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To cite this version:

Sandrine Lemaire, Françoise van Bambeke, Paul M. Tulkens. Activity of finafloxacin, a novel fluoroquinolone with increased activity at acid pH, towards extracellular and intracellular , and. International Journal of Antimicrobial Agents, 2011, 10.1016/j.ijantimicag.2011.03.002 . hal-00703149

HAL Id: hal-00703149

https://hal.science/hal-00703149

Submitted on 1 Jun 2012

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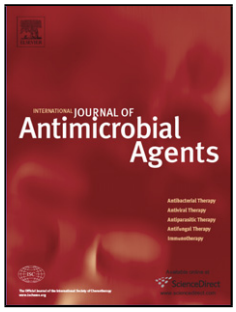

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PII: S0924-8579(11)00136-1
DOI: doi:10.1016/j.ijantimicag.2011.03.002
Reference: ANTAGE 3581

To appear in: *International Journal of Antimicrobial Agents*

Received date: 5-1-2011
Revised date: 24-2-2011
Accepted date: 2-3-2011

Please cite this article as: Lemaire S, Van Bambeke F, Tulkens PM, Activity of finafloxacin, a novel fluoroquinolone with increased activity at acid pH, towards extracellular and intracellular *Staphylococcus aureus*, *Listeria monocytogenes* and *Legionella pneumophila*, *International Journal of Antimicrobial Agents* (2010), doi:10.1016/j.ijantimicag.2011.03.002

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Activity of finafloxacin, a novel fluoroquinolone with increased activity at acid pH, towards extracellular and intracellular *Staphylococcus aureus*, *Listeria monocytogenes* and *Legionella pneumophila* ☆

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ARTICLE INFO

Article history:

Received 5 January 2011

Accepted 2 March 2011

Keywords:

Fluoroquinolones

Staphylococcus aureus

Listeria monocytogenes

THP-1 macrophages

Acid pH

Intracellular

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☆ Part of this work has been presented as a poster at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 12–15 September 2009, San Francisco, CA [A1-1940].

ABSTRACT

Finafloxacin, an 8-cyano-substituted fluoroquinolone, expresses enhanced activity at acidic pH and is less susceptible to several fluoroquinolone resistance determinants. In this study, finafloxacin and ciprofloxacin were compared for (i) activity against ciprofloxacin-susceptible and -resistant *Staphylococcus aureus* as well as wild-type and Lde efflux-positive (Lde+) *Listeria monocytogenes*, (ii) accumulation in THP-1 macrophages and (iii) intracellular activity towards phagocytised *S. aureus*, *L. monocytogenes* and *Legionella pneumophila* (developing in acidic, neutral and mildly acidic environments, respectively), using a pharmacological approach assessing drug potencies and maximal relative efficacies (E_{max}). Finafloxacin minimum inhibitory concentrations (MICs) were two-fold lower than those of ciprofloxacin against meticillin-susceptible *S. aureus* ATCC 25923, were only modestly increased in an isogenic strain overexpressing NorA and were ≤0.25 mg/L for community-acquired meticillin-resistant *S. aureus*. No loss of activity was seen in Lde+ *L. monocytogenes*. An acidic pH decreased the MIC of finafloxacin and increased that of ciprofloxacin both for *S. aureus* and *L. monocytogenes*, in parallel with corresponding changes in drug accumulation (tested with *S. aureus* ATCC 25923 only). Finafloxacin accumulated less than ciprofloxacin in THP-1 cells, but the situation was reversed by exposure of cells to acid pH. In *S. aureus*-infected cells, acid pH increased the potency of finafloxacin without change of E_{max}, whilst decreasing the potency and the maximal relative efficacy of ciprofloxacin (less negative E_{max}). Finafloxacin was more potent and showed larger E_{max} than ciprofloxacin against phagocytised *L. pneumophila*, but was less potent against

phagocytised *L. monocytogenes*. Finafloxacin appears to be an acid pH-favoured antibiotic that may find useful applications in infections where the local pH is low.

1. Introduction

Treating intracellular bacterial infections remains a challenge as the causative organisms are sheltered from many of the immune and innate defence mechanisms and show decreased susceptibility to many antibiotics (see [1–4] for selected reviews), making it necessary to assess novel antibiotics in this context. Finafloxacin is an investigational broad-spectrum fluoroquinolone characterised by a 7-pyrrolo-oxazinyl moiety and an 8-cyano substituent (Fig. 1). It expresses markedly enhanced activity under acidic conditions where other fluoroquinolones are inactivated [5,7–9]. This may confer advantages to finafloxacin for infections occurring not only in acidic body sites such as the skin, vagina and urinary tract or those rendered acidic by an inflammatory response to infection, but also against bacteria sojourning within acidic subcellular organelles (phagosomes and phagolysosomes).

Finafloxacin may be less susceptible than ciprofloxacin to several known fluoroquinolone resistance determinants (alone and in combination) in *Escherichia coli* [8]. Having a bulky substituent in position 7 somewhat similar to that of moxifloxacin, it could also be less susceptible to efflux by the bacterial multidrug transporter NorA [10], which affects the activity of ciprofloxacin but less so that of moxifloxacin [11,12]. In this study, the activity of finafloxacin was examined against a panel of ciprofloxacin-susceptible and -resistant *Staphylococcus aureus* isolates and its accumulation by THP-1 human macrophages and activity towards susceptible extracellular and intracellular *S. aureus* at neutral and acidic pH were studied. In parallel, its activity against intracellular *Listeria monocytogenes* and *Legionella pneumophila*, representative of intracellular

organisms sojourning and multiplying in neutral (cytosol [13]) and mildly acidic (phagosomes [14]) environments, respectively, was also determined.

2. Material and methods

2.1. Antibiotics and main reagents

Finafloxacin and ciprofloxacin were obtained as microbiological standards from MerLion Pharmaceuticals GmbH (Berlin, Germany) and Bayer HealthCare AG (Wuppertal, Germany), respectively. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA) and other reagents from Sigma-Aldrich Inc. (St Louis, MO) or Merck KGaA (Darmstadt, Germany).

2.2. Bacterial strains and susceptibility testing

Tables 1 and 2 show the strains used in the present study. Unless indicated otherwise, minimum inhibitory concentration (MIC) determinations were made in Mueller–Hinton broth (pH 7.4; 24 h) for *S. aureus*, in tryptic soy broth (pH 7.4; 24 h) for *L. monocytogenes* and in α-ketoglutarate-buffered yeast extract broth (pH 6.9; 48 h) for *L. pneumophila*.

2.3. Uptake of fluoroquinolones by Staphylococcus aureus

Staphylococcus aureus strain ATCC 25923 was grown to mid exponential growth phase [optical density at 620 nm (OD_{620}) = 0.5], harvested by centrifugation (4000 rpm, 7 min,

4 °C) and re-suspended in pH-adjusted broth containing 100 mg/L of fluoroquinolone. After 30 min, bacteria were collected by centrifugation (4000 rpm, 7 min, 4 °C), washed free of antibiotic by four successive rinses with ice-cold phosphate buffered saline (PBS) and lysed by three successive freeze–thaw cycles (5 min at –80 °C followed by 5 min at 37 °C). The cellular content of antibiotic was measured by the disk plate assay using Antibiotic medium 2 (pH 6.7) and *E. coli* strain ATCC 25922 as the test organism [lowest limit of detection and linearity of the response: finafloxacin, 1 mg/L and 1–32 mg/L (R^2 = 0.994); ciprofloxacin, 0.25 mg/L and 0.25–16 mg/L (R^2 = 0.969)] and was expressed by reference to the total protein content in the sample.

2.4. Cell lines and assessment of cell viability

Experiments were conducted with human THP-1 cells (ATCC TIB-202; American Tissue Culture Collection, Manassas, VA) as described previously [17]. Viability of cells exposed to different conditions was determined by trypan blue exclusion assay (<10% stained cells).

