bone (OD600) of 1 and PVL was assessed with a specific ELISA at all times.

Results are expressed as means of incubation at 37°C, the number of viable microorganisms was determined. Analyses were computed with SPSS v19.0 software.

**RESULTS**

**In vitro study.** (i) MIC determinations. As expected, all mecA+ strains were resistant to oxacillin, and all strains were susceptible to vancomycin, rifampin, and ceftobiprole (Table 1).

(ii) Antibiotic effect on bacterial growth. CFU counts from 3 different experiments with the LAC strain after incubation of microplate cultures without or with subinhibitory antibiotic concentrations (0.125×, 0.25×, and 0.5× MIC) are shown in Table 2. As expected from previous experiments, growth was inhibited (bacterial inoculum loss exceeded 1 log10 CFU) when oxacillin or vancomycin, at 2 and 4 times their MICs, to detect potentially emerging resistant mutants after 24, 48, and 72 h of incubation at 37°C. When bacterial growth was observed, MICs were determined using the Etest method (AB Biodisk, Solna, Sweden). Mutants were defined as having 3-fold-higher MICs than the initial strain.

### TABLE 2 Antibiotic effects on bacterial growth

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No antibiotic</th>
<th>0.125× MIC</th>
<th>0.25× MIC</th>
<th>0.50× MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>9 ± 0.3</td>
<td>8 ± 0.2</td>
<td>7.5 ± 0.3</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>Ceftobiprole</td>
<td>9 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>7.5 ± 0.6</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>9 ± 0.5</td>
<td>8.5 ± 0.3 A</td>
<td>8 ± 0.4</td>
<td>7 ± 0.4</td>
</tr>
<tr>
<td>Rifampin</td>
<td>9 ± 0.3</td>
<td>9 ± 0.4 A</td>
<td>8.7 ± 0.3 A</td>
<td>8 ± 0.3</td>
</tr>
</tbody>
</table>

a The S. aureus LAC strain was incubated in CCY medium without or with oxacillin, ceftobiprole, vancomycin, or rifampin (at 0.50×, 0.25×, and 0.125× MIC) according to the CLSI standard procedures for 24 h at 37°C without shaking. Results of 3 experiments are expressed as mean ± SD log10 CFU of bacteria. Statistical significance is shown by capital letters: A, P < 0.05 versus oxacillin; B, P < 0.05 versus ceftobiprole.

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Antibiotic effect on PVL production by 22 S. aureus strains. Strains were incubated on CCY-containing microplates without (●) or with antibiotics (oxacillin [◇], vancomycin [△], rifampin [■], and ceftobiprole [□]) at 0.50 ×, 0.25 ×, and 0.125 × MIC according to CLSI standard procedures for 24 h at 37°C without shaking. Samples were taken for bacterial counting and PVL quantification by ELISA. Results are expressed as the ratio of mean µg of PVL/ log10 CFU of bacteria grown at the indicated antibiotic concentration to that of bacteria cultured without antibiotic (control). Results are the means of 3 independent experiments for each strain, and horizontal bars indicate the median values. *, P < 0.05 (ANOVA), antibiotic versus control; atb, antibiotic; OXA, oxacillin; VAN, vancomycin; RIF, rifampin; CEF, ceftobiprole.

(iii) Antibiotic effect on PVL production in microculture (Fig. 1). PVL production by all strains increased when grown with 0.125 or 0.25 times the oxacillin MIC. The amplitude of the enhancement was strain dependent and ranged from 1.2 to 7.2 times, compared to control PVL production. Thus, the medians increased significantly by 1.86-fold for 0.125 × MIC and 2.15-fold for 0.25 × MIC (P < 0.05). When cultured with 0.5 × MIC, overall PVL production was not modified.

PVL production increased for only 15/22 strains cultured with 0.25 or 0.125 times the ceftobiprole MIC. The amplitude increase was strain dependent and ranged from 1.7 to 4.8 times, compared to the control PVL production. Thus, the medians rose significantly by 1.43-fold for 0.125 × MIC and 1.18-fold for 0.25 × MIC (P < 0.05). At 0.125 × or 0.25 × ceftobiprole MIC, induced PVL production levels were significantly lower than those observed with oxacillin (P < 0.05). As for oxacillin at 0.5 × MIC, the overall PVL production was not modified in the presence of 0.5 × ceftobiprole MIC.

