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Contrasting Effects of Acidic pH on the Extracellular and Intracellular Activities of the Anti-Gram-Positive Fluoroquinolones Moxifloxacin and Delafloxacin against *Staphylococcus aureus*▽†

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In contrast to currently marketed fluoroquinolones, which are zwitterionic, delafloxacin is an investigational fluoroquinolone with an anionic character that is highly active against Gram-positive bacteria. We have examined the effect of acidic pH on its accumulation in *Staphylococcus aureus* and in human THP-1 cells, in parallel with its activity against extracellular and intracellular *S. aureus*. Moxifloxacin was used as a comparator. Delafloxacin showed MICs 3 to 5 $\log_2$ dilutions lower than those of moxifloxacin for a collection of 35 strains with relevant resistance mechanisms and also proved to be 10-fold more potent against intracellular *S. aureus* ATCC 25923. In medium at pH 5.5, this difference was further enhanced, with the MIC decreasing by 5 $\log_2$ dilutions. In infected cells incubated in acidic medium, the relative potency was 10-fold higher than that at neutral pH and the maximal relative efficacy reached a bactericidal effect at 24 h. These results can be explained by a 10-fold increase in delafloxacin accumulation in both bacteria and cells at acidic pH, making delafloxacin one of the most efficient drugs tested in this model. Opposite effects were seen for moxifloxacin with respect to both activity and accumulation. As reported for zwitterionic fluoroquinolones, delafloxacin was found associated with the soluble fraction in homogenates of eukaryotic cells. Taken together, these properties may confer to delafloxacin an advantage for the eradication of *S. aureus* in acidic environments, including intracellular infections.

Fluoroquinolones are one of the first families of fully synthetic antibiotics with large clinical use, and they represent a wide range of molecules, which permits the establishment of in-depth structure-activity relationships (16, 17). Over the last 20 years, most fluoroquinolone research efforts have been focused on new molecules with improved activity toward Gram-positive cocci because of the increasing spread of multiresistant staphylococci and streptococci. The 8-methoxy fluoroquinolone moxifloxacin is the first example of this new generation to reach the commercial market. It combines many desirable microbiological and pharmacokinetic properties (rapid bactericidal activity, a spectrum covering pertinent pathogens, excellent oral bioavailability, a large tissue distribution, and a propensity to accumulate within eukaryotic cells [see reference 45 for a review]). Moxifloxacin shows extensive activity toward bacteria thriving in the cytosol (*Listeria monocytogenes* [14]), phagosomes (*Legionella pneumophila*, *Mycobacterium avium*, and *Mycobacterium tuberculosis* [5, 9, 46]), and phagolysosomes (*Staphylococcus aureus* [6]), suggesting that it easily diffuses between different subcellular compartments. Yet moxifloxacin activity is partially defeated by the acidic pH prevailing in vacuolar subcellular compartments (6).

Delafloxacin [RX-3341; 1-(6-amino-3,5-difluoropyridin-2-yl)-8-chloro-6-fluoro-7-(3-hydroxyazetidin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] is an investigational fluoroquinolone originally described by Wakunaga Pharmaceutical Co. (Hiroshima, Japan) as WQ-3034 (35) and then brought to preclinical development by Abbott Laboratories (Abbott Park, IL) as ABT-492. It shows potent activity against Gram-positive organisms, with MICs considerably lower than those of other fluoroquinolones (2, 34). Since 2006, delafloxacin development has been pursued by Rib-X Pharmaceuticals Inc., and successful phase II trials have been completed for community-acquired pneumonia, bronchitis (Rib-X Pharmaceuticals Inc.; data on file [39]), and complicated skin and skin structure infections (36). Delafloxacin also demonstrated very high potency against a large, contemporary collection of Gram-positive clinical isolates, including methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) *S. aureus* (11). From a chemistry perspective, delafloxacin differs from the other fluoroquinolones currently used in the clinic in the absence of a protonatable substituent, which confers to the molecule a weak acid character (see Fig. 1 for chemical structural and main physicochemical properties). This property further increases delafloxacin's potency in acidic environments (35, 43), as also observed for *N*-sulfanilylpiperazinyl fluoroquinolones (1, 3) and finafloxacin (21). This could be an advantage with respect to *S. aureus* infections, since this bacterium, which shows a high tolerance to low pH, survives and multiplies in mild acidic environments (47), such as the skin, the vagina, or the urinary tract, and within the phagolysosomes of infected cells (6), where the pH is about 5 to 5.5 (19). Thus, delafloxacin contrasts with what is observed for many of the current antistaphylococcal antibiotics for which activity is reduced when the pH is lowered, as seen for other fluoroquinolones (e.g., norfloxa-

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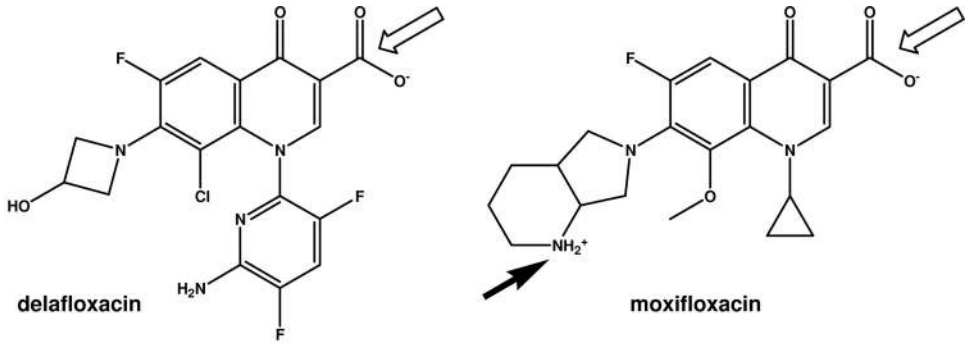

Drug	pKa_1 [a]	pKa_2 [a]	species in solution (calculated ratio) [b]		calculated logP [a]	calculated logD (pH 7.4) [a]
			(%; pH 7.4)	(%; pH 5.4)		
moxifloxacin	6.3	9.3			1.90	-0.63
$^{+}H_2N....COOH$			7	**89**		
$^{+}H_2N....COO^{-}$			**92**	11		
$HN....COO^{-}$			1	0		
delafloxacin	5.4	-			0.94	-0.58
N....COOH			1	**50**		
$N....COO^{-}$			**99**	50		

