Factors influencing the intracellular activity of fluoroquinolones: a study using levofloxacin in a *Staphylococcus aureus* THP-1 monocyte model

Hoang Anh Nguyen¹, Jean Grellet¹*, Delphine Paillard², Véronique Dubois², Claudine Quentin² and Marie-Claude Saux¹

¹EA 525, Laboratoire de Pharmacocinétique et de Pharmacie clinique, Faculté de Pharmacie, Université Victor Ségalen Bordeaux 2, 146 rue Léo-Saignat, 33076 Bordeaux cedex, France; ²EA 525, Laboratoire de Microbiologie, Faculté de Pharmacie, Université Victor Ségalen Bordeaux 2, Bordeaux, France

Received 26 September 2005; returned 23 December 2005; revised 2 February 2006; accepted 17 February 2006

**Objectives:** Recent studies have raised the question of whether the intracellular activity of quinolones is optimal with respect to their cellular accumulation. The aim of this study was to compare the intracellular and extracellular activities of a commonly used quinolone, levofloxacin, and to examine the causes of the possible inconsistency between intracellular and extracellular effects.

**Methods:** The bactericidal activity of levofloxacin at therapeutic levels, alone or in combination with various efflux-pump inhibitors or alkalinizing agents, was studied against *Staphylococcus aureus* ATCC 25923 in Mueller–Hinton (MH) broth and in a THP-1 monocyte cell model, using intracellular salt medium (ISM) mimicking the phagolysosomal environment, and in cell lysate.

**Results:** Levofloxacin accumulation was 2-fold higher in uninfected than in infected cells. Intracellular activity was significantly lower than extracellular activity (decrease in the inoculum of $\leq 1$ log$_{10}$ cfu/mL at 4 or 8 mg/L versus $\geq 2$ log$_{10}$ units at $\leq 1$ mg/L in MH broth over 5 h). Persisters remained fully susceptible to the drug. The efflux pump inhibitors verapamil and gemfibrozil did not affect killing of intracellular bacteria, although gemfibrozil increased cellular accumulation of levofloxacin 1.7-fold. The lysosomotropic alkalinizing agents chloroquine and ammonium chloride significantly enhanced intracellular killing by levofloxacin. The bactericidal activity of levofloxacin, abolished in ISM, was partially restored when the pH was neutralized from 5.0 to 7.4. Binding to intracellular components (20%) substantially decreased the efficiency of levofloxacin.

**Conclusions:** Levofloxacin exhibited substantially lower intracellular activity than extracellular activity. Cellular compartmentalization of the drug, phagolysosomal environment and antibiotic binding to cellular components most likely contribute to this failure.

Keywords: intracellular infections, cellular pharmacokinetics, intracellular pharmacodynamics

**Introduction**

All microorganisms, even the typically extracellular host parasites such as *Staphylococcus aureus*, stimulate the inflammatory response and thus undergo an intracellular phase within professional phagocytes, which encompass neutrophils and cells of the monocytic/macrophage lineage. From a clinical standpoint, the ability of microorganisms to survive and multiply within phagocytic cells yields insights into mechanisms relevant to persistent and recurrent infections and into a subset of instances where infections are refractory to antimicrobial therapy. An effective treatment of the infections caused by intracellular bacterial forms can only be achieved using an antibiotic that can concentrate sufficiently at the site of microbial residence and further maintain activity in the intracellular environment.

