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Influence of pH on the activity of finafloxacin against extracellular and intracellular *Burkholderia thailandensis, Yersinia pseudotuberculosis*, and *Francisella philomiragia* and on its cellular pharmacokinetics in THP-1 monocytes

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ABSTRACT

Objectives: *Burkholderia pseudomallei, Yersinia pestis*, and *Francisella tularensis* are facultative intracellular bacteria causing life-threatening infections. We have (i) compared the activity of finafloxacin (fluoroquinolone in development showing improved activity at acidic pH) with that of ciprofloxacin, levofloxacin, and imipenem against the extracellular and intracellular (THP-1 monocytes) forms of infection by attenuated surrogates of these species (*B. thailandensis*, *Y. pseudotuberculosis*, *F. philomiragia*) (ii) assessed finafloxacin cellular pharmacokinetics (accumulation, distribution, efflux).

Methods: Bacteria in broth or in infected monocytes were exposed to antibiotics at pH 7.4 or 5.5 for 24h. Maximal relative efficacies (E_{max}) and static concentrations (C_s) were calculated using the Hill equation (concentration-response curves). Finafloxacin pharmacokinetics in cells at pH 7.4 or 5.5 was investigated using [^{14}C]-labelled drug.

Results: Extracellularly, all drugs sterilized the cultures, with finafloxacin being 2-6 times more potent at acidic pH. Intracellularly, E_{max} reached the limit of detection (4-5 $\log_{10}$ cfu decrease) for finafloxacin against all species, but only against *B. thailandensis* and *F. philomiragia* for ciprofloxacin and levofloxacin, while imipenem caused less than 2 $\log_{10}$ cfu decrease for all species. At acid pH, C_s shifted to 2-5 times lower values for finafloxacin and to 1-4 times higher values for the other drugs. Finafloxacin accumulated in THP-1 cells by approximately 5-fold at pH 7.4 but up to 20-fold at pH 5.5, and distributed in the cytosol.

Conclusions: Fluoroquinolones have proven to be effective in reducing the intracellular reservoirs of *B. thailandensis*, *Y. pseudotuberculosis*, and *F. philomiragia*, with finafloxacin demonstrating an additional advantage in acidic environments.

Introduction

Burkholderia pseudomallei, Yersinia pestis, and *Francisella tularensis* are potential biothreat agents [1;2] causing lethal infections (melioidosis, plague, and tularemia) and requiring potent, rapidly effective antibiotics. First line therapies include ceftazidime or carbapenems for melioidosis [2], aminoglycosides for plague and tularemia, and fluoroquinolones as alternatives for the latter two diseases [1]. All three pathogens are facultative intracellular organisms, thriving in the cytosol [3], the phagolysosomes [4] or in both [5]. Validated *in-vitro* pharmacodynamic models [6] have demonstrated that β-lactams and aminoglycosides fail to eradicate intracellular bacteria, irrespective of their subcellular localization [6-8]. Fluoroquinolones display better intracellular efficacy [7-10] but have not been evaluated in pharmacodynamic models against these biothreat agents. Here, we compare three fluoroquinolones (finafloxacin, ciprofloxacin, and levofloxacin) with imipenem for activity in human THP-1 monocytes [10] infected by *Burkholderia thailandensis*, *Yersinia pseudotuberculosis*, and *Francisella philomiragia*, which are appropriate surrogates for the corresponding biothreat agents with regards to their intracellular fate [11-13]. Finafloxacin has been shown to have enhanced activity at mild acidic pH [14;15], which could confer an advantage when considering bacteria sojourning in phagolysosomes and other acidic microenvironments [16]. We also studied the cellular pharmacokinetics of finafloxacin.

Materials and Methods

Antibiotics

Levofloxacin and imipenem were procured as TAVANIC® and TIENAM®, respectively. Finafloxacin HCl (potency, 84.19%) was from MerLion Pharmaceuticals GmbH (Berlin, Germany), ciprofloxacin (potency, 98%) from Sigma-Aldrich (St-Louis, MO), and gentamicin sulfate (potency, 60.7%) from VWR, Radnor, PA. [^{14}C]-labelled finafloxacin (specific activity 57 mCi/mmol) was produced by Pharmeron UK Ltd, Cardiff, UK.