FIG. 1. Structural formula and physicochemical properties of moxifloxacin and delafloxacin. The open arrows point to the acidic function of fluoroquinolones and the plain arrow to the basic function of moxifloxacin, which is protonated at physiological pH. The table shows the calculated pK_as, log *P* (partition coefficient), and log *D* (distribution coefficient) at neutral pH together with the ratio of species in solution at neutral and acidic pHs. In columns labeled with a superscript a, sources of values are as follows. pK_a, reference 22 for moxifloxacin; data are on file from Abbott Laboratories for delafloxacin. Log *P* and log *D*, Scifinder Scholar software program, version 2007. Values in the column labeled with a superscript b were calculated as follows. Moxifloxacin, % fully protonated form ($^{+}H_2N....COOH$) = $100/(1 + 10^{pH - pKa_1} + 10^{2 \cdot pH - pKa_1 - pKa_2})$; % zwitterion ($^{+}H_2N....COO^{-}$) = $100/(1 + 10^{pKa_1 - pH} + 10^{pH - pKa_2})$; % fully deprotonated form ($HN....COO^{-}$)$^{-}$ = 100 − % ($^{+}H_2N....COO^{-}$)$^{-}$ % ($^{+}H_2N....COOH$). Delafloxacin, % uncharged form (N....COOH) = $100/(1 + 10^{pH - pKa_1})$: % deprotonated form (N....COO^{-}) = 100 − % (N....COOH). Each value in bold is the most abundant form for each drug at the considered pH.

cin, ciprofloxacin, or moxifloxacin [6, 48]), macrolides (26), clindamycin (37), and gentamicin (7).

The aim of the present study was therefore to compare the effect of pH on delafloxacin and moxifloxacin with respect to their activities against extracellular and intracellular forms of *S. aureus* and to their accumulation in both bacteria and eukaryotic cells. We found that acidic pH increases the activity of delafloxacin against both extracellular and intracellular bacteria, in part due to an increased accumulation in both cell types. Opposite effects were observed for moxifloxacin.

MATERIALS AND METHODS

Antibiotics and main reagents. Delafloxacin (RX-3341) and moxifloxacin were obtained as microbiological standards from Rib-X Pharmaceuticals Inc. (New Haven, CT) and Bayer SA-NV (Brussels, Belgium), respectively. Clarithromycin and telithromycin were kindly given by Cempra pharmaceuticals (Chapel Hill, NC), and quinupristin-dalfopristin (3/7 [wt/wt] ratio) was given by Nordic Pharma Ltd. (Paris, France). Linezolid, gentamicin, vancomycin, and rifampin were obtained as the corresponding branded product (Zyvoxid, Geomycin and Vancocin, and Rifadine) distributed for human use in Belgium (Pfizer NV/SA, Ixelles, Belgium; GlaxoSmithKline SA/NV, Genval, Belgium; and Merrell Dow Pharmaceuticals Inc., Strasbourg, France). Penicillin G and monensin were purchased from Sigma-Aldrich (St. Louis, MO). Human serum for opsonization was obtained from healthy volunteers (Lonza Verviers SA, Verviers, Belgium) and stored at −80°C as pooled samples until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA), and other reagents were from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

Assay of fluoroquinolones. The moxifloxacin concentration was determined by fluorimetric assay in eukaryotic cells, as previously described (29) (lowest limit of detection, 50 ng/ml; linear response between 50 and 250 ng/ml [$r^2 = 0.99$]), and by disc plate microbiological assay in bacteria (with interference of bacteria material being observed when using the fluorescence assay) using as a test organism *Escherichia coli* ATCC 25922 (lowest limit of detection, 1 mg/liter; linear response, 1 and 16 mg/liter [$r^2 = 0.99$]) or *S. aureus* ATCC 25923 (lowest limit of detection, 0.5 mg/liter; linear response between 0.5 and 8 mg/liter). We showed previously that these two methods gave matching values (42) and confirmed their consistency under the conditions of the present study. With delafloxacin being intrinsically poorly fluorescent, its concentration was determined by microbiological assay only, with *S. aureus* ATCC 25923 as a test organism (lowest limit of detection, 0.1 mg/liter; linear response between 0.1 and 8 mg/liter; $r^2 = 0.99$).

Bacterial strains, susceptibility testing, and 24-h dose-response studies in broth. The bacterial strains used in the present study are listed in Table 1. MIC determinations and 24-h dose-response studies using the acellular medium were performed in Mueller-Hinton broth (MHB) as described previously (6).

Determination of accumulation of fluoroquinolones in *S. aureus*. *S. aureus* ATCC 25923 was grown until reaching the mid-exponential phase of growth (optical density at 620 nm [OD_{620}] = 0.5), harvested by centrifugation (3,220 × *g*, 7 min), and resuspended in pH-adjusted MHB containing delafloxacin or moxifloxacin. After 30 min at 37°C, bacteria were collected by centrifugation (3,220 × *g*, 4°C, 3 to 4 min), washed four times with cold phosphate-buffered

TABLE 1. Bacterial strains

Phenotype[a]	Strain identification(s)	Origin
Methicillin-sensitive *S. aureus*	ATCC 25923	ATCC
	SA-1	ATCC (derived from strain ATCC 25923; overexpression of the NorA transporter)
	SA040	Clinical isolate; Hershey Medical Center, Hershey, PA
	SA040L	*In vitro* linezolid-resistant mutant of SA040
Hospital-acquired MRSA	ATCC 33591, ATCC 33592, ATCC 43300	ATCC
	NRS100 (COL)	NARSA[b]
	N4112910, N4120032	Clinical isolates; Cliniques Universitaires de l'UCL, Mont Godinne, Belgium
	RS118, RS119	Linezolid-resistant clinical isolates; Rib-X Pharmaceuticals Inc., New Haven, CT
	SA238	Clinical isolate; Hershey Medical Center
	SA238L	*In vitro* linezolid-resistant mutant of SA238
Community-acquired MRSA	NRS192, NRS384 (USA300)	NARSA
	SA312, SA346	Clinical isolates (SCCmec IVa); Hershey Medical Center
	DM11104, DM8279, EB17917, MEH22256	Clinical isolates; National University of Singapore
	N4090440, N4042228	Clinical isolates; Cliniques Universitaires de l'UCL
	STA268, STA44, CHU1	Clinical isolates; Chang Gung Children's Hospital, Taiwan
MRSA strains with characterized Mutations in QRDR	SA036, SA124, SA481, SA069	Ciprofloxacin-resistant clinical isolates; Hershey Medical Center, PA; Sa036 *gyrA*(*S84L*) *grlA*(*S80F*) *gyrB*(*R404L*); SA069 *gyrA*(*S84L*) *grlA*(*S80F*); SA124 *gyrA*(*S84L*) *grlA*(*E84K*); SA481 *gyrA*(*S84L*) *grlA*(*S80Y E84G*)
MRSA strains associated with both health care and community infection	NRS386 (USA700)	NARSA
MRSA and VISA	NRS18	NARSA
MRSA of animal origin	N7112046	Clinical isolate; Cliniques Universitaires de l'UCL

[a] QRDR, quinolone resistance determining region; VISA, vancomycin-intermediate *S. aureus*.
[b] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

saline (PBS) (pH 7.4), resuspended in 500 μl of Milli-Q water, and lysed by three successive freeze-thaw cycles (5 min at −80°C, followed by 5 min at 37°C). The cellular concentration in antibiotics was expressed by reference to the total protein content in the sample, as determined by the method of Lowry (27).

