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Activities of Ceftobiprole and Other Cephalosporins against Extracellular and Intracellular (THP-1 Macrophages and Keratinocytes) Forms of Methicillin-Susceptible and Methicillin-Resistant *Staphylococcus aureus*▽†

Sandrine Lemaire,[1] Youri Glupczynski,[2] Valérie Duval,[3] Bernard Joris,[3] Paul M. Tulkens,[1]* and Françoise Van Bambeke[1]

Unité de Pharmacologie Cellulaire et Moléculaire, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium[1]; Laboratoire de Microbiologie, Cliniques Universitaires UCL de Mont-Godinne, Yvoir, Belgium[2]; and Centre d'Ingénierie des Protéines, Université de Liège, Sart-Tilman, Belgium[3]

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Staphylococcus aureus **is an opportunistic intracellular organism. Although they poorly accumulate in eukaryotic cells, β-lactams show activity against intracellular methicillin (meticillin)-susceptible *S. aureus* (MSSA) if the exposure times and the drug concentrations are sufficient. Intraphagocytic methicillin-resistant *S. aureus* (MRSA) strains are susceptible to penicillins and carbapenems because the acidic pH favors the acylation of PBP 2a by these β-lactams through pH-induced conformational changes. The intracellular activity (THP-1 macrophages and keratinocytes) of ceftobiprole, which shows almost similar in vitro activities against MRSA and MSSA in broth, was examined against a panel of hospital-acquired and community-acquired MRSA strains (MICs, 0.5 to 2.0 mg/liter at pH 7.4 and 0.25 to 1.0 mg/liter at pH 5.5) and was compared with its activity against MSSA isolates. The key pharmacological descriptors {relative maximal efficacy (E_{max}), relative potency (the concentration causing a reduction of the inoculum halfway between E_0 and E_{max} [EC_{50}]), and static concentration (C_s)} were measured. All strains showed sigmoidal dose-responses, with E_{max} being about a 1 $\log_{10}$ CFU decrease from the postphagocytosis inoculum, and EC_{50} and C_s being 0.2 to 0.3× and 0.6 to 0.9× the MIC, respectively. Ceftobiprole effectively competed with Bocillin FL (a fluorescent derivative of penicillin V) for binding to PBP 2a at both pH 5.5 and pH 7.4. In contrast, cephalexin, cefuroxime, cefoxitin, or ceftriaxone (i) were less potent in PBP 2a competitive binding assays, (ii) showed only partial restoration of the activity against MRSA in broth at acidic pH, and (iii) were collectively less effective against MRSA in THP-1 macrophages and were ineffective in keratinocytes. The improved activity of ceftobiprole toward intracellular MRSA compared with the activities of conventional cephalosporins can be explained, at least in part, by its greater ability to bind to PBP 2a not only at neutral but also at acidic pH.**

Restricted to the hospital setting for many years, the methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) epidemic is now reaching an increasing variety of other environments (12), such as patients in the community in various parts of the world (16, 35, 41) and animals (21, 40). Beyond its spectacular ability to adapt and to develop resistance to most antimicrobial agents (9), including drugs of last resort, such as vancomycin, linezolid, and daptomycin (5, 28, 31), the capacity of *S. aureus* to invade, sojourn, and thrive intracellularly (8, 23, 34) creates an additional challenge since intracellular forms tend to be poorly susceptible to most available antibiotics (38). Evaluations of new antistaphylococcal agents directed against resistant *S. aureus* strains must therefore include an assessment of their ability to control intracellular infections. While animal models of staphylococcal infection are being developed (32), models of cultured cells remain useful because they offer the possibility to explore in detail the pharmacological parameters governing the response of the intracellular bacteria to the drug in the absence of host factors (7, 38). In this context, we observed that, contrary to most original assumptions (36), the poor accumulation of β-lactams in phagocytic cells does not preclude the observation of significant activity against intracellular methicillin-susceptible *S. aureus* (MSSA). This is actually dependent on the time of exposure (12 to 24 h) and whether the extracellular concentration is maintained at a sufficiently large but still clinically pertinent level (7, 20). We previously reported that intraphagocytic MRSA isolates regain almost full susceptibility to penicillins and carbapenems, due to the acidic pH prevailing in phagolysosomes (19). This finding has been rationalized by the observation that acidic pH improves the accessibility to and the acylation of PBP 2a by penicillins within a time frame relevant to the growth rate of MRSA through protein conformational changes (17). This triggered us to study ceftobiprole in this context. Ceftobiprole, also known as BAL9141 and Ro 63-9141 (4), is the first clinically developed cephalosporin that shows almost similar activities against MRSA and MSSA isolates in conventional in vitro tests (15, 42). It has now been approved for clinical use in some countries and has been studied in a large array of preclinical and clinical settings (see references 3 and 43 for recent reviews).

* Corresponding author. Mailing address: Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, UCL 73.70 Avenue E. Mounier 73, Brussels B-1200, Belgium. Phone: 32-2-7622136. Fax: 32-2-7647373. E-mail: tulkens@facm.ucl.ac.be.

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TABLE 1. Characteristics of strains used in this study

Phenotype[a] and strain no.	Origin	SCC*mec* group	MIC (mg/liter)	
			pH 7.4	pH 5.5
MSSA				
ATCC 25923 (β-lactamase −)	Laboratory standard[b]	NA[g]	0.5	0.25
ATCC 11632 (β-lactamase +)	Laboratory standard[b]	NA	0.5–1	0.25–0.5
NRS52 (VISA)	Clinical (bile infection)[c]	NA	1	0.5
Geometric mean			0.72	0.36
HA-MRSA				
ATCC 33591 (inducible)	Laboratory standard[b]	III	2	0.5
ATCC 33592	Laboratory standard[b]	ND[h]	2	0.5–1
ATCC 43300	Laboratory standard[b]	ND	1	0.5
N4120210	Clinical (wound infection)[d]	I	2	1
N4112910	Clinical (nasal swab)[d]	ND	0.5–1	0.25–0.5
N4120032	Clinical (urinary tract infection)[d]	ND	2	0.5
NRS18 (VISA)	Clinical (wound, skin and soft tissue infection)-[c]	II	0.5–1	0.5
NRS126 (VISA)	Clinical (bloodstream infection)[c]	II	1–2	0.25–0.5
VRS1 (VRSA)	Clinical (catheter exit site)[c]	II	2	0.5
VRS2 (VRSA)	Clinical (wound infection)[c]	II	1–2	0.5
Geometric mean			1.45	0.43
CA-MRSA				
NRS192 (PVL +)	Clinical (pneumonia, septic arthritis)[c]	IVa	2	1
NRS384 (PVL +)	Clinical (wound, skin and soft tissue infection)[c]	IVa	2	1
N4090440 (PVL +)	Clinical (wound infection)[d]	IVa	1	0.5
N4042228 (PVL +)	Clinical (septicemia sec. to soft-tissues abscess)[d]	IVa	1	0.5
STA 44 (PVL +)	Clinical[e]	V	2	1
STA 268 (PVL +)	Clinical[e]	V	2	1
CHU (PVL +)	Clinical[e]	V	2	1
MEH2225605 (PVL +)	Clinical[f]	IVa	2	1
NRS386 (PVL −)	Clinical (bloodstream infection)[c]	IVa	2	0.5–1
Geometric mean			1.71	0.83

[a] VISA, vancomycin intermediate *S. aureus* (MICs, >2 and <8 mg/liter); VRSA, vancomycin-resistant *S. aureus* (MICs, >8 mg/liter); PVL, Panton-Valentine leucocidin; −, negative; +, positive.
[b] From the American Type Culture Collection.
[c] From the Network on Antimicrobial Resistance in *Staphylococcus aureus* (operated by Eurofins Medinet, Inc., Herndon, VA).
[d] Clinical collection (Y. Glupczynski, Cliniques Universitaires UCL de Mont-Godinne, Yvoir, Belgium).
[e] Clinical collection (Y. C. Huang, Chang Gung Children's Hospital, Taiwan).
[f] Clinical collection (L. Y. Hsu, Singapore General Hospital, Singapore).
[g] NA, not applicable.
[h] ND, not determined.