However, it has been observed repeatedly that many microbial pathogens are protected inside the cell from antibiotics to which they are susceptible in *vitro*. This observation may also be true.
in the case of fluoroquinolones,\textsuperscript{5–7} despite their having a number of useful properties such as high bactericidal potency\textsuperscript{8} and substantial accumulation in phagocytic cells.\textsuperscript{9,10} In a previous study, we have reported a discrepancy between intracellular and extracellular activities of moxifloxacin in an \textit{S. aureus}-infected THP-1 cell model. Indeed, moxifloxacin was not bactericidal intracellularly at extracellular therapeutic and bactericidal concentrations, despite a 6-fold accumulation of the drug in the cells and the susceptibility of the persisting bacteria.\textsuperscript{11} Other studies on the intracellular accumulation and activity of trovafloxacin,\textsuperscript{7} ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin,\textsuperscript{12} using different cellular models of \textit{S. aureus}-infected phagocytes, have also reported a bacteriostatic rather than a bactericidal effect of fluoroquinolones. These concordant data clearly indicate that as yet unknown factors must be at work to decrease the intracellular antibiotic efficiency of fluoroquinolones. For antibiotics with a high penetration rate within eukaryotic cells, the lack of intracellular microbiocidal activity may be explained by (i) inadequate subcellular compartmentalization of antibiotics, (ii) intracellular inactivation, in particular due to acidic pH or binding to cellular components, and (iii) diminished susceptibility of intracellular forms of pathogens.\textsuperscript{2,10}

In our present work, using the previously designed \textit{S. aureus} THP-1 model, we have examined the intracellular accumulation and activity of another commonly used fluoroquinolone, levofloxacin.\textsuperscript{13}

Materials and methods

Cell line

The THP-1 human monocytic cell line (ECACC 88081201; Wiltshire, UK)\textsuperscript{14} was used for the intracellular experiments. Cells were grown in RPMI 1640 medium, pH 7.4 (Sigma, Saint Quentin Fallavier, France), supplemented with 10% decomplemented fetal calf serum (Gibco BRL, Life Technologies, Paisley, Scotland) at 37°C in an atmosphere of 5% CO\textsubscript{2}. Cells were subcultured every fourth day at an initial density of 2 \times 10^5 cells/mL.

Bacterial strain

The \textit{S. aureus} strain ATCC 25923 was used to assess the activity of levofloxacin. Bacterial cells surviving after exposure to levofloxacin in extracellular and intracellular assays (defined as persisters) were systematically collected. All these organisms were stored in 15% glycerol–trypticase broth at −80°C and cultured on Mueller–Hinton (MH) agar and broth (Sanofi Diagnostics Pasteur, Paris, France) at 37°C for 18 h.

Antibiotic and other chemical agents

Levofloxacin was kindly provided as reference powder (potency 99.2%) by Aventis Pharma (Romainville, France). Verapamil (as hydrochloride salt), gemfibrozil, chloroquine (as diphosphate salt) and ammonium chloride were purchased from Sigma Chemicals (L’Ile d’Abeau, France), and the other chemicals were purchased from Prolabo (Briare le Canal, France).

Measurement of extracellular activity of levofloxacin

MICs were determined using the broth dilution method in tubes under standard conditions (MH broth, final inoculum of 5 \times 10^2 cfu/mL).\textsuperscript{15} Minimum bactericidal concentrations (MBCs) were determined as recommended and were defined as the lowest concentrations that killed 99.9% of the inoculum. These end-points were also determined using the same medium adjusted to acidic pH (5.0) or supplemented with various chemical agents as indicated in the following section. Results represented the modal values of three independent experiments.

Bactericidal studies were performed at eight concentrations ranging from 0.125 to 16 mg/L using a mean inoculum of 5 \times 10^5 cfu/mL in the logarithmic phase in MH broth. Viable bacteria were enumerated after 0, 1, 3, 4, 5 and 24 h of incubation at 37°C, by spreading 0.1 mL samples of 10-fold dilutions into distilled water onto MH agar. Results were expressed as percentage of survival versus initial inoculum and represented the mean value of three different experiments. The limit of sensitivity was set at 1 log_{10} cfu/mL to avoid any carryover effect.

In some experiments, killing curves were carried out in cell lysate, obtained as indicated in the section on binding of levofloxacin to cellular components. Levofloxacin was added in cell lysate to final concentrations of 0.125, 0.5 or 2 mg/L, and the mixtures were incubated in a shaking water bath at 37°C for 1 h to allow binding to take place. Then the bacterial inoculum was added, and the time–kill curve experiments were performed. Appropriate controls, either MH broth or cell lysate alone, were included.