2.5. Accumulation of fluoroquinolones within THP-1 cells

Cellular accumulation of fluoroquinolones was measured using uninfected cells, as the lack of radiolabelled finafloxacin and the fact that finafloxacin is poorly fluorescent compared with other fluoroquinolones forced us to use the microbiological assay described above. This imposed the use of a large extracellular concentration of antibiotics (50 mg/L) that would have prevented intracellular growth of the bacteria. For

ciprofloxacin, both a fluorometric assay (described in detail previously [18,19]; lowest limit of detection and linearity of the response, 20 ng/mL and 20–100 ng/mL) and the microbiological assay were used. Cells incubated with the antibiotics were collected after gentle pelleting and washing in ice-cold PBS. For pH dependence studies, cells were incubated with buffered media adjusted to specific pH values (the exact pH of each medium was measured before and after incubation and was found to not vary by more than 0.1 pH unit during the experiment). Cell lysates were used for determination of antibiotic and total protein content (Folin–Ciocalteu/Biuret method [20]) and the apparent cellular concentration was calculated using a conversion factor of 5 μL of cell volume per mg of cell protein.

2.6. Determination of extracellular and intracellular activities

Concentration–response studies were measured in pH-adjusted Mueller–Hinton broth for *S. aureus* as described previously [21]. Intracellular activities were measured towards bacteria phagocytosed by THP-1 cells following the general procedures described in an earlier publication for *S. aureus* [21], *L. monocytogene*s [19] and *L. pneumophila* [22]. Typical initial inocula were ca. 1–3 $\times$ 10^6 colony-forming units (CFU) per mL of broth or per mg of cell protein (THP-1) [21,23,24]. The large dilution of the cellular material made during collection and actual spread on plates ensured absence of interference with CFU counts by the presence of carried-over antibiotics.

2.7. Curve fitting and statistical analyses

Data were used to fit sigmoidal functions (Hill equation) using GraphPad Prism® version 4.03 (GraphPad Software, San Diego, CA) to obtain, for each condition, numeric values of four key pharmacological descriptors (see [21] for details), namely: (i) the minimal relative efficacy (E_{min}) in log_{10} units, corresponding to the increase in the number of CFU for an infinitely low concentration of antibiotic compared with the original inoculum; (ii) the maximal relative efficacy (E_{max}) in log_{10} units, corresponding to the decrease in the number of CFU for an infinitely large concentration of antibiotic compared with the original inoculum; (iii) the relative potency (EC_{50}), in mg/L or in multiples of the MIC, corresponding to the concentration of antibiotic yielding a value of CFU half-way between E_{min} and E_{max}; and (iv) the static concentration (C_s), in mg/L or multiples of the MIC, corresponding to the concentration of antibiotic causing no apparent change in CFU compared with the original inoculum. Statistical analyses of the differences between experimental groups for E_{min}, E_{max} and EC_{50} values were made with GraphPad InStat version 3.06 (GraphPad Software), using the mean and standard error values provided by the non-linear regression analysis (with log-transformed values for EC_{50}) (see Supplementary Tables 1 and 2 for the tests used).

3. Results

3.1. Susceptibility testing

Table 1 shows the MICs of finafloxacin and ciprofloxacin against a panel of laboratory and clinical isolates of *S. aureus* and against laboratory strains of *L. monocytogenes*

and *L. pneumophila*. Finafloxacin was twice as active as ciprofloxacin against the meticillin-susceptible *S. aureus* (MSSA) strain ATCC 25923 and its MIC was increased by only 2–3 $\log_2$ dilutions against the isogenic strain SA-1 overexpressing NorA (5 $\log_2$ dilutions increase for ciprofloxacin). For the community-acquired meticillin-resistant *S. aureus* (CA-MRSA) included in the panel, both ciprofloxacin and finafloxacin showed low and quite similar MICs (0.125–1 mg/L). For hospital-acquired meticillin-resistant *S. aureus* (HA-MRSA), finafloxacin and ciprofloxacin showed similar MICs towards the two ciprofloxacin-susceptible laboratory strains. For the clinical isolates (Belgian or US) highly resistant to ciprofloxacin (MICs of 32–128 mg/L), the MICs of finafloxacin were only 4–16 mg/L. For *L. monocytogenes* and *L. pneumophila*, the MICs of ciprofloxacin and finafloxacin were similar (1–2 mg/L and 0.01 mg/L, respectively).

3.2. Influence of pH on minimum inhibitory concentrations and bacterial accumulation

The influence of pH on the activity and accumulation of finafloxacin and ciprofloxacin was examined using *S. aureus* strain ATCC 25923. Fig. 2A shows that the MIC of finafloxacin was considerably decreased when the pH was brought from 7.4 to 5.5, whereas the opposite was seen for ciprofloxacin. Fig. 2B shows that the change in MIC was coincident with a corresponding change in drug accumulation. However, Fig. 3C shows that the change in MIC for finafloxacin across pH was associated with a considerably larger change in accumulation than for ciprofloxacin over the same pH range.

To what extent acid pH would also modulate the activity of finafloxacin and ciprofloxacin towards other strains was then examined. For these experiments, *S. aureus* strain SA-1 (overexpressing NorA) and its isogenic wild-type strain (basal expression) as well as two *L. monocytogenes* strains, namely a wild-type strain (EGD) and a ciprofloxacin-resistant clinical isolate (CLIP21369) overexpressing the Lde efflux system [16], were selected. The results are presented in Table 2. For all strains, a decrease in pH caused a decrease in the MICs of finafloxacin and an increase in those of ciprofloxacin. Of interest, finafloxacin maintained its poor susceptibility to NorA across the entire pH change, resulting in its MIC being 7 $\log_2$ dilutions lower than that of ciprofloxacin against SA-1 strain at pH 5.5. Finafloxacin also appeared to be largely immune to the defeating effect exerted by the Lde transporter on ciprofloxacin in *L. monocytogenes*.

3.3. Influence of pH and ammonium chloride on cellular pharmacokinetics in THP-1 cells

Fig. 3A shows that both fluoroquinolones accumulated quickly within THP-1 cells, with an apparent equilibrium being reached within <2 h. However, ciprofloxacin achieved a larger intracellular to extracellular concentration ratio than finafloxacin [ca. 2.4-fold difference; in these experiments, a low concentration (4 mg/L) of ciprofloxacin was used to remain in a microbiologically meaningful range, to allow comparison with our previous work and to ensure a lack of saturation of a potential efflux transporter; measuring the cellular accumulation at a concentration of 50 mg/L as for finafloxacin gave a value for the apparent cellular concentration/extracellular concentration (C_c/C_e) ratio of 10.01 ± 2.21]. Fig. 3B shows that incubation in acid medium reduced the accumulation of

ciprofloxacin to approximately one-half of its value at neutral pH whereas it increased approximately 4-fold the accumulation of finafloxacin. Fig. 3C shows that addition of ammonium chloride (NH_4Cl) (known to neutralise the acid pH of lysosomes and related acidic intracellular organelles) to cells incubated at neutral pH reduced the accumulation of finafloxacin by approximately 60% whilst increasing that of ciprofloxacin approximately two-fold.

