SUPPLEMENT ARTICLE

Activity of Fusidic Acid Against Extracellular and Intracellular *Staphylococcus aureus*: Influence of pH and Comparison With Linezolid and Clindamycin

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Background. Emergence of multidrug-resistant *Staphylococcus aureus* has triggered a reassessment of fusidic acid (CEM-102, sodium fusidate).

Methods. Fusidic acid was examined for (1) activity against recent methicillin-resistant *S. aureus* (MRSA) isolates; (2) modulation of activity by acidic pH; and (3) accumulation by phagocytic cells and intracellular activity against methicillin-susceptible *S. aureus* (MSSA) and MRSA.

Results. About 96% of strains (N = 94) were susceptible (European Committee on Antimicrobial Susceptibility Testing breakpoint [$\leq$ 1 mg/L]). Activity was enhanced at pH 5.5 (6 dilutions decrease for minimum inhibitory concentration) in parallel with an increase of drug bacterial accumulation (opposite effects for clindamycin; linezolid remained unaffected). Fusidic acid accumulated in THP-1 cells (about 5.5 fold), with further accumulation at pH 5.5 vs pH 7.4. The intracellular activity of Fusidic acid was similar to that of clindamycin and linezolid (maximal relative activity, 0.4–0.6 $\log_{10}$ colony-forming unit decrease). No cross-resistance to vancomycin or daptomycin was observed.

Conclusions. Fusidic acid is active against *S. aureus* in broth as well as intracellularly, with no cross-resistance to other antibiotics.

Staphylococcus aureus is both a commensal and a versatile pathogen that causes a variety of infections, most notably when a breach of the skin or mucosal barrier allows it access to underlying tissues or to the bloodstream. It has become an increasingly important threat to worldwide public health, because of a remarkable ability to expand its genome, and, thereby, to acquire resistance mechanisms against whole classes of antibacterial agents. This has resulted in the resistance to major antibiotic classes such as β-lactams [1], fluoroquinolones [2], and macrolides [3], and more recently, of a progressive reduction in susceptibility and even full resistance to vancomycin [4], daptomycin [5], and linezolid [6]. These issues, combined with the emergence of highly virulent isolates, have highlighted the necessity to develop novel anti-staphylococcal agents [7]. While several new molecules have reached the level of late-stage clinical development, they are primarily intravenously administered antibiotics. This has triggered efforts in revisiting older, orally administered drugs with demonstrated anti-staphylococcal activity and to examine how these could provide the clinician with an effective and reasonably safe approach when dealing with multidrug-resistant isolates [8]. In this context, fusidic acid has been brought back into focus, as it has been shown to exhibit low toxicity [9] and potent activity against recent staphylococcal isolates irrespective of their resistance to other antimicrobial classes [10]. Although widely used in Europe, Australia, Canada, and Africa, resistance rates to *S. aureus* have remained low in most countries [11] and are close to zero for US isolates [12].

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Acidic pH has been shown to improve the activity of fusidic acid against *S. aureus* [10]. This may be of particular importance for treatment of staphylococcal infections developing in acidic compartments [13], such as the skin, vagina, and urinary tract, and for eradication of intracellular forms of *S. aureus,* which are also exposed to an acidic pH. The present study examines in more detail the influence of acidic pH on the activity of fusidic acid in extracellular and intracellular forms of *S. aureus*, using an approach developed to examine and compare, in detail, the main pharmacological properties and activity of current and novel antibiotics in vitro [14–18]. In the present study, fusidic acid was compared with clindamycin and linezolid, as typical examples of antibiotics whose activity is either impaired or unmodified by exposure to an acidic pH. In these studies, we used, in part, a pharmacological approach in which the activities of fusidic acid and the comparators were examined using full concentration-effect experiments to obtain information on key parameters, such as maximal relative efficacies and relative potencies, as defined in our previous publications [14] and explained in details hereunder.

MATERIALS AND METHODS

Antibiotics and Main Reagents

Fusidic acid (sodium fusidate, CEM-102) was obtained as laboratory samples for microbiological evaluation from Cempra Pharmaceuticals. Linezolid was obtained as the corresponding branded product (Zyvoxid) distributed in Belgium for clinical use by Pfizer SA/NV. Clindamycin was purchased from Sigma-Aldrich. Cell culture media and sera were purchased from Invitrogen and other reagents (including monensin) from Sigma-Aldrich or Merck KGaA.

Bacterial Strains, Susceptibility Testing, and Concentration-Response Studies in Broth

Susceptibility studies were done with a collection of recent Belgian methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (obtained from D.P. and described in the present study) and 2 American isolates recovered from a patient with persistent bacteremia and endocarditis (obtained from P.C.A. and described previously [19, 20]). Minimum inhibitory concentrations (MICs) were determined by doubling dilutions according to the general recommendations of the Clinical and Laboratory Standards Institute. Susceptibility categorization of isolates for fusidic acid were assessed according to the current clinical breakpoints of the European Committee for Antibiotic Susceptibility Testing (EUCAST) [21]. Concentration-response studies in pH-adjusted Mueller Hinton broth were done as previously described [22, 23]. Pharmacological investigations were made with the methicillin susceptible (MSSA) strain ATCC 25923 and the MRSA strain ATCC 33591 (American Type Culture Collection [ATCC]).

