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Comparative activity of quinolones (ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin) against extracellular and intracellular infection by *Listeria monocytogenes* and *Staphylococcus aureus* in J774 macrophages

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***Objectives*: Quinolones accumulate in eukaryotic cells and show activity against a large array of intracellular organisms, but systematic studies aimed at examining their pharmacodynamic profile against intracellular bacteria are scarce. The present work aims at comparing intracellular-to-extracellular activities in this context.**

***Methods*: We assessed the activities of ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin against the extracellular (broth) and intracellular (infected J774 macrophages) forms of *Listeria monocytogenes* (cytosolic infection) and *Staphylococcus aureus* (phagolysosomal infection) using a range of clinically meaningful extracellular concentrations (0.06–4 mg/L).**

***Results*: All four quinolones displayed concentration-dependent bactericidal activity against extracellular and intracellular *L. monocytogenes* and *S. aureus* for extracellular concentrations in the range 1–4-fold their MIC. Compared at equipotent extracellular concentrations, intracellular activities against *L. monocytogenes* were roughly equal to those that were extracellular, but were 50–100 times lower against *S. aureus*. Because quinolones accumulate in cells (ciprofloxacin, ~3 times; levofloxacin, ~5 times; garenoxacin, ~10 times, moxifloxacin, ~13 times), these data show that, intracellularly, quinolones are 5–10 times less potent against *L. monocytogenes* ($P = 0.065$ [ANCOVA]), and at least 100 times less potent ($P < 0.0001$) against *S. aureus.* Because of their lower MICs and higher accumulation levels, garenoxacin and moxifloxacin were, however, more active than ciprofloxacin and levofloxacin when compared at similar extracellular concentrations.**

***Conclusions*: Quinolone activity is reduced intracellulary. This suggests that either only a fraction of cell-associated quinolones exert an antibacterial effect, or that intracellular activity is defeated by the local environment, or that intracellular bacteria only poorly respond to the action of quinolones.**

Keywords: cellular pharmacodynamics, cellular pharmacokinetics, bactericidal activity, accumulation

Introduction

Effective treatment of intracellular infections remains a challenge in spite of the availability of several classes of antibiotics capable of entering and accumulating in eukaryotic cells. In this context, quinolones are considered as potentially ideal drugs since they combine a number of desirable properties, such as a high bactericidal potency,[1,2] a fair accumulation in phagocytes[3] and an excellent diffusibility throughout subcellular compartments.[4] Accordingly, quinolones have been found to be effective in several types of intracellular infections disregarding the subcellular localization of the organisms, such as those caused by *Listeria monocytogenes* (cytosol),[5–8] *Legionella pneumophila* (phagosomes)[4,9] or *Staphylococcus aureus* (phagolysosomes).[10–13] Systematic comparisons of extracellular and intracellular activities, however, are rarely reported. Nevertheless, recent studies with intracellular *L. monocytogenes* and THP-1 macrophages have shown that cell-associated ciprofloxacin and moxifloxacin are considerably less active against intracellular *L. monocytogenes* than could be anticipated on the basis of their cellular accumulation.[8]

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This has triggered us to enlarge the scope of our studies (i) by examining another Gram-positive organism, namely *S. aureus*, (ii) by expanding the study to two additional quinolones based on their wide clinical usage (levofloxacin) and potential interest for specific activity against Gram-positive organisms (garenoxacin). We examine here the relationship between cell accumulation and intracellular activity of these four quinolones in cells exposed to clinically meaningful drug concentrations.

Materials and methods

Bacterial strains and measurement of extracellular activity of antibiotics

We used a haemolysin-producing strain (EGD serotype 1/2a) for *L. monocytogenes* (obtained from P. Berche, Laboratoire de Microbiologie, Faculté de Médecine, Necker, Paris, France), and a methicillin-susceptible strain of *S. aureus* (ATCC 25923) obtained from the American Tissue Cell Collection (Manassas, VA, USA). MICs and MBCs were determined in tryptic soy broth (*L. monocytogenes*) or Mueller–Hinton broth (*S. aureus*) as in our previous publications.[8,13] MICs for *S. aureus* were also determined in the same medium adjusted to pH 5. For killing-curve experiments, bacteria in logarithmic growth were resuspended at a density of 10^6 cfu/mL in broth. The number of viable bacteria was determined after incubation at 37 °C with antibiotics for suitable periods of time (up to 24 h) by plate assay with appropriately diluted samples.

