

Activity of ceftaroline against extracellular (broth) and intracellular (THP-1 monocytes) forms of methicillin-resistant *Staphylococcus aureus*: comparison with vancomycin, linezolid and daptomycin

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Received 22 August 2012; returned 25 September 2012; revised 26 September 2012; accepted 11 October 2012

Background: Ceftaroline fosamil is approved for treatment of acute bacterial skin and skin structure infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). We examined the activity of its active metabolite (ceftaroline) against intracellular forms of *S. aureus* in comparison with vancomycin, daptomycin and linezolid.

Methods: Two methicillin-susceptible *S. aureus* (MSSA) and 11 MRSA strains with ceftaroline MICs from 0.125 to 2 mg/L [two strains vancomycin- and one strain linezolid-resistant (EUCAST interpretative criteria); VISA and *cfr*+] were investigated. The activity was measured in broth and after phagocytosis by THP-1 monocytes in concentration-dependent experiments (24 h of incubation) to determine: (i) relative potencies (EC_{50}) and static concentrations (C_s) (mg/L and \times MIC); and (ii) relative activities at human C_{max} ($E_{C_{max}}$) and maximal relative efficacies (E_{max}) (change in \log_{10} cfu compared with initial inoculum). Ceftaroline stability and cellular accumulation (at 24 h) were measured by mass spectrometry.

Results: Ceftaroline showed similar activities in broth and in monocytes compared with vancomycin, daptomycin and linezolid, with no impact of resistance mechanisms to vancomycin or linezolid. For all four antibiotics, intracellular $E_{C_{max}}$ and E_{max} were considerably lower than in broth ($\sim 0.5 \log_{10}$ versus $4-5 \log_{10}$ cfu decrease), but the EC_{50} and C_s showed comparatively little change (all values between ~ 0.3 and $\sim 6 \times$ MIC). The mean cellular to extracellular ceftaroline concentration ratios (20 mg/L; 24 h) were 0.66 ± 0.05 and 0.90 ± 0.36 in uninfected and infected cells, respectively.

Conclusion: *In vitro*, ceftaroline controls the growth of intracellular MRSA to an extent similar to that of vancomycin, linezolid and daptomycin for strains with a ceftaroline MIC ≤ 2 mg/L.

Keywords: Hill equation, maximal relative efficacy, static concentration, recursive partitioning analysis, mass spectrometry, VISA, linezolid resistant, *cfr*

Introduction

Ceftaroline,¹ originally known as T-91825, is a novel cephalosporin with *in vitro* activities against methicillin-resistant *Staphylococcus aureus* (MRSA) comparable to those of vancomycin, linezolid and daptomycin towards susceptible strains,^{2,3} and with unimpaired activity against strains non-susceptible (VISA) or resistant (VRSA) to vancomycin.⁴ In *in vitro* time-kill studies, ceftaroline is also more rapidly cidal against MRSA than vancomycin and linezolid.² Developed for clinical use as a water-soluble N-phosphono prodrug [ceftaroline fosamil (TAK-599)],⁵ it has proven efficacious,

thus receiving approval in the USA and the EU for the treatment of acute bacterial skin and skin structure infections caused by susceptible organisms including MRSA.^{6-9a} In these studies a numerically higher clinical response was achieved for ceftaroline over a vancomycin/aztreonam combination at an early stage of treatment.¹⁰ Ceftaroline fosamil has also been approved for the treatment of community-acquired bacterial pneumonia caused by susceptible organisms including methicillin-susceptible *S. aureus* (MSSA) and *Streptococcus pneumoniae*. These studies compared ceftaroline with ceftriaxone and again

ceftaroline clinical response rates were numerically superior to those with the comparator agent.

While historically considered as an extracellular organism only, there is now increasing evidence that *S. aureus* invades, sojourns and thrives intracellularly.^{11,12} This creates a potential therapeutic challenge for clinicians, since it has been clearly documented using *in vitro* and *in vivo* models that most antistaphylococcal agents are considerably less active against intracellular *S. aureus* than expected given their respective intrinsic activity and/or level of cellular accumulation.^{13,14} Evaluation of novel antistaphylococcal agents must therefore include an assessment of their ability to control intracellular infections. In this context, we observed that, contrary to most assumptions, β -lactams actually display significant intracellular activity against *S. aureus*, despite a reported poor cellular accumulation.^{15,16} In a previous study we also showed that ceftobiprole, another anti-MRSA cephalosporin,¹⁷ was equally active against intracellular forms of MSSA and MRSA, regardless of origin (community or hospital acquired) and resistance phenotype towards vancomycin.¹⁸ The present study extends these observations to ceftaroline and compares it with vancomycin, daptomycin and linezolid using a panel of strains with increasing MICs towards these antibiotics [including VISA and linezolid resistant (LZD^R)]. Our study used a previously established pharmacodynamic model of infected human THP-1 monocytes that allows a quantitative assessment of key properties such as intracellular maximal efficacy and potency of antibiotics.¹⁵ We show that all four antibiotics display similar intracellular efficacy, with the activity of ceftaroline remaining essentially unimpaired across all strains investigated up to the highest MIC of ceftaroline observed (2 mg/L).

Materials and methods

Materials

Ceftaroline (potency 85.3%; lot no. FMD-CEF-035) was provided by Forest Laboratories, Inc. (New York, NY, USA). Oxacillin was purchased from Sigma-Aldrich (St Louis, MO, USA). Other antibiotics were obtained as the corresponding branded products for human parenteral use distributed for clinical use in Belgium (gentamicin as Geomycine[®], GlaxoSmithKline, Wavre, Belgium; vancomycin as Vancomycin Sandoz[®], Sandoz n.v., Vilvoorde, Belgium; and linezolid as Zyvoxid[®], Pfizer s.a., Brussels, Belgium) or in France (daptomycin as Cubicin[®], Novartis Europharm Ltd, Horsham, UK). Culture media and sera were from Invitrogen Corporation (Carlsbad, CA, USA) and Becton Dickinson (Franklin Lakes, NJ, USA), and other reagents were from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

Bacterial strains and susceptibility testing

The clinical isolates used in the present study are listed in Table 1, with information on their origin and their resistance phenotypes. MICs were determined following the general recommendations of the CLSI¹⁹ for vancomycin, linezolid and daptomycin (with addition of Ca²⁺ for daptomycin). For ceftaroline, we used both plain and cation-adjusted Mueller-Hinton (MH) broth, but no difference was observed between these two media. There was also no effect of the addition of 2% NaCl to MH broth. MICs were also measured in broth adjusted to pH 5.5 to mimic the phagolysosomal environment where *S. aureus* sojourns after phagocytosis.^{20,21} Strains for which ceftaroline showed an MIC ≥ 1 mg/L were retested using arithmetic dilutions (0.25 mg/L intervals) between

1 and 4 mg/L to determine their MICs in a more accurate fashion in that range than with the conventional geometric (log₂) dilution method.

