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

Azzam Saleh-Mghir,^{a,b} Oana Dumitrescu,^c Aurélien Dinh,^{a,b} Yassine Boutrad,^{a,b} Laurent Massias,^d Émilie Martin,^c François Vandenesch,^c Jérôme Etienne,^c Gérard Lina,^c and Anne Claude Crémieux^{a,b}

Département de Médecine Aiguë Spécialisée, Hôpital Universitaire Raymond Poincaré, Assistance Publique-Hôpitaux de Paris (AP-HP), Garches,^a EA 3647, Faculté de Médecine Paris-Ile-de-France Ouest, Université Versailles Saint-Quentin, Versailles,^b INSERM U851, Centre National de Référence des Staphylocoques, Faculté de Médecine Lyon Est, Université Lyon 1, Lyon,^c and Laboratoire de Toxicologie-Pharmacocinétique, Hôpital Bichat-Claude-Bernard, AP-HP, Université Paris 7-Diderot, Paris,^d France

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-log₁₀-CFU-count decrease. *In vivo*, the mean log₁₀ 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 Pantone-Valentine leukocidin (PVL). The PVL contribution to the course of osteomyelitis, suspected very early by Pantone 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

Received 9 May 2012 Returned for modification 19 June 2012

Accepted 26 August 2012

Published ahead of print 1 October 2012

Address correspondence to Gérard Lina, gerard.lina@univ-lyon1.fr, or Anne Claude Crémieux, anne-claude.cremieux@rpc.aphp.fr.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00926-12

The authors have paid a fee to allow immediate free access to this article.

TABLE 1 Characterization of clinical strains and MICs of selected antibiotics^a

Strain	Sequence type	<i>spa</i> type	<i>mecA</i> status	<i>agr</i> allele	<i>lukSF-PV</i>	Source	MIC (mg/liter) in CCY medium ^b			
							OXA	RIF	CEF	VAN
ST20070572	8	t1911	—	1	+	This study	0.25	0.007	0.5	1
ST20070573	8	t008	—	1	+	This study	0.25	0.015	0.5	1
ST20070575	8	t008	—	1	+	This study	0.25	0.015	0.5	1
ST20070576	8	t008	—	1	+	This study	0.25	0.03	0.5	1
ST20070577	88	t2742	—	3	+	This study	0.25	0.03	0.5	1
ST20070579	121	t645	—	4	+	This study	0.25	0.03	0.5	1
ST20070580	8	t024	—	1	+	This study	0.25	0.007	0.5	1
ST20070581	121	t940	—	4	+	This study	0.25	0.015	0.5	1
ST20070582	8	t024	—	1	+	This study	0.12	0.03	0.5	1
ST20070583	25	t528	—	1	+	This study	0.12	0.03	0.5	1
LUG855	8	ND	—	1	+	10	0.12	0.015	0.5	1
HT20010251	8	t008	+	1	+	This study	4	0.03	1	1
HT20030203	8	t008	+	1	+	This study	16	0.03	1	1
HT20030206	8	t008	+	1	+	This study	4	0.03	1	1
HT20030207	8	t008	+	1	+	This study	8	0.015	1	1
LAC USA300	8	t008	+	1	+	41	8	0.015	1	2
HT20010751	59	t216	+	1	+	This study	8	0.007	1	1
HT20010253	1	t125	+	3	+	This study	16	0.03	1	1
HT20010734	1	t128	+	3	+	10	16	0.007	1	1
HT20010252	1	t175	+	3	+	This study	8	0.03	1	1
HT20010255	1	t175	+	3	+	This study	8	0.015	1	2
HT20020338	1	t128	+	3	+	This study	8	0.007	1	1
ATCC 29213	ND	ND	—	ND	ND	ATCC	0.12	0.007	0.25	0.5

^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.

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 complicated skin and skin structure infections caused by Gram-positive bacteria, including PVL⁺ isolates (29). Its efficacy alone against MRSA experimental endocarditis and localized 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.

(This work was presented, in part, at the 21st European Congress of Clinical Microbiology and Infectious Diseases-27th International 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, or t175 *spa*-type strains belonged to the USA400 lineage; the 1 *agr*-1, ST59, 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 precultured 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 0.50×, 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. Aliquots of each culture (without or with ceftobiprole) were sampled at 6, 8, and 16 h for PVL measurements and bacterial counts. To measure PVL, cultures were adjusted to an optical density at 600 nm (OD_{600}) of 1 and PVL was assessed with a specific ELISA at all times. Experiments were run in triplicate and results are expressed as μ g of PVL/ml of adjusted culture.