Cell infection and measurement of intracellular activity

All experiments were conducted with J774 macrophages, a continuous reticulosarcoma cell line of murine origin,[14] following exactly the procedures described earlier.[13,15] In brief, infection was achieved by incubating macrophages with bacteria for 1 h [5 cfu/cell for *L. monocytogenes* and 0.5 cfu/cell for *S. aureus* (human serum-opsonized)]. Extracellular bacteria were eliminated by washing in PBS (for *S. aureus*, a first washing was made by bathing the cells for 1 h in a medium supplemented by 50 mg/L gentamicin). For experiments in which infected cells were maintained in culture 24 h post-phagocytosis, gentamicin was added at its MIC (1 mg/L for *L. monocytogenes* and 0.5 mg/L for *S. aureus*) during the whole incubation period to prevent the extracellular growth of released bacteria, and the ensuing fast acidification of the medium and subsequent loss of cell viability.[13]

Cellular accumulation of quinolones

Infected and uninfected cells were collected and analysed following the general procedure described earlier.[16] In brief, cell sheets were washed three times with ice-cold PBS and collected by scraping in 0.1 M glycine-NaOH pH 3 buffer for fluorimetric determinations, or in distilled water for radiochemical assays. Cell-associated ciprofloxacin, moxifloxacin and levofloxacin were assayed by fluorimetry (λ_{exc} = 275, 298 and 298 nm; λ_{em} = 450, 504 and 500 nm respectively), as previously described.[8] Garenoxacin was assayed by scintillation counting using ^{14}C-labelled drug. These methods have been fully validated with respect to specificity and reproducibility, and linearity under our conditions of assay. All drug contents in cell samples were expressed by reference to the cell protein (assayed by the Folin-ciocalteu/biuret method),[17] and the apparent cellular-to-extracellular concentration ratio calculated using a conversion factor of 3.08 μL of cellular volume per mg cell protein, as determined for J774 macrophages by the sucrose/urea partition method.[16]

Data analyses

Curve-fitting and statistical analyses [one way analysis of variance (ANOVA), analysis of covariance (ANCOVA) and Tukey's Honestly Significant Difference (HSD) tests (for differences between groups with a confidence interval of 95%)] were made using Prism version 4.02 and InStat version 3.00 (GraphPad Software, San Diego, CA, USA), or XLSTAT version 6.0 (Addinsoft SARL, Paris, France).

Materials

Ciprofloxacin (potency, 85.0%) and moxifloxacin (potency, 91%) were obtained as laboratory samples for microbiological evaluation from Bayer AG (Leverkusen, Germany), and garenoxacin (purity 99.8%) from Bristol Myers Squibb, New Brunswick, CT, USA. Levofloxacin and gentamicin were procured as Tavanic and Geomycin, the registered commercial products available for intravenous administration in Belgium. ^{14}C-labelled garenoxacin (0.8 MBq/mg; 98.3% of radiochemical purity) was obtained from the Bristol Myers Squibb Research Institute, Princeton, NJ, USA. Cell culture media and fetal calf serum (FCS) were purchased from Gibco Biocult (Paisley, Scotland, UK). Human serum for opsonization of *S. aureus* was obtained from healthy volunteers as pooled samples

Table 1. Reported C_{max} (maximal serum concentration observed in healthy volunteers receiving conventional doses as indicated) and measured MICs and MBCs of quinolones for the strains used in this study

			L. monocytogenes		*S. aureus*			
	C_{max}		pH 7		pH 7		pH 5	
Quinolone	mg/L	ref.	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)
Ciprofloxacin	2.3[a]	31	1.25	4	0.125	1	1	2
Levofloxacin	5.4[a]	32	2	4	0.125	0.125	1	1
Moxifloxacin	3.4[b]	33	0.6	2	0.06	0.06	0.25	1
Garenoxacin	4.6[b]	34	0.5	1	0.015	0.03	0.125	1

[a]Single 500 mg oral administration.
[b]Single 400 mg oral administration.

stored as aliquots at −80 °C until use. Unless stated otherwise, all other reagents were of analytic grade and purchased form E. Merck AG (Darmstadt, Germany) or from Sigma-Aldrich (St Louis, MO, USA).