Cell lines, cell infection and determination of cell viability

Experiments were performed with THP-1 cells (ATCC TIB-202; supplied through LGC Promochem Ltd, Teddington, UK), a human myelomonocytic cell line displaying macrophage-like activity,²² maintained in our laboratory as previously described.²³ Cell infection was performed as previously described.^{15,16,18} Briefly, *S. aureus* in the stationary phase (overnight culture) were opsonized in the presence of 10% human serum (Lonza Ltd, Basel, Switzerland) in RPMI-1640 medium and mixed with THP-1 cells (0.5×10^6 cells/mL) for 1 h at a ratio of four bacteria per macrophage after which extracellular and non-internalized bacteria were eliminated by washing and exposure to gentamicin (100 \times MIC; 45 min). This yielded a typical post-phagocytosis bacterial load of $1-3 \times 10^6$ cfu/mg of cell protein. Cells were thereafter incubated for 24 h at 37°C. Cell viability was checked by measuring the release of lactate dehydrogenase and trypan blue exclusion assay.

Determination of the extracellular and intracellular activities of antibiotics (concentration-response curves) and pharmacodynamic descriptors

Extracellular and intracellular activities were measured at a fixed time-point (24 h) using a large array of antibiotic concentrations (typically from 0.01 to 100 \times MIC) to obtain a complete description of the concentration-dependent response, as described in detail in our previous publications.^{15,16,18} For extracellular activity, experiments were performed in MH broth (supplemented with Ca²⁺ for daptomycin) with an initial inoculum of 10^6 cfu/mL and results expressed as the change in cfu/mL from the initial inoculum as measured by colony counting. Bactericidal activity was defined as a reduction of 99.9% (≥ 3 log₁₀ cfu/mL decrease) of the total counts. For intracellular activity, infected THP-1 cells were collected by centrifugation, washed once in PBS and lysed in distilled water. The resulting solution was analysed for protein content (using the Folin-Ciocalteu/biuret method)²⁴ and were plated on Trypticase[™] soy agar (Becton Dickinson) to enumerate bacteria as described in detail in a previous publication (including validation and determination of the lowest limit of detection).¹⁵ Results were expressed as the change in cfu/mg of cell protein. Data were used to fit a sigmoidal function (Hill equation; slope factor = 1) by non-linear regression (GraphPad Prism[®] version 4.03; GraphPad Software, La Jolla, CA, USA) to obtain for each condition numeric values of five key pharmacodynamic descriptors, namely: (i) the increase in the number of cfu for an infinitely low antibiotic concentration [relative minimal efficacy (E_{\min} ; in log₁₀ cfu units)] compared with the original inoculum; (ii) the decrease in the number of cfu for an infinitely large concentration of antibiotic [relative maximal efficacy (E_{\max} ; in log₁₀ cfu units); limit of detection ~ 5.5 log₁₀ cfu decrease from the original inoculum]; (iii) the decrease in the number of cfu at a concentration corresponding to the maximal serum concentration (C_{\max}) of the drug as observed in humans receiving standard therapies ($E_{C_{\max}}$; in log₁₀ cfu); (iv) the concentration of antibiotic yielding a response halfway between E_{\min} and E_{\max} [relative potency (EC_{50} ; in mg/L or in multiples of MIC)]; and (v) the concentration of antibiotic resulting in no apparent bacterial growth compared with the original inoculum [static concentration (C_s ; in mg/L or in multiples of MIC)].

Stability of ceftaroline and measurement of its cellular to extracellular concentration ratio

Ceftaroline stability at 37°C over 24 h in water, broth (adjusted to pH 7.4 and 5.5) and in the cell culture medium (pH ~ 7.4) was checked by

measuring its MIC for *S. aureus* ATCC 25923 using an arithmetic dilution progression (0.1 mg/L intervals) starting from media containing 10 or 100 mg/L. In parallel, broth and culture medium samples incubated with 20 mg/L ceftaroline and cell samples from uninfected and infected THP-1 cells incubated with 20 mg/L ceftaroline for 24 h (collected by centrifugation, followed by washing in PBS and final resuspension in distilled water) were used for measurement of ceftaroline concentrations using liquid chromatography (LC) and tandem mass spectrometry (MS/MS). In brief, samples (100 μ L) were mixed with 1.125 mL of methanol/acetonitrile (4:21, v/v), stored at -20°C for 30 min (to facilitate protein denaturation), thawed and centrifuged. The supernatant was collected, evaporated to dryness under a gentle stream of nitrogen and resuspended in 100 μ L of methanol/water (1:1) with care to obtain full dissolution of any visible material. Samples were then subjected to LC separation using a ThermoFischer LC system equipped with a C18 XBridge column (150 \times 2.1 mm, i.d. 3.5 μ m) (Waters Corp., Milford, MA, USA) and using 100 mM ammonium formate/water/methanol/isopropanol (100:780:80:40, v/v/v/v) as eluent and a flow rate of 0.2 mL/min. Chromatography was performed at 30°C , but samples were maintained at 7°C prior to injection (10 μ L). High collision dissociation spectra were recorded with a Q-Exactive in LC-MS/MS [quadrupole precursor selection with accurate mass (HR/AM) Orbitrap detection; ThermoFisher Scientific, Waltham, MA, USA] at relative collision energy of 40%. Multiple reaction monitoring mode was used for the quantification of the analytes by monitoring the transition m/z 605 \rightarrow 208 by high-resolution mass spectrometry. Calculation of the actual concentration was made using a calibration curve [external standard; linearity 1–5000 ng/mL ($R^2=0.9976$); limit of detection and limit of quantification: 0.1 and 0.5 ng/mL,

respectively] and corrected for actual extraction efficiency from cells by running samples of control cells to which a known amount of ceftaroline had been added and which were then treated exactly as the samples from incubated cells. The cell content in ceftaroline was expressed as ng/mg of cell protein and the ratio of the apparent cellular to extracellular concentrations was calculated using a conversion factor of 5 μ L of cell volume per mg of cell protein, as in our previous publications.¹⁵

Statistical analyses

Statistical analyses of the differences between values of the pharmacological descriptors were made with GraphPad InStat version 3.06 (GraphPad Software). Recursive partitioning analysis was made with JMP version 9.0.3 (SAS Institute, Cary, NC, USA) using a single-pass decision tree method with node splitting based on the LogWorth statistic (see details and justification in the white paper 'Monte Carlo Calibration of Distributions of Partition Statistics', available at <http://www.jmp.com>).

Results

Strains and susceptibility to ceftaroline and comparator antibiotics at neutral and acid pH

Table 1 shows that the MICs of ceftaroline at pH 7.4 for the strains used in this study ranged from 0.125 to 0.25 mg/L for MSSA and from 0.25 to 2 mg/L for MRSA irrespective of their resistance phenotype to vancomycin (range 0.5–4 mg/L),

Table 1. Strains used in this study (origin, resistance phenotype and MICs in broth at neutral and acidic pH)

Strain	Resistance phenotype	MIC (mg/L) ^a							
		ceftaroline		vancomycin		daptomycin		linezolid	
		pH 7.4	pH 5.5	pH 7.4	pH 5.5	pH 7.4	pH 5.5	pH 7.4	pH 5.5
ATCC 25923 ^b	MSSA	0.125	0.125	0.5	0.5–1	0.125–0.25	0.5	1	1–2
34843/33134 ^c	MSSA	0.25	0.125–0.25	1	1	1	1	4	4
ATCC 33591 ^b	MRSA	0.5	0.25	0.5	1	0.25–0.5	0.5	1–2	2
19210/18057 ^c	MRSA	0.5	0.25	0.5	1	0.5	1	2	2
SA 555 ^d	MRSA/VISA	0.5	0.125–0.25	4	2	4	ND	0.5	0.5
SA 1984 ^d	MRSA	0.5	0.25	0.5	0.5	0.25–0.5	2	1	0.5
36065/34090 ^c	MRSA	0.25	0.125–0.25	2	2	1	2	4	2
NRS18 ^e	MRSA/VISA	0.5–1	0.25	2–4	2	1–2	2	0.5–2	0.5
35165/33258 ^c	MRSA	1 (0.75) ^f	0.25	0.5	1	0.5	1–2	4	2
48046/44800 ^c	MRSA	2 (2.25) ^f	0.5	1	1	1	2	2	1
CM05 ^g	MRSA/LZD ^R	2 (1.75) ^f	0.5–1	1–2	0.5–1	0.5–1	0.5–1	4–8	4
062-13091 A ^c	MRSA	2 (1.75) ^f	1	1	2	0.25	1	2	2
062-13101 A ^c	MRSA	2 (2) ^e	0.5	1	2	0.25	1	1–2	2

LZD^R, linezolid resistant; ND, not determined.