3.4. Influence of pH on extracellular and intracellular pharmacodynamics against Staphylococcus aureus

Staphylococcus aureus develops in acid environments, including in phagocytic cells where it mainly localises in phagolysosomes (the pH of which is ca. 5.5). A full pharmacodynamic evaluation [21] of the activities of finafloxacin and ciprofloxacin at neutral and acid pH was therefore performed. In these experiments, *S. aureus* strain ATCC 25923, either in broth (extracellular) or after phagocytosis by THP-1 cells (intracellular), was exposed for 24 h to drug concentrations spanning from ca. 0.01× to 800× (ciprofloxacin) or 1700× (finafloxacin) the MIC (as measured at pH 7.4). Experiments were conducted at pH 7.4 and pH 5.5 using pH-adjusted broth or culture medium. The results of these studies are shown in Fig. 4, with the regression parameters and numerical values of the pharmacological descriptors [minimal and maximal relative efficacies (E_{min} and E_{max}) and relative potencies (EC_{50})] and static concentrations presented in Supplementary Table 1. With regard to extracellular bacteria (Fig. 4, upper panels), both drugs showed essentially similar concentration-–response curves and regression parameters when tested at pH 7.4. Acid pH did not

modify the minimal and maximal relative efficacies but affected, in opposite ways, the relative potencies (EC_{50}) and static concentrations (C_s) when expressed as weight concentrations (mg/L). However, this effect was entirely accounted for by the change in MIC, as both EC_{50} and C_s values became non-statistically different when expressed as multiples of the MIC in the corresponding environment. For intracellular bacteria (Fig. 4, lower panels), it is seen that, as previously described for several other antibiotics [21], the maximal relative efficacies (E_{max}) of both finafloxacin and ciprofloxacin are considerably reduced compared with extracellular bacteria, since the reduction of the inoculum does not exceed 1–1.5 $\log_{10}$ CFU (compared with ≥5 $\log_{10}$ CFU for bacteria in broth). As for extracellular bacteria, acid pH increases the potency of finafloxacin (lower EC_{50} and C_s). The increased potency of finafloxacin against intracellular bacteria when the external pH was acidified appeared to be related to the enhanced MIC under acidic conditions, but other factors such as pH-dependent accumulation of the drug may also be important. For ciprofloxacin, acid pH not only caused a shift of the concentration-dependent curve to higher values but also a significant loss of maximal relative activity (E_{max}), the drug becoming essentially bacteriostatic even at large extracellular concentrations. Acid pH also caused a loss of potency that, again, was largely accounted for by the change in MIC [note that because E_{max} is less negative and E_{min} is slightly more positive at acid pH, the EC_{50} of ciprofloxacin at that pH remains almost unchanged when expressed as weight concentrations, but the loss of potency clearly appears from the change in C_s (in mg/L)].

3.5. Intracellular pharmacodynamics against Listeria monocytogenes and Legionella pneumophila

Finafloxacin and ciprofloxacin were then tested against two other intracellular organisms, developing in neutral (*L. monocytogenes*, cytosol) and in mildly acidic (*L. pneumophila*, phagosomes) environments. The same pharmacodynamic approach as for *S. aureus* was followed, but only cells incubated at neutral pH were used as bacterial growth was too poor in cells exposed to acid pH. Results presented in Fig. 5 (with regression parameters and numerical values of the pharmacological descriptors given in Supplementary Table 2) show that while both fluoroquinolones exerted a marked bactericidal effect against intraphagocytic *L. monocytogenes* (>4 $\log_{10}$ CFU decrease), ciprofloxacin had a greater potency (ca. two-fold lower EC_{50} and C_s), which could not be attributed to a difference in MIC (see Table 1). For *L. pneumophila*, for which little or no intracellular growth was observed in the absence of antibiotic, finafloxacin maximal relative efficacy (E_{max}) was close to a bactericidal effect (–2.7 $\log_{10}$ CFU decrease), whereas that of ciprofloxacin was significantly weaker (less negative E_{max}). Ciprofloxacin relative potency was also lower (higher EC_{50} and C_s) than that of finafloxacin.

4. Discussion

Developed and introduced in clinics since the mid 1980s, fluoroquinolones have represented a milestone in the chemotherapy of bacterial infections thanks to their wide spectrum, intense bactericidal activity and favourable pharmacokinetics. Fluoroquinolones rapidly accumulate in eukaryotic cells [25–27] and display significant activity towards susceptible bacteria present in various subcellular compartments,

including *S. aureus* (phagolysosomes [21,28]), *L. monocytogenes* (cytosol [23,29]) and *L. pneumophila* (phagosomes [30,31]). However, beyond the wide clinical successes of drugs such as ciprofloxacin, levofloxacin and moxifloxacin, a need exists for more focused derivatives that (i) address so far unmet medical needs and (ii) are less susceptible to resistance mechanisms that have reduced the utility of several of the currently clinically available molecules. Finafloxacin has not only demonstrated potent antibacterial activity both towards Gram-positive and Gram-negative organisms in in vitro and in vivo models [32] but, most conspicuously, exhibits significantly enhanced antibacterial activity in acidic media, a situation in which other currently marketed fluoroquinolones are less active. The present study confirms these original observations and extends them in several respects.

Considering the intrinsic activity of finafloxacin, the data shows that finafloxacin: (i) is as active or more active than ciprofloxacin towards ciprofloxacin-susceptible MSSA and CA-MRSA [using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint as interpretative criterion]; (ii) is probably a poor substrate of the two major facilitator superfamily (MFS) multidrug efflux transporters examined (NorA in *S. aureus* and Lde in *L. monocytogenes*); and (iii) shows considerably lower MICs than ciprofloxacin against ciprofloxacin-resistant HA-MRSA, consistent with the phenotype of dissociated resistance observed with moxifloxacin [33] and a few other fluoroquinolones [34]. This first set of observations clearly calls for more extensive surveys as they may help in better defining the potential advantages of finafloxacin in environments where resistance to ciprofloxacin has become critical. The lack of efficient recognition by the

efflux transporters may also point to unanticipated structure–activity relationships in this context. Indeed, examination of the biophysical properties of finafloxacin contradicts the generally accepted rule that it is the hydrophobic character of a fluoroquinolone that allows it to escape recognition and efflux by NorA and related transporters [35]. The data rather suggest that the bulkiness of the substituents at C-7 and C-8 is much more critical [36].

Regarding the enhanced activity of finafloxacin at acid pH in broth, the present study provides a first rational, albeit limited, explanation based on the results of uptake studies. Thus, it is shown that the increased activity of finafloxacin towards *S. aureus* in acidic conditions is associated with an increased drug uptake in the bacteria. This is consistent with previous studies performed on *E. coli* demonstrating a rank order relationship between increased quinolone uptake and improved antibacterial activity (lower MIC values) [37]. However, the underlying mechanisms remain unclear and are probably not related to the biophysical properties of the molecules only. Indeed, ciprofloxacin and finafloxacin do not markedly differ in terms of pKa values of ionisable groups or in terms of global hydrophilicity (see the predicted properties presented in the caption of Fig. 1 and, for pKa values, the published experimental data [5,6]). Thus, the shift in ionisation curves of finafloxacin towards acidic values compared with ciprofloxacin is probably too modest to account for the magnitude of the effects seen, and finafloxacin is, globally, more hydrophilic than ciprofloxacin. More efforts could therefore be directed at other mechanisms, such as those involving active or efflux transporters acting specifically on finafloxacin (and other fluoroquinolones with

enhanced activity at acidic pH [38]). Indeed, transporter activities are known to be markedly influenced by acidic conditions, as shown for NorA in recent analyses using microarray approaches [39].

A major observation from the present study is that pH also modulates the accumulation of fluoroquinolones in eukaryotic cells, resulting, as for bacteria, in an enhanced accumulation of finafloxacin and a decreased accumulation of ciprofloxacin at acid pH. As for bacteria, no simple explanation based on the biophysical properties of the drugs can be put forward, calling for further studies in this context. An interesting observation concerns the modulation of drug accumulation (in opposite ways) seen upon addition of NH_4Cl. As the primary and most conspicuous effect of NH_4Cl is to neutralise the acid pH of intracellular membrane-bounded structures [40], the data suggest different partitioning of finafloxacin and ciprofloxacin between the cytosol on the one hand and lysosomal/phagosomal vacuoles on the other hand. Cell fractionation studies show that the bulk of the ciprofloxacin accumulated by cells is recovered in the cytosol [41,42]. Further studies to define the subcellular localisation of finafloxacin will be required to explore its partitioning in relation to other fluoroquinolones.