Bacteria and culture media

B. thailandensis ATCC 700388, *Y. pseudotuberculosis* ATCC 29833, and *F. philomiragia* ATCC 25015 were from the American Type Culture Collection (Manassas, VA). *B. thailandensis* and *F. philomiragia* were grown on Schaedler agar (Sigma-Aldrich), and *Y. pseudotuberculosis* on trypticase soy or Mueller-Hinton agar (BD Life Sciences, Franklin Lakes, NJ). Mueller-Hinton cation-adjusted broth (CA-MHB; BD Life Sciences) was used for the 3 species. Cell culture media and sera were from Gibco/ThermoFisher Scientific (Waltham, MA).

MIC determination

MICs were determined by broth microdilution in CA-MHB at pH 7.4 or adjusted to pH 6.5 or 5.5 by addition of HCl.

Extracellular antibiotic activity

Concentration-kill curves were determined after 24h of incubation in CA-MHB adjusted to pH 7.4 or 5.5 using a starting inoculum of 10^6 cfu/mL (see **Figure S1**) and antibiotic

concentrations ranging from 0.003 to 100xMIC. Serially diluted aliquots were plated on agar containing 0.4% activated charcoal to adsorb residual antibiotic.

Intracellular models of infection

We adapted the protocol developed for *P. aeruginosa* and THP-1 monocytes [10] (see **Figure S2** for details and model validation). Bacteria (opsonized with human serum) were added to monocytes to obtain suitable bacteria:cell ratios, and incubated at 37°C in 5% CO_2 to allow for phagocytosis. Cells were pelleted by centrifugation, washed, and incubated for 1h with gentamicin at high concentration to kill non-internalized bacteria. Cells were re-pelleted, washed, and resuspended in culture medium. The integrity of the pericellular membrane of THP-1 cells was evaluated (trypan blue), and the remaining sample pelleted, resuspended in water (to lyse cells) and used for protein assay [17] and cfu counting (by plating onto charcoal-supplemented media).

Intracellular antibiotic activity

Infected cells were incubated for 24h with antibiotics over a range of concentrations (0.003 to 100xMIC) in media adjusted to pH 7.4 or 5.5, following which the cells were collected and their integrity, protein content and cfu counts measured as described above.

Finafloxacin pharmacokinetics in THP-1 cells

We used a published protocol [18] with media buffered at pHs ranging from 5.5 to 7.4 and [^{14}C]-labelled finafloxacin (mixed with unlabeled drug to achieve the desired total concentration). For influx studies, cells were incubated with finafloxacin at 37°C, pelleted by centrifugation, washed in cold PBS, pelleted again (at 4°C) and resuspended in distilled water. They were sonicated and used for radioactivity determination (by scintillation counting) and protein assay. For efflux studies, cells were first incubated with finafloxacin for 2h,

pelleted by centrifugation, washed in ice-cold PBS, pelleted again at 4°C, resuspended and then incubated at 37°C in drug-free media (adjusted to the same pH as when studying influx). They were finally collected and used as for the accumulation studies. Drug accumulation (apparent cellular to extracellular concentration ratio) was calculated using a conversion factor of 5 µL of cell volume per mg of cell protein [7].