Results

Determination of MIC and MBC

Table 1 shows the MIC and MBC values of the four quinolones under investigation against the two bacterial strains used in this study. Bearing in mind the results obtained at pH 7, the four drugs were less active against *L. monocytogenes* than *S. aureus*, and levofloxacin or ciprofloxacin were in all cases less active than moxifloxacin and garenoxacin. All MICs, however, were still lower than the maximal serum concentrations observed in healthy volunteers (C_{max}, see Table 1). On investigating the extracellular activities of quinolones, we observed that the MIC/MBC ratios were between 2 and 8, demonstrating the bactericidal activity of these drugs. MICs and MBCs measured at acid pH against *S. aureus* (to mimic the conditions prevailing in lysosomes)[18] were 2 (ciprofloxacin) to 33 (garenoxacin) times higher than at pH 7 but still lower than C_{max}.

Extracellular and intracellular activities of quinolones

The activity of the four quinolones was then examined against extracellular and intracellular forms of *L. monocytogenes* and *S. aureus*. In the first series of experiments (Figure 1), all drugs were compared at a fixed post-phagocytosis time point (5 h for *L. monocytogenes*, 24 h for *S. aureus*; these times were selected based on previous observations showing that intracellular *L. monocytogenes* grows after a lag period of about 1 h only, whereas this lag period extends for up to 8 h for *S. aureus*)[8,13] and at the same extracellular concentration (4 mg/L). We observed that the growth of *L. monocytogenes* was similar in broth and in infected macrophages, whereas that of *S. aureus* was significantly lower intracellularly as compared with broth. On examining the extracellular activities of quinolones, we see that these were only slightly bactericidal towards *L. monocytogenes* (achieving an inoculum reduction from about 1.2 log for ciprofloxacin, 1.5 log for levofloxacin and garenoxacin, and about 2 log for moxifloxacin (these differences were statistically significant). In contrast, they were highly bactericidal against *S. aureus*, achieving an inoculum reduction of about 4 log for ciprofloxacin and levofloxacin, and of 4.5 log for moxifloxacin and garenoxacin (these differences were statistically significant). However, when considering activities against intracellular bacteria, a global analysis of the results showed that these were always significantly lower than what was seen against extracellular bacteria, the difference being, however, smaller for *L. monocytogenes* (especially for moxifloxacin) than for *S. aureus*. Comparison between drugs showed a ranking (with statistically significant differences) of ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin for *L. monocytogenes*, and of ciprofloxacin = levofloxacin < moxifloxacin = garenoxacin for *S. aureus*.

In the next series of experiments, we examined the influence of varying the concentrations of the quinolones. We concentrated on garenoxacin, for which no previous data were available, and used levofloxacin as a comparator (the results of similar studies with ciprofloxacin and moxifloxacin have been reported previously[8,13,15]). Figure 2 shows the change in bacterial counts observed in broth (left panels) and in cells (right panels) at 5 h for *L. monocytogenes* and at 24 h for *S. aureus* when exposed to drug concentrations up to 4 mg/L. On examining *L. monocytogenes* first (upper panels), we see that activity was concentration-dependent both extracellularly and intracellularly over the whole range investigated. Garenoxacin was significantly more effective than levofloxacin, reflecting their differences of MIC. In sharp contrast, activity against *S. aureus* developed in a narrow concentration range (0–0.25 mg/L), after which no or only little gain was noticeable whether in broth or in cells. Interestingly, the ratio of intracellular to extracellular activities remained almost constant at all concentrations above 0.25 mg/L. The difference between garenoxacin and levofloxacin was not