In vivo study design. (i) Test strain. The ceftobiprole-susceptible LAC strain used in this study was isolated from a patient with a skin abscess.

(ii) Preparation of bacterial inocula. The organisms were stored at -80°C until used. Before experiments, they were cultured in CCY medium at 37°C for 18 h with shaking. After centrifugation, the pellets were washed twice and resuspended in phosphate-buffered saline to the desired bacterial density immediately before inoculation. All inocula were quantified by plating serial dilutions on tryptic soy agar (bioMérieux).

(iii) Experimental model. Norden's method (30) was used to induce osteomyelitis in female New Zealand White rabbits, each weighing 2 to 3 kg. The rabbits were housed in individual cages and received food and water *ad libitum*. The experimental protocol complied with French legislation on animal experimentation and was approved by the Animal Use Committee of Maison Alfort Veterinary School. The animals were anesthetized by intramuscular injection of 25 mg of ketamine/kg of body weight (Vibrac, Carros, France) and 25 mg/kg of 2% xylazine (Rompum; Bayer Santé, Division Santé Animal, Puteaux, France). An 18-gauge needle was inserted percutaneously through the lateral right tibial metaphysis into the medullary cavity. Infection was induced by direct injection of a sclerosing agent (0.1 ml of 3% sodium tetradecyl sulfate (Trombovar; Kreussler Pharma, La Chaussée-Saint-Victor, France), followed by 0.2 ml of CA-MRSA LAC inoculum (3.4×10^7 CFU) and 0.1 ml of saline. Patch analgesia (Durogesic; Janssen-Cilag, Issy-les Moulineaux, France) was given for 7 days following surgery. A 3.4×10^7 -CFU inoculum was selected because previous experiments (data not shown) had shown that it induced persistent osteomyelitis in 90% of animals with low early mortality due to severe sepsis ($<10\%$).

(iv) Treatment and its evaluation. Fourteen days postinfection, rabbits were randomly assigned to the untreated (control) or treated group. The latter received ceftobiprole (40 mg/kg subcutaneously [s.c.] four times a day [q.i.d.], $n = 10$) or vancomycin (60 mg/kg intramuscularly [i.m.] twice a day [b.i.d.]) alone ($n = 11$) or combined with rifampin (10 mg/kg i.m. b.i.d., $n = 11$). Vancomycin and rifampin doses were selected based on previous experiments (33) showing that they achieved concentrations close to those recommended for humans (trough vancomycin concentrations of 15 to 20 μ g/ml and rifampin dose equivalent to 900 mg/day) (25). The ceftobiprole dose was selected based on the pharmacokinetic study described below. Each regimen was administered for 14 days. Control rabbits ($n = 9$) were left untreated. Animals were killed by intravenous (i.v.) injection of pentobarbital 7 days after the end of therapy (day 35) to allow for bacterial regrowth after ending treatment while avoiding the persistence of residual antibiotic in the bone. Control rabbits were also killed on day 35. At the time of death, the right hind limb was dissected, and the tibia and femur were separated from the surrounding soft tissues. For quantitative bacterial counts, the upper third of the tibia (3 cm long), including compact bone and marrow, was isolated, split with a bone crusher, weighed, cut into small pieces, frozen in liquid nitrogen, and crushed in an autopulverizer (Spex 6700; Freezer/Mill Industries Inc., Metuchen, NJ). The pulverized bone was suspended in 10 ml of sterile saline; serial dilutions were made and plated on tryptic soy agar. After 24 h of incubation at 37°C , the number of viable microorganisms was determined. Results are expressed as means $\pm$ standard deviations (SD) $\log_{10}$ CFU/g of bone and as the percentage of animals with sterile bone. Bone was considered sterile when the culture showed no growth after incubation for 48 h at 37°C and the number of CFU was recorded as the lowest detectable bacterial count (1.10 to 1.30 CFU/g of bone, depending on the weight of the sample).