^aTriplicate determinations using conventional 1 log₂ dilution progression unless specified otherwise (lower and higher values shown in case of divergence).

^bFrom the ATCC collection (Manassas, VA, USA).

^cFrom JMI Laboratories (North Liberty, IA, USA); the first number is the strain number and the second is the bank number.

^dFrom K. Kosowska-Shick and P. C. Appelbaum (Hershey Medical Center, Hershey, PA, USA); VISA phenotype based on vancomycin MIC determination.

^eFrom the Network on Antimicrobial Resistance in *S. aureus* (managed by the Eurofins Global Central Laboratory, Chantilly, VA, USA; supported under NIAID/NIH contract no. HHSN2722007 00055C); SCCmec group II.

^fValues in parentheses refer to MICs measured using arithmetic dilutions (0.25 mg/L intervals over the 1–4 mg/L range).

^gFrom J. Quinn (Pfizer, Groton, CT, USA); *cfr+* mechanism of resistance.

daptomycin (range 0.125–4 mg/L) or linezolid (range 0.5–8 mg/L). Strains 35165, 48046, CM05, 062-13091 A and 062-13101 A were retested using arithmetic dilutions (0.25 mg/L intervals between 1 and 4 mg/L), but the values (reported in Table 1) were always within the corresponding $\pm 1 \log_2$ range of the progression scale of the conventional assay method.

The MICs of ceftaroline for MRSA were lower (1–2 \log_2 dilutions) when assayed at pH 5.5, especially for strains with a higher MIC. Conversely, acid pH caused an increase in the MICs of daptomycin, no or discordant changes for vancomycin and non-systematic decreases for linezolid. Of note, strains 062-13091 A and 062-13101 A, which have previously been reported to display ceftaroline MICs of 4 mg/L, consistently showed an MIC of 2 mg/L in our assays.

Stability of ceftaroline

As β -lactams in general, and ceftaroline in particular, are known to be potentially unstable when exposed to 37°C, we checked for recovery of the antibiotic from broth and culture media after 24 h incubation using both microbiological and analytical (LC-MS/MS) assays. Recovery was $\sim 66\%$ using LC-MS/MS determination, and $>75\%$ and $\sim 50\%$ by the microbiological method for water or broth and the cell culture medium, respectively.

Pharmacological descriptors of the activity of ceftaroline and comparator antibiotics against extracellular and intracellular forms of MRSA strain ATCC 33591

In this first series of experiments, 24 h concentration–responses were examined for ceftaroline in comparison with vancomycin, daptomycin and linezolid against both extracellular and intracellular forms of the reference MRSA strain ATCC 33591. Data are presented graphically in Figure 1 with the corresponding pharmacological descriptors and regression parameters shown in Table 2. In all cases, a single sigmoidal function could be fitted to the data, in accordance with the pharmacological model previously described for the fully susceptible MSSA strain ATCC 25923 and various antibiotics.¹⁵ In broth (Figure 1, left panel), maximal or close to maximal effects (E_{\max}) were observed for all four antibiotics when their concentration reached a value corresponding to their serum peak concentrations in patients (C_{\max} ; total drug). Ceftaroline, vancomycin and daptomycin were highly bactericidal, yielding calculated E_{\max} values corresponding to the actual limit of detection. In contrast, linezolid did not achieve a mean 3 \log_{10} cfu/mL decrease. As expected for this bacteriostatic agent, linezolid was statistically significantly inferior to the other antibiotics when evaluating the rate of bacterial kill. Moving to the intracellular forms (Figure 1, right panel), maximal relative activities (E_{\max}) were considerably lower (less negative) for all four antibiotics, as

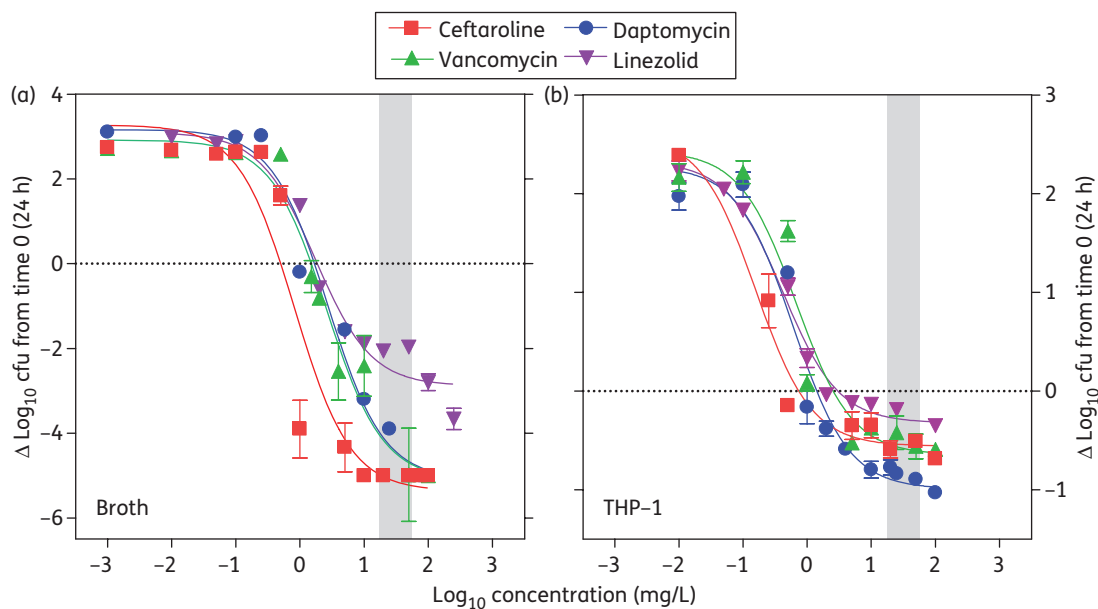


Figure 1. Concentration-dependent activities of four antistaphylococcal antibiotics against extracellular [MHB broth pH 7.4 (a)] and intracellular [THP-1 monocytes (b)] forms of *S. aureus* strain ATCC 33591 (MRSA). For these experiments, broths or infected cells were incubated for 24 h in the presence of increasing concentrations of antibiotic (total drug; abscissa). The ordinates show the change in the number of cfu (\log_{10}) per mL of medium (broth) or per mg or cell protein (THP-1). Note that because of the marked difference in the amplitude of the change between bacteria in broth versus bacteria in THP-1 cells, the scale extends from -6 to 4 in panel (a) and from -1 to 3 in panel (b), with the broken horizontal line showing the zero value (no apparent change from the initial, post-phagocytosis inoculum). All values are means \pm SD ($n=2$ or 3 ; when not visible, the SD bars are smaller than the size of the symbols). The lowest limit of detection corresponds to a cfu decrease of 5 \log_{10} units compared with the original inoculum. The grey zone shows the range of maximal serum concentrations observed in humans for the antibiotics (20–57 mg/L based on the following reported C_{\max} values: ceftaroline, 21 mg/L; vancomycin, 20–50 mg/L; daptomycin, 57 mg/L; and linezolid, 15–20 mg/L; see footnote c in Table 2).