Regarding infected cells, these studies show that while finafloxacin and ciprofloxacin have similar intracellular activities against *S. aureus* when cells are incubated at neutral pH, the two molecules can clearly be differentiated when experiments are conducted in acid media. The increased relative potency (EC_{50} and C_s) of finafloxacin observed in cells incubated at pH 5.5, without change in its maximal relative efficacy (E_{max}), may

result from and is consistent with the increased accumulation of the drug and a decrease of its MIC at acid pH, which has been discussed earlier (this, however, also assumes that the phagolysosomal pH of cells incubated at acid pH is lower than in cells incubated at neutral pH). The situation with ciprofloxacin is more complex as with cells incubated at acid pH we see not only a shift of the concentration–effect curve, indicating a loss of relative potency (essentially detected by an increased C_s, probably originating from the combined effects of reduced accumulation and an increased MIC), but also a loss of maximal relative efficacy (less negative E_{max}, the drug becoming essentially static). This effect of acid pH on intracellular ciprofloxacin should be interpreted as indicating that a substantial proportion of the intracellular bacteria (numerically corresponding to the original, post-phagocytosis inoculum) have become insensitive and/or tolerant to the drug. Of interest, a similar loss of maximal relative efficacy has been observed in the same model when testing the activity of moxifloxacin against CA-MRSA with an MIC (measured at pH 7.4) >0.125 mg/L [43]. Here we see that ciprofloxacin becomes ill effective when the pH condition is such that its MIC also exceeds a similar value. This may have a broad clinical significance as it may point to an intrinsic limitation in the use of ciprofloxacin and moxifloxacin to fight intracellular infections. This is all the more important as, indeed, *S. aureus* is found intracellularly within phagolysosomes [44,45] of most eukaryotic cells where the pH is around 5–5.5. Finafloxacin might be spared such limitation. In this context, the experiments with intracellular *L. monocytogenes* (developing in the neutral environment of the cytosol [13,46]) and *L. pneumophila* (sojourning, at least in part, in mildly acidic vacuoles [47,48]) help in better delineating the effects of local pH on the activities of

fluoroquinolones. Although we cannot exclude other mechanisms, the simplest interpretation of our results (finafloxacin being less potent against *L. monocytogenes* than ciprofloxacin, whilst the reverse is true for *L. pneumophila*) is that they are due to difference in local pH, as shown in the susceptibility testing studies for *L. monocytogenes* (similar experiments could not be conducted with *L. pneumophila* owing to failure to grow in broth at acid pH).

In conclusion, the present set of studies confirms and rationalises the increased potency of finafloxacin against pathogens at acid pH, which could represent a promising alternative for the treatment of infected body sites such as the skin, mouth, cervical mucus, vagina, urine or abscesses. The combination of a decreased MIC and a reduced effect of MFS efflux transporters may lead to maintenance of sufficient susceptibility against ciprofloxacin-resistant organisms at acid pH. The results also suggest that finafloxacin may be better suited than ciprofloxacin for fighting intracellular organisms such as *S. aureus* when the surrounding pH is acidic. *Staphylococcus aureus* is actually well adapted to an acidic intracellular environment, with extensive modulation of gene expression favouring its intracellular survival [49]. Finafloxacin may also prove useful against *L. pneumophila*, but no advantage can be expected for organisms developing in non-acid compartments.

Acknowledgments

The authors thank P.C. Appelbaum (Hershey Medical Center, Hershey, PA), Y. Glupczynski (Cliniques universitaires de Mont-Godinne, Yvoir, Belgium), L.Y. Hsu

(National University of Singapore, Singapore), Y.C. Huang (Chang Gung Children's Hospital, Taiwan), C. Quentin (Université Victor Ségalan, Bordeaux, France) and P. Courvalin (Institut Pasteur, Paris, France) for the kind gift of bacterial isolates. M.C. Cambier and C. Misson provided dedicated technical assistance throughout this work.

Funding

SL is a Postdoctoral Researcher and FVB is a Senior Research Associate of the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS). This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale (grant no. 3.597.06) and by a grant-in-aid from MerLion Pharmaceuticals.

Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Structural formula of finafloxacin (IUPAC name 7-[(4aS,7aS)-3,4,4a,5,7,7a-hexahydro-2H-pyrrolo[3,4-b][1,4]oxazin-6-yl]-8-cyano-1-cyclopropyl-6-fluoro-4-oxoquinoline-3-carboxylic acid). Compared with ciprofloxacin and moxifloxacin, finafloxacin displays an 8-cyano substituent (vertical thick open arrow; no substituent in ciprofloxacin; 8-methoxy in moxifloxacin) and a bulky 7 substituent [piperazine in ciprofloxacin; similar [but more hydrophilic due to the presence of an oxygen (vertical closed arrow) and with a different stereoconfiguration of the 7a hydrogen (thin arrow)] to that of moxifloxacin (7-[(4aS,7aS)-1,2,3,4,4a,5,7,7a-octahydropyrrolo[3,4-b]pyridin-6-yl])]. The predicted log P and log D at pH 7 and 5 are 0.397,–1.45 and –2.93 (vs. 1.625, –0.33 and –1.28 for ciprofloxacin, and 1.896, –0.63 and –1.11 for moxifloxacin), and the predicted pKa_1 (acidic) and pKa_2 (basic) of finafloxacin are 5.98 and 7.73 (vs. 6.43 and 8.68 for ciprofloxacin and 6.04 and 10.61 for moxifloxacin). Predicted values are from SciFinder Scholar and are calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (1994–2010 ACD/Labs; the experimentally determined pKa_1 and pKa_2 of finafloxacin and ciprofloxacin are 5.6 and 7.8 [5] and 6.2 and 8.8 [6].

Fig. 2. Influence of pH on (A) the minimum inhibitory concentration (MIC) and (B) intrabacterial accumulation of finafloxacin and ciprofloxacin for *Staphylococcus aureus* ATCC 25923. (A) MICs were determined in pH-adjusted Mueller–Hinton broth (MHB) (microdilution method; results are from three independent samples yielding identical MIC values). (B) Growing bacteria were incubated for 30 min in pH-adjusted MHB with 100 mg/L of antibiotic and were then collected, lysed and used for assay of antibiotic accumulation. Results are the mean ± standard deviation of three independent

determinations). (C) Correlation between the change in accumulation and of MIC at pH 5.5, 6.0, 6.5 and 7.0, both expressed as the ratio of the values observed at pH 7.4.

Fig. 3. Cellular pharmacokinetics of finafloxacin (50 mg/L) and ciprofloxacin (4 mg/L) in human THP-1 macrophages. (A) Kinetics of cellular accumulation [C_c, apparent cellular concentration; C_e, extracellular concentration (both in mg/L)]. (B) Influence of the pH of the culture medium on the accumulation of antibiotics in short-term incubation (30 min). (C) Influence of ammonium chloride (NH_4Cl) on the accumulation of antibiotics at equilibrium (2 h incubation). All values are the mean ± standard deviation (S.D.) of three independent determinations (when not visible, S.D. bars are smaller than the size of the symbols).