Finafloxacin subcellular distribution in uninfected and infected THP-1 cells

Uninfected THP-1 cells were incubated with [^{14}C]-labelled finafloxacin for 30 min, washed, and resuspended in ice-cold 0.25 M sucrose–3 mM Na EDTA–3 mM imidazole (pH 7.4). For infected cells, monocytes were first allowed to phagocytize bacteria (as described above), washed, returned to fresh medium for 2h (to allow for complete bacteria internalization), and incubated for 30 min with [^{14}C]-labelled finafloxacin at a sub-MIC concentration. After washing, they were resuspended in sucrose-EDTA-imidazole for fractionation. The homogenization method, centrifugation conditions (to yield 3 fractions [N: nuclei and unbroken cells; MLP: mitochondria, lysosomes, membranes and other organelles; and S: supernate (cytosol)]), and assay techniques (radioactivity, protein, activity of marker enzymes (cytochrome *c*-oxidase [mitochondria], N-acetyl-β-hexosaminidase [lysosomes], lactate dehydrogenase [cytosol]) were previously described [19].

Finafloxacin accumulation in bacteria

Bacterial suspensions (10^9 cfu/mL) were incubated with [^{14}C]-labelled finafloxacin (1.4 mg/L; 0.2 mCi/L [required for accurate radioactivity detection]), collected by centrifugation (4000 rpm; 7 min) at 4°C and washed 3 times with cold PBS. Aliquots were taken for cfu counts and radioactivity measurement (after lysis by 2 cycles of freezing/thawing (-80°C - room temperature). To mitigate artefacts caused by drug binding to killed bacteria, only

experimental points with <0.5 log cfu reduction of viable bacteria compared to controls were considered.

Curve fitting and statistical analysis

Statistical analyses and curves fitting were performed using GraphPad Prism version 8.1.1 (GraphPad Software, San Diego, CA). Pharmacodynamic parameters were calculated based on Hill equations of concentration-response curves.

Results

Antimicrobial susceptibility

Table S1 shows MICs at pHs ranging from 7.4 to 5.5. The values for finafloxacin decreased upon media acidification while those of the other drugs increased.

Antibiotic activity against extracellular bacteria

Figure 1 shows the concentration-effect relationships for antibiotics against the 3 bacteria in CA-MHB at pH 7.4 or pH 5.5, with the corresponding pharmacodynamic parameters shown in **Table 1**. All antibiotics were highly bactericidal, with cfu counts below the limit of detection at low multiples (2-6-fold) of their MIC. At pH 5.5, the concentration-response curve for finafloxacin was shifted to the left (lower C_s, denoting a greater potency) but to the right (higher C_s, denoting a lower potency) for the other antibiotics (see **Figure S3** for a comparison of C_s values). Incidentally all fluoroquinolones seemed almost equipotent against *F. philomiragia* at pH 5.5 when C_s were expressed in mg/L, but not in multiples of MIC.

Antibiotics activity against intracellular bacteria

Figure 2 shows the concentration-response curves against intracellular bacteria using culture media adjusted to pH 7.4 or pH 5.5, with the corresponding pharmacodynamic parameters presented in **Table 1**. No major loss in cell viability was observed. *F. philomiragia* did not multiply in cells during the 24h incubation. Addition of L-cysteine (promoting intracellular growth of *F. tularensis* [20]) did not improve our results (**Figure S4**) and was, therefore, not implemented.

At neutral pH and for all 3 species, fluoroquinolones were highly bactericidal, with cfu counts below the detection limit at an extracellular concentration of about 10 times their MICs.

Imipenem was much less potent than against extracellular bacteria (C_s > its MIC) and was not bactericidal (E_{max} never < -1.7 $\log_{10}$ cfu).

At pH 5.5, finafloxacin was more potent (lower C_s) than at neutral pH and as effective against all 3 bacteria (cfus below the detection level). The other fluoroquinolones and imipenem were less potent (higher C_s) against all bacteria. Levofloxacin and ciprofloxacin were less effective than finafloxacin against *Y. pseudotuberculosis* (E_{max} above the limit of detection) and imipenem was poorly active against *B. thailandensis* and *Y. pseudotuberculosis* and bacteriostatic against *F. philomiragia*.