Accumulation of Antibiotics by Bacteria

S. aureus strain ATCC 25923 was grown until mid-exponential phase of growth ($OD_{620\ nm} = 0.5$), harvested by centrifugation (4000 rpm, 7 min), and resuspended in pH-adjusted Mueller Hinton broth containing 125 mg/L of the antibiotic under study. After 30 min, bacteria were collected by centrifugation (4000 rpm, 7 min) at 4°C, washed free of antibiotic (4 successive washings with ice-cold phosphate-buffered saline [PBS]), and lysed by 3 successive freeze-thaw cycles (5 min at −80°C, followed by 5 min at 37°C). The cellular content of the antibiotic was measured by the disc-plate assay using Antibiotic Medium 11 (BD) and *S. aureus* strain ATCC 25923 as the test organism (typical values obtained for antibiotics, CEM-102: lowest limit of detection, 2–4 mg/L, linear response between 4 and 500 mg/L [$R^2 = 0.983$]; linezolid: lowest limit of detection, 8 mg/L, linear response between 8–16 and 1000 mg/L [$R^2 = 0.986$]; clindamycin: lowest limit of detection, 2 mg/L, linear response between 4 and 500 mg/L [$R^2 = 0.979$]), and expressed by reference to the total bacterial protein content in the sample.

Cell Lines and Assessment of Cell Viability, and Cellular Accumulation of Antibiotics

Experiments were conducted with human THP-1 cells (ATCC TIB-202), a myelomonocytic cell line displaying macrophage-like activity and maintained in our laboratory as described previously [24]. The viability of cells exposed to the different conditions used in the present study (antibiotics, pH) was checked by a Trypan blue exclusion assay. No significant difference was noted between treated and control cells (<10% of stained cells).

Cellular accumulation of antibiotics was measured with uninfected cells only, as the lack of radiolabeled drug required large extracellular concentrations of antibiotics (≥100 mg/L) to allow satisfactory detection of the corresponding intracellular drug, which prevented intracellular bacterial growth (see Results). Cells were incubated with the antibiotic under study for the desired time (with or without inhibitors), collected by gentle pelleting (1000 rpm; 10 min), and washed free from antibiotic by 4 successive centrifugations in ice-cold PBS. For pH-dependence studies, cells were incubated with buffered media adjusted to specific pH values ranging from 5.0 to 7.4 (the exact pH of each medium was measured before and after incubation, and was found to not vary by more than 0.1 pH unit during the experiment). Cell pellets were resuspended in distilled water; the resulting lysates were used to determine antibiotic content and used in a protein assay utilizing a general technique described previously [14]. Cellular content of antibiotic was expressed by reference to the total cellular protein content and converted into apparent total cell concentrations using a conversion factor of 5 μL/mg of cell protein [16, 23].

Determination of the Intracellular Activity of Antibiotics

Intracellular activities were measured against bacteria phagocytosed by THP-1 macrophages following the general procedure described in our earlier publications [14, 23]. In brief, bacteria were opsonized with nondecomplemented, freshly thawed human serum diluted 1:10 in serum-free culture medium (RPMI 1640; Invitrogen). Phagocytosis was performed at a 4:1 bacteria-macrophage ratio. Elimination of nonphagocytized bacteria and collection of cells at the end of the experiment were accomplished by centrifugation at room temperature (1300 rpm; 8 min). Cells were then exposed to antibiotics using, for concentration-dependent experiments, a wide range of concentrations, typically spanning from 0.01 to 100 times the MIC (as measured in broth). At the end of the experiments, cells were collected by centrifugation, resuspended in PBS, centrifuged again to further remove adherent bacteria, and then lysed in distilled water and processed for colony-forming unit (CFU) counting by plating on agar (previous studies have shown that the amounts of antibiotic that could be carried over from cells into the final assay were too low to significantly interfere with CFU determinations, given the high dilution of the cell content during the sample preparation). Cell proteins were measured in parallel, and results expressed as CFU/mg protein.

Solubility and Chemical Stability of Fusidic Acid

Solubility and chemical stability of fusidic acid in the various conditions used in the present study were assessed by visual inspection and by high pressure liquid chromatography (the latter using a Waters 2690 Alliance System, Waters Corp, equipped with a diode array detection device; chromatography conditions: Lichrosphere 100 RP-18 column [25 × 4 mm, 5 μM; Merck AG]; elution: acetonitrile/phosphate buffer 20 mM pH 3.5 [v/v, 70:30]; typical retention time: 5 min).

Curve Fitting and Statistical Analyses

Curve fittings were accomplished with GraphPad Prism software version 4.03 (GraphPad). For experiments examining the change in CFU as a function of the antibiotic concentration (concentration-dependent experiments), data were used to calculate the pertinent pharmacological descriptors of the biological response, as derived from regression parameters of the corresponding Hill equation (sigmoid; slope factor = 1), namely (1) relative minimal and maximal efficacies (E_{min} and E_{max}, both in $\log_{10}$ units), (2) relative potencies and static concentrations (EC_{50} and C_s, both in either mg/L [weight concentrations; allowing us to relate the value to actual concentrations observed in serum or other compartments] or multiples of the MIC [allowing us to compare different antibiotics having distinct MICs for the antibiotic analyzed or different strains against which a given antibiotic has distinct MICs]. This type of analysis has been described in details in our previous publications with various antibiotics and/or assay conditions [14–16, 22, 23]). In brief, E_{min} is the change in CFU (usually positive) for an infinitely low concentration of antibiotic compared with the original inoculum and describes the growth of the bacteria in absence of antibiotic; E_{max} is the change in CFU (usually negative) for an infinitely large concentration of antibiotic compared with the original inoculum and describes the maximal effect obtained with the antibiotic when, presumably, all its binding sites at the level of the bacterial target(s) have been saturated; EC_{50} corresponds to the drug concentration yielding a change in CFU reduction halfway between E_{min} and E_{max} (this parameter, common in pharmacological evaluations of drugs, essentially addresses differences in apparent affinities of the drug for its target[s]); C_s corresponds to the drug concentration causing no apparent change compared with the original inoculum and is similar (and its value often close) to a conventional MIC. Statistical analyses were made with GraphPad Instat software, version 3.06.