Fig. 4. Pharmacodynamic analysis of the influence of pH on the activities of finafloxacin (left panels) and ciprofloxacin (right panels) towards the extracellular (upper panels) and intracellular (lower panels) forms of *Staphylococcus aureus* strain ATCC 25923. The pH of the broth or of the culture medium was adjusted to pH 7.4 or pH 5.5. The ordinates show the change in the number of colony-forming units (CFU) per mL of broth (extracellular bacteria) or per mg of cell protein (intracellular bacteria) as a function of the extracellular concentration of the corresponding antibiotic. All values are the mean ± standard deviation (S.D.) of three independent experiments (when not visible, S.D. bars are smaller than the size of the symbols). The curves correspond to sigmoidal functions (Hill coefficient = 1) fitted to the data by non-linear regression and allowing determination of four key pharmacological descriptors of antibiotic action, namely the

minimal and maximal relative efficacies (E_{min} and E_{max}, corresponding to the increase and decrease in the number of CFU for an infinitely low and infinitely large antibiotic concentration, respectively), the relative potency (EC_{50}, corresponding to the concentration of antibiotic yielding a value of CFU half-way between E_{min} and E_{max}) and the static concentration (C_s, corresponding to the concentration of antibiotic causing no apparent change in CFU compared with the original inoculum) (see Supplementary Table 1 for numerical values and statistical analysis of the differences observed between experimental groups). The horizontal dotted lines at an ordinate value of 0 (all panels) and –5 (upper panels) indicate an apparent static effect and the limit of quantification, respectively.

Fig. 5. Concentration–response activities of finafloxacin and ciprofloxacin towards the phagocytised *Listeria monocytogenes* (left panel; 24 h incubation) and *Legionella pneumophila* (right panel; 48 h incubation). The pH was adjusted to pH 7.4. The ordinates show the change in the number of colony-forming units (CFU) per mg of cell protein as a function of the extracellular concentration of the corresponding antibiotic. All values are the mean ± standard deviation (S.D.) of three independent experiments (when not visible, S.D. bars are smaller than the size of the symbols). The curves correspond to sigmoidal functions (Hill coefficient = 1) fitted to the data by non-linear regression allowing the determination of four key pharmacological descriptors defined in the legend of Fig. 4 (the corresponding numerical values are shown in Supplementary Table 2, together with statistical analysis of the differences observed between experimental groups) (see Table 4 for goodness of fit and pertinent regression

parameters). The horizontal dotted lines at an ordinate value of 0 indicate an apparent static effect. The limit of quantification corresponds to an ordinate value of –5.

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Table 1

Susceptibility testing of *Staphylococcus aureus* strains with various resistance phenotypes as well as laboratory strains of *Listeria monocytogenes* and *Legionella pneumophila* against finafloxacin and ciprofloxacin. For ciprofloxacin and *S. aureus*, figures in bold for ciprofloxacin indicate minimum inhibitory concentration (MIC) values exceeding the susceptible clinical breakpoint of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org)

Species and phenotype	Collection no.	Origin	MIC (mg/L)	
			Finafloxacin	Ciprofloxacin
S. aureus				
MSSA	ATCC 25923	Laboratory strain [a]	0.06	0.125
	SA-1	NorA-overexpressing strain (derived from ATCC 25923) [b]	0.25–0.5	**4**
CA-MRSA	N4042228	Belgian clinical isolate [c]	0.25	0.25
	NRS192	US clinical isolate [d]	0.25	0.5
	CHU1	Asian clinical isolate [e]	0.125	0.5
	MEH22256	Asian clinical isolate [f]	0.25	1
	N7112046	Animal MRSA (food-animal caregiver) [c]	0.25	0.25
HA-MRSA	COL (NRS100)	Laboratory strain [d]	0.125	0.125
	ATCC 33591	Laboratory strain [a]	0.125	0.25
	N4112910	Belgian clinical isolate [c]	16	**128**

	N4120032	Belgian clinical isolate [c]	4	**128**
HA-MRSA/VISA	NRS18b	US clinical isolate [d]	4	**32**
L. monocytogenes	EGD	Laboratory strain [g]	1	1–2
L. pneumophila	ATCC 33153	Laboratory strain [a]	0.01	0.01

MSSA, meticillin-susceptible *S. aureus*; CA-MRSA, community-acquired meticillin-resistant *S. aureus*; HA-MRSA, hospital-acquired meticillin-resistant *S. aureus*; VISA, vancomycin-intermediate *S. aureus*.

[a] From the American Tissue Culture Collection (Manassas, VA).

[b] From C. Quentin (Université Victor Ségalan, Bordeaux, France [15]).

[c] From Y. Glupczynski (Cliniques universitaires de Mont-Godinne, Yvoir, Belgium).

[d] From the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program (operated by Eurofins Medinet, Inc., Herndon, VA; supported under NIAID/NIH contract no. HHSN272200700055C); details for each strain are available at http://www.narsa.net.

[e] From Y.C. Huang (Chang Gung Children's Hospital, Taiwan).

[f] From L.Y. Hsu (Department of Medicine, National University of Singapore, Singapore).

[g] From P. Berche (Hôpital Necker, Paris, France).

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Table 2

Influence of pH on the minimum inhibitory concentration (MIC) of wild-type and efflux-resistant *Staphylococcus aureus* and *Listeria monocytogenes* strains

pH	MIC (mg/L)							
	Finafloxacin				Ciprofloxacin			
	SA [a]	SA-1 [b]	L.m. EGD [c]	L.m. CLIP [d]	SA [a]	SA-1 [b]	L.m. EGD [c]	L.m. CLIP [d]
7.4	0.0.625	0.25	1	1	0.125	4	1	2
7.0	0.0625	0.25	1	1	0.125	4	1	4
6.7	0.0625	0.25	1	0.5	0.125	4	2	4
6.5	0.03125	0.25	0.5	0.5	0.125	4	2	4
6.0	0.03125	0.125	0.5	0.5	0.25	8	4	4
5.7	0.015625	0.0625	0.5	0.25	0.5	8	4	4
5.5	0.015625	0.0625	0.5	0.5	1	8	8	8

[a] *Staphylococcus aureus* isogenic strain of SA-1 (originally ATCC 25923).

[b] *Staphylococcus aureus* overexpressing NorA (from C. Quentin, Université Victor Ségalan, Bordeaux, France [15]).

[c] *Listeria monocytogenes* wild-type (serotype 1/2a) (from P. Berche, Hôpital Necker, Paris, France).

[d] *Listeria monocytogenes* clinical isolate overexpressing the Lde efflux transporter (from P. Courvalin, Institut Pasteur, France [16]).

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Supplementary Table 1

Pertinent regression parameters (with 95% CI) and statistical analysis of the concentration–response curves of finafloxacin versus ciprofloxacin against *Staphylococcus aureus* ATCC 25923 in broth (extracellular bacteria) and in THP-1 cells (phagocytised bacteria) at pH 7.4 and 5.5 as illustrated in Fig. 4

Condition	Finafloxacin						Ciprofloxacin					
	E_{min} [a]	E_{max} [b]		EC_{50} [c]	C_s [d]	R^2	E_{min} [a]	E_{max} [b]		EC_{50} [c]	C_s [d]	R^2
Broth												
pH 7.4	2.86 a;A (1.82–3.90)	–5.07 a:A (–5.80 to –4.34)	mg/L	0.30 a;A (0.15–0.62)	~0.16	0.96	3.08 a;A (2.10–4.05)	–4.87 a;A (–5.68 to –4.05)	mg/L	0.36 a;A (0.17–0.75)	~0.23	0.98
			× MIC	4.83 a;A (2.34–9.97)	~2.69				× MIC	1.32 a;B (0.55–3.17)	~0.95	
pH 5.5	2.84 a;A (2.09–3.59)	–4.99 a;A (–5.43 to –4.56)	mg/L	0.09 b:A (0.06–0.16)	~0.05	0.99	2.57 a,c;A (1.78–3.36)	–4.79 a;A (–5.65 to –3.94)	mg/L	0.87 b;B (0.44–1.74)	~0.49	0.97
			× MIC	6.39 a:A (4.05–10.10)	~3.73				× MIC	0.84 a,c;B (0.32–2.18)	~0.47	
THP-1												

pH 7.4	2.92 a:B (2.34–3.51)	–1.22 b;B (–1.65 to –0.79)	mg/L	0.32 a;A (0.14–0.69)	~0.77	0.96	2.05 b,c;A (1.77–2.33)	–1.65 b;A (–1.84 to –0.45)	mg/L	0.21 a;A (0.14–0.32)	~0.26	0.99
			× MIC	5.08 b,c;A (2.31–11.18)	~12.7				× MIC	1.66 a;B (1.11–2.49)	~2.12	
pH 5.5	2.99 a;A (1.68–4.31)	–1.38 b;B (–2.04 to –0.72)	mg/L	0.05 b;A (0.01–0.25)	~0.11	0.94	2.53 a,c;A (2.13–2.94)	–0.31 c;A (–0.68–0.06)	mg/L	0.38 a;B (0.14–1.04)	~3.34	0.97
			× MIC	3.34 a,c;A (0.67–16.61)	~7.57				× MIC	0.39 b,c;B (0.14–1.04)	~3.36	

95% CI, 95% confidence interval; CFU, colony-forming units; MIC, minimum inhibitory concentration.