TABLE 2 Antibiotic effects on bacterial growth^a

Antibiotic	Bacterial count, $\log_{10}$ CFU/ml			
	No antibiotic	0.125 $\times$ MIC	0.25 $\times$ MIC	0.50 $\times$ MIC
Oxacillin	9 $\pm$ 0.3	8 $\pm$ 0.2	7.5 $\pm$ 0.3	6.5 $\pm$ 0.3
Ceftobiprole	9 $\pm$ 0.4	8.5 $\pm$ 0.4	7.5 $\pm$ 0.6	6.5 $\pm$ 0.6
Vancomycin	9 $\pm$ 0.5	8.5 $\pm$ 0.3 A	8 $\pm$ 0.4	7 $\pm$ 0.4
Rifampin	9 $\pm$ 0.3	9 $\pm$ 0.4 A	8.7 $\pm$ 0.3 A, B	6.8 $\pm$ 0.3

^a The *S. aureus* LAC strain was incubated in CCY medium without or with oxacillin, ceftobiprole, vancomycin, or rifampin (at 0.50 $\times$, 0.25 $\times$, and 0.125 $\times$ MIC) according to the CLSI standard procedures for 24 h at 37°C without shaking. Results of 3 experiments are expressed as mean $\pm$ SD $\log_{10}$ CFU of bacteria. Statistical significance is shown by capital letters: A, $P < 0.05$ versus oxacillin; B, $P < 0.05$ versus ceftobiprole.

(v) In vivo selection of mutants. Aliquots (0.1 ml) of each undiluted bone homogenate were also plated onto Mueller-Hinton agar (Becton, Dickinson, Rungis, France) containing ceftobiprole, rifampin, 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.

(vi) Serum ceftobiprole levels. Serum antibiotic levels were determined in uninfected rabbits. Dose regimens were selected on the basis of previous experimental studies in rabbits (42) and then adjusted to achieve serum levels equivalent to those obtained in humans. Four rabbits received an s.c. injection of ceftobiprole, and blood was drawn 15 and 30 min and 1, 3, 6, and 8 h later. Ceftobiprole concentrations were measured by high-performance liquid chromatography with Waters Alliance e2695 and Waters 2487 UV detectors. The column was a Nova-Pack C_{18} , 4.0 μ m (150 by 3.9 mm) (Waters, St Quentin-en-Yvelines, France). The mobile phase was composed of a mixture of 0.1 M KH_2PO_4 buffer (pH 6.1) and acetonitrile, 95/5 (vol/vol). The flow rate was 1 ml/min, and ceftobiprole was detected by UV absorbance at 300 nm. The internal standard was ceftazidime. The detection limit was 0.5 mg/liter. The coefficient of variation was <7.5 mg/liter.

Statistical analyses. Bacterial densities in bones from the experimental groups were compared by analysis of variance (ANOVA), followed by Scheffé's test for multiple comparisons. Results are expressed as means $\pm$ SD. For PVL measurements, statistical analyses were based on one-way ANOVA followed, when necessary (samples of unequal variance), by Dunnett's *a posteriori* test. The impact of subinhibitory concentrations of oxacillin, ceftobiprole, vancomycin, and rifampin on bacterial growth was analyzed with Student's *t* test. A P value of <0.05 was considered significant. 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 $\times$, 0.25 $\times$, and 0.5 $\times$ MIC) are shown in Table 2. As expected from previous experiments, growth was inhibited (bacterial inoculum loss exceeded 1 $\log_{10}$ CFU) when oxacillin or ceftobiprole was used at 0.5 $\times$ or 0.25 $\times$ MIC, but also when cultures were incubated with vancomycin or rifampin at 0.5 $\times$ MIC. Bacterial growth in the presence of oxacillin was significantly lower than that with vancomycin at 0.125 $\times$ MIC ($P = 0.045$) or rifampin at 0.125 $\times$ MIC ($P = 0.017$) and 0.25 $\times$ MIC ($P = 0.007$). It was also lower in the presence of ceftobiprole than in the presence of rifampin at 0.25 $\times$ MIC ($P = 0.028$). For all other antibi-

FIG 1 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/ $\log_{10}$ 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.

otic concentrations, bacterial counts were comparable to control values.