Bacterial studies were also carried out in a minimal medium formulated to mimic the eukaryotic phagolysosomal environment.\textsuperscript{16} This intracellular salt medium (ISM) contained potassium phosphate (170 mM), magnesium phosphate (0.5 mM), calcium chloride (1 mM), potassium sulphate (6 mM), ammonium chloride (5 mM), sodium chloride (5 mM), glucose (0.4%), 2-mercaptoethanol (100 mM) and nicotinic acid (2 mM). The pH was adjusted to 5.0 or 7.4 by varying the relative concentrations of monobasic and dibasic potassium phosphate salts.

Cell infection and assessment of intracellular activity of levofloxacin

Infection of THP-1 cells by \textit{S. aureus}, treatment with levofloxacin (from 0.125 to 16 mg/L) and enumeration of viable intracellular bacteria were carried out as described previously.\textsuperscript{11} In some experiments, an inhibitor of P-gp (verapamil, 20 µM) or an inhibitor of organic anion transport (gemfibrozil, 0.25 mM) or lysosomotropic agents (chloroquine, 1 mg/L or ammonium chloride, 1 mg/mL) were added to the culture medium with or without levofloxacin. The lack of cytotoxic effect of these agents at the concentrations used was confirmed at the end of the infection using the Trypan Blue dye exclusion test. Results were expressed as the percentage of surviving \textit{S. aureus} versus the initial inoculum, and represented the mean value of three different experiments.

Levofloxacin uptake by THP-1 cells

Uninfected THP-1 cells were incubated in RPMI 1640 medium with 0.125–32 mg/L levofloxacin. Then, infected cells were incubated with 8 mg/L levofloxacin, and in some experiments with the chemical agents at the concentrations indicated above introduced into the medium 30 min previously. After 5 h of incubation at 37°C, cells were separated from the extracellular medium using differential centrifugation through a water-impermeable silicone–paraffin oil barrier and lysed into orthophosphoric acid. Extracellular and cell-associated levofloxacin were determined using high-performance liquid chromatography (HPLC) with fluorescence detection (λ\textsubscript{ex} = 296 nm and λ\textsubscript{em} = 504 nm), as reported previously,\textsuperscript{17} with adaptation for intracellular samples. Intracellular concentrations of levofloxacin were determined by dividing the amount of drug in the lysate
**Factors influencing levofloxacin intracellular activity**

### Table 1. Levofloxacin susceptibility of *S. aureus* ATCC 25923 and persisters under various culture conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture conditions</th>
<th>MIC (mg/L)</th>
<th>MBC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>MH, pH 7.4</td>
<td>0.5 (0.5–0.5)</td>
<td>1 (0.5–1)</td>
</tr>
<tr>
<td></td>
<td>MH, pH 5.0</td>
<td>2 (2–4)</td>
<td>4 (4–8)</td>
</tr>
<tr>
<td></td>
<td>MH, pH 7.4 + verapamil (20 μM)</td>
<td>0.25 (0.25–0.5)</td>
<td>0.5 (0.5–0.5)</td>
</tr>
<tr>
<td></td>
<td>MH, pH 7.4 + gemfibrozil (0.25 mM)</td>
<td>0.5 (0.5–0.5)</td>
<td>1 (0.5–1)</td>
</tr>
<tr>
<td></td>
<td>MH, pH 7.4 + chloroquine (1 mg/L)</td>
<td>0.5 (0.5–0.5)</td>
<td>1 (1–1)</td>
</tr>
<tr>
<td></td>
<td>MH, pH 7.4 + ammonium chloride (1 mg/mL)</td>
<td>0.5 (0.5–0.5)</td>
<td>0.5 (0.5–0.5)</td>
</tr>
<tr>
<td>Persisters</td>
<td>time–kill curves</td>
<td>MH, pH 7.4</td>
<td>0.5 (0.5–0.5)</td>
</tr>
<tr>
<td></td>
<td>intracellular assays</td>
<td>MH, pH 7.4</td>
<td>0.5 (0.5–1)</td>
</tr>
</tbody>
</table>