Cell lines. Unless stated otherwise, experiments were conducted with human THP-1 cells (ATCC TIB-202 [American Tissue Culture Collection, Manassas, VA]), a myelomonocytic cell line displaying macrophage-like activity (44). Fractionation studies were performed with J774 murine macrophages. Both cell lines were maintained in our laboratory as previously described, using RPMI medium supplemented with 10% fetal calf serum (15, 29).

Assessment of eukaryotic cell viability. Cell viability and growth capacity were assessed by evaluating their metabolic activity using the MTT assay (reduction in mitochondria of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium in purple formazan crystal) (31). Briefly, cells exposed to increasing concentrations of fluoroquinolones, media at different pHs, or monensin were incubated for 1 h with 0.2 mg/ml MTT, and the reaction was stopped by addition of dimethyl sulfoxide (DMSO). The OD was measured at 590 and 660 nm. Using this procedure, no significant difference (<10%) was detected between treated cells and controls or between infected cells incubated at neutral or acidic pH.

Determination of accumulation of fluoroquinolones in THP-1 cells. We used the general protocol described in our previous publications (6, 26) for uninfected cells. For pH dependence studies, cells were incubated with media adjusted to specific pH values, ranging from 5.0 to 7.4, using 5 mM phosphate buffer. 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. After suitable times of incubation, cells incubated in the presence of antibiotics were harvested by low-speed centrifugation (340 × *g*), washed free from antibiotics (using cold PBS), and finally resuspended in milli-Q water. Cell lysates obtained after sonication of the samples were used for determination of antibiotic content and protein assay, with all cellular drug contents expressed by reference to the total cell protein content and converted into apparent total cell concentrations using a conversion factor of 5 μl per mg of cell protein (14).

Cell fractionation studies in J774 cells. Cells were incubated with 100 mg/liter delafloxacin or moxifloxacin for 2 h, washed free from antibiotics in 0.25 M sucrose–1 mM EGTA–3 mM imidazole (pH 7.4), and finally collected by gentle scraping in the same medium. Cells were then homogenized with a Dounce tissue grinder, and a cytoplasmic extract free of nuclei was obtained after three successive low-speed centrifugations (770, 625, and 500 × *g*, 10 min). The resulting cytoplasmic extract was further fractionated into a "granule/membrane" fraction (containing the bulk of the cell organelles and membranes; MLP fraction) and a final supernatant fraction (S fraction) by high-speed centrifugation (145,000 × *g*) for 30 min (Ti 50 rotor; Beckman Instruments, Inc., Fullerton, CA). Antibiotic concentrations and activities of marker enzymes of the main organelles (cytochrome *c* oxidase for mitochondria, *N*-acetyl-beta-glucosaminidase for lysosomes, and lactate dehydrogenase for cytosol) were measured in each fraction (38, 41).

Cell infection and assessment of antibiotic intracellular activities. Infection of THP-1 cells and assessment of the intracellular activity were performed exactly as described earlier (6). The postphagocytosis inoculum was typically 1.0 to 2.5 × 10^6 CFU per mg of cell protein. As discussed previously (33), the large dilution of samples before spreading on agar plates for CFU counting ensured the absence of a carryover effect. To further check for the absence of a carryover effect, we examined the capacity of cell lysates obtained from cells incubated with delafloxacin to impair the growth of *S. aureus* ATCC 25923. Cells were incubated for 24 h with delafloxacin (0.1 to 50 mg/liter), washed 3 times in cold PBS, and collected in water. Half of the samples were then further incubated for 10 min with 12.5 mg/liter charcoal, and all samples were plated on agar previously inoculated with *S. aureus*; CFU were counted after incubation. No significant

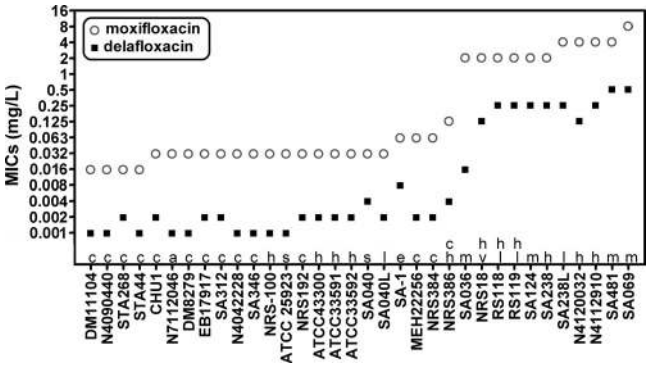

FIG. 2. Comparative susceptibilities of various *S. aureus* isolates to moxifloxacin (circles) or delafloxacin (squares). MICs were measured at pH 7.4, and strains are ranked based on their susceptibility to moxifloxacin. Resistance phenotypes and/or strain source are designated by lowercase letters along the *x* axis: a, animal MRSA; c, CA-MRSA; e, efflux (NorA); h, HA-MRSA; l, linezolid-resistant; m, characterized mutations in fluoroquinolone targets; s, MSSA.

differences in counts (≤0.2 log CFU) were seen between samples treated by activated charcoal (Sigma-Aldrich) or not or between samples coming from cells that had been incubated with delafloxacin or not, thus ruling out any potential carryover effect.

Curve fitting and statistical analyses. Curve-fitting analyses were made using the GraphPad Prism software program, version 4.03 (GraphPad Software, San Diego, CA). Data were used to fit sigmoidal functions (Hill equation [see reference 6 for details]) to obtain numeric values of relative efficacy (E_{max}, CFU decrease in $\log_{10}$ units at 24 h compared to the original inoculum, as determined for infinitely large antibiotic concentrations) and static concentration (C_{static}, the concentration of antibiotic resulting in no apparent bacterial growth compared to the original inoculum) for each condition. Statistical analyses were made with the GraphPad Instat software program, version 3.06 (GraphPad Software).