Cephalosporins active against MRSA are characterized by the presence of a bulky hydrophobic moiety in position 3 (27) (vinyl pyrrolidinone in the case of ceftobiprole; see the supplemental material for the structural formula), which increases interactions with PBP 2a and induces conformational changes that render the protein more susceptible to acylation by drugs, even at neutral pH (11, 22, 39). In the present study, the intracellular activity of ceftobiprole was examined against a panel of hospital-acquired and community-acquired MRSA strains. We then studied its activity against MRSA and MSSA strains in broth and its properties of binding to PBP 2a at neutral and acidic pHs and compared its activity and binding with those of selected conventional cephalosporins approved for use for the treatment of staphylococcal infections. We also compared the activity of ceftobiprole to the activities of conventional cephalosporins against intracellular MSSA and MRSA in THP-1 macrophages as a model of phagocytic cells, which are known to harbor persisting *S. aureus* cells for long periods and to help disseminate the organism (1, 23), and in keratinocytes, which may also host *S. aureus* (24) and which is a model pertinent for the use (and current approval) of ceftobiprole for complicated skin and soft tissue infections (10).

MATERIALS AND METHODS

Antibiotics, purified PBP 2a, and other main reagents. Ceftobiprole (the active form of the compound used clinically [ceftobiprole medocaril]) was obtained as the microbiological standard from Johnson & Johnson Pharmaceutical Research & Development, Raritan, NJ. The comparators, chosen to represent narrow-spectrum, expanded-spectrum, and broad-spectrum cephalosporins (cephalexin, cefuroxime, and ceftriaxone, respectively) and cephamycins (cefoxitin), were obtained from Sigma-Aldrich, St. Louis, MO, or Teva Pharma, Wilrijk, Belgium. Gentamicin, used to control extracellular growth in the absence of another antibiotic (7), was obtained as Geomycin (distributed in Belgium by Glaxo-SmithKline SA, Genval, Belgium); Bocillin FL (a fluorescent derivative of penicillin V [13]) was obtained from Invitrogen Corp., Carlsbad, CA. Staphylococcal PBP 2a (soluble form) was obtained from *Escherichia coli* Rosetta 2(DE3) transformed with plasmid pET28a carrying the truncated PBP 2a-coding sequence (ΔM1-Y23 PBP 2a). The protein was purified on an S-Sepharose HP column and then on a phenyl-Sepharose column (Amersham plc, Little Chalfont, United Kingdom), and the purity of the working sample was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with Coomassie blue staining and fluorescence visualization (14). Additional details on the production and purification procedure are given in the supplemental material. Cell culture medium and serum were from Invitrogen or Becton Dickinson, Franklin Lakes, NJ. Unless stated otherwise, all other reagents were obtained from Merck AG (Darmstadt, Germany) or Sigma-Aldrich.

Cell lines. Experiments were performed with (i) THP-1 cells (ATCC TIB-202; American Type Culture Collection, Manassas, VA), a human myelomonocytic cell line that displays macrophage-like activity and that is maintained in our

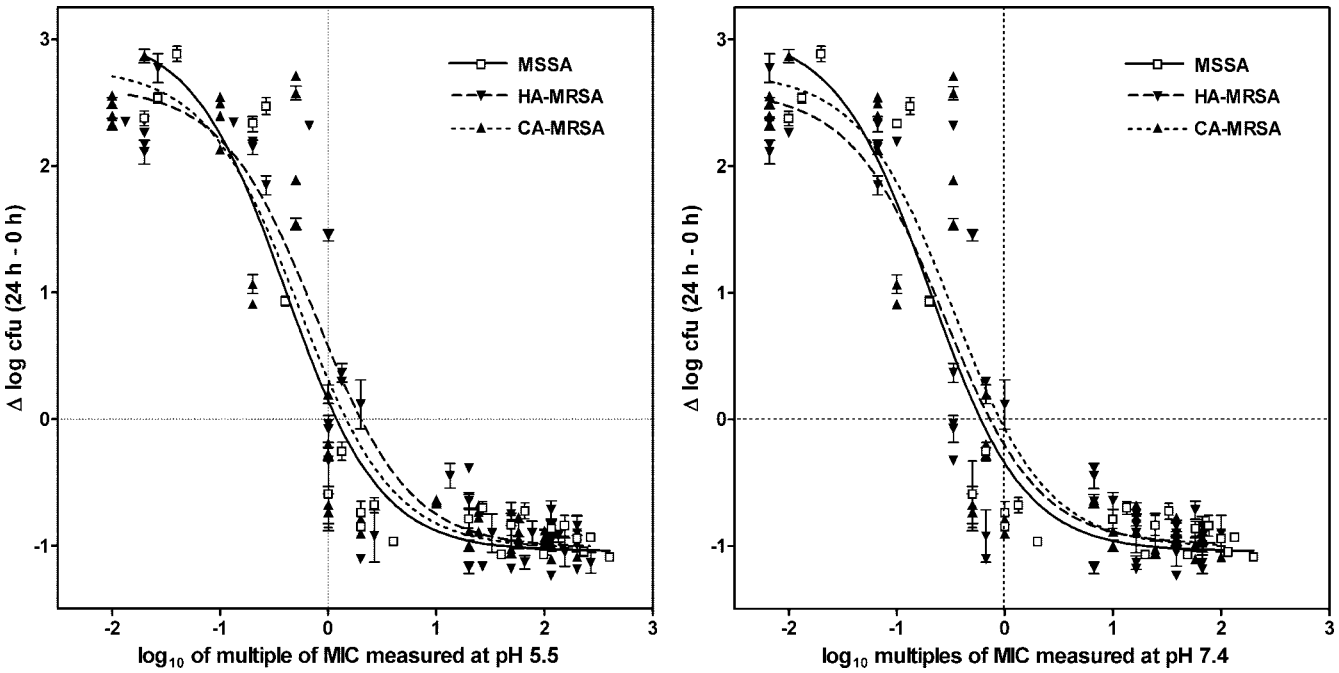

FIG. 1. Dose-response curves of ceftobiprole against MSSA (strains ATCC 25293, ATCC 11632, and NRS52), HA-MRSA (strains ATCC 33591, ATCC 33592, ATCC 433000, NRS18, NRS126, and VRS1), and CA-MRSA (strains NRS192, N4090440, N4042228, STA44, STA228, and MEH22256-05) phagocytized by human THP-1 macrophages after 24 h of incubation of the cells in the presence of increasing concentrations of the antibiotic. The ordinate shows the change in the number of CFU (means ± standard deviations; $n = 3$; several standard deviation bars are smaller than the symbols) per mg of cell protein. The abscissa is the multiple of the MIC (in $\log_{10}$ units) obtained for each strain when it was tested in broth at pH 5.5 or pH 7.4 (for strains for which different MICs were obtained [Table 1], calculations were made on the basis of the means of these values). Data from dose-response experiments performed with each strain (see the supplemental material) of each of the three phenotypes indicated above were pooled and used to fit one single sigmoidal function. The equation used, the goodness of fit, the pertinent pharmacological descriptors, and a statistical analysis of their differences (between groups and between mode of plots) are shown in Table 2.

laboratory, as described previously (7), and (ii) human skin keratinocytes, obtained as primary human keratinocytes (catalog no. 12332-011; Gibco, Invitrogen Corporation, Invitrogen SA, Merelbeke, Belgium) and cultivated in defined keratinocyte–serum-free medium (Invitrogen, Carlsbad, CA).