MH, Mueller–Hinton broth.
*Data are modal values and range (three independent assays).

by the volume of monocytes, which was estimated as 1 μL for 1 × 10⁶ cells.¹⁸ The uptake of levofloxacin by THP-1 cells was expressed as the cellular-to-extracellular (C/E) ratio.

**Binding of levofloxacin to cellular components**

The extent of intracellular binding of levofloxacin to cellular components was determined in cell lysate using the discontinuous ultrafiltration method.¹⁹ Cells were suspended in phosphate buffer pH 7.0 and lysed by rapid freezing–thawing followed by sonication. This procedure completely destroyed the cells, as checked microscopically. The rare intact cells and membranes were eliminated using centrifugation (14 000 g, 20 min at 4°C) and the supernatants were collected as cell lysate (at a final protein concentration of 5 mg/mL, as determined using a Lowry modified micromethod). Levofloxacin was added to cell lysate to obtain drug concentrations of 0.125, 0.5 and 2 mg/L. The mixture was incubated for 1 h at 37°C and then an aliquot of 0.3 mL was ultrafiltered using a micropartition system (Microcon YM 10000; Millipore, Saint Quentin en Yvelines, France) with a membrane filter of 10 000 MW at 4000 g for 20 min at 37°C. The ultrafiltrate was analysed using HPLC, as described in the previous section. The concentration of quinolone bound to intracellular macro-components was calculated from the difference between the concentration in the ultrafiltrate and the known total concentration in lysate. Preliminary control experiments indicated that the adsorption of the drug to the ultrafiltration membrane was ≤5%, so no corrections were made.

**Statistical analysis**

A bilateral *t*-test was performed for comparison between groups, and analysis of covariance for comparison between curves. Significance was defined as *P* < 0.05.

**Results**

In vitro susceptibility of *S. aureus* ATCC 25923 and persisters to levofloxacin

MICs and MBCs of levofloxacin for *S. aureus* ATCC 25923 were 0.5 and 1 mg/L, respectively, under standard conditions (Table 1). They were 4-fold higher when the pH was decreased to 5.0. In contrast, the addition of verapamil (20 μM), gemfibrozil (0.25 mM), chloroquine (1 mg/L) or ammonium chloride (1 mg/mL) to the MH medium did not change the MIC and MBC values. *S. aureus* ATCC 25923 and the persisters exhibited the same susceptibility to levofloxacin.

**Cellular accumulation of levofloxacin in THP-1 cells**

Levofloxacin penetrated rapidly into THP-1 cells. At equilibrium (after 1 h), cellular concentrations of levofloxacin were ~2-fold greater than the extracellular ones in uninfected cells (C/E ratio: 2.1 for 8 mg/L, Table 2) and were independent of extracellular concentrations between 0.1 and 32 mg/L (data not shown). Accumulation of levofloxacin in infected cells (C/E ratio: 2.0) did not differ significantly from the accumulation in uninfected THP-1 cells. Incubation of infected cells with verapamil, chloroquine or ammonium chloride at the concentrations cited above 30 min prior to the addition of levofloxacin did not change the intracellular accumulation of the antibiotic. In contrast, previous incubation with gemfibrozil (0.25 mM) statistically increased the uptake of levofloxacin 1.7-fold (Table 2).