Table 2. Pharmacological descriptors, goodness of fit and statistical analysis of the concentration–response studies of the antibiotics against strain ATCC 33591 (MRSA) in broth and in THP-1 monocytes (24 h incubation)

Condition/antibiotic	Pharmacological descriptor							Goodness of fit (R^2)
	E_{\min}^a	E_{\max}^b	$E_{C_{\max}}^c$	EC_{50}^d		C_s^e		
				mg/L	\times MIC ^f	mg/L	\times MIC ^f	
MH broth (extracellular bacteria)								
ceftaroline	3.28 A;a (1.91–4.64)	−5.37 A;a (−6.50 to −4.23)	−5.04	0.82 A;a (0.33–2.06)	1.64 A;a (0.65–4.12)	~0.48	~1.01	0.934
vancomycin	2.92 A;a (2.08–3.76)	−5.10 A;a (−6.20 to −3.99)	−4.55	2.62 B;a (1.43–4.81)	5.24 B;a (2.85–9.61)	~1.54	~3.04	0.970
daptomycin	3.17 A;a (2.27–4.06)	−5.09 A;a (−5.94 to −4.25)	−4.72	2.70 B;a (1.34–5.42)	5.39 B;a (2.69–10.84)	~1.67	~3.28	0.978
linezolid	3.10 A;a (2.40–3.80)	−2.89 B;a (−3.48 to −2.28)	−2.34	1.74 A,B;a (0.92–3.28)	1.74 A;a (0.92–3.28)	~1.98	~1.89	0.970
TH P-1 monocytes (intracellular bacteria)								
ceftaroline	2.60 A;a (1.92–3.27)	−0.56 A;b (−0.82 to −0.29)	−0.53	0.16 A;b (0.08–0.35)	0.32 A;b (0.14–0.71)	~0.76	~1.45	0.969
vancomycin	2.43 A;a (1.70–3.16)	−0.65 A;b (−1.08 to −0.21)	−0.59	0.67 B;b (0.23–1.9)	1.35 B;b (0.47–3.88)	~2.61	~5.22	0.943
daptomycin	2.28 A;b (1.76–2.79)	−0.99 B;b (−1.27 to −0.71)	−0.96	0.61 B;b (0.32–1.18)	2.46 B;b (1.28–4.70)	~1.40	~6.02	0.963
linezolid	2.33 A;b (2.10–2.55)	−0.32 A,C;b (−0.47 to −0.17)	−0.26	0.42 B;b (0.27–0.63)	0.38 A;b (0.23–0.61)	~3.55	~3.41	0.990

Data are from Figure 1.

Statistical analysis: comparison of regression parameters (E_{\max} and EC_{50}). Figures with different letters are significantly different ($P \leq 0.05$) from all others in the same group. Uppercase letters: comparison between antibiotics (i) in broth (upper four rows) or (ii) in THP-1 monocytes (lower four rows) by analysis of variance (with Tukey–Kramer multiple comparisons test if $P < 0.05$) comparing the four values; lowercase letters: comparison between broth and THP-1 monocytes for the same antibiotic (unpaired two-tailed t -test comparing the two values).

^acfu increase (in \log_{10} units) at 24 h from the corresponding initial inoculum as extrapolated for an infinitely low antibiotic concentration.

^bcfu decrease (in \log_{10} units) at 24 h from the corresponding initial inoculum as extrapolated for an infinitely large antibiotic concentration.

^ccfu decrease (in \log_{10} units) at 24 h from the corresponding initial inoculum as interpolated (using the Hill equation) for a concentration of antibiotic corresponding to the maximal serum concentration observed in humans receiving conventional therapy (C_{\max}). Values chosen for this table are: ceftaroline, 21 mg/L; vancomycin, 35 mg/L; daptomycin, 57 mg/L; and linezolid, 17.5 mg/L (based on mean values in the corresponding US labelling for ceftaroline at its registered dosage of 600 mg every 12 h in the USA and the corresponding rationale document from EUCAST for the most common registered dosages of the other antibiotics in Europe).

^dConcentration [in mg/L or \times MIC (total drug)] causing a reduction halfway between E_{\min} and E_{\max} , as obtained from the Hill equation (slope factor of 1).

^eConcentration (in mg/L or \times MIC [total drug]) resulting in no apparent bacterial growth, as determined by graphical interpolation.

^fMeasured at pH 7.4 in broth (see Table 1; for linezolid, an MIC of 1 mg/L was used for calculations).