PVL production decreased in the presence of 0.5 or 0.25 times the rifampin MIC for all strains and for most of the strains at 0.125 times its MIC. The reduction amplitude was strain dependent and ranged from 2 to 20 times below control growth. Thus, the medians decreased significantly by 1.45-fold for 0.125 × MIC and 5-fold for 0.25 × MIC, and 16.67-fold for 0.5 × MIC (P < 0.05).

Vancomycin, at any concentration tested, did not significantly affect PVL production, which was significantly lower with vancomycin than with ceftobiprole at 0.125 ×, 0.25 ×, and 0.5 × MIC (P < 0.006).

(iv) Ceftobiprole effect on LAC PVL production in time-kill cultures. Figure 2 shows the transient PVL production rise of 1.91 and 1.84 times control growth after 6 and 8 h of incubation with ceftobiprole at its MIC. After 16 h, S. aureus cultured with ceftobiprole showed a 2-fold PVL production decline concomitant with a 2-log10-CFU decrease of bacterial counts in time-kill analyses.

Experimental CA-MRSA (LAC) osteomyelitis study. Following a single s.c. injection of ceftobiprole (40 mg/kg), its mean peak plasma concentration was 100.1 ± 24.7 µg/ml, the mean trough concentration was 4.53 ± 2.07 µg/ml, and area under the curve from 0 to 8 h was 283 mg · h/liter (n = 4). Those parameters are close to those obtained in humans given the 1,000-mg dose three times a day (t.i.d.) (27).

Control animals infected with LAC had a mean bacterial count of 4.57 ± 1.09 log10 CFU/g of bone (Fig. 3). In the vancomycin-treated group, 5/11 animals had sterile bone and the mean bacterial count in bone (2.37 ± 1.22 log10 CFU/g) differed significantly from that of controls. In the ceftobiprole-treated group, 7/10 animals had sterile bone and the mean bone bacterial density (1.44 ± 0.40 log10 CFU/g) was significantly lower than those of controls.
and vancomycin-treated rabbits. Compared to each primary antibiotic alone, the addition of rifampin significantly enhanced the efficacies of ceftobiprole (1.16 ± 0.04 log₁₀ CFU/g of bone, P = 0.056) and vancomycin (1.23 ± 0.06 log₁₀ CFU/g of bone, P = 0.011), and all animals had sterile bone. No ceftobiprole- or vancomycin-resistant strain emerged in the bones of treated animals.

**DISCUSSION**

In this study, we tested *in vitro* ceftobiprole activity against a large representative sample of the major clones of PVL⁺ CA-MRSA. We also evaluated its *in vivo* efficacy in a PVL⁺ CA-MRSA experimental osteomyelitis model as monotherapy and combined with rifampin.

*In vitro*, all tested strains were susceptible to vancomycin, rifampin, and ceftobiprole, in accordance with other *in vitro* studies that observed ceftobiprole MICs between 0.5 and 2 µg/ml against 100 CA-MRSA strains (23).

Here, PVL production by strains cultured with 0.12× or 0.25× MIC of ceftobiprole or oxacillin increased, while it was not modified by vancomycin. However, the ceftobiprole-induced PVL rise was smaller than that induced by oxacillin. We recently showed that PVL induction by beta-lactams was triggered by penicillin-binding protein 1 (PBPI) inhibition. Therefore, only beta-lactams inhibiting PBPI were able to increase PVL expression (11). Cef-tobiprole is a broad-spectrum cephalosporin with high affinity for PBPs a with a 50% inhibitory concentration (**IC₅₀**) of about 0.5 mg/liter for MRSA (13). Cef-tobiprole has good affinity for MSSA PBPs 1 to 3, with an **IC₅₀** of about 1 mg/liter (7). However, oxacillin affinity for PBP1 is about 20 times higher than that of ceftobiprole (26), thereby explaining their different PVL-inducing abilities.