**Extracellular and intracellular activities of levofloxacin**

The extracellular activity of levofloxacin against *S. aureus* ATCC 25923 was further evaluated in MH broth, using the recommended inoculum of 5 × 10⁵ cfu/mL, at eight therapeutic concentrations (0.125–16 mg/L). Results were consistent with MIC and MBC data (Figure 1a). Indeed, levofloxacin could only delay growth at 0.125 mg/L, had an inhibitory effect at
0.25 and 0.5 mg/L, and was bactericidal (reduction of the bacterial inoculum by \(\frac{3}{10}\) log10 units after incubation for 24 h) at concentrations \(\leq 1\) mg/L. These concentrations (1–16 mg/L) led to highly similar curves: they produced a rapid initial decrease in viable bacteria, with the maximum effect obtained after 5 h of incubation, and a subsequent bacterial persistence of 0.5% of the inoculum during the following 19 h.

In the THP-1 model, just after infection, the mean infestation rate was \(4.7 \times 10^5\) bacteria per \(10^6\) cells. In the absence of antibiotic, the number of viable bacteria increased by \(\frac{1}{10}\) log10 unit (mean percentage survival 796–373%) after 5 h of incubation compared with the number at time zero (Figure 1b). This was 1.5 log10 units fewer bacteria than were present at the same time in MH broth. Levofloxacin, at concentrations of 0.125 and 0.25 mg/L in the extracellular fluid, did not inhibit bacterial multiplication (mean percentage survival 908 ± 473% and 464 ± 257%, respectively). Addition of levofloxacin to the extracellular medium at concentrations \(\geq 0.5\) mg/L resulted in a concentration- and time-dependent decrease in the cell-associated S. aureus. After 5 h of incubation in the presence of 1, 2 and 4 mg/L extracellular levofloxacin, mean percentages of viable bacteria were 120 ± 18%, 38.2 ± 2.4% and 7.2 ± 1.3%, respectively. The maximum decrease in the inoculum was 1.0 log10 cfu/mL for concentrations \(>4\) mg/L. The percentage survival of ingested staphylococci exposed to a levofloxacin concentration of 8 mg/L was 5.6 ± 1.1% at 5 h, whereas this percentage at the same concentration in the cell-free system was 0.65 ± 0.25%, indicating that the antibacterial activity of levofloxacin against S. aureus ingested by THP-1 monocytes was significantly less than that against the same bacteria in broth.

To better compare the intracellular activity and extracellular activity, taking into account the accumulation of levofloxacin in THP-1 cells, the percentage change in the bacterial inoculum was expressed as a function of extracellular and calculated intracellular concentrations (Figure 2). This analysis highlighted that the activity of levofloxacin reached a plateau considerably lower (\(~20\)-fold) within THP-1 cells than in the extracellular MH broth medium.

**Influence of efflux pump inhibitors on the intracellular activity of levofloxacin**

The addition of verapamil or gemfibrozil modified neither the intracellular growth of bacteria nor the effect of levofloxacin on the staphylococcal strain at both concentrations tested (2 and 8 mg/L) (Figure 3a and b). In particular, doubling extracellular concentrations (from 2 to 4 mg/L) increased the intracellular killing by levofloxacin (38.2 ± 2.4% versus 7.2 ± 1.3% after 5 h, \(P < 0.05\)), while the use of gemfibrozil, despite doubling intracellular concentrations, did not (33.3 ± 4.5%) (Figure 3b).

**Influence of phagolysosomal pH on the intracellular activity of levofloxacin**

The lysosomotropic alkalinizing agents (LAAs) chloroquine and ammonium chloride did not affect the intracellular growth of S. aureus (Figure 4a). However, the antibacterial activity of levofloxacin at an extracellular concentration of 8 mg/L was enhanced when LAAs were incorporated into the incubation medium.
medium. The mean percentages (± SD) of bacterial survival after 5 h of incubation were 6.9 ± 1.0% and 3.2 ± 0.7% (P = 0.044 and P = 0.016) when levofloxacin was combined with chloroquine or ammonium chloride, respectively, versus 12.1 ± 3.1% when levofloxacin was used alone (Figure 4a). The enhancement of antibacterial activity was also observed at lower concentrations of levofloxacin (2 and 4 mg/L) with ammonium chloride (Figure 4b).