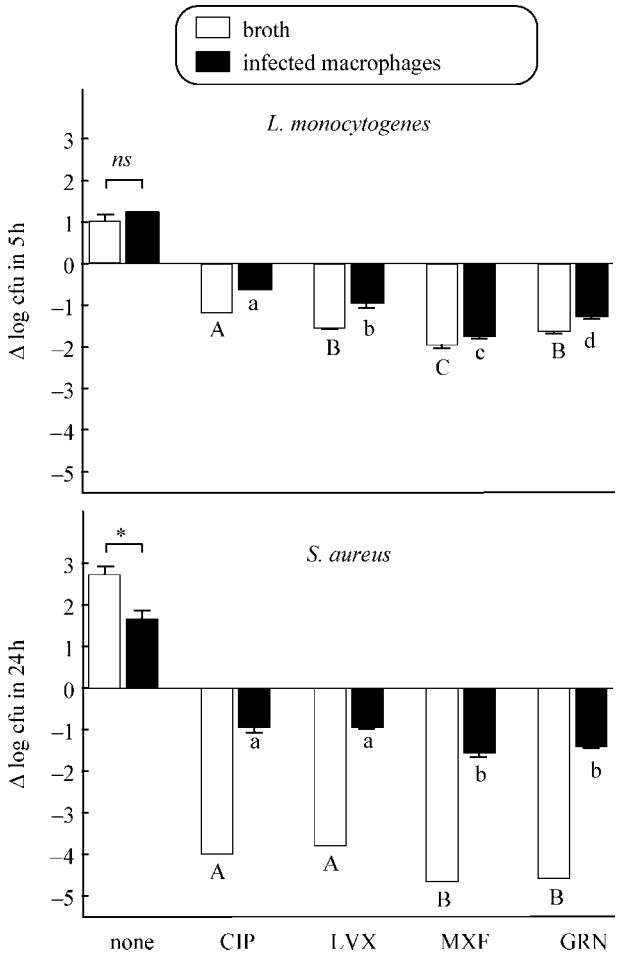

Figure 1. Change in the number of cfu of *L. monocytogenes* or *S. aureus* upon incubation with quinolones (CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GRN, garenoxacin). Open bars, bacteria in broth at pH 7; closed bars, intracellular bacteria in J774 macrophages. Upper panel, experiments conducted for 5 h; lower panel, experiments conducted for 24 h. All quinolones were present at a concentration of 4 mg/L. Each value is the mean ± S.D. of three independent determinations. Statistical analysis: (i) comparison between extracellular and intracellular growth of bacteria in cells unexposed to antibiotics [Tukey's (HSD)], no significant difference for *L. monocytogenes* (ns), $P < 0.01$ for *S. aureus* (∗); (ii) comparison between extracellular and intracellular activities for each quinolone [Tukey's (HSD), $P < 0.01$ for both *L. monocytogenes* and *S. aureus*; (iii) comparison between quinolones (one way ANOVA), bars labelled with different letters denote values with significant differences ($P < 0.01$); upper case, extracellular activity; lower case, intracellular activity].

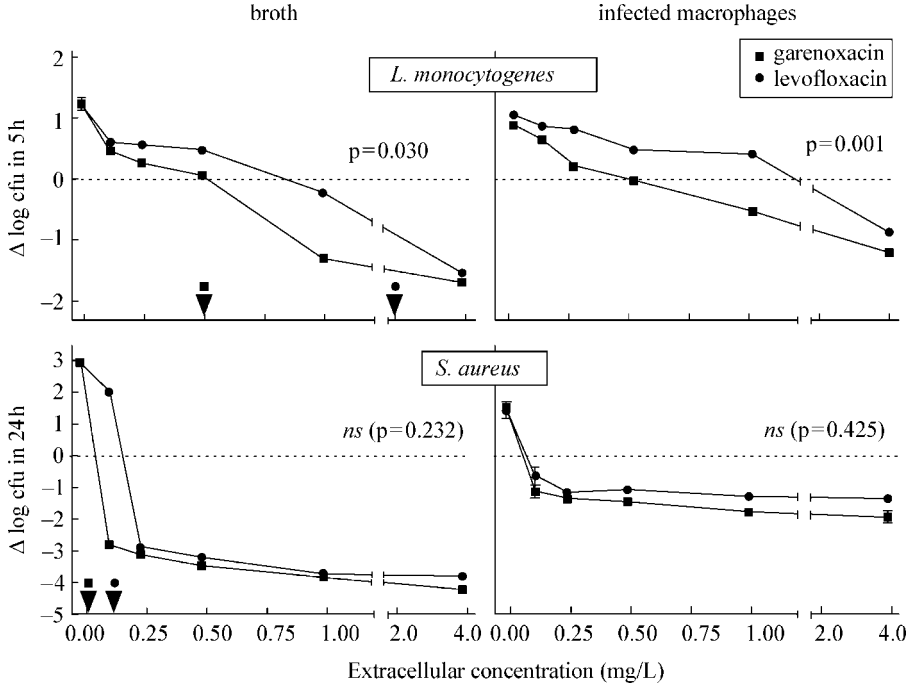

Figure 2. Change in the number of cfu of *L. monocytogenes* (upper panels) or *S. aureus* (lower panels) upon incubation in broth (left panels) or in infected J774 macrophages (right panels) with quinolones at increasing extracellular concentrations. Circles, levofloxacin; squares, garenoxacin. The dotted horizontal line corresponds to the original inoculum and shows a bacteriostatic effect. The arrowheads point to the MIC of the two quinolones (identified by the same symbol as in the graph). Each point corresponds to the mean ± S.D. of three independent determinations (when not visible, S.D. values are smaller than the symbols). The *P* values shown on the graphs are those obtained by ANCOVA for global analysis of the differences between garenoxacin and levofloxacin in each condition (ns, non-significant).