Bacterial strains and susceptibility testing. The strains used in this study, their main characteristics, and their origins are listed in Table 1. They were obtained from the American Type Culture Collection, the Network on Antimicrobial Resistance in *Staphylococcus aureus* (operated by Eurofins Medinet, Inc., Hern-

TABLE 2. Pharmacological descriptors, goodness of fit, and statistical analysis of studies of the dose-response of ceftobiprole against MSSA, HA-MRSA, and CA-MRSA strains[a]

Data plot function and resistance pattern	Pharmacological descriptor[b]				Goodness of fit (R^2)
	E_0[c] (95% CI[d])	E_{max}[e] (95% CI)	EC_{50}[f] (95% CI)	C_s[g]	
Data plotted as a function of the MIC measured at pH 5.5					
MSSA	3.06 (2.49 to 3.64) AC,a	−1.04 (−1.27 to −0.81) A,a	0.41 (0.23 to 0.70) AC,a	1.19	0.93
HA-MRSA	2.63 (2.27 to 2.98) B,a	−1.01 (−1.20 to −0.82) A,a	0.77 (0.51 to 1.17) B,a	1.97	0.91
CA-MRSA	2.78 (2.34 to 3.211) BC,a	−1.02 (−1.27 to −0.77) A,a	0.54 (0.33 to 0.88) C,a	1.45	0.87
Data plotted as a function of the MIC measured at pH 7.4					
MSSA	3.06 (2.49 to 3.64) AC,a	−1.04 (−1.27 to −0.81) A,a	0.20 (0.12 to 0.35) A,b	0.60	0.92
HA-MRSA	2.60 (2.19 to 3.01) B,a	−0.99 (−1.21 to −0.77) A,a	0.28 (0.17 to 0.45) AB,b	0.74	0.88
CA-MRSA	2.74 (2.27 to 3.21) BC,a	−1.01 (−1.29 to −0.73) A,a	0.33 (0.19 to 0.56) BC,b	0.91	0.84

[a] Data are for 24 h of incubation and are from Fig. 1.

[b] The equation for the sigmoidal dose-response is as follows: $y = E_{max} + [(E_0 - E_{max})/1 + 10^{(\log EC_{50} - x)}]$, where x is the concentration (in mg/liter). Statistical analyses were performed as follows: (i) data for the parameters (E_0, E_{max}, and EC_{50}) between MSSA, HA-MRSA, and CA-MRSA for pH 5.5 (upper half of table) or 7.4 (lower half of table) were compared by one-way analysis of variance with the Tukey-Kramer multiple-comparisons test (data with different uppercase letters were significantly different from each other [$P < 0.05$]) and (ii) data for the same parameters but between values observed at pH 5.5 (upper half of table) and with those observed at pH 7.4 (lower half of table) were compared by unpaired t test, two tailed (data with different lowercase letters are significantly different from each other [$P<0.05$]).

[c] Change in $\log_{10}$ CFU per mg of cell protein from the original postphagocytosis inoculum for an infinitely low ceftobiprole extracellular concentration.

[d] CI, confidence interval.

[e] Change in $\log_{10}$ CFU per mg of cell protein from the original postphagocytosis inoculum for an infinitely large ceftobiprole extracellular concentration.

[f] Ceftobiprole concentration (in multiples of the MIC [Table 1; for strains for which two different values were obtained, the mean value was used]) giving a response halfway between E_0 and E_{max}.

[g] Apparent static concentration (in multiples of the MIC [Table 1; for strains for which two different values were obtained, the mean value was used]), as determined by graphical intrapolation of the corresponding function.

TABLE 3. MICs of cephalosporins against selected MSSA and MRSA strains at pH 7.4 and 5.5 in broth and pH-induced decreases in MICs

Cephalosporin	MSSA ATCC 25923			MRSA ATCC 33591			Decrease in MRSA MIC/MSSA MIC ratio	MRSA MIC/MSSA MIC ratio at pH 5.5
	MIC (mg/liter)		pH-induced MIC decrease (fold)	MIC (mg/liter)		pH-induced MIC decrease (fold)		
	pH 7.4	pH 5.5		pH 7.4	pH 5.5			
Cephalexin	2	1	2	256	16	16	8	16
Cefuroxime	2	0.125	16	128	1	128	8	4
Cefoxitin	1	0.25	4	512	1	512	128	4
Ceftriaxone	2	0.125	16	512	1	512	32	4
Ceftobiprole	0.5	0.25	2	2	0.5	4	2	2

don, VA; supported under NIAID/NIH contract no. HHSN2722007 00055C), or clinical collections. MICs were measured by the microdilution method, as described earlier (19). For assays performed at a specified pH, the broth was adjusted to that pH prior to inoculation, and we checked that this pH had been maintained at its original value (±0.1 pH unit) at the end of the experiment. When *S. aureus* was tested at pH 5, it grew more slowly than it did at higher pHs, but it grew sufficiently to allow the accurate determination of the MIC. The MRSA phenotype of each strain was confirmed by detection of *mecA* by PCR (19) and the staphylococcal chromosome cassette *mec* (SCC*mec*) subgroup of most of the strains was established as described previously (18).

Cells, cell infection, and assessment of intracellular activity of antibiotics. Cell infection was performed exactly as described previously (7, 18, 19), and the postphagocytosis inoculum was set at 1.5×10^6 to 3.0×10^6 CFU per mg of cell protein. For both cell types, the intracellular growth of *S. aureus* within 24 h in the presence of gentamicin at an extracellular concentration of 0.5× its MIC in broth (to fully prevent extracellular growth [7, 33]) was about 1 $\log_{10}$ CFU/mg protein. The changes in CFU from the CFU of the postphagocytosis inoculum was taken as the response to the antibiotics and was plotted as a function of the antibiotic extracellular concentration. As shown earlier (7, 20), a sigmoidal function (Hill function) can be fitted to the data if both coordinates are subjected to logarithmic transformation. The use of logarithmic transformation for concentrations is in line with what is commonly used to describe pharmacological dose-responses when the doses span several orders of magnitude, as is the case here. The change in CFU also needs to be treated logarithmically because chemotherapeutic responses, unlike enzyme inhibition, for instance, progress by fractional and not constant changes upon finite increases in the drug concentration.

PBP 2a binding of ceftobiprole and other cephalosporins. The PBP 2a binding of ceftobiprole and the other cephalosporins tested was assessed by competition experiments by following the general method described for ^{3}H-labeled penicillin G (29, 37) but by using Bocillin FL (a boron-dipyrromethene [bodipy] derivative of penicillin V [13]) as the reporter antibiotic. The purified PBP 2a was incubated at 37°C for 25 min with increasing concentrations of the cephalosporins under study, after which 100 μM Bocillin FL was added to the samples for an additional 25 min at 37°C. The reaction was terminated by the addition of SDS-loading buffer, and the samples were then subjected to SDS-PAGE. Following electrophoresis, Western blotting was performed with an anti-rabbit monoclonal antibody directed against the bodipy moiety of Bocillin FL (antibodipy primary antibody [1/500; catalog no. A5770; Invitrogen, Carlsbad, CA]) and goat anti-rabbit immunoglobulin G labeled with horseradish peroxidase (catalog no. 65-6120; Invitrogen [Zymed, Carlsbad, CA]; this method has been validated against the conventional assay by using the fluorescent properties of Bocillin FL [17]). Bands were revealed with the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL), and scanned films were subjected to densitometric analysis (Image J software, version 1.3.1; available from the Research Service Branch of the National Institute of Mental Health at http://rsb.info.nih.gov/ij).

Statistical analyses. Curve-fitting analyses were performed with Prism (version 4.02) software for Windows and statistical analyses with Instat (version 3.06) software (GraphPad Prism Software, San Diego, CA).

RESULTS

Strains and susceptibility to ceftobiprole at neutral and acidic pHs in broth. Table 1 shows that the MICs of the strains used in this study ranged from 0.5 to 1, 0.5 to 2, and 1 to 2 mg/liter for MSSA, hospital-acquired MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA) strains when they were tested in broth. With the exception of strain NRS18, the MICs were globally 1 twofold dilution (MSSA, CA-MRSA) to 2 twofold dilutions (HA-MRSA) lower when they were measured at pH 5.5.