Antibacterial activity of levofloxacin in the ISM

In the ISM broth at pH 5.0, the growth of S. aureus was delayed and was considerably lower than in the MH broth at pH 7.4, reaching only 2-fold the initial inoculum after 24 h of incubation (Figure 5a). Under these conditions, levofloxacin at 2 and 8 mg/L only inhibited bacterial growth and did not exert any bactericidal activity (120.3 ± 17.7% and 121.3 ± 23.1%, respectively). However, when the pH of ISM was adjusted to 7.4, the killing effect of levofloxacin was partially restored, yielding a percentage of viable bacteria of 15.0 ± 4.9% and 5.0 ± 2.1% of the inoculum for 2 and 8 mg/L levofloxacin, respectively (Figure 5b). Nevertheless, these values remained higher than those observed in the MH broth.

Factors influencing levofloxacin intracellular activity

Based on ultrafiltration studies, levofloxacin tested at three concentrations (0.125, 0.5 and 2 mg/L) appeared to bind to intracellular components at ~20% (21.3 ± 1.6%, 20.8 ± 1.2% and 22.1 ± 0.6%, respectively). The bacterial growth of S. aureus ATCC 25923 was highly similar in cell lysate and in MH broth (Figure 6). The killing curves of levofloxacin were significantly different (≥1.0 log₁₀ difference) at all concentrations tested in cell lysate compared with MH broth (18.6 ± 4.2% versus 1.8 ± 0.1%, respectively, after 24 h of incubation with 0.5 mg/L levofloxacin) (Figure 6a and b).

Discussion

Fluoroquinolones, like macrolides, tetracyclines and rifampicin, are active against intracellular pathogens; these antibiotics are successfully used to treat various infections caused by both obligate and facultative intracellular bacteria.20 However, the key question remains whether this activity is optimal and whether
that this is a common trait of fluoroquinolones.7,11,12 Intracellular accumulation and intracellular activity, confirming bacterial eradication. Thus, there was a discrepancy between less potent than the extracellular activity, yielding an incomplete bactericidal effect against the test organism and accumulated ~2-fold in the cells, the proportion of bacteria killed did not exceed 95% of the initial population of intracellular bacteria. In fact, the intracellular activity of levofloxacin was 20 times less potent than the extracellular activity, yielding an incomplete bacterial eradication. Thus, there was a discrepancy between intracellular accumulation and intracellular activity, confirming that this is a common trait of fluoroquinolones.7,11,12 Intracellular bacterial pathogens that are not eliminated by lethal concentrations of the antibiotic can resume growth immediately upon removal of the drug10 and may cause antibiotic therapy failures and lead to infection relapses.21-23

The reason for the decreased activity of quinolones in the intracellular environment is probably multifactorial.1,2 However, three broad issues should be taken into account: the respective compartmentalization of the drug in the cell, the inactivation of the drug in the local intracellular environment and a decreased intracellular susceptibility of the organism.24

The subcellular compartmentalization of levofloxacin is probably a major limiting factor. Indeed, to become active, the intracellular portion of the antibiotic must come into direct contact with S. aureus, which resides mainly in phagolysosomes of phagocytic cells. Fluoroquinolones are able to diffuse in various subcellular compartments,9 but Vazifeh et al.25 have suggested that only a small fraction of intracellular levofloxacin will interact with phagolysosomal bacteria. This hypothesis is consistent with the lower activity of levofloxacin against intracellular S. aureus in THP-1 cells. In this context, the reduction of the intracellular bacterial count when extracellular concentrations increased from 0.5 to 4 mg/L is related to an increase in the intraphagolysosomal concentration of levofloxacin resulting from a diffusion equilibrium between extracellular, cytoplasmic and phagolysosomal compartments. Surprisingly, increasing intracellular concentrations obtained by doubling the outside antibiotic levels from 4 to 8 or to 16 mg/L did not produce enhanced activity against S. aureus. Intracellular activity reached a plateau for extracellular concentrations higher than 4 mg/L and never reached the maximal effect of levofloxacin seen extracellularly.