RESULTS

Susceptibility to delafloxacin and moxifloxacin of staphylococcal isolates with clinically relevant mechanisms of resistance. In the first series of experiments, we compared the MICs of the two fluoroquinolones against a series of clinical strains of *S. aureus* of worldwide origins (Table 1) and with pertinent mechanisms of resistance. Delafloxacin MICs were systematically 3- to 5-fold $\log_2$ dilutions lower than those of moxifloxacin for all the strains tested, whatever their phenotype of resistance (Fig. 2), with all values remaining ≤0.5 mg/liter.

Influence of pH on the accumulation of fluoroquinolones inside bacteria and on their intrinsic activity. Because of the different acidobasic characteristics of the two molecules, we next examined (in parallel experiments) the influence of broth pH on drug accumulation inside bacteria after 30 min and the MICs of both fluoroquinolones using *S. aureus* ATCC 25923. At neutral pH, accumulations of the two drugs inside the bacteria were equivalent (cellular to extracellular concentration ratio of approximately 50 ng · mg protein^{-1}/μg · ml^{-1}). The left panel of Fig. 3 shows a 10-fold increase in the accumulation of delafloxacin when the pH was decreased from 7.0 to 6.0, with half of the effect obtained at a pH of 6.5. This was accompanied by a decrease in the MIC (Fig. 3, right panel), which gained 4 to 5 dilutions over the same drop of pH (MIC value decreasing from 0.00094 mg/liter at pH 7.4 to 0.00006 mg/liter at pH 5.5). In contrast, moxifloxacin accumulation decreased 2-fold over the same pH range, and its MIC increased 2 dilutions (0.03 mg/liter at pH 7.4 to 0.12 mg/liter at pH 5.5) over the same pH range.

Influence of pH on bactericidal effects of fluoroquinolones in acellular media. To further characterize the effect of pH on the activities of both molecules, we compared their activities against *S. aureus* ATCC 25923 after 24 h of incubation with different concentrations in media at neutral or acidic pH (Fig. 4). The upper panel compares data at selected concentrations. At acidic pH, delafloxacin gained 3 logs of activity at 0.001 mg/liter and became bactericidal at 0.05 mg/liter. At neutral pH, a bactericidal effect was reached at 0.1 mg/liter. In contrast, moxifloxacin was inactive at both pHs for a low concentration (0.005 mg/liter), lost activity at higher concentrations in acidic medium, and reached a bactericidal effect only at concentrations of 0.5

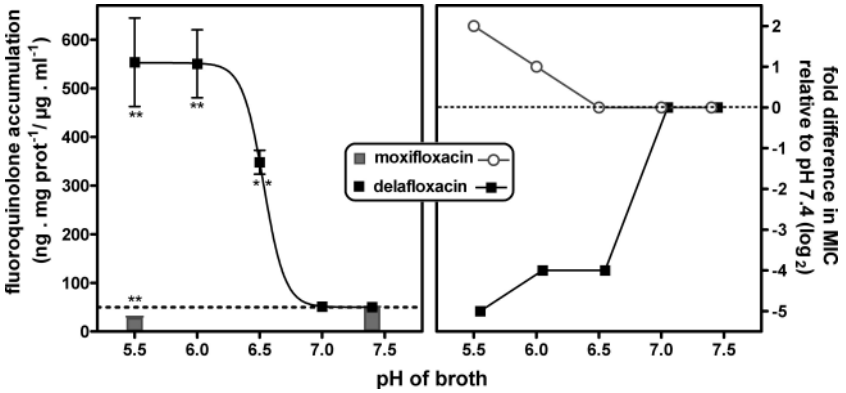

FIG. 3. Accumulation in *S. aureus* ATCC 25923 (left) or MIC (right) of fluoroquinolones in broth at different pHs. Left: growing bacteria were incubated for 30 min in pH-adjusted broth with delafloxacin (100 mg/liter) or moxifloxacin (50 mg/liter). Values are expressed as the cellular-to-extracellular concentration ratio and are shown as means ± SD for three independent samples. The dotted line shows the value measured for both drugs at pH 7.4. For statistical analysis, data with asterisks are significantly different from those for controls (pH 7.4; $P < 0.01$), as determined by one-way analysis of variance (ANOVA) (Dunnett multiple-comparison test). Right: MICs of delafloxacin and moxifloxacin (expressed as the change [in $\log_2$ units] from the MIC determined at pH 7.4) in pH-adjusted broth. Values are means for three independent samples (yielding 3 identical values).

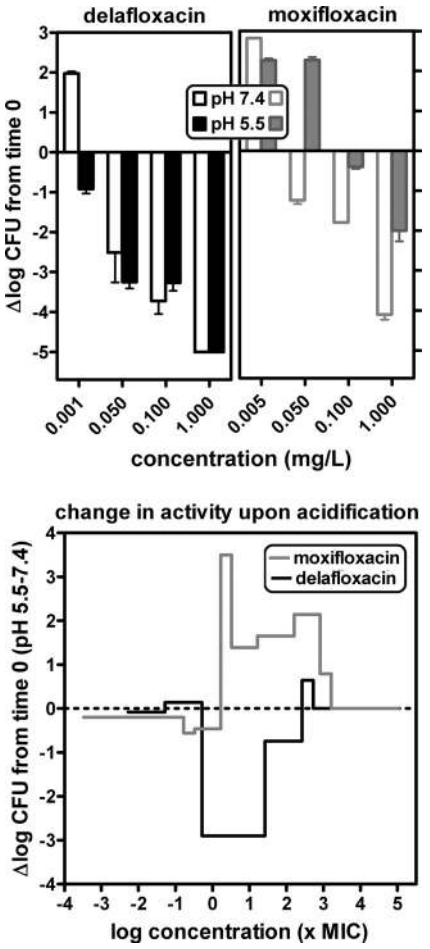

FIG. 4. Activities of delafloxacin and moxifloxacin against *S. aureus* ATCC 25923 after 24 h of incubation in broth at pH 5.5 or 7.4. Top: change in CFU from time zero (log scale) for 4 selected concentrations. Limit of detection is set at −5 log CFU. Bottom: difference in the change of CFU (log scale) measured at pH 5.5 and pH 7.4 over a wide range of antibiotic concentrations. All values are means ± SD for three independent samples (when not visible, SD bars are smaller than the size of the symbols). Positive values denote a loss and negative values a gain in activity in acidic medium.

mg/liter (not shown on the graph) or higher at neutral pH. The lower panel plots the gain in activity obtained at pH 5.5 versus pH 7.4. The pH effect was significant for concentrations of fluoroquinolones ranging from 1 to 1,000 × their MICs, with a gain (delafloxacin) or loss (moxifloxacin) of activity reaching 3 to 3.5 logs at low multiples of the MIC.