RESULTS

Solubility and Chemical Stability of Fusidic Acid

Visible particulate matter (evidence of incomplete dissolution) was only observed in acidic broth (pH 5.5) when fusidic acid concentrations exceeded 200 mg/L. No chemical instability (>92% recovery of intact drug) was observed in any experimental conditions used here, with no significant influence of pH and no appearance of abnormal elution profiles that would have heralded the appearance of degradation product(s).

MIC Distribution of Fusidic Acid for Recent MRSA Isolates

In the first series of experiments, we examined the susceptibility of a recent (2008) collection of hospital-acquired MRSA isolates (N = 94) to fusidic acid, as obtained from patients with skin and skin structure infections, bacteremia, and endocarditis. Results are shown in Figure 1. Regardless of the type of infection, about 96% of the strains were susceptible according to EUCAST clinical breakpoints for fusidic acid (S ⩽ 1 mg/L), with only 1 strain showing a high level of resistance (8 mg/L). The MICs of fusidic acid against the MSSA strain ATCC 25923 and the MRSA strain ATCC 33591 were 0.25 mg/L (1 dilution higher than the modal MIC value of the clinical MRSA isolates), further demonstrating that fusidic acid was unaffected by the methicillin-resistance phenotype of the clinical strains. The MSSA ATCC 25923 strain was used for most subsequent experiments, and we included MRSA ATCC 33591 in our assessment of intracellular activity (described below).

Influence of pH on the Intrabacterial Concentration and MIC of Antibiotics

In a second series of experiments, we examined the effect of pH on the accumulation of fusidic acid by *S. aureus* and on the

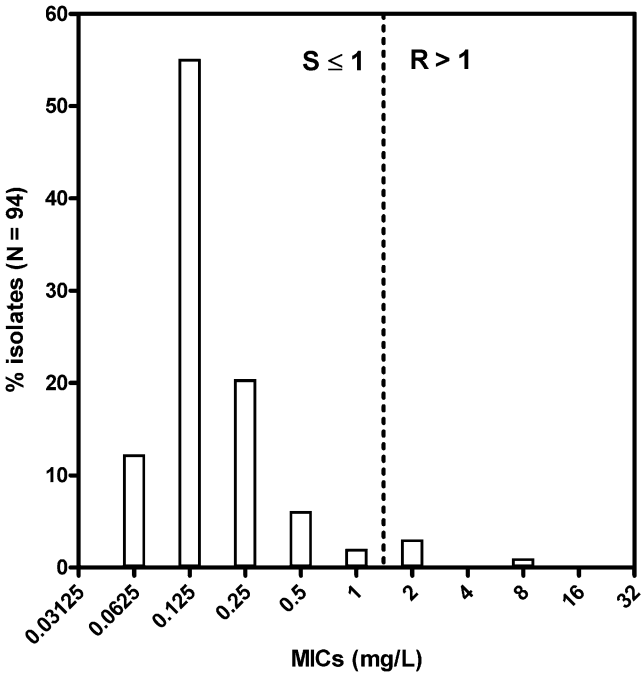

Figure 1. Minimum inhibitory concentration (MIC) distribution of fusidic acid (CEM-102) against a panel of recent (2008) methicillin-resistant *S. aureus* isolates from patients suffering from skin and skin structure infections, bacteremia, and endocarditis. The vertical dotted line separates isolates considered susceptible and resistant according to clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing [21].

corresponding MIC using the ATCC 25923 strain. Clindamycin and linezolid were used as comparators. Figure 2A shows that the accumulation of fusidic acid was enhanced when the pH was decreased from 7.4 to 5.5, in sharp contrast to clindamycin, for which an opposite behavior was seen (marked decrease at acidic pH), and to linezolid (not significant, consistent effect). These changes in accumulation were consistent with changes in MIC over the same pH range (Figure 2B). Thus, fusidic acid had a considerably lower MIC at acidic than at neutral pH (with a value as low as .0078 mg/L at pH 5.5). In contrast, clindamycin showed an increase in MIC (up to 4 mg/L at pH 5.5), and the MIC of linezolid remained unchanged (2 mg/L) throughout the whole range of pH values investigated.

Influence of pH on the Dose-Response Activity of Antibiotics in Broth

To further characterize the influence of pH on the activity of fusidic acid vs clindamycin and linezolid, we performed detailed concentration-dependence studies in broth adjusted to pH values spanning from 5.5 to 7.4 and using a fixed exposure time of 24 h. Data are presented in Figure 3, with pertinent regression parameters presented in Table 1.

The static concentrations (C_s) of fusidic acid and clindamycin, expressed as mg/L, were markedly decreased and increased, respectively, when the pH of the broth was shifted from 7.4 to 5.5. Conversely, no or only minimal changes were seen in E_{min} (not shown) and E_{max} parameters, indicating that, while the change of pH affected the potency, it did not alter bacterial growth nor the maximal relative efficacy of the drugs. This change in potency, observed in these experiments, was directly proportional to the change in MIC seen in Figure 2, as all curves and the corresponding C_s values become almost indistinguishable when the same data are presented as a function of the multiples of MIC as measured at the corresponding pH.

Cellular Accumulation of Antibiotics and Influence of pH and Monensin (Lysosomal/Cytoplasmic pH Gradient Collapser)

In the following series of experiments, we examined to what extent fusidic acid accumulates in THP-1 macrophages in comparison to other antibiotics, and whether this accumulation would be modified by monensin, a known collapser of the lysosomal-cytoplasmic pH gradient [25]. Figure 4A shows that fusidic acid and clindamycin accumulated within THP-1 cells (reaching an apparent cellular to extracellular concentration ratio of about 5), whereas the intracellular concentration of linezolid was only twice as high as its extracellular concentration. Decreasing the pH of the incubation medium resulted in a marked increase in fusidic acid accumulation, whereas the opposite effect was seen for both clindamycin and linezolid (Figure 4B). The addition of monensin drastically reduced the accumulation of clindamycin and linezolid, whereas it only slightly increased the accumulation of fusidic acid.