In this context, the present work demonstrated that virulent S. aureus multiplying intracellularly within THP-1 monocytes was protected from the bactericidal activity of levofloxacin. Although levofloxacin used at therapeutic levels exerted a bactericidal effect against the test organism and accumulated ~2-fold in the cells, the proportion of bacteria killed did not exceed 95% of the initial population of intracellular bacteria. In fact, the intracellular activity of levofloxacin was 20 times less potent than the extracellular activity, yielding an incomplete bacterial eradication. Thus, there was a discrepancy between intracellular accumulation and intracellular activity, confirming that this is a common trait of fluoroquinolones.7,11,12 Intracellular bacterial pathogens that are not eliminated by lethal concentrations of the antibiotic can resume growth immediately upon removal of the drug10 and may cause antibiotic therapy failures and lead to infection relapses.21-23

The reason for the decreased activity of quinolones in the intracellular environment is probably multifactorial.1,2 However, three broad issues should be taken into account: the respective compartmentalization of the drug in the cell, the inactivation of the drug in the local intracellular environment and a decreased intracellular susceptibility of the organism.24

The subcellular compartmentalization of levofloxacin is probably a major limiting factor. Indeed, to become active, the intracellular portion of the antibiotic must come into direct contact with S. aureus, which resides mainly in phagolysosomes of phagocytic cells. Fluoroquinolones are able to diffuse in various subcellular compartments,9 but Vazifeh et al.25 have suggested that only a small fraction of intracellular levofloxacin will interact with phagolysosomal bacteria. This hypothesis is consistent with the lower activity of levofloxacin against intracellular S. aureus in THP-1 cells. In this context, the reduction of the intracellular bacterial count when extracellular concentrations increased from 0.5 to 4 mg/L is related to an increase in the intraphagolysosomal concentration of levofloxacin resulting from a diffusion equilibrium between extracellular, cytoplasmic and phagolysosomal compartments. Surprisingly, increasing intracellular concentrations obtained by doubling the outside antibiotic levels from 4 to 8 or to 16 mg/L did not produce enhanced activity against S. aureus. Intracellular activity reached a plateau for extracellular concentrations higher than 4 mg/L and never reached the maximal effect of levofloxacin seen extracellularly. This plateau could result either from the loss of pharmacodynamic potency (discussed below) or from the saturation of levofloxacin accumulation in phagosomes. Interestingly,
Factors influencing levofloxacin intracellular activity

A different impact on intracellular activity was observed between increasing total cell concentration by doubling levofloxacin extracellular concentrations (from 2 to 4 mg/L) and increasing it using gemfibrozil, which generated an almost 2-fold increase in levofloxacin uptake without increasing antibacterial activity. A similar effect of gemfibrozil on ciprofloxacin activity has been reported against intracellular *S. aureus* in J774 macrophages. It has been proposed that quinolones could be sequestered within phagolysosomes by means of organic anion transporters located on the phagosomal membranes. In this case, inhibition of organic anion transporters by gemfibrozil would result in the antibiotic being confined within the cytosol and in decreased drug diffusion in the vacuoles. This might explain why, despite doubling whole-cell concentrations of levofloxacin, gemfibrozil did not enhance the intracellular activity of levofloxacin. Nevertheless, such a mechanism implies a significant penetration of levofloxacin in phagolysosomes, in disagreement with the assumption of Vazifeh et al. The sequestration of quinolones into intracellular vacuoles and the impact of the expression of the efflux pumps on this process and consequently on antibacterial activities against intracellular pathogens are complex mechanisms that need to be investigated further.