Accumulation of fluoroquinolones in THP-1 macrophages. In the next series of experiments, we compared the accumulation of both fluoroquinolones in THP-1 macrophages and examined how accumulation is influenced by pH and by agents known to modulate the pH of acidic subcellular compartments. After 2 h of incubation, delafloxacin accumulated only 2- to 3-fold, while moxifloxacin accumulated approximately 7-fold at neutral pH (Fig. 5, left panel). When cells were incubated for 30 min in medium at decreasing pHs (Fig. 5, middle panel), the cellular accumulation of delafloxacin was markedly increased (12-fold) over the 7.2 to 5.7 range, with half of the effect being obtained at pH 6.2. Over the same range of pH, the accumulation of moxifloxacin was decreased 2- to 3-fold. To investigate the potential role of pH gradients between the extracellular and intracellular milieus, we then measured the accumulation of fluoroquinolones in the presence of NH_4Cl (to neutralize the lysosomal pH) and of monensin (to collapse the pH gradient between the extracellular and intracellular compartments (23, 28). Opposite effects were observed for the two fluoroquinolones under these two conditions. Accumulation of delafloxacin was 2-fold lower in the presence of NH_4Cl and 1.7-fold higher in the presence of monensin, while accumulation of moxifloxacin was 2-fold increased by NH_4Cl and 2-fold decreased with monensin (Fig. 5, right panel).

Subcellular distribution of fluoroquinolones in J774 macrophages. We then compared the subcellular distributions of these two drugs in J774 macrophages incubated during 2 h with 100 mg/liter of delafloxacin or moxifloxacin. The delafloxacin distribution was determined in cells incubated at both neutral and acidic pHs. Homogenates were separated into a nuclear fraction (containing nuclei and unbroken cells), a granular fraction (containing the bulk of the organelles, including acidic vacuoles), and a soluble fraction (containing cytosol and broken organelles). Activities of enzyme markers of the cytosol, lysosomes, and mitochondria were determined in parallel (Fig. 6). As in THP-1 macrophages, delafloxacin accumulated to about 10-fold-higher levels when cells were incubated in acidic medium. The distribution of delafloxacin and moxifloxacin was very similar and was not modified by the pH of the culture medium. Eighteen to 25% of delafloxacin was associated with the nuclear-unbroken cells fraction, 10 to 17% with the organelle fraction, and 62 to 73% with the soluble fraction. Marker enzymes showed the expected distribution (38), attesting to the quality of the preparations. In particular, 85% of lactate dehydrogenase (LDH) activity was detected in the soluble fraction, suggesting that both fluoroquinolones were mostly localized in the cytosol and that only a minimal proportion was associated with organelles.

Cellular activities of fluoroquinolones in THP-1 macrophages infected by *S. aureus*. We then examined the intracellular activities of delafloxacin and moxifloxacin against phagocytosed *S. aureus* ATCC 25923 (Fig. 7; pertinent pharmacological parameters are presented in Table 2). Because of the contrasting effects of extracellular pH on the cellular accumulation of the two antibiotics, 24-h dose-response experiments were performed in culture medium at pH 7.4 or in medium adjusted to pH 5.5. To facilitate explanation, the upper panels of Fig. 7 compare the drugs at each pH, and the lower panels compare the effect of pH on each drug. Sigmoidal functions could be fit to all data sets except those obtained with delafloxacin at neutral pH, for which an improved fit was obtained by the use of two successive sigmoidal functions. In cells incubated at pH 7.4, delafloxacin reached a static effect at concentrations about 30-fold lower than moxifloxacin and both fluoroquinolones caused a more than 2-log decrease in CFU. For delafloxacin, a plateau value could not be reached even at the highest concentration tested; thus, maximal efficacy may be underestimated based on sigmoidal regression. When the culture medium was made acidic, delafloxacin was bacteriostatic against intracellular *S. aureus* for extracellular concentrations as low as 1 μg/liter and was bactericidal (3-log decrease from the

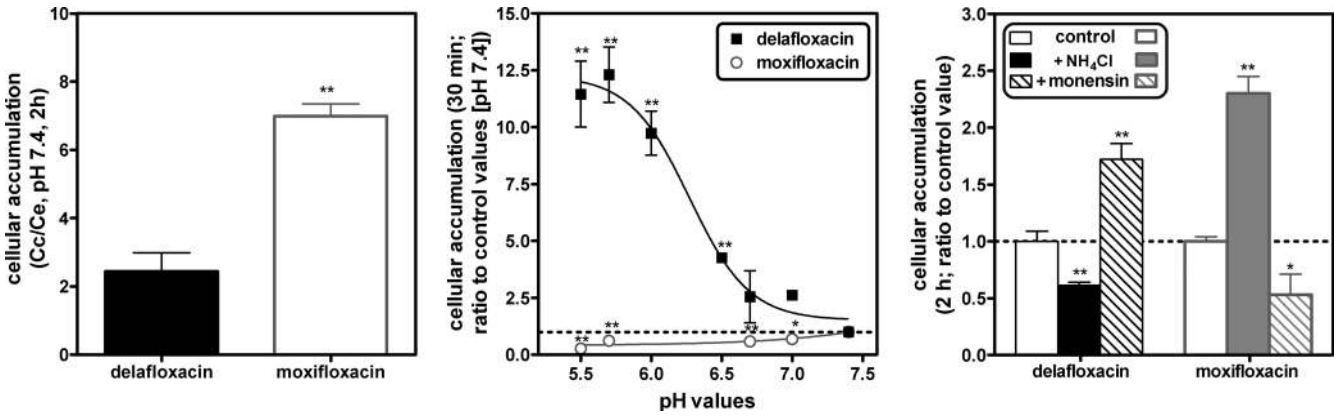

FIG. 5. Cellular accumulation of delafloxacin versus moxifloxacin at 37°C (both drugs were tested at an extracellular concentration of 20 mg/liter) within THP-1 macrophages. Left: accumulation ratio (Cc, cellular concentration; Ce, extracellular concentration) of both antibiotics after 2 h of incubation. Middle: influence of the pH of the culture medium on the accumulation (30 min) of delafloxacin (closed squares) and moxifloxacin (open circles). For statistical analyses, data with asterisks are significantly different from control data. Right: influence of ammonium chloride (10 mM) or monensin (50 μM) on the cellular accumulation of both antibiotics (2 h). All values are means ± SD for three independent samples (when not visible, SD bars are smaller than the size of the symbols). For statistical analyses, data with asterisks are significantly different from those for controls (*, $P < 0.05$; **, $P < 0.01$, as determined by unpaired *t* test [left panel] or one-way ANOVA [Dunnett posthoc test; middle and right panels]).