Intracellular Activity of Antibiotics

In these experiments, we first measured the activities of fusidic acid, clindamycin, and linezolid against phagocytized *S. aureus* ATCC 25923 by performing full concentration-dependent experiments (Figure 5). All 3 antibiotics showed similar maximal relative efficacies (E_{max}) at around –0.4 to –0.6 log10 CFU decrease (compared with the postphagocytosis inoculum). When considering the relative potencies (EC_{50}), no significant differences were observed between the responses in broth vs THP-1 cells for fusidic acid and clindamycin, but a lower value (higher potency) was noted for linezolid. However, these EC_{50} values corresponded to drug concentrations at which significant intracellular growth was observed. When examining the intracellular static concentration (C_s), one sees that these are ~2.5, ~0.25, and ~1× the corresponding MIC in broth at pH 7.4 for fusidic acid, clindamycin, and linezolid, respectively.

We then made a direct comparison between the MSSA ATCC 25923 and MRSA ATCC 33591 strains for susceptibility to fusidic acid after phagocytosis by THP-1 cells, using the same protocol as that for the comparison between antibiotics. The results are presented in Figure 6 and show that the 2 strains could not be distinguished from each other regardless of the

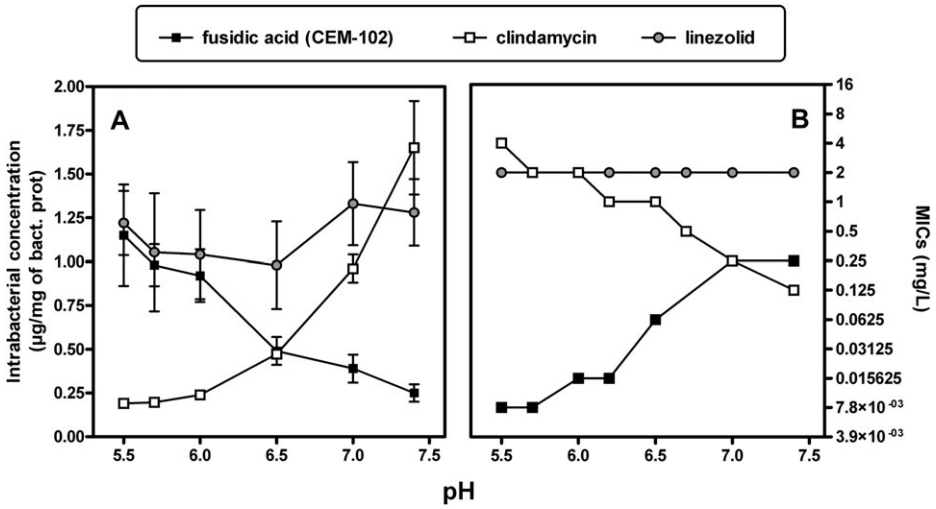

Figure 2. Influence of pH on the intrabacterial accumulation (*A*) and minimum inhibitory concentration (MIC) (*B*) of fusidic acid (CEM-102), clindamycin, and linezolid against *Staphylococcus aureus* strain ATCC 25923. A: growing bacteria were incubated for 30 min in pH-adjusted broth with antibiotic (125 mg/L). Results are expressed as the intrabacterial drug content (values are means ± SD of 3 independent determinations). B: MICs as measured in pH-adjusted Mueller Hinton broth. Values are means of 3 independent samples (yielding 3 identical values). Statistical analysis (1-way analysis of variance [ANOVA] with Dunnett's multiple comparisons posttest for examining the variation of the response according to pH change): fusidic acid accumulation and MIC, $P < .0001$; clindamycin accumulation and MIC, $P < .0001$; linezolid accumulation and MIC, not significant.

pharmacological parameter examined (E_{max}, E_{50}, or C_s), demonstrating an equivalence in the response.

We then used 2 clinical isolates obtained from a patient with persistent staphylococcal bacteremia (HMC 546, aortic valve; HMC 549, blood isolate [resistant to vancomycin and daptomycin] [19, 20]) and compared the intracellular activity of fusidic acid to that of vancomycin, linezolid, and daptomycin, using a fixed extracellular concentration of antibiotic corresponding to each antibiotic's reported human C_{max}. As shown in Figure 7, the intracellular activity of fusidic acid was quite similar against these clinical strains compared with the fully susceptible strain ATCC 25923, and similar to that of vancomycin and linezolid. The intracellular activity of daptomycin was greater for the laboratory strain ATCC 25923 but similar to or lower than that of fusidic acid for the HMC 546 and HMC 549 isolates.

DISCUSSION

The 3 decades that followed the clinical introduction of penicillin G witnessed wide and fruitful efforts in identifying and characterizing a large number of molecules endowed with potent antibacterial activity. Many of these were soon forgotten, or only sparingly used, because of the commercial success of a few major classes of antibiotics still in regular use today. Yet, as with vancomycin use, due to the emergence of MRSA epidemics in the late 1980s [26], other "old" antibiotics may now become increasingly indispensable if their mode of action ensures activity against strains that have acquired resistance mechanisms to currently used antibiotics. This is clearly illustrated for fusidic acid which, though discovered in the early 1960s [27] and soon shown to be effective against staphylococcal bacteremia [28], was not used widely for many years. Although the antibiotic has been used in Canada for many years, it has not yet been introduced into the United States. Yet, as shown in a survey of recent Belgian hospital-acquired MRSA, as well as from recent US surveys [12], fusidic acid maintains constant activity against these types of isolates (including, based on our data, a vancomycin- and daptomycin-resistant clinical isolate). This, as such, may justify careful microbiological and clinical reinvestigation of fusidic acid in an environment where the susceptibility of *S. aureus* to other drugs is decreasing. In this context, pharmacodynamic studies may help in better delineating the conditions of the clinical use of fusidic acid by pinpointing some of its potential advantages as well as weaknesses. The present study is the first, to our knowledge, to address some of these issues in the context of pH influence and assessment of intracellular activity of fusidic acid. In summary, the data show that (1) fusidic acid activity is enhanced in acidic media; (2) it accumulates in eukaryotic cells; and (3) its intracellular activity, although weaker than anticipated in view of its increased activity at an acidic pH and its cellular accumulation, is comparable to that of linezolid, including against a methicillin-, vancomycin-, and daptomycin-resistant isolate.