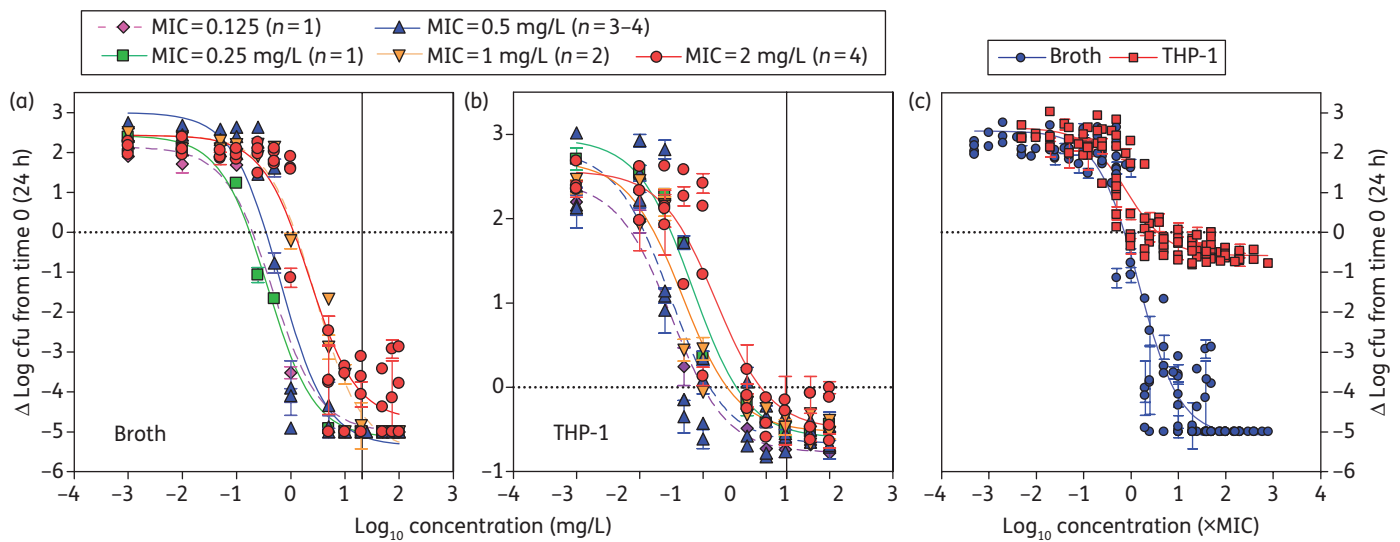


Figure 2. 24 h concentration-dependent activity of ceftaroline against *S. aureus* isolates with differing susceptibilities. Left (a) and middle (b) panels: activity in broth and in THP-1 monocytes, respectively, as a function of drug weight concentration [mg/L (total drug)]: diamonds, MIC=0.125 mg/L (strain ATCC 25923; MSSA); squares, MIC=0.25 mg/L (strain 34843; MSSA); triangles, MIC=0.5 mg/L [strains ATCC 33591 (MRSA), 19210 (MRSA); not tested in broth], SA 555 (MRSA/VISA) and SA 19834 (MRSA); all MRSA]; inverted triangles, MIC=1 mg/L [strains NRS18 (MRSA/VISA) and 35165 (MRSA)]; circles, MIC=2 mg/L [strains 48046 (MRSA), CM05 (MRSA/LZD^R), 062-13101 A (MRSA) and 062-13091 A (MRSA)]; see Table 1 for more details. Right panel (c): activity in broth (circles) and in THP-1 monocytes (squares) as a function of multiples of MIC (total drug). The ordinates of all graphs show the change in cfu (log₁₀) per mL of medium (broth) or per mg of cell protein (THP-1) at 24 h compared with the original post-phagocytosis inoculum (horizontal broken line). Note that because of the marked difference in the amplitude of the change between bacteria in broth versus bacteria in THP-1 cells, the scale extends from -6 to 3 in panel (a) and from -1 to 3 for THP-1 cells in panel (b). All values are means \pm SD (with each strain tested in duplicate); when not visible, the SD bars are smaller than the size of the symbols). The lowest limit of detection corresponds to a cfu decrease of 5 log₁₀ units compared with the original inoculum. The vertical continuous line in the left and middle panels indicates the maximal serum concentration of ceftaroline commonly observed in humans (C_{max}).

these achieved only a ~ 0.5 to ~ 1 log cfu decrease compared with the original inoculum. Statistically significant but quite small differences of intracellular E_{max} were observed, with daptomycin being more active than ceftaroline and vancomycin, and linezolid being the least active. Considering the relative potencies (EC_{50}) and the static concentrations (C_s), ceftaroline appeared to be the most potent, whether in broth or in cells, with values systematically about 2- to 4-fold less than for the other antibiotics, whether expressed as weight concentration (mg/L) or, except for linezolid, as multiples of the MIC in broth (pH 7.4). Interestingly enough, all values of EC_{50} and C_s were quite similar or lower for intracellular bacteria compared with bacteria in broth, denoting an unimpaired potency in the intracellular milieu. The numerical values of these parameters were also close to the MICs of the corresponding antibiotics.

Extracellular and intracellular activity of ceftaroline against *S. aureus* isolates with differing susceptibilities

In these experiments we compared the 24 h concentration-responses of a series of strains of *S. aureus* with ceftaroline MICs ranging from 0.125 to 2 mg/L (strains with higher MICs could not be identified). The results are presented graphically in Figure 2 with changes in cfu/mL shown as a function of the drug weight concentration for bacteria in broth (left panel) and in THP-1 monocytes (middle panel), and as a function of multiples

of MIC for both (right panel). Considering first cfu/mL changes as a function of weight concentrations (mg/L), there was a gradual shift of the curves to higher concentrations as a function of the MIC for the strains, resulting in increases in the EC_{50} and C_s parameters. This was accompanied by a small (but variable among strains) decrease in the activity (less-negative values) observed at a concentration corresponding to the drug C_{max} ($E_{C_{max}}$) or of the maximal relative activities (E_{max}). These changes are illustrated in Figure S1 (available as Supplementary data at JAC Online). The data were then used for recursive partitioning analysis of each descriptor on the basis of the MIC (broth; pH 7.4; log₂ dilution) of the strains, with the results shown in Table 3 (and individual graphs shown in Figure S2, available as Supplementary data at JAC Online). The method used (single-pass decision tree) yielded a dichotomous split at an MIC of 1 mg/L (split between <1 and ≥ 1) for all four descriptors, but yielded statistically significant differences for the EC_{50} and C_s parameters with bacteria in broth only (differences were at the limit of statistical significance for $E_{C_{max}}$ with bacteria in THP-1 cells). Using MICs determined by arithmetic dilutions (0.25 mg/L intervals) for strains with MICs ≥ 1 mg/L (see Table 1) did not significantly change the results of the analysis and its conclusions.

Lastly, when changes in cfu/mL data were plotted as a function of multiples of the MIC of the corresponding strains, data for all strains could be analysed as single functions for bacteria in broth and bacteria in THP-1 cells, respectively, as presented graphically in the right panel of Figure 2 with the corresponding

Table 3. Recursive partitioning analysis of the values of the pharmacological descriptors of the concentration-dependent responses of bacterial strains with increasing MICs of ceftaroline in broth or in THP-1 cells as a function of the corresponding MIC (pH 7.4)

Descriptor	Optimal candidate split value [MIC (mg/L)] ^a	Parameter values (below/above MIC split value)	LogWorth ^b /P value ^c
1. Broth (n=11)			
E_{max} ($\Delta\log_{10}$ cfu) ^d	<1/ \geq 1	$-5.24 \pm 0.10 / -4.89 \pm 0.88$	0.09/0.81
E_{Cmax} ($\Delta\log_{10}$ cfu) ^e	<1/ \geq 1	$-5.04 \pm 0.12 / -4.14 \pm 0.79$	0.97/0.11
EC_{50} (mg/L) ^f	<1/ \geq 1	$0.54 \pm 0.16 / 2.41 \pm 0.50$	8.05/<0.01*
C_s (mg/L) ^g	<1/ \geq 1	$0.28 \pm 0.12 / 1.24 \pm 0.40$	3.56/<0.01*
2. THP-1 (n=12)			
E_{max} ($\Delta\log_{10}$ cfu)	<1/ \geq 1	$-0.68 \pm 0.14 / -0.55 \pm 0.22$	0.19/0.64
E_{Cmax} ($\Delta\log_{10}$ cfu)	<1/ \geq 1	$-0.62 \pm 0.16 / -0.36 \pm 0.15$	1.35/0.04*
EC_{50} (mg/L)	<1/ \geq 1	$0.38 \pm 0.21 / 1.44 \pm 1.11$	0.85/0.14
C_s (mg/L)	<1/ \geq 1	$1.83 \pm 1.55 / 6.51 \pm 4.35$	1.00/0.10

Analysis was made on the basis of the data shown in Figure 2 (left and middle panels), but considering the individual values of each strain.

Asterisks indicate results considered statistically significant on the basis of the P value.

^aValues of MIC separating datasets in two categories based on minimization of the sum of squared errors across the whole data as a function of the MIC (further splitting was unsuccessful because of the limited number of independent values).

^bNode splitting is based on the LogWorth statistic (values >2 indicate that the variable used in the branch is significant and should be included in the decision tree).

^cCalculated based from LogWorth value [$P = 10^{(-\text{LogWorth})}$].

^dcfu decrease (in \log_{10} units) at 24 h from the corresponding initial inoculum, as extrapolated from infinitely large concentrations of antibiotics.

^ecfu decrease (in \log_{10} units) at 24 h from the corresponding initial inoculum, as intrapolated (using the Hill equation) for a concentration of antibiotic corresponding to the maximal serum concentration observed in humans receiving conventional therapy (C_{max}).