initial inoculum) for extracellular concentrations of ≥1 mg/liter. In contrast, the moxifloxacin static concentration shifted to a 10-fold-higher value at acidic pH, and its maximal effect was not modified. In a last set of experiments, we compared the concentration needed to reach a static intracellular effect (C_{static}) and maximal relative efficacy (E_{max}) for a variety of antistaphylococcal agents against *S. aureus* ATCC 25923, namely, clarithromycin, telithromycin, linezolid, vancomycin, gentamicin, rifampin, moxifloxacin, and quinupristin-dalfopristin (see Fig. S1 in the supplemental material). Delafloxacin was clearly the most potent (lower C_{static}) and showed the higher efficacy (E_{max}) among all agents tested in this intracellular infection model, probably due to its exceptionally low MIC and its high bactericidal character at acidic pH.

DISCUSSION

By comparing two fluoroquinolones that differ in acid-base properties, namely, the weakly acidic compound delafloxacin and the zwitterionic compound moxifloxacin, the present article illustrates that the ionization status of these antibiotics is determinative of their activity and cellular accumulation in both bacteria and eucaryotic cells.

First, we have shown that delafloxacin is intrinsically very active against all *S. aureus* strains tested, whatever their resistance phenotypes, including moxifloxacin-resistant strains. MIC values for delafloxacin are indeed systematically 3 to 5 $\log_2$ dilutions lower than those of moxifloxacin, usually considered a highly potent anti-Gram-positive fluoroquinolone (45). Delafloxacin's increased potency could result from a higher accumulation in bacteria or an improved interaction with the drug target. The first reason is not consistent with the observation that the concentrations of the two molecules in bacteria are similar at neutral pH (i.e., under the conditions under which MICs are normally measured; Fig. 3). An increased interaction with the target cannot be related to the acidic character of delafloxacin only, since improved antistaphylococcal activity, compared to that of moxifloxacin, is observed not only for *N*-sulfanilylpiperazinyl fluoroquinolones (which share with delafloxacin the presence of a single ionizable group [3, 4]) but also for new zwitterionic fluoroquinolones, such as JNJ-Q2 (30). In a broader context, delafloxacin also proves more potent than other bactericidal antistaphylococcal agents, like synercid, rifampin, or lipoglycopeptides, against a series of MRSA strains used in the present study (6, 8, 24).

Second, we have demonstrated that acidic pH markedly enhances the uptake of delafloxacin in both bacteria and eukaryotic cells, which is accompanied by an increased activity against both extracellular and intracellular forms of *S. aureus.* Moxifloxacin shows an opposite behavior. These changes in activity under different pH conditions are likely due to changes in cell permeation. The contrasting effect of pH on the two molecules can indeed be explained by a transmembrane diffusion model, in which uncharged (neutral or zwitterionic) species cross the membranes more easily than those having a net charge. Figure 1 indicates that at acidic pH, 50% of delafloxacin is under a neutral form, while only 11% of moxifloxacin is present as a zwitterion, which should favor the uptake of delafloxacin rather than that of moxifloxacin. Yet once inside the bacteria, where pH is neutral, delafloxacin will be deprotonated and less diffusible than moxifloxacin (which will remain mainly zwitterionic) and therefore more likely to remain inside the bacterial cell. The same model has been applied to explain the improved activity and higher accumulation inside bacteria of *N*-sulfanilylpiperazinyl fluoroquinolones (4). It also explains why the gain in activity is observed at low multiples of the MIC, since the higher accumulation of the drug causes a shift of the MIC to lower values. So, despite the likelihood that a simple membrane diffusion effect accounts for the increase in delafloxacin accumulation at reduced pHs, we cannot exclude the possibility that an acidic pH may affect influx or efflux transporters, since some rely on membrane potential (ψ) for activity.

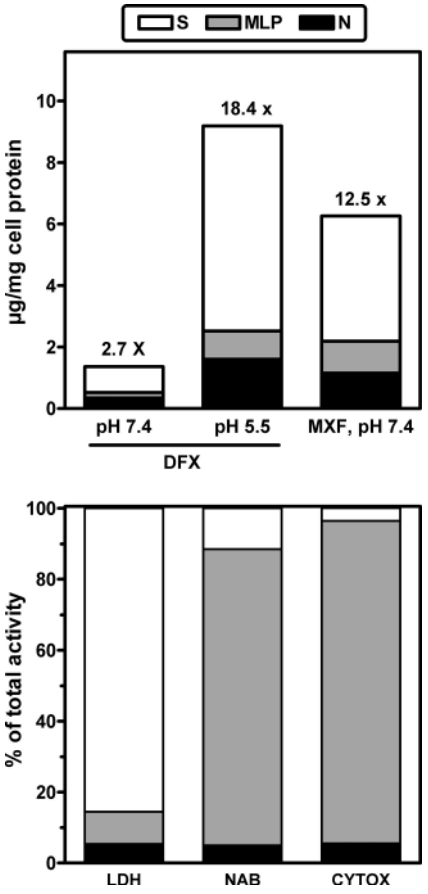

FIG. 6. Accumulation and subcellular distribution of delafloxacin (DFX) and moxifloxacin (MXF) in J774 mouse macrophages incubated for 2 h with 100 mg/liter of the drugs at the indicated pHs. The upper panel shows the antibiotic content in the nuclear (N), granule (MLP), and soluble (S) fractions expressed as μg/mg of protein in the unfractionated homogenate. Values at the top of each bar correspond to the apparent cellular accumulation of the antibiotic (cellular-to-extracellular concentration ratio, calculated using a conversion factor of 5 μl of cell volume per mg of cell protein). The lower panel shows the distribution of lactate dehydrogenase (marker of the soluble proteins) (LDH), *N*-acetyl-β-hexosaminidase (marker of lysosomes) (NAB), and cytochrome *c*-oxidase (marker of mitochondria) (CYTOX). All enzymes were assayed for each experimental condition; the graph shows the mean values.