The enhancement of the activity of fusidic acid by an acidic pH was documented both on the basis of conventional MIC measurements and detailed pharmacological studies. The latter clearly showed that an acidic pH enhances the potency of the

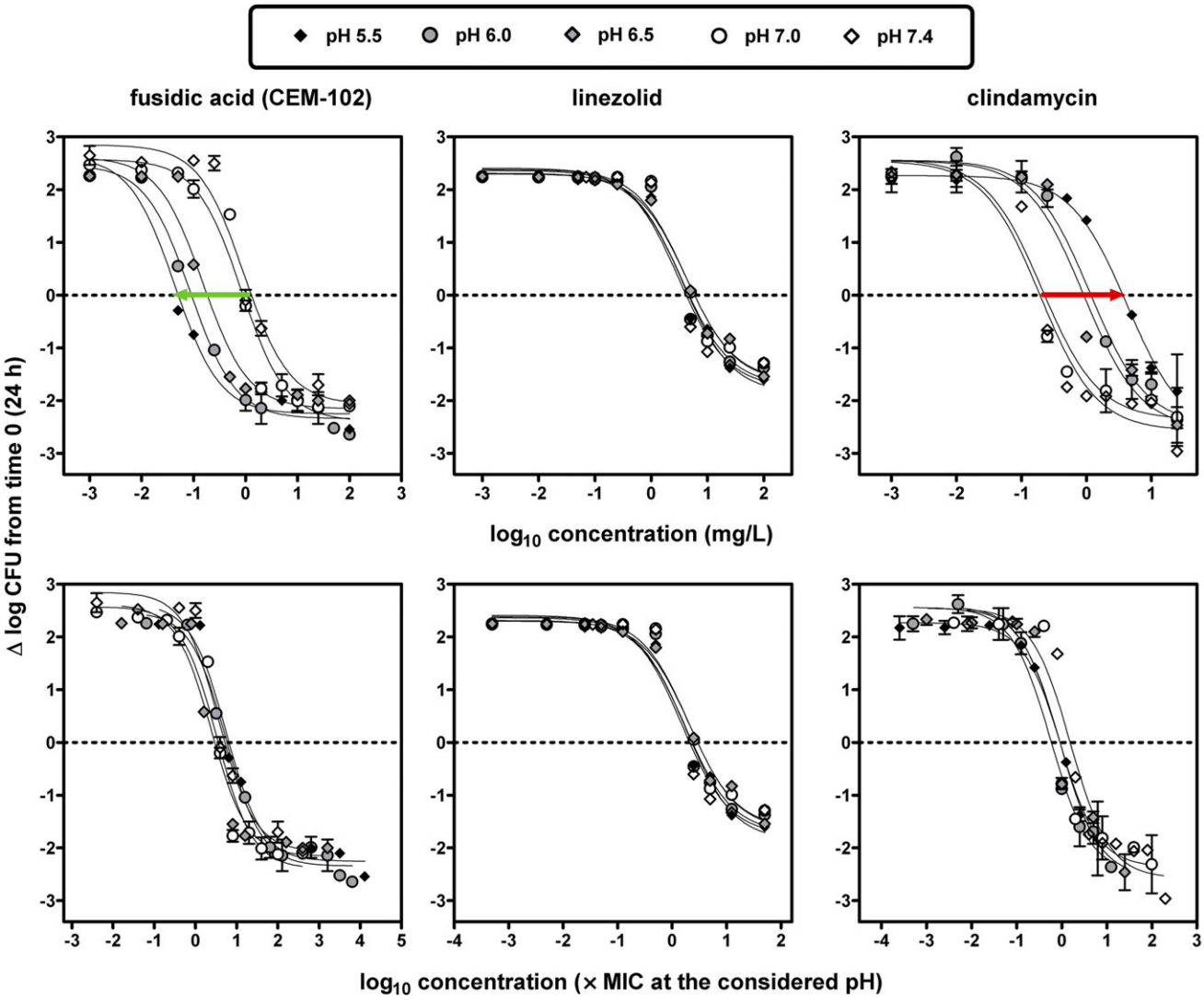

Figure 3. Concentration response of the activity of fusidic acid (CEM-102), clindamycin, and linezolid against *Staphylococcus aureus* strain ATCC 25923 in pH-adjusted Mueller Hinton broth. Bacteria (initial inoculum, 10^6 colony-forming unit [CFU]/mL) were incubated for 24 h with increasing concentrations of antibiotics (total drug; upper panels, weight concentrations; lower panels, multiple of their minimum inhibitory concentrations [MICs] at the considered pH). The ordinates show the change in the number of CFU (log scale) per milliliter of broth. All values are means ± SD of 3 independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Data were used to fit a Hill function (sigmoid; slope factor = 1) for each antibiotic. The green and red horizontal arrows show the shift in static concentrations (C_s) observed for fusidic acid (CEM-102) and clindamycin, respectively, when changing the pH from 7.4 to 5.5. See Table 1 for numerical values and statistical analyses.