(iii) Antibiotic effect on PVL production in microplate cultures (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, 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, 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

FIG 2 Ceftobiprole effect on PVL production by *S. aureus* LAC. The *S. aureus* LAC strain was cultured in CCY broth under time-kill conditions, without (black bars) or with ceftobiprole at its MIC (white bars). Aliquots were taken at 6, 8, and 16 h for PVL measurement after being adjusted to an OD_{600} of 1. Experiments were run in triplicate, and results are expressed in μg of PVL/ml of adjusted culture (mean $\pm$ SD). *, $P < 0.05$ (ANOVA), with antibiotic versus without antibiotic.

with a 2- $\log_{10}$ -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 $100.1 \pm 24.7 \mu\text{g/ml}$, the mean trough concentration was $4.53 \pm 2.07 \mu\text{g/ml}$, and area under the curve from 0 to 8 h was $283 \text{ mg} \cdot \text{h/liter}$ ($n = 4$). 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 $4.57 \pm 1.09 \log_{10}$ CFU/g of bone (Fig. 3). In the vancomycin-treated group, 5/11 animals had sterile bone and the mean bacterial count in bone ($2.37 \pm 1.22 \log_{10}$ 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 ($1.44 \pm 0.40 \log_{10}$ CFU/g) was significantly lower than those of controls

FIG 3 Antibiotic effect against a rabbit model of CA-MRSA LAC osteomyelitis. Rabbits were injected for 14 days with ceftobiprole (Cef; 40 mg/kg, s.c., q.i.d.) or vancomycin (Van; 60 mg/kg, i.m., b.i.d.) alone or combined with rifampin (Rif; 10 mg/kg, i.m., b.i.d.). Bone was considered sterile when the culture showed no growth after incubation for 48 h at 37°C and the number of CFU was recorded as the lowest detectable bacterial count (1.10 to 1.30 CFU/g of bone, depending on the weight of the sample).

and vancomycin-treated rabbits. Compared to each primary antibiotic alone, the addition of rifampin significantly enhanced the efficacies of ceftobiprole ($1.16 \pm 0.04 \log_{10}$ CFU/g of bone, $P = 0.056$) and vancomycin ($1.23 \pm 0.06 \log_{10}$ 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 μ g/ml against 100 CA-MRSA strains (23).

Here, PVL production by strains cultured with 0.12 $\times$ or 0.25 $\times$ 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 (PBP1) inhibition. Therefore, only beta-lactams inhibiting PBP1 were able to increase PVL expression (11). Ceftobiprole is a broad-spectrum cephalosporin with high affinity for PBP2a with a 50% inhibitory concentration (IC₅₀) of about 0.5 mg/liter for MRSA (13). Ceftobiprole has good affinity for MSSA PBP1 to PBP4, 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 USA300 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 μ g/ml) might have favored ceftobiprole. Indeed, it was previously shown that elevated MICs (1.5 to 2 μ g/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 μ g/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 μ g/ml) (42), ceftobiprole was able to sterilize 100% of the animals compared to 73% of vancomycin- or linezolid-treated animals. Notably, ceftaroline, another cephalosporin with anti-MRSA bactericidal 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 $\times$ 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.

ACKNOWLEDGMENTS

This work was supported in part by a research grant from Janssen-Cilag, Issy-les-Moulineaux, France. O.D., F.V., J.E., and G.L. were supported by grants from the European Community EC 222718 and Pfizer Holding, Paris, France.