Susceptibilities of intracellular (THP-1 macrophages) MSSA, HA-MRSA, and CA-MRSA strains to ceftobiprole. In a first series of experiments, 24-h dose-responses studies were performed with 15 strains chosen from among the isolates listed in Table 1 (MSSA, $n = 3$; HA-MRSA, $n = 6$; CA-MRSA, $n = 6$) and with a wide range of extracellular concentrations of antibiotics. The data were used to fit sigmoidal functions (Hill's equation [see reference 7 for details]), yielding for each strain the values of the two key pharmacological descriptors of antibiotic activity defined previously (7), namely, the relative maximal efficacy (E_{max}) and the relative potency (the concentration causing a reduction of the inoculum halfway between E_0 and E_{max} [EC_{50}]) of ceftobiprole, together with an important characteristic of the model, that is, bacterial growth in the absence of antibiotic [E_0]). The data were plotted as multiples of the MIC to allow comparison of the activity of ceftobiprole at equipotent concentrations. As shown in the

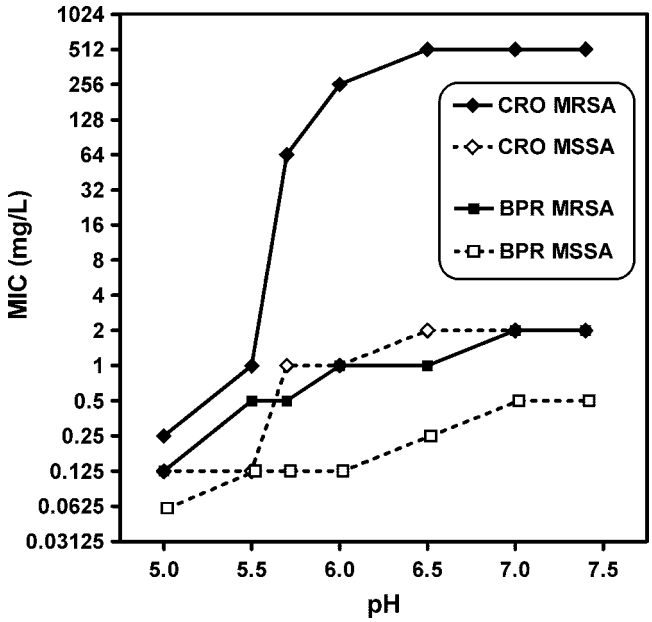

FIG. 2. Influence of pH on MICs of ceftobiprole (BPR) and ceftriaxone (CRO) for MSSA ATCC 25923 and MRSA ATCC 33591, as measured in broth.

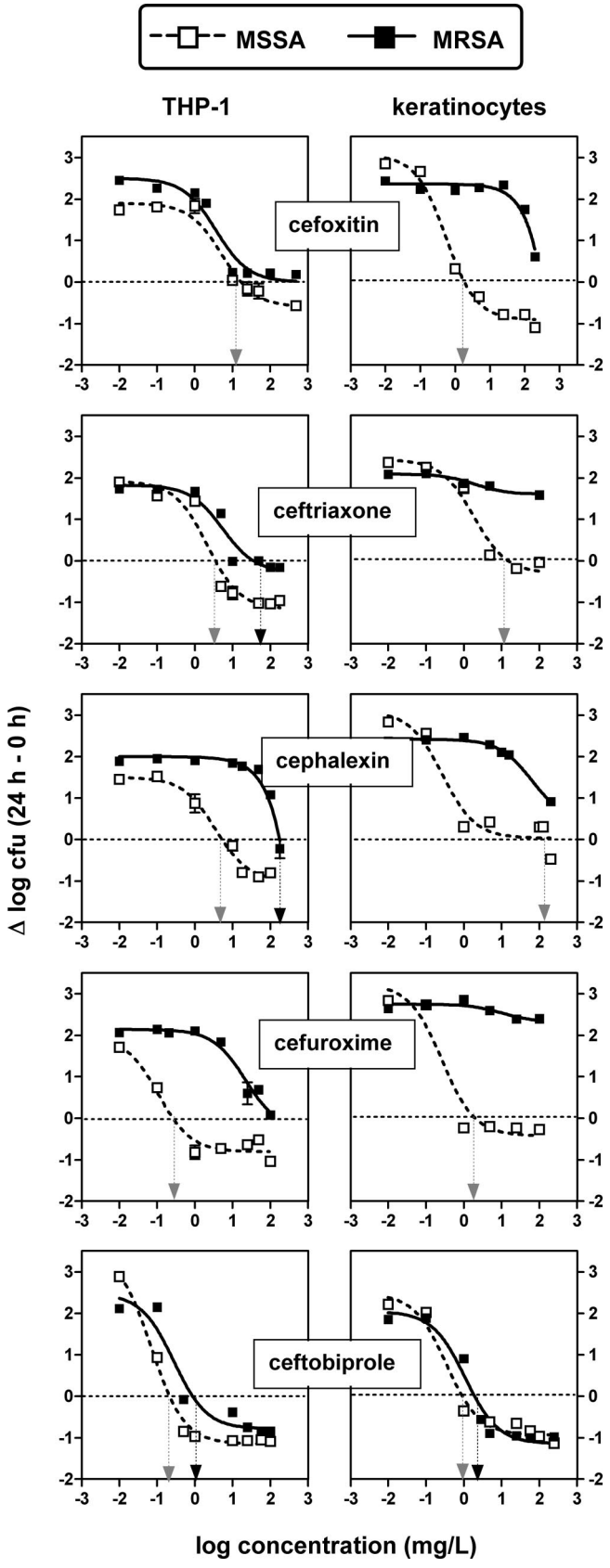

FIG. 3. Dose-response curves of cephalosporins against MSSA (ATCC 25293) and MRSA (ATCC 33591) phagocytized by human THP-1 macrophages or skin keratinocytes after 24 h of incubation of the cells in the presence of increasing concentrations of the antibiotics. The ordinate shows the change in the number of CFU (means ± standard deviations; $n = 3$; several standard deviation bars are smaller than the symbols) per mg of cell protein. The vertical arrows point to the C_s for each condition (gray arrow, MSSA; black arrow, MRSA). The goodness of fit, the pertinent pharmacological descriptors, and a statistical analysis of their differences (between antibiotics and between MSSA and MRSA) are shown in Table 4.

supplemental material, sigmoid dose-responses were obtained for each strain, and the values of E_{max} (~1 $\log_{10}$ CFU decrease), EC_{50} (close to the corresponding MICs, as determined in broth at pH 5.5), and E_0 (~2.5 $\log_{10}$ CFU increase) were similar or closely similar. The data for all strains were then pooled by phenotype (MSSA, HA-MRSA, or CA-MRSA), and the results are graphically shown in Fig. 1 (with numerical data and the results of the statistical analyses presented in Table 2). This shows that ceftobiprole globally exerted similar activity against all strains, regardless of their resistance phenotypes, although its relative potency was slightly, albeit statistically significantly, higher (lower EC_{50}) against MSSA than against HA-MRSA and, to some extent, CA-MRSA.

Susceptibilities of MSSA and MRSA to conventional cephalosporins compared with that to ceftobiprole at neutral and acidic pH in broth and after phagocytosis by THP-1 macrophages and keratinocytes. We previously found that acidic pH allowed the almost complete recovery of the activities of penicillins and carbapenems against MRSA strains when they were tested at acidic pH (pH 5.5) in broth or after phagocytosis by macrophages or keratinocytes (18, 19). This was therefore investigated in the present study with conventional cephalosporins, and the results were compared with those obtained with ceftobiprole.

Table 3 shows that acidic pH (pH 5.5) also markedly reduced the MICs of conventional cephalosporins for MRSA in broth but that the MIC of ceftobiprole remained the lowest of all drugs tested at that pH. To ensure that this was not related to differences in the thresholds at which a significant change in activity would occur, full pH dependence curves were made over the pH 5 to pH 7.5 range. These showed an abrupt decrease in the MICs of conventional cephalosporins between pH 6.5 and pH 5.5 for MRSA but a shallow decrease in the MICs ceftobiprole over the whole pH range that paralleled that seen for all drugs when their activities against MSSA were tested (see a typical example for the results for ceftriaxone versus those for ceftobiprole in Fig. 2; see Fig. SP2 in the supplemental material for data for each individual cephalosporin tested).