drug while leaving its maximal activity unchanged. This strongly suggests that the acidic pH neither modifies the mode of action nor changes the target of fusidic acid, but acts by increasing the drug's accessibility to its target. This is consistent with our observation that acidic pH increases the bacterial accumulation of fusidic acid. Fusidic acid is a weak acid (pK_a ~4.7 to 5.3) with increased lipophilicity when the pH is brought from 7 to 5 [29, 30]. Bacteria maintain a pH gradient across their membranes through their proton motive respiratory chain system [31] (making the pH more alkaline inside), which increases when exposed to an acidic pH [32, 33]. Thus, at pH 5, fusidic acid will more easily diffuse into bacteria and will more effectively be trapped compared with bacteria exposed to a neutral pH (see the general model of accumulation of weak organic acid and weak organic bases in membrane-bounded compartments where a pH gradient is maintained with the surrounding medium [34]). An inverse phenomenon is anticipated for clindamycin, a weak organic base with pK_a ~8.7 [30]), and is what we observed. No effect of acidic pH was seen with linezolid, also an organic base, probably because of its very low pK_a (~5 to 5.7 [30, 35]). These results suggest that fusidic acid may have a potential advantage over clindamycin and linezolid when used against staphylococci

Table 1. Regression Parameters, Pharmacological Descriptors, and Statistical Analyses of the Concentration-Response Curves Illustrated in Figure 2 (Broth) and Figure 5 (THP-1 Cells) (*Staphylococcus aureus* ATCC 25923)

	Broth pH 7.4					Broth pH 5.5					THP-1 Cells				
Antibiotic	E_{max}[a]		EC_{50}[b]	C_s[c]	R^2	E_{max}[a]		EC_{50}[b]	C_s[c]	R^2	E_{max}[a]		EC_{50}[b]	C_s[c]	R^2
Fusidic acid (CEM-102)	−2.07 (−2.9 to −1.28) a,c;A	mg/L	.94 (.42–2.11) c;A	1.32	.98	−2.25 (−2.87 to 1.64) a,c;A	mg/L	.04 (.02–.10) b;B	.05	.98	−.63 (−.78 to −.47) a,c;B	mg/L	.87 (.63–1.20) a;A	3.34	.99
		× MIC	3.76 (1.68–8.44) b;A	5.43			× MIC	5.58 (2.36–13.19) b;A	6.41			× MIC	3.50 (2.54–4.81) a;A	13.5	
Clindamycin	−2.57 (−3.24 to −1.89) a;A	mg/L	.19 (.08–.45) a;A	.20	.95	−2.66 (−3.23 to −2.08) a;A	mg/L	4.26 (2.84–6.40) a;B	3.72	.99	−.63 (−.99 to−.27) a;B	mg/L	.08 (.04–.18) b;A	.33	.97
		× MIC	1.56 (.67–3.62) a;A	1.59			× MIC	1.07 (.71–1.60) a;A	.92			× MIC	.74 (.35–1.57) b;A	.35	
Linezolid	−1.75 (−2.49 to −1.01) b,c;A	mg/L	3.05 (1.27–7.28) b;A	4.02	.96	−1.85 (−2.36 to −1.35) b;cA	mg/L	3.70 (2.12–6.44) a;A	4.55	.99	−.39 (−.55 to −.22) b,c;B	mg/L	.68 (.47–1.00) a;B	4.21	.98
		× MIC	1.52 (.64–3.64) a;A	2.10			× MIC	1.85 (1.06–3.22) a;A	2.45			× MIC	.34 (.23–.50) b;B	2.17	

NOTE. CFU, colony-forming unit; MIC, minimum inhibitory concentration. Statistical analyses were performed as follows: (1) analysis per column (1-way analysis of variance with Tukey's posttest for multiple comparisons between each parameter of all drugs): data with different lowercase letters are significantly different from each other ($P < .05$); (2) analysis per row (1-way analysis of variance with Tukey's posttest for multiple comparisons between broth pH 7.4, broth pH 5.5, and THP-1 cells): data with different uppercase letters are significantly different from each other ($P < .05$).

[a] Maximal relative efficacy: CFU decrease (in $\log_{10}$ units) at 24 h from the corresponding initial (broth) or postphagocytosis (THP-1 cells) inoculum, as extrapolated for an infinitely large antibiotic concentration as obtained from the Hill equation (slope factor = 1).

[b] Relative potency: concentration (in mg/L or in multiples of MIC; broth: as determined at the corresponding pH; THP-1 cells: as determined in broth at pH 7.4) causing a reduction of the inoculum halfway between the E_{min} (increase of CFU for an infinitely low antibiotic concentration) and E_{max}, as obtained from the Hill equation.

[c] Static concentration (in mg/L or in multiples of MIC; broth: as determined at the corresponding pH; THP-1 cells: as determined in broth at pH 7.4) for which no apparent bacterial growth was detected (no change from the initial inoculum), as determined by graphical interpolation.