The same reasoning can be applied to explain the effect of extracellular pH on fluoroquinolone accumulation in eukaryotic cells. It is also interesting to note that both moxifloxacin and delafloxacin are found in the soluble fraction of the cells, with only a minimal amount detected in the organelles, as already observed for other fluoroquinolones (12, 41). This is in opposition to what is seen with weak bases like macrolides or the biaryloxazolidinone radezolid, which are associated in large proportion with acidic vacuoles such as lysosomes (13, 25). While such molecules show a marked decrease of accumulation when the pH gradient between subcellular compartments and the extracellular milieu is collapsed with monensin (25), the weak acid character of delafloxacin results in an opposite behavior. Moxifloxacin behaves similarly to the weak bases in this respect, probably due to the presence of a protonatable amino group. The effect of NH_4Cl on the accumulation of both fluoroquinolones remains unexplained, yet it is consistent with data in the literature about moxifloxacin, which show an increased accumulation of the drug upon short incubation under these conditions (20).

Third, we also extend our previous observations that fluoroquinolones are highly effective against intracellular *S. aureus* (6, 40) in spite of their apparent localization in the cytosol. This suggests a capacity to rapidly redistribute inside the cell to reach their target, which is consistent with their capacity to easily diffuse through membranes (29) and to their strong binding to the DNA-topoisomerase complex in bacteria (45). In infected cells incubated in neutral medium, delafloxacin shows a very low static concentration and reaches a near-bactericidal effect, making it one of the most active drugs against intracellular *S. aureus* in this model. In particular, it is more potent (displaying a lower static concentration) than moxifloxacin despite its lower accumulation within the cells under these conditions, thanks to its lower MIC. Maximal efficacies of the two drugs are difficult to compare, since the plateau of activity could not be reached for delafloxacin at the concentrations tested in these experiments. Of interest, the dose-effect relationship best fits a bimodal sigmoid, as previously observed with the lipoglycopeptide oritavancin (32). This was interpreted in the latter case as suggesting a dual mode of action or a dual target for the drug. In this respect, it is interesting to note that delafloxacin is nearly an equipotent inhibitor of cleavable complex formation by DNA gyrase and topoisomerase IV, with only a 3-fold preference for the gyrase enzyme (34). Moxifloxacin was shown to behave similarly (10), with a 3-fold preference for topoisomerase IV. Such data contrast with those for ciprofloxacin, for example, where an 8× to 10× preference for topoisomerase IV is reported (34). These results, coupled with microbiological data showing equal shifts in delafloxacin MICs for a single gyrase mutant and a single topoisomerase IV mutant compared to data for the isogenic wild type, suggest that delafloxacin is a dually targeting quinolone with balanced activity against both enzymes (43). Alternatively, the bimodal shape of the delafloxacin curve could result from nonlinear cellular pharmacokinetics over the range of concentrations tested. The current lack of a sensitive assay (using radiolabeled drug, for example) to measure delafloxacin accumulation in the cell at the lowest extracellular concentrations prevented us from investigating these observations further.

When infected cells are incubated in acid medium, the relative potency of delafloxacin is further improved, possibly in relation to the higher accumulation of the drug within the cells under these conditions. Moreover, the maximal efficacy of delafloxacin now clearly appears as bactericidal, making it the most active of the series of drugs tested here. This could be ascribed to the drastic increase in activity for this drug at acidic pH (with *S. aureus* being localized in phagolysosomes in THP-1 cells [6], where the pH is acidic). Notably, also, only the second part of the bimodal sigmoid remains visible over the concentration range investigated, because a static effect is already reached at the lowest concentration tested.

Pending a more detailed analysis of drug-target interactions at the molecular level, we show in this report a potential advantage of delafloxacin over moxifloxacin or other commer-

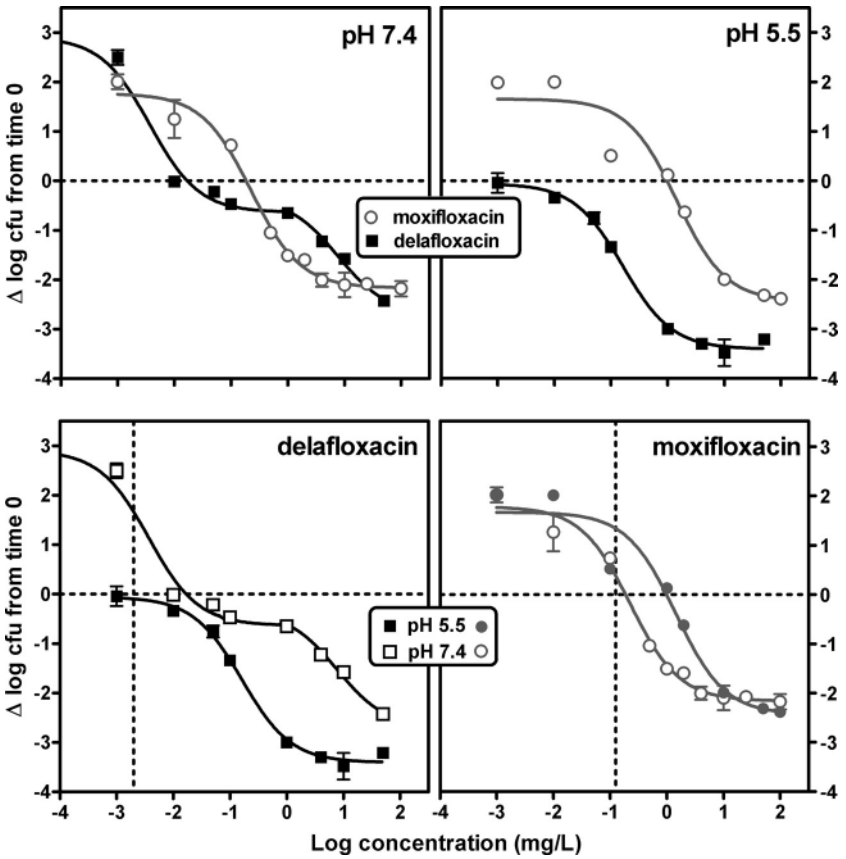

FIG. 7. Dose-response activities of delafloxacin and moxifloxacin against the intracellular forms of *S. aureus* strain ATCC 25923 (THP-1 macrophages). Cells were incubated with increasing concentrations of antibiotic (total drug) for 24 h in RPMI 1640 medium adjusted to pH 7.4 or 5.5. The ordinate shows the change in the number of CFU (log scale) per mg of cell protein. The horizontal dotted line corresponds to an apparent static effect, and the vertical line in the lower panel corresponds to the MIC of the drug. The upper panels compare the two drugs at both pHs, and the lower panels compare each drug at both pHs. All values are means ± SD for three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). The pertinent pharmacological descriptors of the curves are presented in Table 2.