The LAC strain selected for the *in vivo* study belongs to the USA 300 lineage, the major PVL⁺ CA-MRSA clone in the United States. To mimic growth conditions similar to the experimental setting, we determined PVL production by the LAC strain, during exposure to ceftobiprole at its MIC. PVL production rose transiently after 6 and 8 h of culture but declined thereafter, probably linked to the antibiotic’s bacterial growth-inhibitory effect. These *in vitro* data support using ceftobiprole as an appropriate beta-lactam to treat CA-MRSA infection, as its overall observed effect combined suppression of bacterial growth and that of PVL expression.

Among the antibiotics tested, only rifampin, at sub-MICs, decreased PVL production for all tested strains, including LAC, while, as shown previously, vancomycin had no effect on PVL expression (10).

*In vivo*, ceftobiprole was significantly more effective than vancomycin at decreasing bone LAC counts. However, the clinical significance of that observation merits being discussed in its context. First, the vancomycin MIC of the tested LAC strain (2 µg/ml) might have favored ceftobiprole. Indeed, it was previously shown that elevated MICs (1.5 to 2 µg/ml) enhanced the likelihood of vancomycin therapeutic failure (25). Second, none of the monotherapies was able to sterilize 100% of the animals. Moreover, the good ceftobiprole efficacy found in our model agrees with other experimental studies performed with different staphylococcal strains.

In experimental endocarditis due to MRSA strain COL (vancomycin MIC, 1 µg/ml) (37), ceftobiprole led to significantly lower bacterial counts in vegetations than did vancomycin. Also, in a rabbit MRSA osteomyelitis model (vancomycin MIC, 0.78 µg/ml) (42), ceftobiprole was able to sterilize 100% of the animals compared to 73% of vancomycin- or linezolid-treated animals. Notably, cefaroline, another cephalosporin with anti-MRSA bacterial activity, performed better than vancomycin against MRSA rabbit endocarditis and acute osteomyelitis (18, 19). The previously reported good *in vivo* activity of ceftobiprole monotherapy, noted early in experimental endocarditis (13), remains only partially understood; its bactericidal effect against MRSA might play a role. In our *in vivo* study, plasma ceftobiprole dosage yielded trough concentrations twice the MIC. Authors of previous *in vitro* time-kill studies reported ceftobiprole to be bactericidal against MRSA at 2× MIC at 24 h (24). Another explanation could be good antibiotic diffusion at the infection site. However, to our knowledge, ceftobiprole bone penetration has not been studied. The absence of ceftobiprole-resistant strains after monotherapy in our model is also pertinent in the perspective of its use in clinical situations.

Combination with rifampin is well known to enhance the efficacy of treatment of experimental bone-and-joint infections because of its bactericidal activity against slow-growing bacteria (44). However, this remarkable *in vivo* efficacy could be hampered by the emergence of rifampin-resistant strains, even when rifampin is used in combination (21, 40). In our study, combination with rifampin improved the efficacy of ceftobiprole or vancomycin and sterilized 100% of the rabbits.

In addition to its bactericidal effect on bone-enclosed bacteria, the antibiotic impact on the capacity of *S. aureus* to release PVL could interfere with its *in vivo* efficacy against PVL⁺ CA-MRSA osteomyelitis. Indeed, we previously showed that PVL plays a role in the persistence and rapid local extension of CA-MRSA (LAC) rabbit osteomyelitis (6). In our *in vitro* model, ceftobiprole sub-MICs slightly increased LAC PVL production. Rifampin sub-MICs significantly lowered PVL production by LAC, by as much as 20 times less than the controls. Early effective suppression of PVL expression by rifampin combined with ceftobiprole or vancomycin might explain the therapeutic successes of these regimens in our *in vivo* model.

The experimental model used here, induced by 10⁷ CFU of LAC, reproduces CA-MRSA hematogenous osteomyelitis in children with early cortical bone involvement and local extension to muscles and joints (6). However, also described in children (22, 39) is a more severe systemic disease with potential lethal sepsis that can be experimentally reproduced by using a higher inoculum (35). The results obtained here cannot be extrapolated to those cases that warrant further therapeutic studies.

Unlike vancomycin, which must be closely monitored to avoid treatment failures and toxicity (25), beta-lactam antibiotics, with their extensive history of use, are generally considered safe and easy to administer. Taken together, our *in vitro* and *in vivo* findings suggest that beta-lactams active against MRSA, like ceftobiprole, should be of interest in treating CA-MRSA osteomyelitis.

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