[a] Minimal relative efficacy: CFU increase (in $\log_{10}$ units) at 24 h from the original inoculum (per mL of broth or per mg of cell protein for THP-1 cells), as extrapolated for an infinitely low antibiotic concentration using the Hill equation (slope factor = 1). These figures must be interpreted as the bacterial growth that can be observed in the absence of antibiotic under the corresponding conditions.

[b] Maximal relative efficacy: CFU decrease (in $\log_{10}$ units) at 24 h from the original inoculum (per mL of broth or per mg of cell protein for THP-1 cells), as extrapolated for an infinitely large antibiotic concentration using the Hill equation (slope factor = 1). These figures must be interpreted as the maximal antibacterial effect that can be obtained by the antibiotic under the corresponding conditions.

[c] Relative potency: concentration [in mg/L (upper row) and in multiples of MIC measured at the corresponding pH (7.4 or 5.5) (lower row)] causing a reduction of the inoculum at 24 h halfway between the minimal (E_{min}) and maximal (E_{max}) relative efficacies as derived from the Hill equation.

[c] Concentration [in mg/L (upper row) and in multiples of MIC measured at the corresponding pH (7.4 or 5.5) (lower row)] resulting in no apparent bacterial growth at 24 h compared with the initial inoculum (time = 0 h), as determined by graphical interpolation using the Hill equation.

Statistical analysis (using raw values for E_{min} and E_{max}, and log-transformed values for EC_{50}):

- analysis per column: values with different lowercase letters are significantly different ($P < 0.05$) from each other [one-way analysis of variance (ANOVA) with Tukey's post test for multiple comparisons];
- analysis per row: values with different upper case letters are significantly different ($P < 0.05$) from each another within the pertinent comparison group (finafloxacin vs. ciprofloxacin for E_{min}, E_{max} and EC_{50}, respectively; unpaired, two-tailed *t*-test).

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Supplementary Table 2

Pertinent regression parameters (with 95% CI) and statistical analysis of the concentration–response curves of finafloxacin versus ciprofloxacin against *Listeria monocytogenes* and *Legionella pneumophila* in THP-1 cells (phagocytised bacteria) at pH 7.4 as illustrated in Fig. 5

Bacteria	Finafloxacin					Ciprofloxacin				
	E_{min} [a]	E_{max} [b]	EC_{50} [c]	C_s [d]	R^2	E_{min} [a]	E_{max} [b]	EC_{50} [c]	C_s [d]	R^2
L. monocytogenes	3.60 A (2.85–4.36)	–4.73 A (–6.44 to –3.02)	5.62 A (2.47–12.81)	~4.21	0.96	3.16 A (2.77–3.55)	–4.06 A (–4.57 to –3.54)	2.12 B (1.52–2.96)	~1.67	0.99
L. pneumophila	0.53 A (–0.20–1.27)	–2.70 A (–3.34 to –2.05)	0.42 A (0.11–1.58)	~0.08	0.90	0.24 A (–0.23–0.71)	–1.98 B (–2.77 to –1.19)	2.86 B (0.63–12.8)	~0.35	0.84

95% CI, 95% confidence interval; CFU, colony-forming units.

[a] Minimal relative efficacy: CFU increase (in $\log_{10}$ units) at 24 h from the original inoculum per mg of cell protein as extrapolated for an infinitely low antibiotic concentration using the Hill equation (slope factor = 1). This figure must be interpreted as the bacterial growth that can be observed in the absence of antibiotic.

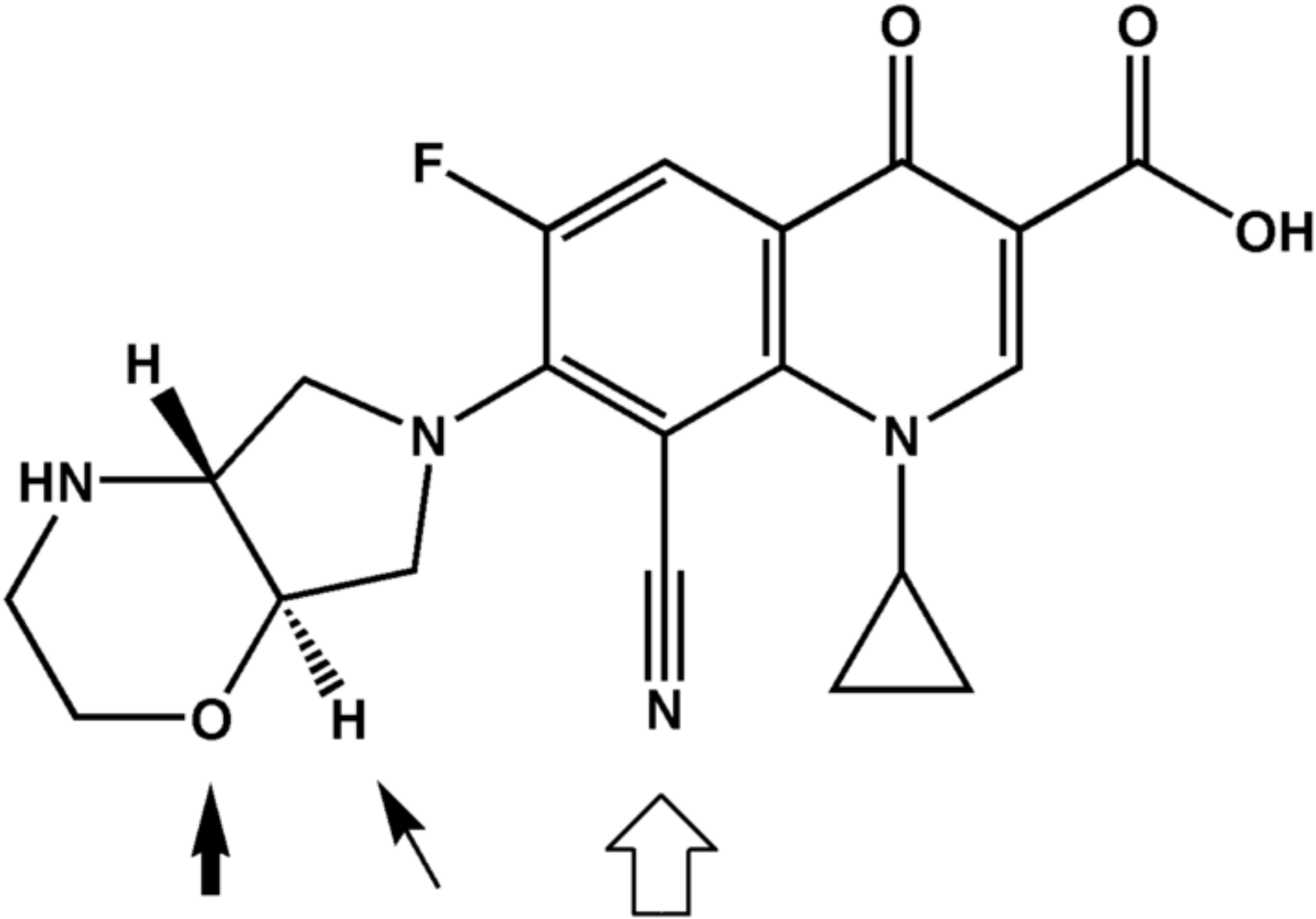
[b] Maximal relative efficacy: CFU decrease (in $\log_{10}$ units) at 24 h from the original inoculum per mg of cell protein as extrapolated for an infinitely large antibiotic concentration using the Hill equation (slope factor = 1). This figure must be interpreted as the maximal antibacterial effect that can be obtained by the antibiotic.