statistically significant when examined globally (ANCOVA), although the plateau of activity in broth was obtained at a two-fold lower concentration for garenoxacin compared with levofloxacin, reflecting their differences of MIC.

In the last series of experiments, we examined the time-dependency of the killing activities of the quinolones towards *L. monocytogenes* and *S. aureus*, again concentrating on garenoxacin and using levofloxacin as comparator. Figure 3 shows that there was no significant difference in extracellular and intracellular growth of *L. monocytogenes* in controls, as previously reported.[19] In contrast, *S. aureus* grew significantly more slowly intracellularly, with a definite lag period of at least 5 h, as also observed previously.[13] The Figure also shows that the activity of the two quinolones was time-dependent with, however, a slower rate for intracellular as compared with extracellular bacteria.

Accumulation of quinolones in uninfected and infected cells

Table 2 compares the apparent cellular accumulations of the four quinolones in both uninfected and infected cells, under the conditions used for the experiments described in Figure 1. Marked differences were observed among drugs, with the following ranking: ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin. Differences between data obtained at 5 and 24 h in uninfected cells were either not significant (ciprofloxacin, moxifloxacin) or small (levofloxacin, garenoxacin), indicating that an apparent steady state had been reached or was close to being obtained. Differences between uninfected and infected cells were also not significant (ciprofloxacin, garenoxacin) or small (levofloxacin, moxifloxacin).

Discussion

It has been known for a long time that quinolones accumulate in eukaryotic cells,[4,10,12,16] and this property has, generally speaking, been considered an important asset as far as activity against intracellular organisms is concerned.[8,13,20,21] Surprisingly, however, few studies have directly compared the extracellular and intracellular activities of quinolones in models where (i) the influence of time and concentration can be fully assessed; (ii) the results can be directly correlated with levels of cellular accumulation. Together with recent studies by our group,[8,13,15] the experiments reported here are part of a systematic approach in this direction.

Two main conclusions emerge from the present data. First, whereas quinolones appear to be concentration-dependent drugs for *L. monocytogenes* (whether extra- or intracellularly), this seems not to be the case for *S. aureus.* However, closer examination of the data suggests that concentration-dependency is observed for both organisms but may be limited to a range of concentrations that span from the MIC up to a maximum of ~4 times the MIC. Future studies will need to explore these limits in more detail, perhaps by using strains of *S. aureus* with higher MICs.

Second, and surprisingly, our data show that the intracellular activity of quinolones against both *L. monocytogenes*