REFERENCES

- Badiou C, et al. 2008. Pantone-Valentine leukocidin is expressed at toxic levels in human skin abscesses. *Clin. Microbiol. Infect.* 14:1180–1183.
- Bocchini CE, et al. 2006. Pantone-Valentine leukocidin genes are associated with enhanced inflammatory response and local disease in acute hematogenous *Staphylococcus aureus* osteomyelitis in children. *Pediatrics* 117:433–440.
- Centers for Disease Control and Prevention. 2007. Severe methicillin-resistant *Staphylococcus aureus* community-acquired pneumonia associated with influenza—Louisiana and Georgia, December 2006–January 2007. *MMWR Morb. Mortal. Wkly. Rep.* 56:325–329.
- Chua K, Laurent F, Coombs G, Grayson ML, Howden BP. 2011. Antimicrobial resistance: not community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)! A clinician's guide to community MRSA—its evolving antimicrobial resistance and implications for therapy. *Clin. Infect. Dis.* 52:99–114.
- Clinical and Laboratory Standards Institute. 2009. Performance standards for antimicrobial susceptibility testing. Approved standard M100-S14. Clinical and Laboratory Standards Institute, Wayne, PA.
- Crémieux AC, et al. 2009. Pantone-Valentine leukocidin enhances the severity of community-associated methicillin-resistant *Staphylococcus aureus* rabbit osteomyelitis. *PLoS One* 4:e7204. doi:10.1371/journal.pone.0007204.
- Davies TA, et al. 2007. Binding of ceftobiprole and comparators to the penicillin-binding proteins of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* 51:2621–2624.
- Deleo FR, Otto M, Kreiswirth BN, Chambers HF. 2010. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet* 375:1557–1568.
- Dohin B, et al. 2007. Pediatric bone and joint infections caused by Pantone-Valentine leukocidin-positive *Staphylococcus aureus*. *Pediatr. Infect. Dis. J.* 26:1042–1048.
- Dumitrescu O, et al. 2007. Effect of antibiotics on *Staphylococcus aureus* producing Pantone-Valentine leukocidin. *Antimicrob. Agents Chemother.* 51:1515–1519.
- Dumitrescu O, et al. 2011. Beta-lactams interfering with PBP1 induce Pantone-Valentine leukocidin expression by triggering *sarA* and *rot* global regulators of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 55:3261–3271.
- Enright M, Day N, Davies C, Peacock S, Spratt B. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* 38:1008–1015.
- Entenza JM, Hohl P, Heinze-Krauss I, Glauser MP, Moreillon P. 2002. BAI9141, a novel extended-spectrum cephalosporin active against methicillin-resistant *Staphylococcus aureus* in treatment of experimental endocarditis. *Antimicrob. Agents Chemother.* 46:171–177.
- Gerber JS, Coffin SE, Smathers SA, Zaoutis TE. 2009. Trends in the incidence of methicillin-resistant *Staphylococcus aureus* infection in children's hospitals in the United States. *Clin. Infect. Dis.* 49:65–71.
- Gillet Y, et al. 2002. Association between *Staphylococcus aureus* strains carrying gene for Pantone-Valentine leukocidin and highly lethal necrotizing pneumonia in young immunocompetent patients. *Lancet* 359:753–759.
- Gonzalez BE, et al. 2005. Severe staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant *Staphylococcus aureus*. *Pediatrics* 115:642–648.
- Harmsen D, et al. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J. Clin. Microbiol.* 41:5442–5448.
- Jacqueline C, et al. 2010. Efficacy of the new cephalosporin ceftaroline in the treatment of experimental methicillin-resistant *Staphylococcus aureus* acute osteomyelitis. *J. Antimicrob. Chemother.* 65:1749–1752.
- Jacqueline C, et al. 2007. In vivo efficacy of ceftaroline (PPI-0903), a new broad-spectrum cephalosporin, compared with linezolid and vancomycin against methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus* in a rabbit endocarditis model. *Antimicrob. Agents Chemother.* 51:3397–3400.
- Jarraud S, et al. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect. Immun.* 70:631–641.
- John AK, et al. 2009. Efficacy of daptomycin in implant-associated infection due to methicillin-resistant *Staphylococcus aureus*: importance of combination with rifampin. *Antimicrob. Agents Chemother.* 53:2719–2724.
- Kaplan SL. 2010. Acute hematogenous osteomyelitis in children: differences in clinical manifestations and management. *Pediatr. Infect. Dis. J.* 12:1128–1129.
- Leonard SN, Cheung CM, Rybak MJ. 2008. Activities of ceftobiprole, linezolid, vancomycin, and daptomycin against community-associated and hospital-associated methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 52:2974–2976.