[c] Relative potency: concentration (in mg/L) causing a reduction of the inoculum at 24 h halfway between the minimal (E_{min}) and maximal (E_{max}) relative efficacies as derived from the Hill equation.

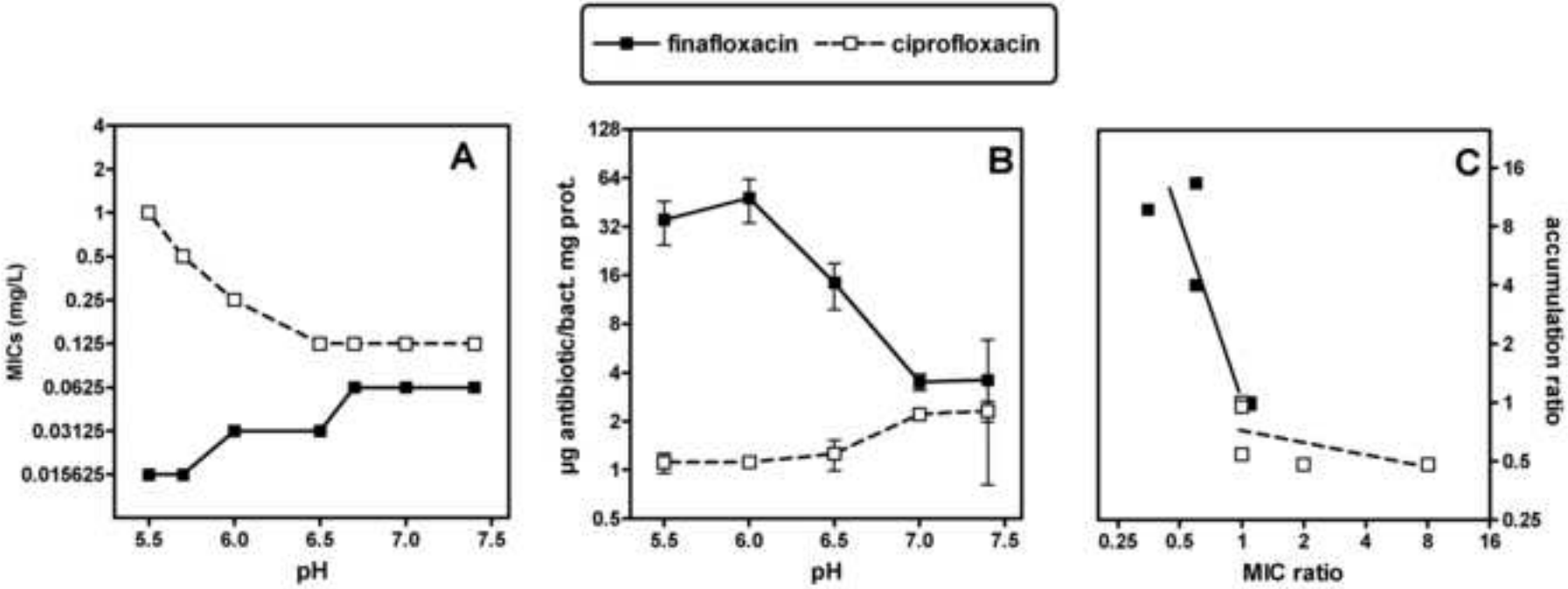
[d] Concentration (in mg/L) resulting in no apparent bacterial growth at 24 h compared with the initial inoculum (time = 0 h), as determined by graphical interpolation using the Hill equation.

Statistical analysis (analysis per row, using raw values for E_{min} and E_{max}, and log-transformed values for EC_{50}): values with different upper case letters are significantly different ($P < 0.05$) from each another within the pertinent comparison group (finafloxacin versus ciprofloxacin for E_{min}, E_{max} and EC_{50}, respectively; unpaired, two-tailed *t*-test).