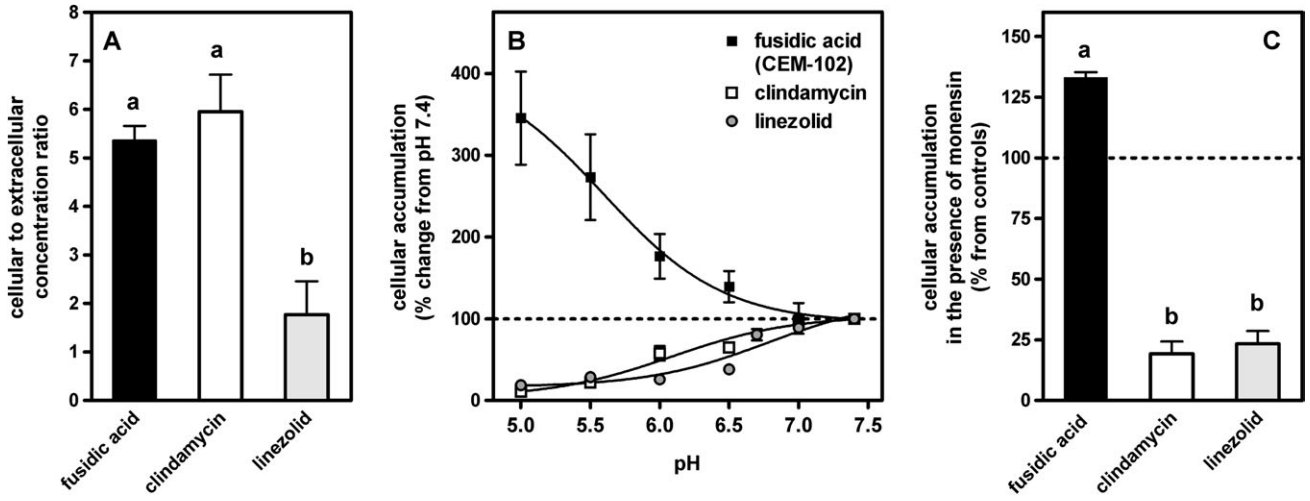

Figure 4. Cellular accumulation of antibiotics within THP-1 macrophages and influence of pH and monensin. All antibiotics were tested at an extracellular concentration of 250 mg/L to allow for satisfactory quantitative detection of the intracellular antibiotic concentrations. All values are means ± SD of 3 independent determinations (when not visible, SD bars are smaller than the size of the symbols). A: cellular to extracellular concentration ratio after 24 h incubation. Statistical analysis (1-way analysis of variance [ANOVA] with Tukey-Kramer multiple comparisons posttest): bars with different letters are significantly different from each other ($P < .05$). B: influence of pH of the cell culture medium on the accumulation of antibiotics (30 min incubation). Statistical analysis (1-way ANOVA with Dunnett's multiple comparisons posttest for examining the variation of the response according to pH change): all 3 antibiotics show significant changes of accumulation upon pH change in the range investigated ($P < .05$). C: Influence of the presence of 50 μM monensin on the cellular accumulation of antibiotics (2 h incubation). Statistical analysis (1-way ANOVA with Dunnett's multiple comparisons posttest): bars with different letters are significantly different from each other ($P < .05$).

in acidic compartments [13], such as the skin (pH 4.2–5.9), the mouth (pH 5–7), the vagina (pH 4.2–6.6), or the urinary tract (pH 4.6–7), with no difference expected between MSSA and MRSA, as long as they show the same MICs in susceptibility testing studies. This will need to be confirmed in appropriate animal models or clinical studies.

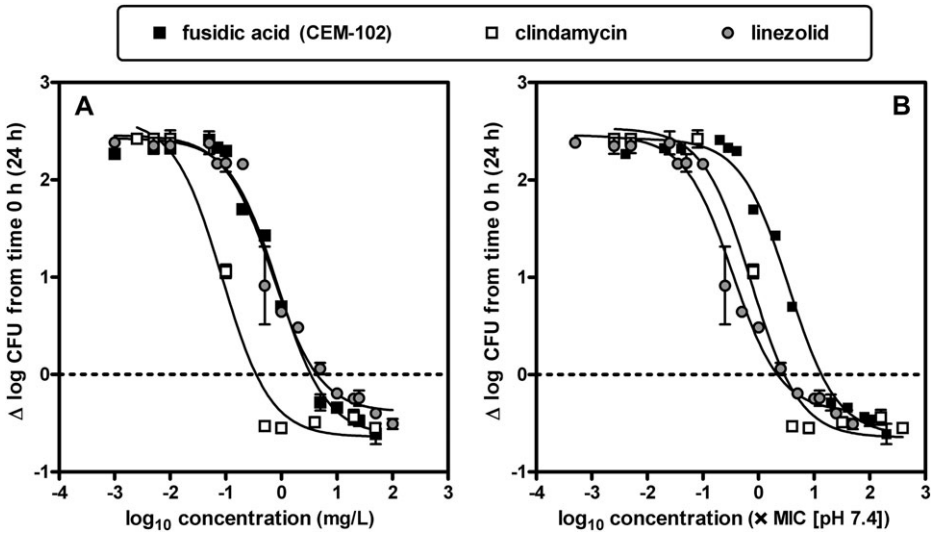

Figure 5. Concentration response of the activity of fusidic acid (CEM-102), clindamycin, and linezolid against *Staphylococcus aureus* strain ATCC 25923 phagocytized by THP-1 macrophages. After phagocytosis and removal of nonphagocytized bacteria (initial inoculum: $\sim 10^6$ colony-forming unit [CFU]/mg of cell protein), cells were incubated for 24 h with increasing concentrations of antibiotics (total drug; panel *A*, weight concentrations; panel *B*, multiple of the minimum inhibitory concentration [MIC] as measured in broth at pH 7.4). The ordinates show the change in the number of CFU (log scale) per milligram of cell protein. All values are means ± SD of 3 independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Data were used to fit a Hill function (sigmoid; slope factor = 1) for each antibiotic. See Table 1 for numerical values of the corresponding parameters and statistical analyses.