In addition, the phagolysosomal environment is apt to inactivate fluoroquinolones. Indeed, phagolysosomes are characterized by a low pH (~4.8–5.0), and fluoroquinolones, including levofloxacin, are known to have a reduced activity in acidic medium. Accordingly, in our study, the MIC/MBC of levofloxacin against *S. aureus* ATCC 25923 was increased 4-fold, from 0.5/1.0 mg/L at the standard pH 7.4 to 2.0/4.0 mg/L at pH 5.0. Alkalization of the *S. aureus*-containing vacuoles was carried out using chloroquine at 1 mg/L, which raises the lysosomal pH to 5.7, and ammonium chloride at 1 mg/mL, which elevates the lysosomal pH to 6.85. Although these LAAs had no antibacterial activity against the test organism and did not modify the antibiotic uptake, phagolysosomal alkalization slightly, but significantly, enhanced the intracellular activity of levofloxacin. Similarly, a correlation between killing rate of amikacin against intracellular *S. aureus* and phagolysosomal pH has been described previously. Nevertheless, levofloxacin did not achieve a bactericidal effect, indicating that the modification of the phagolysosomal pH was insufficient to completely restore the efficacy of levofloxacin under our experimental conditions. In addition to acidic pH, intracellular vacuoles provide a particular environment which was mimicked using the artificial ISM. When the test organism was grown in the ISM adjusted to pH 7.4, the bacterial growth reduced and the activity of levofloxacin was less than in MH broth. This points to a role of the phagolysosomal environment in decreasing the intracellular activity of levofloxacin. When the ISM was adjusted to pH 5.0, the bacterial growth and the killing effect of levofloxacin were completely abolished even for a high concentration of antibiotic (8 mg/L), demonstrating that intraphagolysosomal composition and pH act together negatively on intracellular activity.

Binding to macromolecules might also contribute to the decreased intracellular activity of levofloxacin. Ultrafiltration experiments showed that ~20% of intracellular levofloxacin was bound to cellular components. A similar percentage of intracellular binding has been reported previously for pefloxacin in human blood monocytes. Kill curve experiments revealed that the antimicrobial activity of levofloxacin was inhibited by whole-cell lysate. Thus, our results and those of other investigators confirm that binding to cellular components, leading to a decreased intracellular concentration of free drug, might hamper bacterial killing by antibiotics. Quinolones probably bind to precipitable molecules such as proteins, but also to non-precipitable molecules such as DNA. However, the impact of binding to macromolecules, such as proteins, on the pharmacodynamics of fluoroquinolones remains unclear.

Finally, intracellular organisms might have a reduced susceptibility to fluoroquinolones. Bacteria surviving after initial exposure to levofloxacin did not exhibit an increased MIC/MBC, and thus were not resistant mutants. They gave a normal colony when subcultured, ruling out the selection of less susceptible ‘small colonies variants’. Intracellular growth of *S. aureus* in control cells proceeded at a slow rate, indicating that staphylococci in phagolysosomes were not dormant and fluoroquinolones are capable of killing slow-growing and even resting cells. The physiological status of intracellular—and intraphagolysosomal—microorganisms and its relationship to *in situ* antimicrobial susceptibility remain to be elucidated.

In conclusion, using a well-controlled cell model, we demonstrated that levofloxacin exhibited a partial loss of intracellular activity against *S. aureus*. The compartmentalization of the drug within phagolysosomes and intracellular environment factors such as the acidic pH and the particular composition of the vacuoles as well as antibiotic binding to cellular components can explain this failure, at least in part. Further studies on the sequestration of quinolones into intracellular vacuoles, the major binders of quinolones within monocytic cells and the persister state among intracellular *S. aureus* are required. It remains to be seen whether this phenomenon is common to all fluoroquinolones.

Acknowledgements

We thank Catherine André for expert technical assistance. This work was supported by a PhD grant to H. A. N. from the French government, via the Foreign Office, and from the Ministry of National Education and Research (EA 525), University of Bordeaux 2, Bordeaux, France.

Transparency declarations

None to declare.

References

6. Seral C, Van Bambekte F, Tulkens PM. Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and