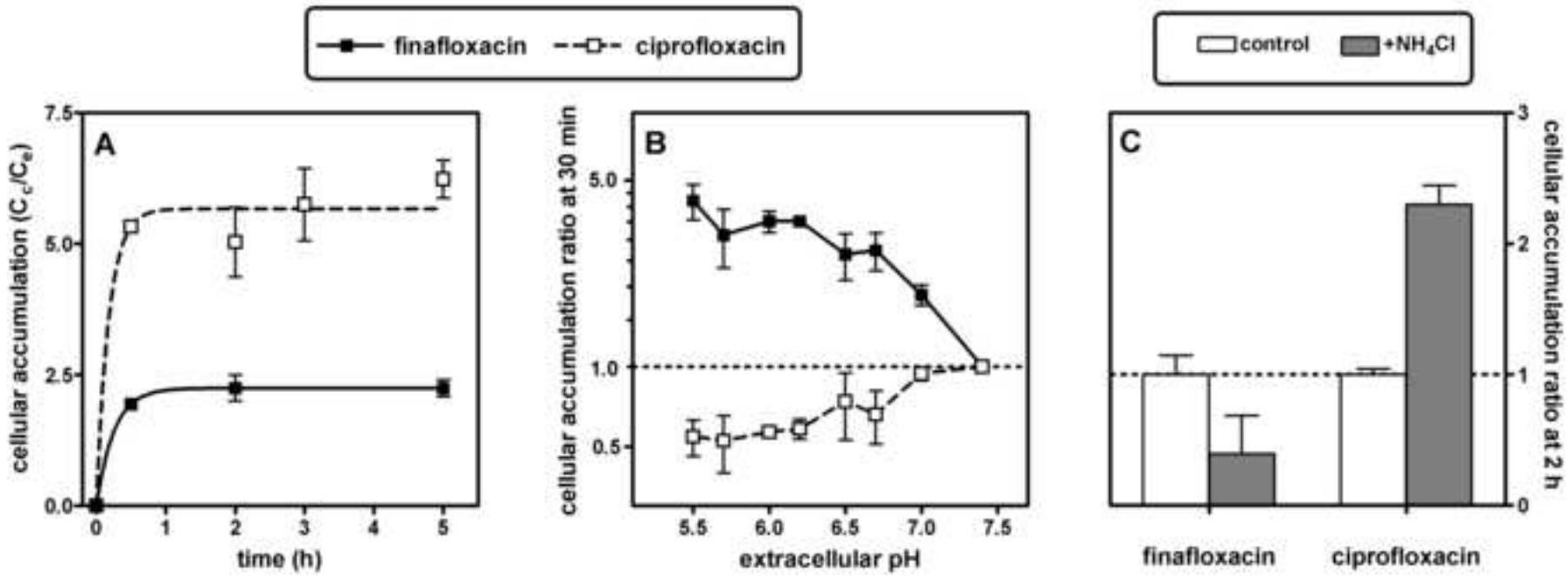
Edited Figure 1



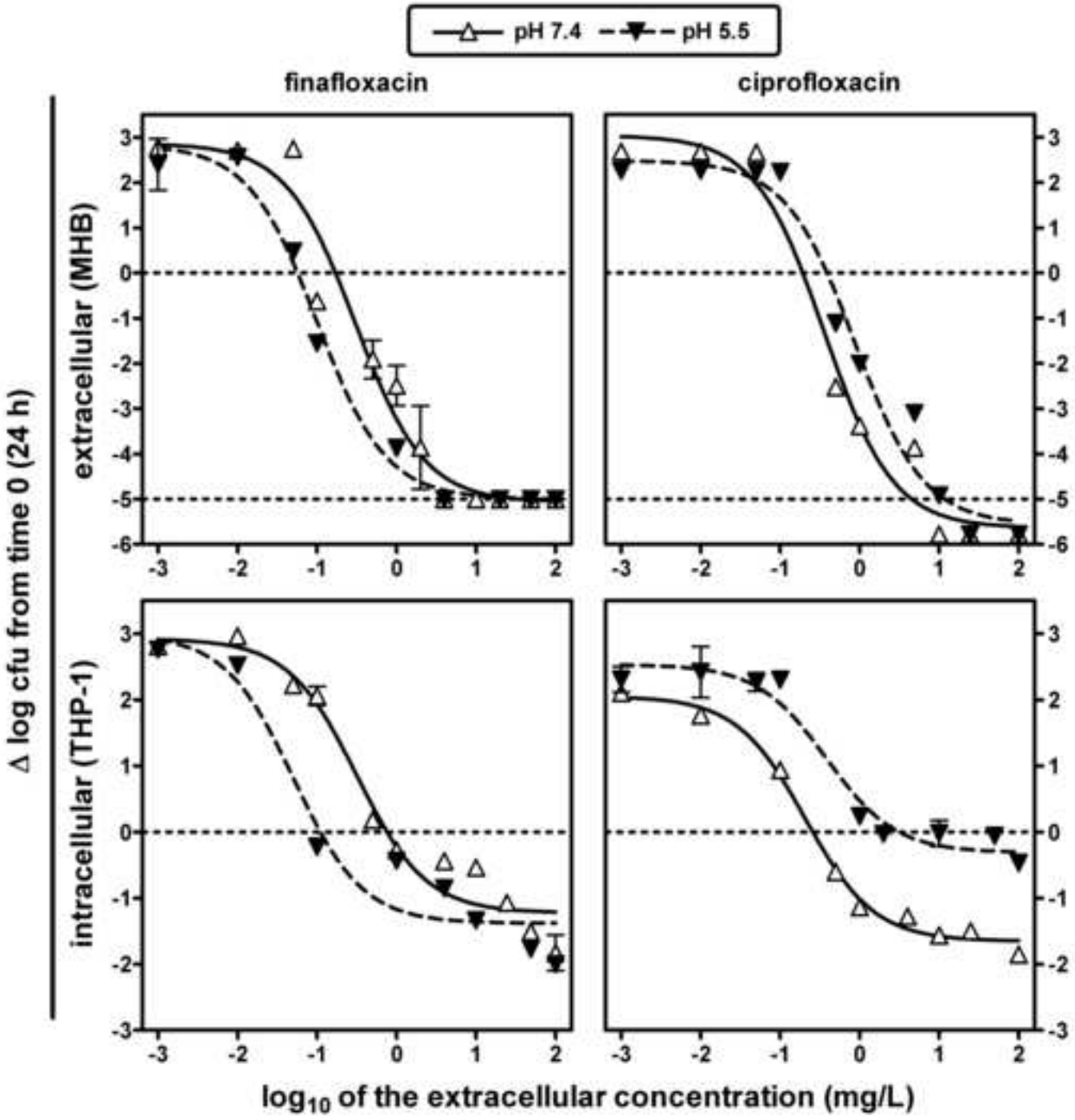
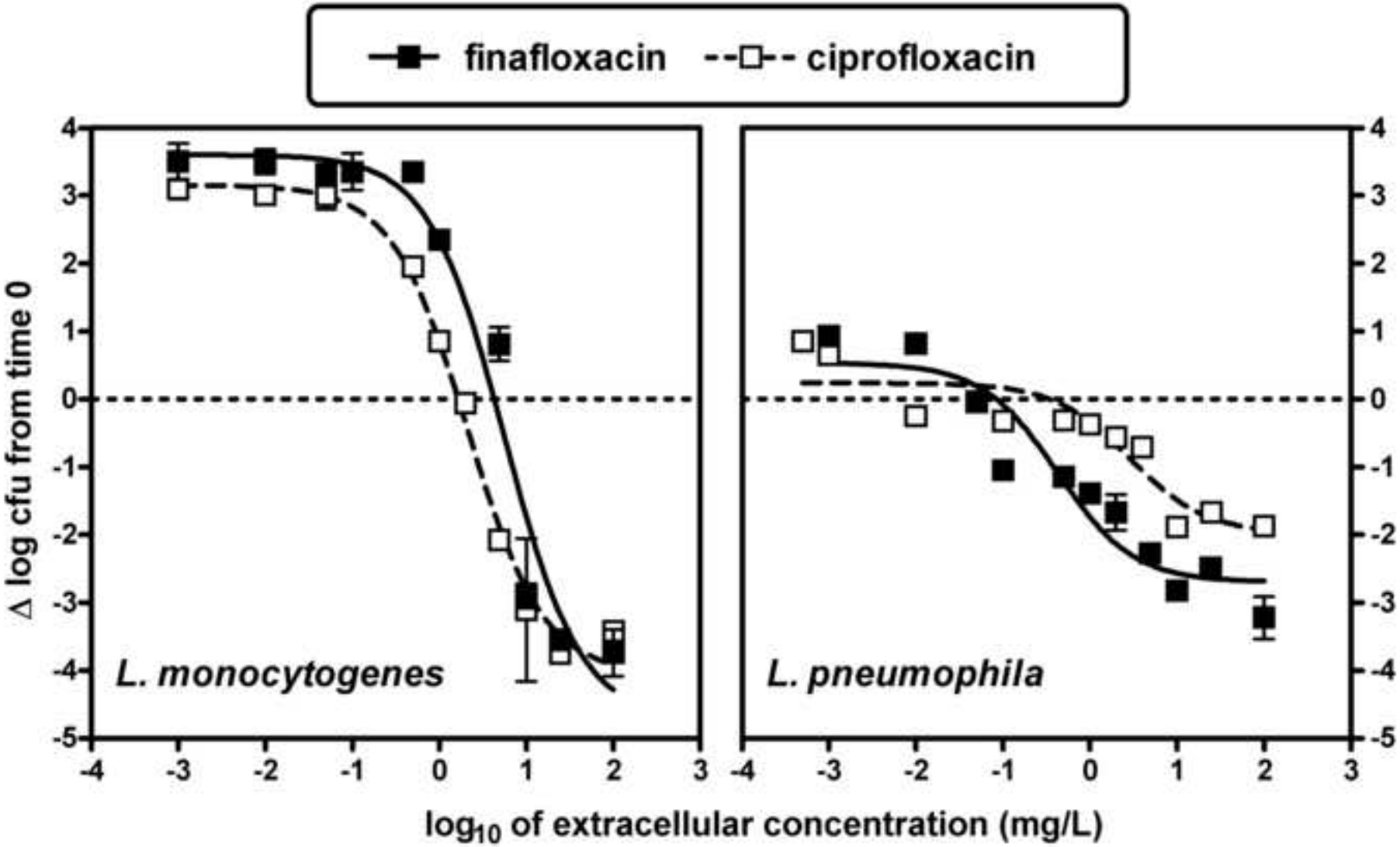
Edited Figure 2



Edited Figure 3



ACCEPTED MANUSCRIPT



Edited Figure 5