^fConcentration (total drug) causing a reduction halfway between E_{min} and E_{max} , as obtained from the Hill equation (slope factor of 1).

^gConcentration (total drug) resulting in no apparent bacterial growth, as determined by graphical interpolation.

Table 4. Pharmacological descriptors, goodness of fit and statistical analysis of the concentration-response studies of ceftaroline against strains with increasing MICs (from 0.125 to 2 mg/L; see list and MICs in the caption of Figure 2) in broth and in THP-1 monocytes (24 h incubation)

Condition	Pharmacological descriptor				Goodness of fit (R^2)
	E_{max} ^a	E_{Cmax} ^b	EC_{50} (\times MIC) ^{c,d}	C_s (\times MIC) ^{d,e}	
MH broth (extracellular bacteria), n=11	-5.09 a (-5.34 to -4.84)	-4.60	1.44 a (1.14-1.83)	0.71	0.938
THP-1 monocytes (intracellular bacteria), n=12	-0.58 b (-0.74 to -0.43)	-0.46	0.81 a (0.57-1.16)	3.62	0.855

Data shown are from Figure 2 (right panel).

Statistical analysis: comparison of parameters (E_{max} and EC_{50}) between broth and THP-1 monocytes. Figures with different letters are significantly different ($P \leq 0.05$) from each other (unpaired t-test two-tailed).

^acfu decrease (in \log_{10} units) at 24 h from the corresponding initial inoculum as extrapolated from infinitely large concentrations of antibiotics.

^bcfu decrease (in \log_{10} units) at 24 h from the corresponding initial inoculum as interpolated for an antibiotic concentration (total drug) corresponding to the reported human C_{max} of ceftaroline (21 mg/L).

^cConcentration [multiples of MIC (total drug)] causing a reduction halfway between E_{min} and E_{max} , as obtained from the Hill equation (slope factor of 1).

^dConcentration (multiple of MIC [total drug]) resulting in no apparent bacterial growth, as determined by graphical interpolation.

^eMIC determined in broth at pH 7.4.

pharmacological descriptors shown in Table 4. While this further confirmed the major differences in efficacy (E_{max} and E_{Cmax}) of ceftaroline against bacteria in broth versus bacteria in THP-1 monocytes, the corresponding relative potencies (EC_{50}) were

not markedly different from each other. The static concentration (C_s) value was close to 1 \times the MIC for bacteria in broth, as anticipated, and was only about 4-fold higher for bacteria in THP-1 monocytes.

Cellular accumulation of ceftaroline

The accumulation of ceftaroline was first measured after 24 h incubation at 20 mg/L in both uninfected and infected (strain ATCC 33591) cells using an LC-MS/MS assay. The apparent cellular to extracellular concentration ratio was 0.66 ± 0.05 ($n=6$) and 0.90 ± 0.36 ($n=3$) in uninfected and infected cells, respectively.

Discussion

There is a clear need to discover and develop novel antibiotics to meet the increased resistance of bacteria to currently registered drugs.^{25,26} Although the most blatant lack of progress is for anti-Gram-negative agents, the situation with Gram-positive organisms remains of concern since several new, potentially promising compounds were not approved by the regulatory authorities,²⁷⁻²⁹ are facing toxicity and emergence of resistance issues³⁰⁻³³ or may require higher doses than originally foreseen.³⁴ In this context, ceftaroline fosamil may represent a useful alternative, especially in light of the decreased susceptibility of contemporary isolates to vancomycin (including heteroresistance),^{35,36} and the emergence of both chromosomal and transferable resistance to linezolid.^{37,38}

Ceftaroline *in vitro* activity against MRSA is related to its high affinity for PBP2a,^{39,40} an affinity that is enhanced in the presence of a cell wall structural surrogate.⁴¹ In the present study we confirm the unimpaired *in vitro* activity of ceftaroline against MRSA with non-susceptibility or resistance mechanisms to vancomycin and linezolid, as previously reported by others.^{4,42} In this context, the present study adds important information about the intracellular activity of ceftaroline in comparison with vancomycin, linezolid and daptomycin. We first show that ceftaroline compares in almost every respect to these antibiotics for a susceptible reference strain (ATCC 33591), but that their relative efficacy is considerably reduced compared with what is observed in broth. This is based on a comprehensive pharmacodynamic analysis comparing static concentrations, relative potencies, effects at concentrations corresponding to the C_{max} in humans and maximal effects. Of note, daptomycin, reported to be highly bactericidal *in vitro*,⁴³ did not prove superior to ceftaroline in THP-1 monocytes or in broth. Thus, for all four agents tested here, it clearly appears that intracellular bacteria are protected to some degree against the antibacterial activities of these different classes of antibiotics (a similar observation was made for ceftobiprole, another cephalosporin with activity against MRSA).¹⁸ This cannot be due to a lack of bacterial growth in cells (as E_{min} values show that there is an ~100-fold growth of bacteria in cells in the absence of antibiotic) and is not related to a loss of potency (the EC_{50} and C_s parameters being essentially similar for bacteria in broth and in THP-1 monocytes). Thus, ceftaroline as well as vancomycin and daptomycin seem to become essentially bacteriostatic against intracellular bacteria and compare, in this respect, to linezolid. This is in contrast to lipoglycopeptides (telavancin and oritavancin), fluoroquinolones or quinupristin/dalfopristin, which show maximal relative efficacy (E_{max}) values between -2 and $-3 \log_{10}$ cfu/mL in the same model⁴⁴⁻⁴⁸ and may therefore be considered as exerting a near-to-bactericidal intracellular activity based on commonly accepted criteria of cidalty.¹⁹

In our study we also examined whether a decrease in the susceptibility of *S. aureus* to ceftaroline (as measured by its MIC in broth) would be accompanied by a decrease or loss of intracellular activity. This approach was triggered by a previous successful attempt to define an intracellular breakpoint for moxifloxacin in the same model.⁴⁷ With ceftaroline, we see that the intracellular activity of antibiotics is primarily driven by the MIC for the phagocytosed organism, confirming previous observations made with other antibiotics.^{18,48-50} However, the changes in the values of the pharmacodynamic descriptors of ceftaroline over the range of MICs investigated (0.125–2 mg/L) were small. Thus, although the recursive partitioning analysis suggested a breakpoint at an MIC value of 1 mg/L, this was only significant for bacteria grown in broth and for parameters related to potency (EC_{50} and C_s), while efficacy parameters (E_{Cmax} and E_{max}) remained unaffected. This may be due to the too narrow MIC range investigated (4 \log_2 dilutions only) or may simply indicate that changes in efficacy parameters will only become visible at higher MICs. However, strains displaying increased ceftaroline MICs seem very difficult to raise⁵¹ and, when observed, may show only limited changes⁵² as those we used here (*mecA*-independent high-level ceftaroline-resistant mutants have been recently generated by serial passage *in vitro*,⁵³ but these were not available to us during our study). The fact that the parameters of intracellular activity are even less affected by an increase in MIC may also be related to the fact that the MIC of ceftaroline is lowered by about 1–2 \log_2 dilutions when tested at pH 5.5 (to mimic the phagolysosomal environment),^{20,21,44} especially for strains against which ceftaroline is less active [thus further narrowing the MIC range (3 \log_2 dilutions only)]. Interestingly enough, this increased activity at acid pH may partly compensate for the weak cellular accumulation of ceftaroline, and account for its effect on intracellular bacteria as previously observed for other β -lactams.⁵⁴ This is also consistent with our observation that acidic pH causes conformational changes of PBP2a that improve its acylation by β -lactams within a time frame relevant to the growth rate of MRSA.⁵⁵ Thus, the present data suggest that ceftaroline activity against intracellular *S. aureus* may remain unimpaired for organisms with an MIC of 2 mg/L, even though it becomes adversely affected for extracellular bacteria when the MIC exceeds 1 mg/L.