The antibacterial activity of ceftobiprole was then tested against the intracellular forms of MSSA (ATCC 25923) and MRSA (ATCC 33591) phagocytized by professional phagocytes (THP-1 macrophages) and nonprofessional phagocytes (skin keratinocytes) and was compared with the activities of conventional cephalosporins. The results are shown graphically in Fig. 3, with numerical data and the results of the statistical analyses presented in Table 4. For THP-1 macrophages and MSSA, all cephalosporins showed similar or nearly similar relative efficacies (E_{max}s) and 50% effective concentrations

TABLE 4. Pharmacological descriptors, goodness of fit, and statistical analysis of the dose-response studies of cephalosporins against MSSA and MRSA[a]

Cell line and antibiotic[b]	MSSA ATCC 25923				
	E_0[c] (95% CI[d])	E_{max}[e] (95% CI)	EC_{50}[f] (95% CI)	C_s[g]	R^2
THP-1 macrophages					
FOX	1.90 (1.47–2.33) ad,A	−0.59 (−1.13-−0.06) ac,A	5.30 (1.47–19.1)	16.4	0.971
CRO	1.91 (1.30–2.53) a,A	−1.17 (−1.67-−0.67) b,A	2.13 (0.70–6.46)	0.26	0.956
CFX	1.50 (1.17–1.84) ad,A	−1.00 (−1.39-−0.61) ab,A	3.49 (1.25–9.71)	5.50	0.984
CXM	1.97 (1.02–2.92) ad,A	−0.80 (−1.14-−0.47) abce,A	0.11 (0.03–0.43)	0.26	0.959
BPR	3.49 (2.84–4.14) c,A	−1.15 (−1.34-−0.95) b,A	0.07 (0.04–0.12)	0.21	0.991
Keratinocytes					
FOX	3.06 (2.530–3.588) bc,A	−0.91 (−1.24-−0.59) ae,A	0.55 (0.27–1.13)	1.84	0.989
CRO	2.44 (1.75–3.13) bc,A	−0.30 (−1.03–0.43) ce,A	1.83 (0.39–8.52)	14.8	0.967
CFX	3.09 (1.82–4.36) bc,A	0.035 (−0.57–0.64) d,A	0.27 (0.03–2.19)	~ 200	0.928
CXM	3.23 (1.52–4.93) bc,A	−0.42 (−1.37–0.52) e,A	0.27 (0.02–2.96)	0.26	0.943
BPR	2.48 (1.82–3.14) be,A	−0.94 (−1.25-−0.63) ab,A	0.35 (0.13–0.95)	0.91	0.975

[a] Data are from Fig. 4 for 24 h of incubation. See footnote *b* of Table 2 for the equation used for modeling. Statistical analysis was as follows: for analysis of the data in each column (one-way analysis of variance with the Tukey test for multiple comparisons), data with different lowercase letters are significantly different from each other ($P < 0.01$); for analysis of the data in each row (unpaired, two-tailed *t* test between corresponding parameters for MSSA and MRSA), data with different uppercase letters are significantly different from each other ($P < 0.01$). No statistical analysis was performed for the parameters EC_{50} and C_s, as these are related to weight concentrations that cannot be directly compared between antibiotics (see Discussion for the correlation with clinically achievable concentrations in serum).

[b] FOX, cefoxitine; CRO, ceftriaxone; CFX, cephalexin; CXM, cefuroxime; BPR, ceftobiprole. See Table 3 for the MICs.

[c] CFU increase (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for an infinitely low concentration of cephalosporin.

[d] CI, confidence interval.

[e] CFU decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for the antibiotic concentration at an infinitely high concentration.

[f] Concentration (mg/liter; total drug) causing a reduction of the inoculum halfway between E_0 and E_{max}, as obtained from the Hill equation (by using a slope factor of 1).

[g] Concentration (mg/liter; total drug) resulting in no apparent bacterial growth (the number of CFU was identical to that of the original inoculum), as determined by graphical interpolation.

[h] (1), no meaningful calculation was possible since data points were obtained for the upper part of the sigmoidal function only.

[i] (2), since there was only a minimal decrease in CFU within the limits of the experiment, the E_{max}, EC_{50}, and C_s descriptors, as calculated from the Hill equation, become meaningless in the context of antimicrobial activity.

(EC_{50}s). Moreover, all C_s values were within the limits of the concentrations clinically achievable in the serum of humans. Against MRSA, cefoxitin and, to a lesser extent, ceftriaxone showed activities roughly similar to those obtained against MSSA, whereas cephalexin and cefuroxime were largely ineffective. In MSSA-infected keratinocytes, only cefoxitin and ceftobiprole showed activities similar to those observed in THP-1 macrophages. For MRSA, ceftobiprole was the only effective antibiotic (and had activity against MRSA similar to that against MSSA), whereas none of the conventional cephalosporins could prevent the intracellular growth of the bacteria.

Influence of pH on the binding of ceftobiprole and conventional cephalosporins to PBP 2a. To determine the influence of pH on the binding of ceftobiprole and conventional cephalosporins to PBP 2a, we looked for the impairment of Bocillin FL binding to PBP 2a after exposure to cephalosporins. We first characterized our system by running experiments in which increasing concentrations of ceftobiprole were added before countermarking was done with a fixed concentration of Bocillin FL (100 μM) at pH 5.5 and 7.4. Ceftobiprole exerted a marked impairment of Bocillin FL binding and had 50% inhibitory concentrations of about 8 μM at pH 7.4 and 1.9 μM at pH 5.5. Conventional cephalosporins were less effective (25 to 50% impairment only at 10 μM, but also with an enhancement at pH 5.5; however, this was not consistently seen for all cephalosporins) (see Fig. SP3 in the supplemental material).

DISCUSSION

The present work extends our knowledge concerning the activities of β-lactams against intracellular *S. aureus* isolates in two main directions. First, we confirmed that cephalosporins have limited although significant activity against MSSA in THP-1 macrophages, as was previously found for penicillins and carbapenems (7, 20). Second, we showed that the intracellular activity of ceftobiprole is only very modestly affected by the methicillin resistance phenotype (MSSA versus MRSA; differences in MICs of about 1 to 2 $\log_2$ dilutions persist, however, between the two types of strains). This is not the case for the other cephalosporins tested, especially when experiments are conducted with keratinocytes. This lower level of activity or even a lack of activity of conventional cephalosporins against intracellular MRSA is surprising at first glance. Earlier studies indeed show that acidic pH favors the activities of penicillins and carbapenems not only in broth (19, 30) but also in cells (in THP-1 macrophages and keratinocytes) to the point of making them equally active against MRSA and MSSA (18, 19).

Restoration of the susceptibility of MRSA to β-lactams has been ascribed to a conformational change in PBP 2a consistent with the opening of its active site from a closed conformation when it is exposed to these antibiotics at acidic pH (17). Although acidic pH also improves the activities of conventional cephalosporins against MRSA, (i) these molecules actually poorly compete with Bocillin FL (the microbiologically active part of which is penicillin V) for binding to PBP 2a, and (ii) at

TABLE 4—*Continued*

MRSA ATCC 33591				
E_0 (95% CI)	E_{max} (95% CI)	EC_{50} (95% CI)	C_s	R^2
2.50 (1.98–3.01) abc,B	−0.001 (−0.49–0.49) a,B	3.64 (1.10 to 12.0)	>200	0.951
1.82 (1.35–2.30) c,A	−0.28 (−0.79–0.23) ab,B	5.45 (1.44 to 20.7)	35.5	0.934
1.99 (1.75–2.23) ac,B	(1)[h] B	(1)	~ 170	0.955
2.14 (1.90–2.38) ac,A	(1) B	(1)	>100	0.968
2.47 (1.26–3.68) b,A	−0.78 (−1.37-−0.20) c,B	0.27 (0.05 to 1.43)	0.89	0.939
2.36 (2.12–2.61) abc,B	(1) B	(1)	>200	0.946
2.09 (1.86–2.33) ac,A	(2)[i] B	(2)	>200	0.947
2.42 (2.33–2.50) b,A	(1) B	(1)	~ 200	0.993
2.75 (2.51–2.99) b,A	(1) B	(1)	~ 200	0.773
2.04 (1.44–2.65) ac,A	−1.14 (−1.60-−0.69) c,A	1.08 (0.42 to 2.77)	1.95	0.964