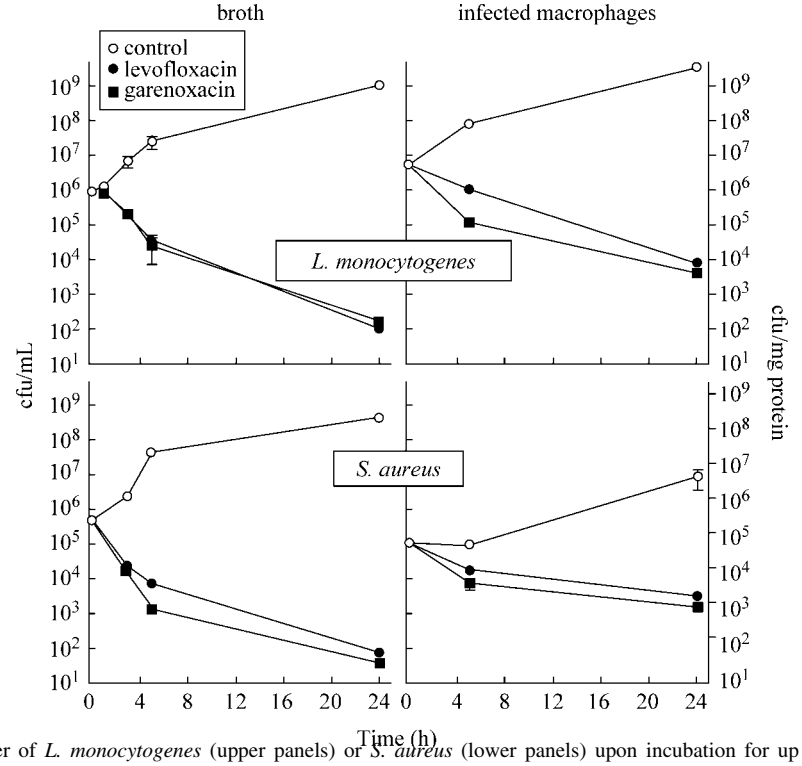

Figure 3. Change in the number of *L. monocytogenes* (upper panels) or *S. aureus* (lower panels) upon incubation for up to 24 h in broth (left panels) or infected J774 macrophages (right panels). Closed circles: levofloxacin (4 mg/L); closed squares: garenoxacin (4 mg/L); open circles: controls (infected macrophages that were not incubated with a quinolone were exposed to gentamicin at its MIC [1 mg/L for *L. monocytogenes*; 0.5 mg/L for *S. aureus*] during the whole incubation to avoid extracellular contamination. Each point corresponds to the mean ± S.D. of three independent determinations (when not visible, S.D. values are smaller than the symbols). Statistical analysis (ANCOVA): differences between controls and antibiotics are significant ($P < 0.02$), but those between antibiotics are not significant.

Table 2. Cellular accumulation of quinolones upon incubation of uninfected and infected J774 macrophages with an extracellular quinolone concentration of 4 mg/L

	Cellular to extracellular concentration ratio			
	Uninfected		Infected	
Quinolone	5 h	24 h	*L. monocytogenes* (5 h)	*S. aureus* (24 h)
Ciprofloxacin	3.3 ± 0.2	3.9 ± 0.8	3.6 ± 0.2	3.9 ± 0.3
Levofloxacin	5.2 ± 0.2	4.7 ± 0.3*	5.0 ± 0.7	5.7 ± 0.5**
Moxifloxacin	13.6 ± 6.0	14.1 ± 1.0	16.2 ± 5.2	16.6 ± 0.5**
Garenoxacin	10.2 ± 0.2	12.2 ± 0.6*	9.6 ± 0.5	10.9 ± 0.7

Statistical analysis (Student's *t*-test; $P < 0.05$): *Significantly different between 5 h and 24 h; **significantly different between uninfected and infected cells.

and *S. aureus* is only a fraction of what could be anticipated if their apparent accumulation in cells is taken into account. Thus, whereas all quinolones used in the present study show higher concentrations in cells compared with medium, all are also characterized by somewhat weaker activity against intracellular *L. monocytogenes* and drastically reduced activity against intracellular *S. aureus* in comparison with broth. To substantiate this conclusion further, we used all data generated in this study—pooling them with a series of data obtained previously with the same models—[13,15] to compare activities in broth and in cells after normalization of concentrations for differences in MIC (Figure 4). Thus, the activity of quinolones against intracellular *L. monocytogenes* was lower (five- to 20-fold depending on the concentration) than against the extracellular forms, but the global difference was at the limit of statistical significance ($P = 0.065$ by ANCOVA). For *S. aureus*, the difference is at least 100-fold and was highly significant. Several factors could account for such a loss of activity. For instance, we know that intracellular *L. monocytogenes* is surrounded by a thick layer of actin that confers motility to the bacteria,[22,23] but could also partially protect it from antibiotics. For *S. aureus*, the slow intracellular growth of this organism may make it poorly sensitive to quinolones, as suggested from studies in broth with slowly growing bacteria.[24–26] (This could result from the decreased