The present study also has several limitations that need to be underlined. First, assessment of intracellular activity was made with cells (THP-1 monocytes) that, in contrast to fully differentiated macrophages, do not quickly kill phagocytosed *S. aureus*. This was by design, as we aimed to analyse the pharmacodynamic properties of ceftaroline without undue interference from host defence mechanisms. Future studies may need to examine how and to what extent ceftaroline cooperates with these mechanisms. Other cell types also capable of harbouring *S. aureus*, such as keratinocytes or endothelial cells, could also be used. Second, we did not examine the effect of time on the response to ceftaroline (all experiments used a fixed 24 h time-point), which, again, could be the subject of future studies similar to those made recently with extracellular bacteria.⁵⁶ However, we know from previous studies¹⁵ that killing of intracellular *S. aureus* by β -lactams is a slow process. Thus, shorter exposure time would yield only minimal changes in cfu that prove non-significant. Third, we did not monitor the expression of Pantone-Valentine leucocidin toxin (PVL), which could affect

the host cell viability. However: (i) our previous studies failed to find evidence of an impact of the presence of the PVL-encoding genes on the intracellular behaviour and antibiotic susceptibility of *S. aureus* in the model used;⁴⁷ (ii) the production of PVL and other toxins is maximal at the stationary stage,⁵⁷ which is not reached for intracellular bacteria under the conditions of our experiment; and (iii) PVL presence was not a primary determinant of outcome in patients with complicated skin and skin structure infections due to either MRSA or MSSA in the clinical studies assessing the efficacy of ceftaroline.⁵⁸ Lastly, all comparisons were made using total drug concentrations and using nominal ones. We know that only free concentrations are usually considered for *in vivo* activity assessment and for clinical breakpoint setting.⁵⁹ However, our culture medium contains only 10% serum, which means that most drugs will be free, as previously documented for β -lactams with high protein-binding.¹⁶ But this will not influence much the behaviour of ceftaroline since it has a low protein binding ($\sim 20\%$) in 100% serum.¹ Conversely, our model may lead to an overestimation of daptomycin activity, as it is impaired in human serum in comparison with broth.⁶⁰ Loss of activity during incubation was also not taken into account because it is likely to be progressive and did not, over the 24 h duration of our experiments, exceed 50%, which is less than the 2-fold MIC change that is considered significant in conventional susceptibility testing.

In conclusion, we present *in vitro* evidence that ceftaroline is capable of controlling the growth of intracellular *S. aureus* to an extent similar to that of vancomycin, daptomycin and linezolid, irrespective of the presence of resistance mechanisms to conventional β -lactams (methicillin resistance), vancomycin (VISA) or linezolid (*cfrr*), and for strains for which ceftaroline shows a MIC ≤ 2 mg/L. These results may now trigger the performance of *in vivo* animal and human studies aimed at better delineating the potential use of ceftaroline in difficult-to-treat infections where the persistence of an intracellular inoculum may be a critical determinant.¹²

Acknowledgements

We thank K. Kosowska-Shick, P. C. Appelbaum, J. Quinn, JMI Laboratories and the Network on Antimicrobial Resistance in *S. aureus* (NARSA) for the gift of bacterial strains. We are grateful to M.-C. Cambier for dedicated technical assistance.

Funding

This work was supported by Belgian *Fonds de la Recherche Scientifique Médicale* (FRSM; grant nos. 3.4.597.06 and 3.4530.12), the Belgian *Fonds de la Recherche Scientifique* (FRS-FNRS; grant no. 1.5118.11) and a grant-in-aid from Cerexa, Inc., a wholly owned subsidiary of Forest Laboratories, Inc. Editorial assistance was funded by Forest Research Institute, Inc.

L. G. G. is *Boursière* of the Belgian *Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture* (FRIA). D. D. is the recipient of a post-doctoral fellowship of the Belgian *Fonds de la Recherche Scientifique* (FRS-FNRS; grant no. 1.5118.11). S. L. and F. V. B. are *Chargé de Recherche* and *Maître de Recherche* of the FRS-FNRS, respectively.

Transparency declarations

No conflicts of interest to declare.

Cerexa, Inc. was involved in the design and decision to present these results, but had no involvement in the collection, analysis or interpretation of data.

Editorial assistance was provided by Scientific Therapeutics Information, Inc.

Supplementary data

Figures S1 and S2 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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Supplementary data