- Lin G, Appelbaum PC. 2008. Activity of ceftobiprole compared with those of other agents against *Staphylococcus aureus* strains with different resistotypes by time-kill analysis. *Diagn. Microbiol. Infect. Dis.* 60:233–235.
- Liu C, et al. 2011. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children: executive summary. *Clin. Infect. Dis.* 52:285–292.
- Moisan H, Pruneau M, Malouin F. 2010. Binding of ceftaroline to penicillin-binding proteins of *Staphylococcus aureus* and *Streptococcus pneumoniae*. *J. Antimicrob. Chemother.* 65:713–716.
- Murthy B, Schmitt-Hoffmann A. 2008. Pharmacokinetics and pharmacodynamics of ceftobiprole, an anti-MRSA cephalosporin with broad-spectrum activity. *Clin. Pharmacokinet.* 47:21–33.
- Narita S, et al. 2001. Phage conversion of Pantone-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. *Gene* 268:195–206.
- Noel GJ, et al. 2008. Results of a double-blind randomized trial of ceftobiprole treatment of complicated skin and skin structure infections caused by Gram-positive bacteria. *Antimicrob. Agents Chemother.* 52:37–44.
- Norden CW. 1970. Experimental osteomyelitis. I. A description of the model. *J. Infect. Dis.* 122:410–418.
- Pantone PN, Valentine FCO. 1932. Staphylococcal toxin. *Lancet* i:506–508.
- Proctor RA. 2008. Role of folate antagonists in the treatment of methicillin-resistant *Staphylococcus aureus* infection. *Clin. Infect. Dis.* 46:584–593.
- Saleh-Mghir A, et al. 2002. Combination of quinupristin-dalfopristin (Synercid) and rifampin is highly synergistic in experimental *Staphylococcus aureus* joint prosthesis infection. *Antimicrob. Agents Chemother.* 46:1122–1124.
- Saleh-Mghir A, et al. 1998. Efficacy of teicoplanin and autoradiographic diffusion of pattern [¹⁴C]teicoplanin in experimental *Staphylococcus aureus* joint prosthesis infection. *Antimicrob. Agents Chemother.* 42:2830–2835.
- Saleh-Mghir A, et al. 2011. Pantone-Valentine leukocidin (PVL) increases hematogenous lung lesions but not mortality of severe sepsis associated with community associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) rabbit acute osteomyelitis (AOM), abstr. B-054. Abstr. 51st Intersci. Conf. Antimicrob. Agents Chemother.
- Saleh-Mghir A, et al. 2011. Ceftobiprole efficacy in vitro on Pantone-Valentine leukocidin production and in vivo in a rabbit community-associated methicillin-resistant *Staphylococcus aureus* osteomyelitis model, poster P-1068. 21st Eur. Congr. Clin. Microbiol. Infect. Dis.-27th Int. Congr. Chemother.
- Stryjewski ME, et al. 2007. Use of vancomycin or first-generation cephalosporins for the treatment of hemodialysis-dependent patients with methicillin-susceptible *Staphylococcus aureus* bacteremia. *Clin. Infect. Dis.* 44:190–196.
- Tattevin P, Basuino L, Bauer D, Diep BA, Chambers HF. 2010. Ceftobiprole is superior to vancomycin, daptomycin, and linezolid for treatment of experimental endocarditis in rabbits caused by methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 54:610–613.
- Tseng CW, et al. 2009. *Staphylococcus aureus* Pantone-Valentine leukocidin contributes to inflammation and muscle tissue injury. *PLoS One* 4:e6387. doi:10.1371/journal.pone.0006387.
- Vander Have KL, et al. 2009. Community-associated methicillin-resistant *Staphylococcus aureus* in acute musculoskeletal infection in children: a game changer. *J. Pediatr. Orthop.* 29:927–931.
- Vergidis P, et al. 2011. Treatment with linezolid or vancomycin in com-

- bination with rifampin is effective in an animal model of methicillin-resistant *Staphylococcus aureus* foreign body osteomyelitis. Antimicrob. Agents Chemother. 55:1182–1186.
41. Voyich J, et al. 2006. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J. Infect. Dis. 194:1761–1770.
 42. Yin LY, Calhoun JH, Thomas JK, Shapiro S, Schmitt-Hoffmann A. 2008. Efficacies of ceftobiprole medocaril and comparators in a rabbit model of osteomyelitis due to methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 52:1618–1622.
 43. Yun HC, Ellis MW, Jorgensen JH. 2007. Activity of ceftobiprole against community-associated methicillin-resistant *Staphylococcus aureus* isolates recently recovered from US military trainees. Diagn. Microbiol. Infect. Dis. 59:463–466.
 44. Zimmerli W, Trampuz A, Ochsner PE. 2004. Prosthetic-joint infections. N. Engl. J. Med. 351:1645–1654.