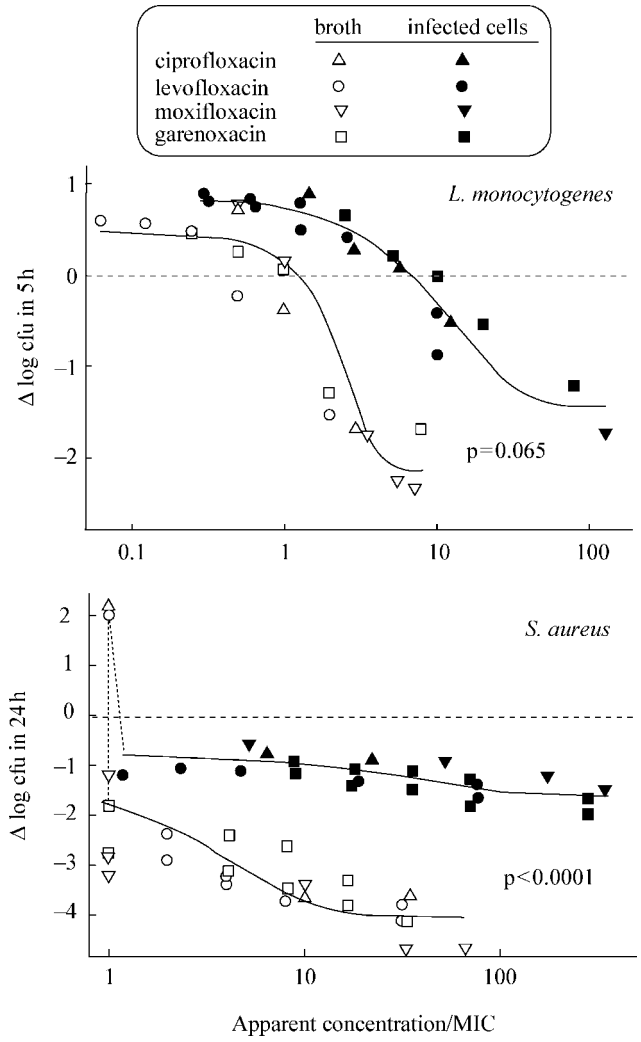

Figure 4. Change in log scale in the number of cfu of *L. monocytogenes* (upper panel) or *S. aureus* (lower panel) upon exposure to increasing concentrations of quinolones in broth or in cells. The concentration refers to the actual concentration used for broth or the measured intracellular concentration for infected cells. The MIC refers to the MIC measured in broth (pH 7) for extracellular bacteria and for intracellular *L. monocytogenes*, and to the MIC measured at pH 5 for intracellular *S. aureus* (to mimic the situation prevailing in phagolysosomes). This figure is constructed based on all the data generated in the present study and all usable data from our previous publications with the same models.[13,15] Triangles, ciprofloxacin; circles, levofloxacin; inverted triangles, moxifloxacin; squares, garenoxacin. Open symbols, experiments conducted in broth (pH 7); closed symbols, experiments conducted in infected J774 macrophages. The horizontal dotted line shows a bacteriostatic effect. For *L. monocytogenes*, a variable slope sigmoidal dose-response curve was fitted to the data (R^2, 0.72 for broth; 0.92 for macrophages). For *S. aureus*, the curves shown are 'best fit' but no attempt to define a given function was made since the response to low concentration/MIC values could not be explored (these values being not clinically meaningful within the context of an antibiotic treatment in humans). The *P* values shown on the graphs are those obtained by ANCOVA for global analysis of the differences between broth and infected cells.

expression of quinolone targets as recently found for topoisomerase IV).[27] Part of the drastic reduction in activity against intracellular *S. aureus* could also be due to the acidic environment of phagolysosomes, which is unfavourable to their activity (see Table 1). Moreover, we have to consider that we do not know with certainty where the drugs are located intracellularly. Whereas in fractionation studies the bulk of cell-associated ciprofloxacin is recovered in the cytosol,[15] we cannot exclude a binding to proteins[28] or lipids,[29] or simply the formation of complexes with ions.[30] These various, non-mutually exclusive hypotheses are now open to experimental evaluation.

The present data may have important implications for the correct assessment of existing and newly developed quinolones. First, they stress the importance of comparing extracellular and intracellular activities of antibiotics in a systematic fashion and using appropriate models, so as to refrain from simplistic conclusions such as those equating cell accumulation and activity (or denying a relationship between them). In this context, the selection of 'best candidates', within a given drug class, must be based on both MIC and cellular accumulation considerations. This is exemplified here by the behaviour of garenoxacin and moxifloxacin, which always showed larger intracellular activity in comparison with levofloxacin and ciprofloxacin. Finally, it is essential to perform experimental confirmation studies at clinically meaningful concentrations, since doing otherwise may lead to erroneous conclusions as far as the potential clinical applications are concerned.

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