Figure S1

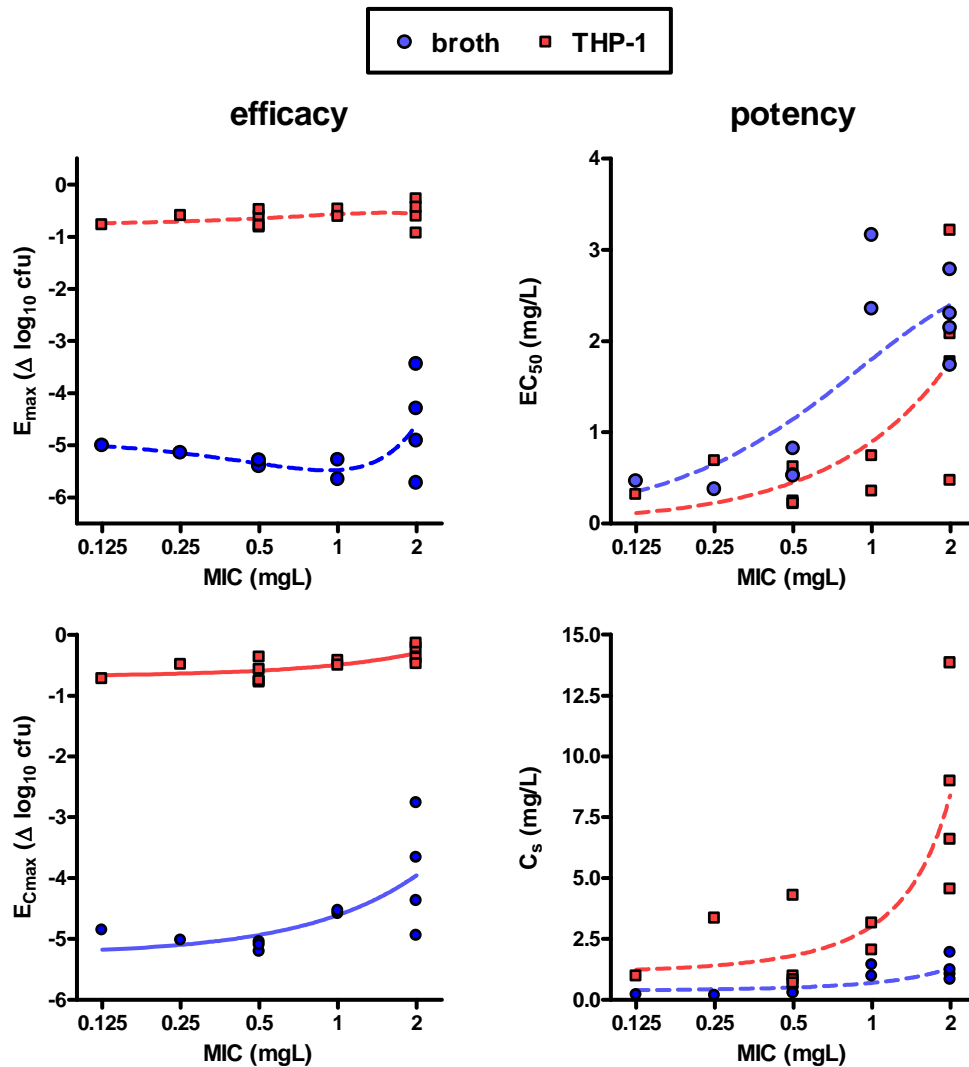


Figure S1: Pharmacological descriptors of the concentration-dependent responses of *Staphylococcus aureus* strains with increasing MICs to ceftaroline. Circles: bacteria in broth (MHB pH 7.4: 11 strains); squares: bacteria in THP-1 monocytes (12 strains). Data are from the experiments illustrated in Figure 2 (24 h incubation). E_{max} (upper left): change in cfu (in \log_{10} units) from the original inoculum for an infinitely large antibiotic concentration; E_{Cmax} (lower left): change in cfu (in \log_{10} units) for a ceftaroline concentration corresponding to its maximal serum concentration in patients receiving standard therapy (21 mg/L); EC_{50} (upper right): concentration (in mg/L) yielding a change in cfu half way between E_{min} (change in cfu from the original inoculum for an infinitely low antibiotic concentration) and E_{max} ; C_s (lower right): concentration (in mg/L) resulting in an apparent static effect (no change from the original inoculum). E_{max} and EC_{50} are the parameters of the sigmoidal function (Hill equation; slope factor = 1) fitted to the data (non-linear regression); E_{Cmax} and C_s are determined by graphical intrapolation using the corresponding Hill equations (each individual point corresponds to the value observed for one strain). Curves are "best fitting" with no specific underlying model.

Figure S2

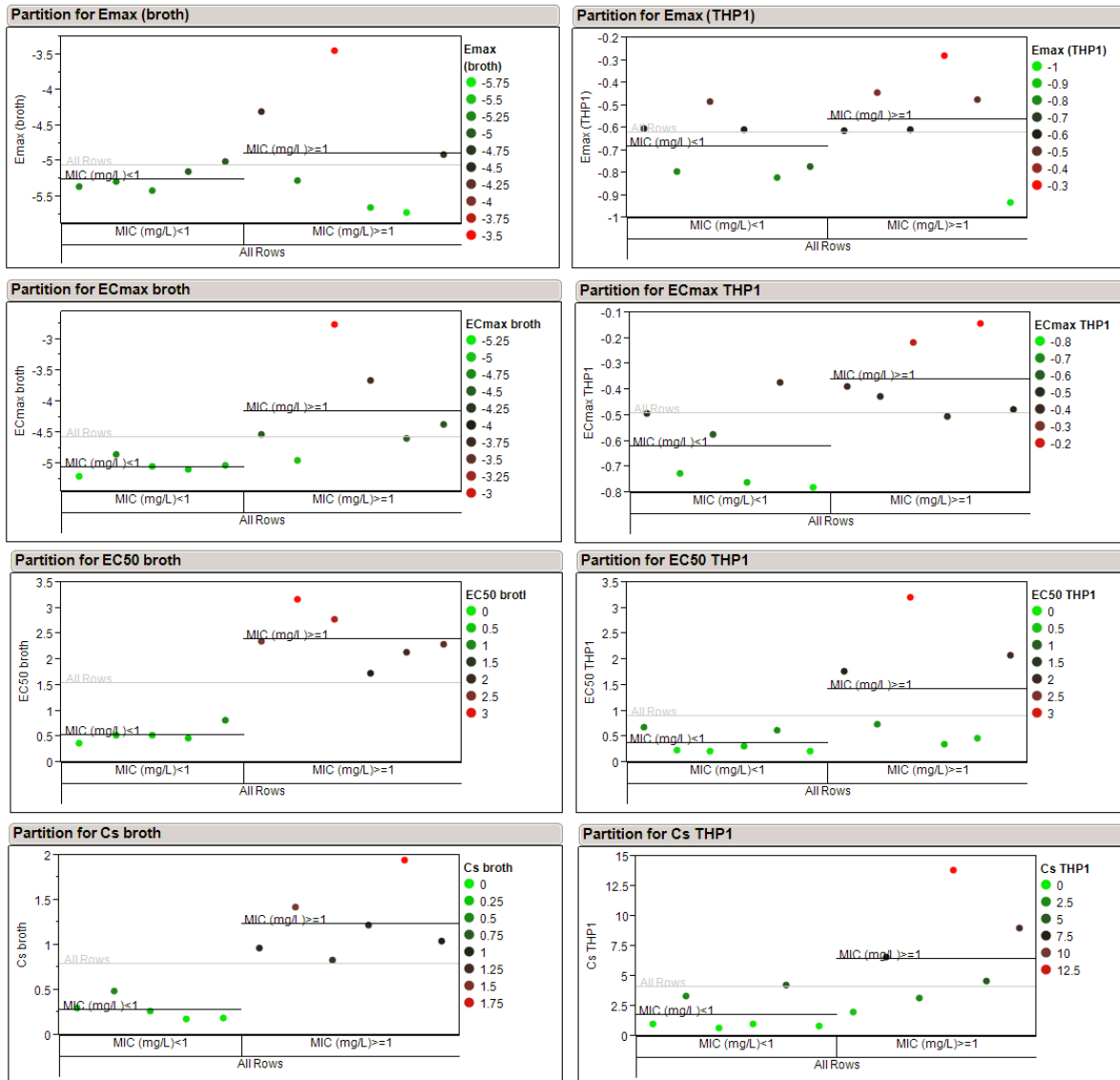


Figure S2: Graphical representation of the results of the recursive partitioning analysis of the pharmacological descriptors derived from the concentration-dependent responses experiments using bacterial strains with increasing MIC to ceftaroline in broth (left) or in THP-1 cells (right). The ordinate of each graph shows the values of the corresponding pharmacological descriptor (E_{max} [upper row]: cfu decrease [in \log_{10} units] at 24 h from the corresponding initial inoculum, as extrapolated from infinitely large concentrations of antibiotic; E_{cmax} [2d row]: cfu decrease [in \log_{10} units] at 24 h from the corresponding initial inoculum, as intrapolated (using the Hill equation) for a concentration of antibiotic corresponding to the maximal serum concentration observed in humans receiving conventional therapy (C_{max} [21 mg/L total drug]); EC_{50} [3d row]: concentration [in mg/L; total drug] causing a reduction halfway between E_{min} (cfu change at 24 h [in \log_{10} units] from the corresponding initial inoculum as extrapolated for an infinitely low antibiotic concentration) and E_{max} ; C_s [lower row]: concentration [in mg/L; total drug] resulting in no apparent bacterial growth, as determined by graphical interpolation (using the Hill equation)). The abscissa separates the data in two groups according to the MIC values (broth; pH 7.4) of the corresponding strains as falling below or above the best split value (1 mg/L). The horizontal bars show the mean of the values for each group. Data points are coloured (from green to black to red) according to the scale on the right of each graph to highlight the differences between them (points with similar colour are numerically close to each other).