least one of them (cephalexin) keeps fairly elevated MICs for MRSA compared to those for MSSA at pH 5.5. This strongly suggests that conventional cephalosporins are collectively less able than penicillins or carbapenems to cooperate with acidic pH to induce the necessary conformational change in PBP 2a for effective acylation. This possibility needs to be examined experimentally, but it is consistent with the observations made in the present study as well as in our previous studies (18, 19). Conversely, ceftobiprole, like other anti-MRSA cephalosporins (11), causes a conformational change consistent with the opening of the PBP 2a active site even at neutral pH (22). Ceftobiprole may therefore be expected to behave at neutral pH somewhat as penicillins and carbapenems do at acidic pH, i.e., to display MICs for MRSA close to those observed for MSSA, as reported by the discoverers of ceftobiprole (4, 15) and as confirmed here for various HA-MRSA and CA-MRSA isolates. The acidic pH may further facilitate this process, since the MICs of ceftobiprole for MRSA are still further lowered when pH is brought from 7.4 to 5.5. These MICs nevertheless remain slightly higher than those observed for MSSA, which can be interpreted either (i) as corresponding to the energy required to induce the conformational change in PBP 2a from its closed to its open state or (ii) as a competition between other PBPs for binding, given that it is known that only binding to PBP 2a is effective for impairing bacterial growth. In all cases, however, the MICs of ceftobiprole for the strains studied here remain in the range of those for which eradication was observed in clinical trials (2, 26), equal to or less than those corresponding to a target attainment rate of 100% in pharmacokinetic/pharmacodynamic evaluation (25), and less than the clinical breakpoints (4 mg/liter) approved so far for skin and skin structure infections.

Modulation of the activity against MRSA by acidic pH also probably explains the observations made with infected THP-1 macrophages for cefoxitin and ceftriaxone, since these cephalosporins eventually display low MICs when they are tested at acidic pH (only fourfold higher than those for MSSA). Conversely, the failure of cephalexin to control MRSA infections in the same cells can be explained by the fact that its MIC remains elevated even at acidic pH. There is, however, some inconsistency for cefuroxime, since it showed low a MIC for MRSA at acidic pH in broth but was nevertheless unable to control MRSA infection in THP-1 macrophages. More extensive structure-activity relationship studies are probably needed in this context.

The situation is quite different for infected keratinocytes, in which all conventional cephalosporins tested almost totally failed to control infection with MRSA (which ceftobiprole does) but showed a response similar to that of ceftobiprole against MSSA. Potential reasons may include (i) a lower level of acidification of the phagolysosomes in infected keratinocytes than in infected THP-1 macrophages, which would then affect all cephalosporins except ceftobiprole; (ii) the differential handling of ceftobiprole compared with that of the other cephalosporins by cells, especially keratinocytes; and (iii) the higher levels of susceptibility of cephalexin and cefuroxime to the β-lactamase of MRSA ATCC 33591 compared to the susceptibilities of the other cephalosporins and ceftobiprole when they are exposed to the intracellular milieu (but a simple effect of pH can be ruled out, since cefuroxime has an MIC as low as that of ceftobiprole at pH 5.5). These hypotheses could not be tested in the present work, as they represent major undertakings requiring the availability of radiolabeled compounds to track the intracellular fate of the drugs and their degradation products.

The cell culture models used in the present study suffer from many limitations that have been analyzed in previous studies (7, 18–20). We may also need to expand our models to other cell types, such as endothelial and epithelial cells, which could handle *S. aureus* in a different fashion. Yet, the models, as designed so far, allow the objective comparison of meaningful pharmacological properties between antibiotics against intracellular infections, which is an important step for the proper design and interpretation of the results of more elaborate in vitro and in vivo studies. Thus, despite all the uncertainties mentioned above, the data reported here clearly demonstrate and rationalize the superiority of ceftobiprole over conventional cephalosporins for controlling intracellular infections caused by MRSA in two cell types that are probably important for consideration when clinicians are dealing with persistent staphylococcal infections. Of note, however, is the fact that in all cases the reduction of the intracellular CFU load over the

postphagocytosis inoculum never exceeded about 1 log unit. The reason for this limited intracellular efficacy, which has been observed for all β-lactams studied so far, has no simple explanation that can be offered at this stage. The model used indeed allows the observation of the reduction of the intracellular inoculum down to 2 to 3 log CFU achieved with other antibiotics against *S. aureus*, including MRSA (6). Further studies will need to establish whether it represents an intrinsic limitation of β-lactams with clinical significance.

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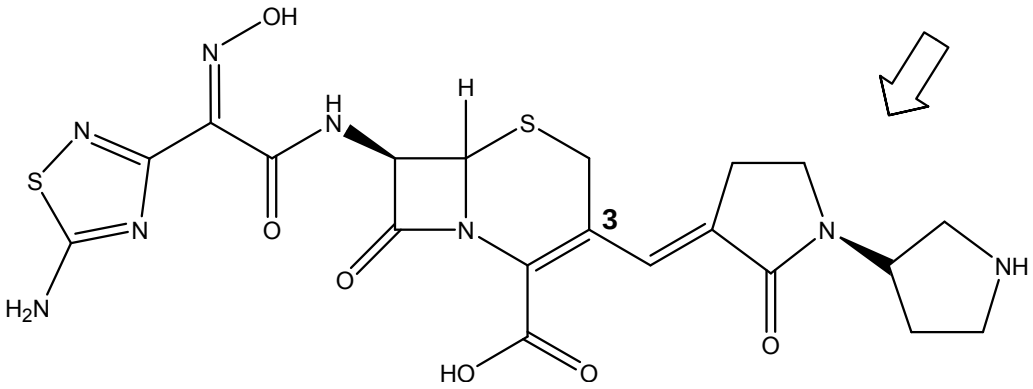
Activity of ceftobiprole and other cephalosporins against extracellular and intracellular (THP-1 macrophages, keratinocytes) forms of Methicillin-Sensitive (MSSA) and Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Supplemental Material

Structural formula of ceftobiprole



IUPAC denomination:

(6R,7R)-7-[[(2Z)-2-(5-amino-1,2,4-thiadiazol-3-ylidene)-2- nitrosoacetyl]amino]-8-oxo-3-[(E)-[2-oxo-1-[(3R)-pyrrolidin-3- yl]pyrrolidin-3-ylidene]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2- ene-2-carboxylic acid

also known as BAL 9141, BAL 9141-000, or Ro 63-9141 (4).