cially available antistaphylococcal agents for eradication of *S. aureus* in this *in vitro* model and with respect to acidic environments. Further studies should document whether this advantage is also observed *in vivo*, especially in persistent or recurrent infections for which intracellular reservoirs of bacteria may play a determinant role (18) but also in abscesses (pH 6.2 to 7.3) or infection of specific territories, like the skin (pH 4.2 to 5.9), mouth (pH 5 to 7), urinary tract (pH 4.6 to 7),

TABLE 2. Pertinent regression parameters[a] (with confidence intervals) and statistical analyses of the dose-response curves for delafloxacin and moxifloxacin at pHs 7.4 and 5.5

Extracellular pH	Delafloxacin				Moxifloxacin			
	r^2	C_{static}[b]	E_{max}[c] (CI[d])	Stat[f]	r^2	C_{static}	E_{max} (CI)	Stat
7.4[e]	0.982	0.015	−0.64 (−1.13 to −0.14)	A				
	0.986	NA[g]	−2.71 (−3.36 to −2.06)	B, a	0.988	0.19	−2.17 (−2.40 to −1.93)	A, a
5.5	0.933	0.001	−3.41 (−3.59 to −3.23)	C, a	0.954	1	−2.44 (−3.26 to −1.62)	A, a

[a] By use of all data for antibiotic concentrations from 10^{-3} to 10^{2} mg/liter. The parameters shown in the table were derived from analysis of the data shown in Fig. 5.
[b] C_{static}, static concentration: concentration (total drug; mg/liter) resulting in no apparent bacterial growth (number of CFU identical to the initial inoculum), as determined by graphical intrapolation.
[c] E_{max}, relative maximal efficacy: CFU decrease (in $\log_{10}$ units) at time 24 h from the corresponding original inoculum, as extrapolated for an infinitely large antibiotic concentration.
[d] CI, confidence interval.
[e] Two successive sigmoidal regressions were used to fit to data with delafloxacin.
[f] Stat, statistical analyses. Analysis per column (one-way analysis of variance [ANOVA] with Tukey's posttest for multiple comparisons between each parameter for delafloxacin [3 groups]; unpaired, two-tailed *t* test for moxifloxacin): values with different uppercase letters are significantly different from each other ($P < 0.05$). Analysis per row (unpaired, two-tailed *t* test between corresponding parameters of intracellular and extracellular activities): values with different lowercase letters are significantly different from each other ($P < 0.05$).
[g] NA, not applicable.

vagina (pH 4.2 to 6.6) (47), or other different body fluids and tissues which become acidic upon bacterial infection (21).

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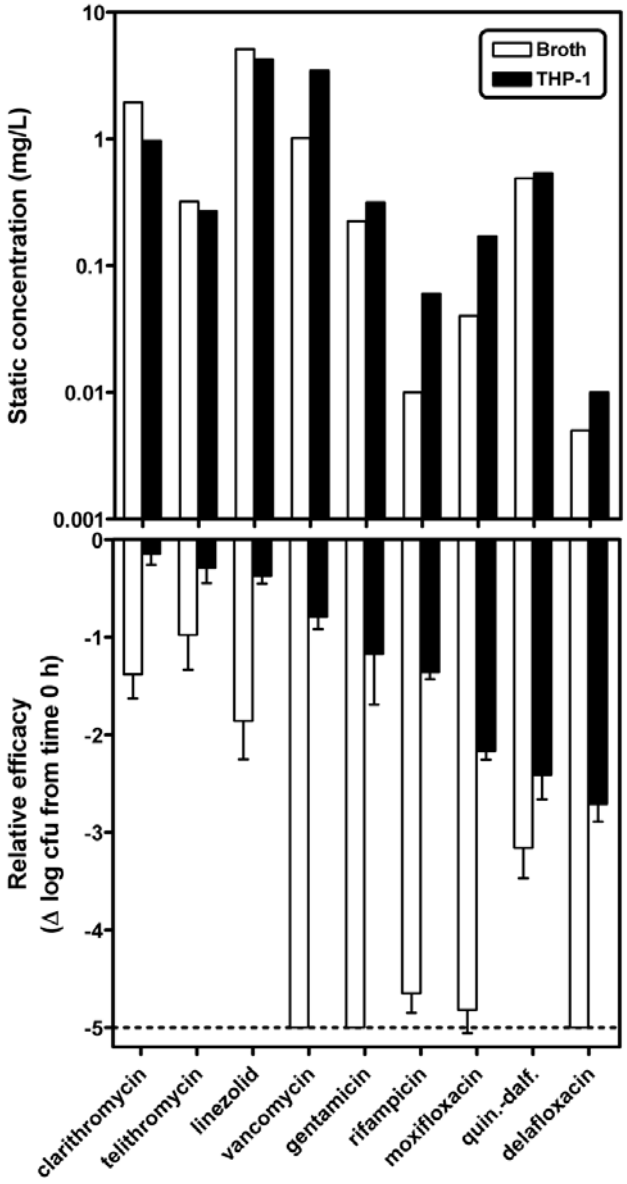
Supplemental material – Figure 1



Comparative potency (Static concentration, upper panel) and efficacy (Emax, lower panel) of antibiotics towards both the extracellular (broth, MHB pH 7.4 [open bars]) and intracellular forms (THP-1 macrophages [closed bars]) of *S. aureus* strain ATCC 25923. Cs and Emax values were determined by interpolation from sigmoidal regression of data obtained in concentration-effects relationships. MICs of antibiotics (pH 7.4) were as follows:
clarithromycin, 0.5 mg/L; telithromycin, 0.5 mg/L; linezolid, 1-2 mg/L; vancomycin, 1 mg/L; gentamicin, 0.125 mg/L; rifampicin, 0.03 mg/L; quinupristin-dalfopristin, 0.5 mg/L; moxifloxacin, 0.03 mg/L; delafloxacin, 0.001 mg/L. Antibiotics are classified by increasing efficacy.