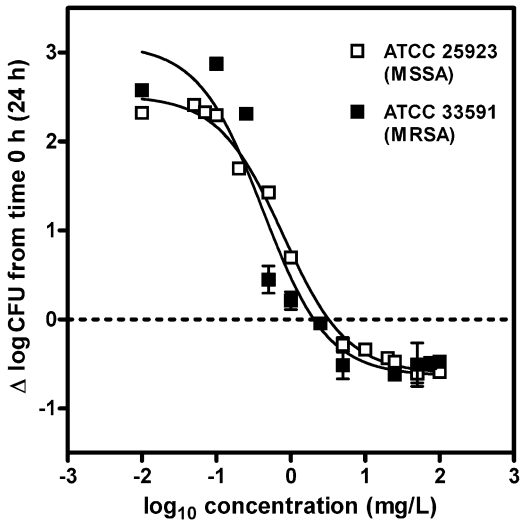

Figure 6. Concentration response of the activity of fusidic acid (CEM-102), against methicillin-susceptible *Staphylococcus aureus* (MSSA) strain ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591 phagocytized by THP-1 macrophages (minimum inhibitory concentration [MIC] in broth at pH 7.4 and pH 5.5: 0.25 mg/L and 0.01 mg/L for both strains). After phagocytosis and removal of non-phagocytized bacteria (initial inoclum: $\sim 10^6$ colony-forming unit [CFU]/mg of cell protein), cells were incubated for 24 h with increasing concentrations of antibiotics (mg/L; total drug). The ordinates show the change in the number of CFU (log scale) per milligram of cell protein. All values are means ± SD of 3 independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Data were used to fit Hill functions (sigmoid; slope factor = 1) for each strain. There are no significant differences between the 2 functions taken globally or between their main parameters (E_{min}, E_{max}, and EC_{50}); the C_s is similar (3.1 mg/L and 2.0 mg/L, respectively).

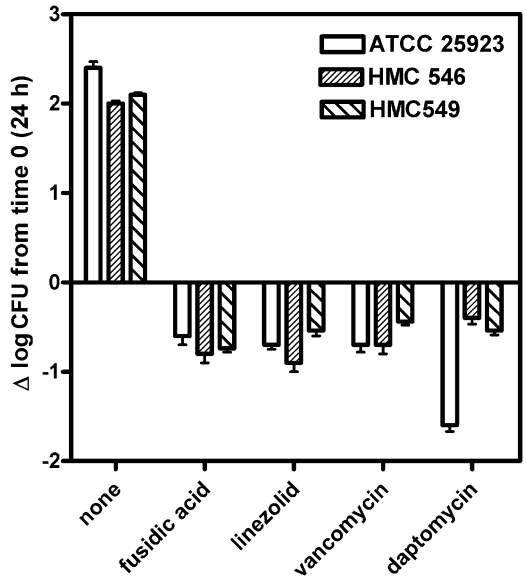

Figure 7. Intracellular activity of antibiotics tested after 24 h incubation of THP-1 cells at an extracellular concentration corresponding to the human C_{max} (total drug; fusidic acid [CEM-102], 20 mg/L; linezolid, 20 mg/L; vancomycin, 50 mg/L; daptomycin, 77 mg/L) against *Staphylococcus aureus* strain ATCC 25923, HMC 546, and HMC 549 (minimum inhibitory concentrations [MICs]: fusidic acid [CEM-102], 0.25, 0.5, and 0.5 mg/L; linezolid, 1 mg/L for all 3 strains; vancomycin, 1, 1, and 4 mg/L; daptomycin, 0.25, 2 and 4 mg/L). The ordinates show the change in the number of CFU (log scale) per milligram of cellular protein. All values are means ± SD of 3 independent determinations (when not visible, the SD bars are smaller than the size of the symbols).

Accumulation of fusidic acid in eukaryotic cells was described about 25 years ago using polymorphonuclear leukocytes and lymphocytes, with values roughly similar to what was observed in this study [36]. Early studies also showed that fusidic acid does not alter monocyte oxidative metabolism [37], suggesting that its accumulation is not detrimental to host defenses. Yet, the volume of distribution of fusidic acid in humans, as determined in early studies with healthy adults, is low (~.3 L/kg [38]). Thus, it is possible that cellular accumulation of fusidic acid is restricted to phagocytic cells. This should be investigated in the future. The mechanism of this accumulation, as well as the subcellular distribution of the drug, also should be further examined. However, the available data already suggest that the cellular accumulation of fusidic acid must rely on very different mechanisms than those operating for clindamycin and linezolid. Indeed, we observe divergent effects with incubation at acidic pH and with co-incubation with monensin. The behavior of clindamycin and linezolid is consistent with a (partial) trapping in lysosomes and related intracellular acidic vacuoles [15, 35, 39, 40]. As a weak organic acid, fusidic acid would be expected to accumulate in the basic, membrane-bounded compartments of eukaryotic cells, such as mitochondria with a resting pH around 8 [41]. This will need to be experimentally studied, but requires the availability of labeled compound.

Accumulation of fusidic acid in THP-1 cells was not associated with a larger activity than in broth, despite the demonstrated cellular accumulation of the drug and its anticipated increased potency at an acidic pH. On the contrary, we saw a reduction of both drug potency (as measured by its C_s) and E_{max}. Reduction of maximal relative efficacy toward the intracellular forms of *S. aureus* has been observed for most antibiotics studied so far [14, 20, 23, 42]. In the present situation, this affected all 3 antibiotics studied, suggesting that it is more related to an intrinsic resistance of the intracellular bacteria than to a specific property of each of these drugs. We know other molecules for which the maximal relative activity is less impaired, allowing them to yield true intracellular bactericidal activity [14, 43]. As described in this study, the lower than anticipated activity of fusidic acid against intraphagocytic bacteria cannot be related to instability or degradation. A possible explanation could be that the subcellular localization of fusidic acid in mitochondria makes it partly unavailable for activity against the intracellular forms of *S. aureus* that thrive in

phagolysosomes [14, 44]. Alternatively, it is possible that *S. aureus* transiently shuts down the metabolic functions targeted by fusidic acid once it reaches the intracellular milieu, in parallel with its reduced intracellular bacterial replication. At this point, however, it is safe to say that the intracellular activity of fusidic acid is not globally different from that of clindamycin, daptomycin, or linezolid, and does not seem affected by the resistance mechanisms specific to these antibiotics or by methicillin resistance.

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