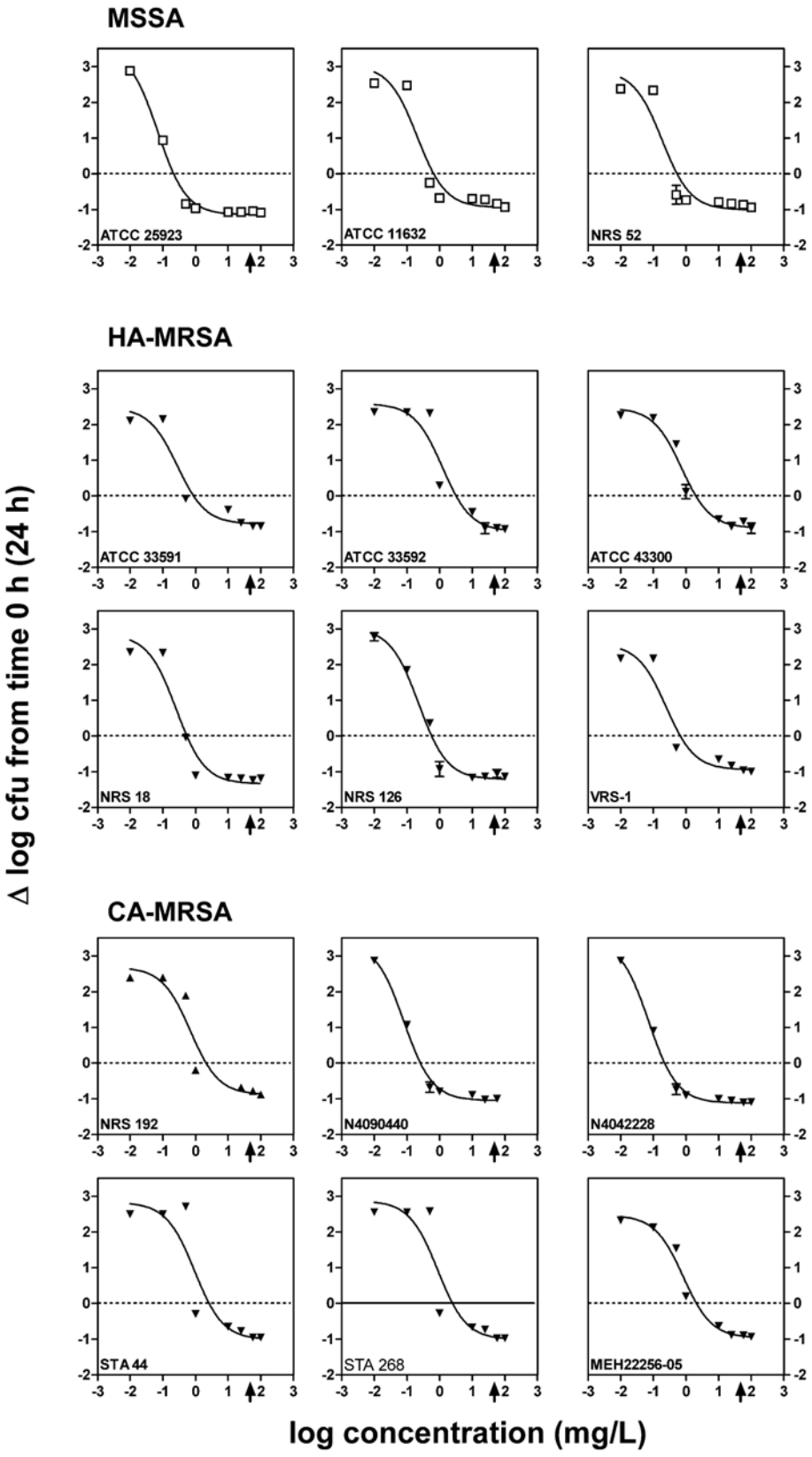
The arrow points to the bulky hydrophobic moiety in position 3 responsible for activity against MRSA.

Note that the clinically-used form of the drug is ceftobiprole medocaril ([[(5-methyl-2-oxo-1,3-dioxo-4-yl)-methoxy]carbonyl]; a pro-drug ester) because of the low water solubility of ceftobiprole.

MATERIALS AND METHODS

Preparation and purification of PBP 2a (soluble form). Staphylococcal PBP 2a was prepared and purified under its soluble form using pET28a vector for overproduction of PBP 2a. The truncated PBP 2a coding sequence (ΔM1-Y23 PBP 2a) was amplified by PCR from the *S. aureus* ATCC 43300 genomic DNA using AccuTaq LA DNA polymerase (Sigma-Aldrich) and the following primers : NcoI-mecA 5'-CCATGGCTTCAAAAGATAAAGAAATTATAATAC-3' and XhoI-mecA 5'-CTCGAGTTATTCATCTATATCGTATTTTTTATTAC-3' (purchased from Eurogentec SA, LIEGE Science Park, Seraing, Belgium). The fragment generated corresponding to the PBP 2a coding sequence within the restriction sites for *Nco*I and *Xho*I was cloned into the pGEM T-Easy vector and the identity of the sequence verified. Next, the vector was digested with *Nco*I and *Xho*I restriction enzymes and the fragment corresponding to the *mecA* gene was cloned into the pET28a to generate the pCIP461. The PBP2a sample was prepared by growing *E. coli* Rosetta 2 (DE3) transformed with the pCIP461 vector in Terrific Broth (BD, Franklin Lakes, NJ). Bacteria were grown at 28°C to an A_{600nm} of 1.5 and 0.5 mM isopropyl β-D-1-thiogalactopyranoside added for the induction of the protein expression. After 6 h, cells were harvested by centrifugation and suspended in buffer A (20 mM NaH_2PO_4/Na_2HPO_4, 200 mM NaCl, pH 6.4). Cells were disrupted by passage through an Inceltech desintegrator (Inceltech, Toulouse, France). The soluble fraction was obtained by centrifugation at 40,000 g and loaded onto a S-Sepharose HP column (Amersham Pharmacia) equilibrated with the buffer A. The protein was eluted with a gradient of NaH_2PO_4/Na_2HPO_4, 1 M NaCl, pH 6.4 (buffer B). The fractions containing the PBP 2a were dialyzed against 20 mM NaH_2PO_4/Na_2HPO_4, 1.5M $(NH_4)_2SO_4$, 200 mM NaCl, pH 8 (buffer C). The insoluble fraction was discarded by centrifugation at 9,000g for 15 minutes. The soluble fraction was injected onto a Phenyl-Sepharose® column (Amersham plc, Little Chalfont, UK) equilibrated with buffer C and the PBP 2a was eluted with a gradient of buffer A. The fractions containing the PBP2a were dialysed against 20 mM NaH_2PO_4/Na_2HPO_4, 0.5M NaCl, pH 7.5 for storage.

Figure SP1


MSSA
ATCC 25923
ATCC 11632
NRS 52
HA-MRSA
ATCC 33591
ATCC 33592
ATCC 43300
NRS 18
NRS 126
VRS-1
CA-MRSA
NRS 192
N4090440
N4042228
STA 44
STA 268
MEH22256-05
Δ log cfu from time 0 h (24 h)
log concentration (mg/L)

Caption of Figure SP1: Individual dose-response curves of cefobiprole against the intracellular forms of the 15 *S. aureus* strains described in Table 1 after phagocytosis by THP-1 macrophages. The ordinate of each graph show the change in the number of cfu (means ± SD; n=3; most SD bars are smaller than the symbols) per mg of cell protein. The abscissa is the $\log_{10}$ of the ceftobiprole extracellular concentration (mg/L). The horizontal dotted line in each graph corresponds to an apparent static effect. Data were used to fit individual sigmoidal functions (for all functions, a Hill coefficient =1 was used because of insufficient data points in each individual experiment; see Table SP1 in this supplementary material for pertinent regression parameters (pharmacological descriptors) and goodness of fit and for each dose-response function).

Figure SP2

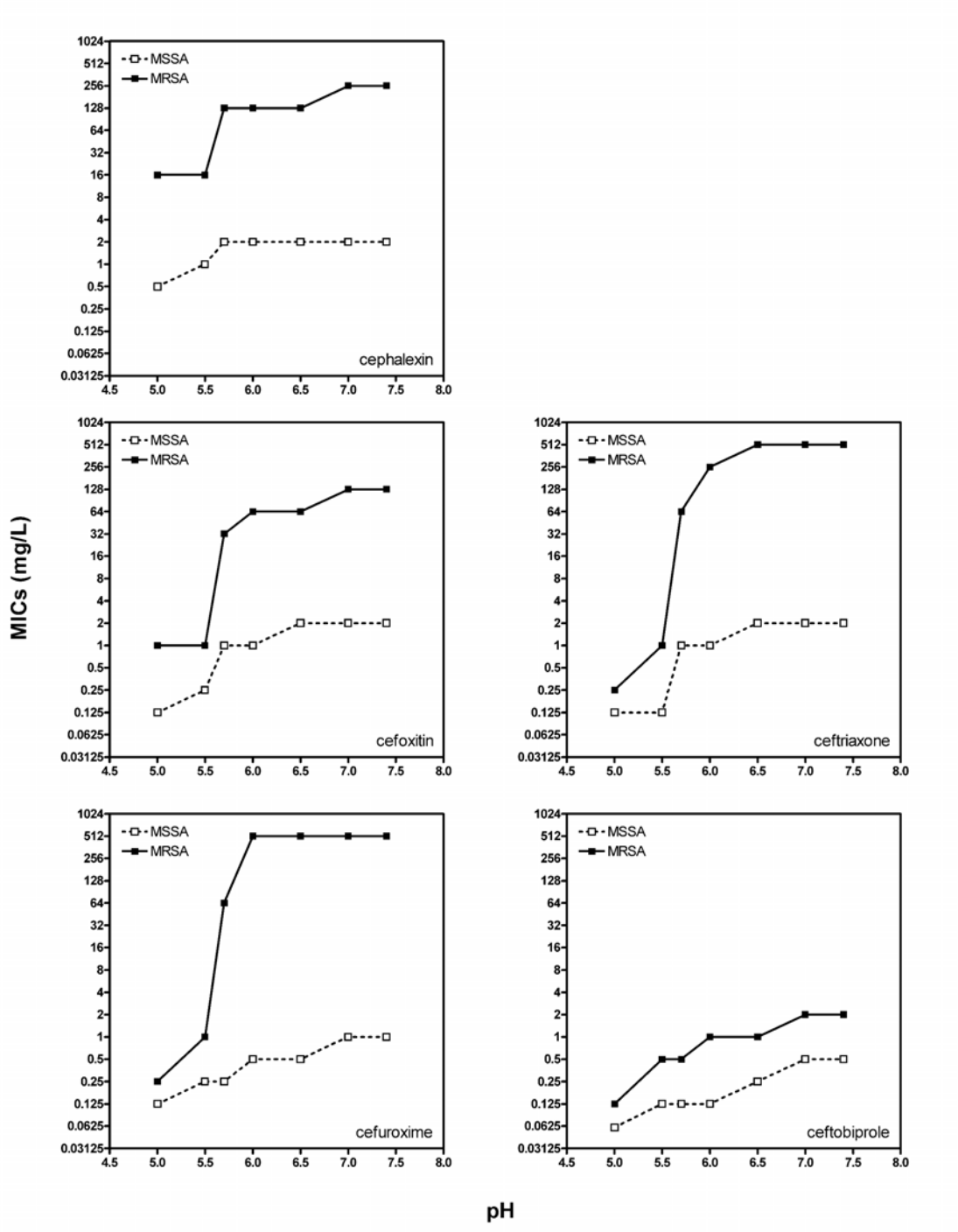


Figure SP2: Influence of the pH on the MICs of ceftobiprole (CFB) compared to 4 conventional cephalosporins towards MSSA ATCC 25923 and MRSA ATCC 33591 as measured in broth.

Figure SP3

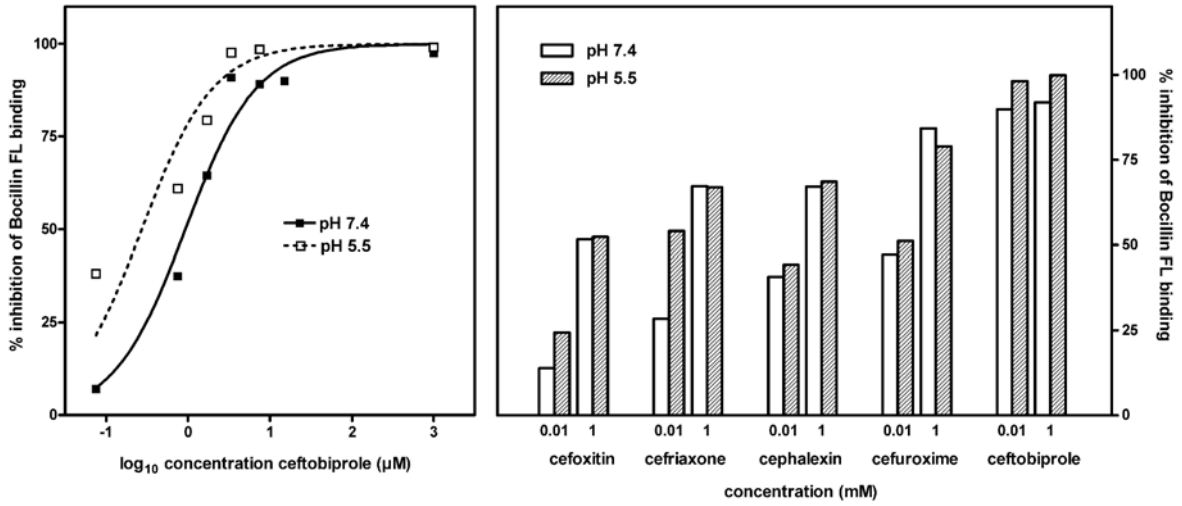


Figure SP3: Impairment of Bocillin FL (100 µM) binding to PBP 2a by pre-exposure to cephalosporins. Left panel: ceftobiprole was added at at increasing concentrations at pH 7.4 (closed symbols) and pH 5.5 (open symbols); IC_{50} values are 0.27 µM [CI: 0.11 to 0.65] at pH 5.5 and 0.93 µM [CI: 0.63 to 1.35] at pH 7.4. Right panel: cephalosporins were added at two fixed concentrations (0.01 and 1 mM) at pH 5.4 (open bars) or pH 7.4 (striped bars).

Table SP1: Pharmacological descriptors and goodness of fit of the dose-response of each of the individual experiments shown in Figure SP1 (24 h incubation).

Phenotype and strain no.	Pharmacological descriptor [a]			goodness of fit (R^2)
	E_0 [b] (95 % CI)	E_{max} [c] (95 % CI)	EC_{50} [d] (95 % CI)	
MSSA				
ATCC259323	3.49 (2.84 to 4.14)	-1.15 (-1.34 to -0.95)	0.070 0.042 to 0.12	0.99
ATCC11632	3.03 (1.682 to 4.387)	-0.95 (-1.56 to -0.34)	0.205 (0.05 to 0.80)	0.93
NRS52	2.89 (1.37 to 4.42)	-1.01 (-1.68 to -0.35)	0.19 (0.04 to 0.88)	0.91
HA-MRSA				
ATCC33591	2.47 (1.26 to 3.68)	-0.78 (-1.37 to -0.20)	0.28 (0.05 to 1.43)	0.94
ATCC33592	2.58 (1.58 to 3.58)	-0.99 (-1.70 to -0.27)	1.23 (0.28 to 5.34)	0.93
ATCC43300	2.46 (1.86 to 3.06)	-0.90 (-1.28 to -0.52)	0.73 (0.32 to 1.64)	0.97
NRS18	2.83 (1.62 to 4.05)	-1.34 (-1.92 to -0.75)	0.26 (0.08 to 0.84)	0.94
NRS126	3.02 (2.26 to 3.78)	-1.21 (-1.56 to -0.86)	0.23 (0.11 to 0.47)	0.98
VRS1	2.57 (1.21 to 3.93)	-0.95 (-1.58 to -0.31)	0.25 (0.05 to 1.31)	0.94
CA-MRSA				
NRS192	2.68 (1.35 to 4.01)	-0.89 (-1.80 to 0.03)	0.70 (0.13 to 3.74)	0.92
N4090440	3.39 (2.73 to 4.04)	-1.05 (-1.29 to -0.812)	0.08 (0.05 to 0.14)	0.99
N4042228	3.45 (2.98 to 3.91)	-1.12 (-1.26 to -0.98)	0.07 (0.05 to 0.10)	0.99
STA44	2.82 (1.17 to 4.48)	-1.02 (-2.12 to 0.09)	0.94 (0.12 to 7.5)	0.86
STA268	2.87 (1.32 to 4.43)	-1.01 (-2.03 to 0.01)	0.88 (0.13 to 5.85)	0.88
MEH22256-05	2.47 (1.92 to 3.02)	-0.97 (-1.32 to -0.61)	0.82 (0.39 to 1.72)	0.98

[a] using a sigmoidal dose response function: $y = E_0 + \frac{E_{\min} - E_{\max}}{1+10^{(\log EC_{50} - x)}}$ where x is the concentration (in mg/L)

[b] change of $\log_{10}$ cfus per mg of cell protein from the original postphagocytosis inoculum for an infinitely low ceftobiprole extracellular concentration

[c] change of $\log_{10}$ cfus per mg of cell protein from the original post-ohagocytsosis inoculum for an infinitely large ceftobiprole extracellular concentration

[d] ceftobiprole concentration (mg/L) giving a response half-way between E_0 and E_{max}