Finafloxacin pharmacokinetics in THP-1 cells

Figure 3 (upper panels) shows the accumulation and efflux kinetics of finafloxacin in cells incubated at pH 7.4 or 5.5. Finafloxacin uptake was very rapid at neutral pH and quasi-instantaneous at acidic pH, reaching apparent cellular to extracellular concentration ratios of approximately 5 and 18 at neutral and acidic pH, respectively. Efflux proceeded at a similar rate as influx at neutral pH, but was markedly slower at acidic pH. **Figure 3** (lower left panel) shows that the cellular accumulation of finafloxacin increased almost linearly when the pH of the medium was reduced from 7.4 to 5.5. **Figure 3** (lower right panel) shows that finafloxacin accumulation was almost constant at both pHs up to an extracellular concentration of 100 mg/L (and 350 mg/L at pH 5.5 [not shown]).

Finafloxacin subcellular distribution in uninfected and infected THP-1 cells

Figure 4 shows the distribution of [^{14}C]-labelled finafloxacin and of markers of mitochondria (cytochrome *c*-oxidase), lysosomes (N-acetyl-β-hexosaminidase) and cytosol (lactate dehydrogenase) between the isolated fractions. Lactate dehydrogenase was mainly recovered in the S fraction, and the two other enzymes in the MLP fraction. A similar

155 proportion of all enzymes was also found in the N fraction, corresponding most probably to
156 unbroken cells. The distribution of finafloxacin was very similar to that of lactate
157 dehydrogenase, suggesting a distribution in the cytosol, which was similar at both pHs. This
158 experiment was then performed with cells infected with *B. thailandensis*. Again finafloxacin
159 was mainly recovered in the S fraction (**Figure S5**) while bacteria were recovered in the MLP
160 fraction (probably sedimenting with the organelles due to their high density).
161
162 **Finafloxacin accumulation in bacteria**
163 Accumulation levels were variable between experiments (2-4 fold inter-experiment
164 difference; n = 2 to 4) but accumulation at pH 5.5 was consistently larger than at pH 7.4 (1.2-
165 2 fold for *B. thailandensis,* 1.5 to 2.2-fold for *Y. pseudotuberculosis* and 2.3-2.5-fold for
166 *F. philomiragia* (significant only for the two latter species [t-test; $p < 0.05$]).

Discussion

This study evaluates the activity of three fluoroquinolones and imipenem against the extracellular and intracellular forms of surrogates of three biothreat pathogens, using a pharmacodynamic model validated with a large array of bacterial species and panels of antibiotics [7;9;10;18;19;21;22]. While all investigated drugs were highly effective against the extracellular forms of these bacteria, the fluoroquinolones were also very effective against their intracellular forms (with finafloxacin being superior to levofloxacin and ciprofloxacin) but imipenem was largely ineffective. For finafloxacin, we included measurements of its accumulation within bacterial cells and its distribution in THP-1 monocytes. Five main observations deserve in-depth analysis.

Firstly, finafloxacin demonstrated an increased activity at low pH while ciprofloxacin, levofloxacin and imipenem lost activity in acidic media. Similar observations have been made with other bacteria [9;14;15;23;24], but we show here it is associated with an increased accumulation of the drug within bacteria. We tentatively ascribe this to a higher proportion of zwitterionic form of finafloxacin at acid pH (see **Table S2** and **Figure S6** for chemical structures and comparative ionization data).

Secondly, in contrast to other zwitterionic fluoroquinolones [25;26], finafloxacin accumulates more rapidly and to a greater extent in cells incubated in an acidic medium. Here also, this could be ascribed to its higher proportion of zwitterionic species. At neutral pH, efflux proceeded at a rate similar to that of influx, suggesting diffusion as the only process involved (as for moxifloxacin [26]). In contrast, finafloxacin efflux was much slower than influx at pH 5.5. The intervention of an influx transporter active at acidic pH seems unlikely as there was no saturation in finafloxacin accumulation at equilibrium over a broad

range of extracellular concentrations. Slow apparent efflux at pH 5.5 may therefore result from an immediate reuptake of released molecules that become zwitterionic again when diffusing out of the cells, reaching an acidic environment.

Thirdly, all three fluoroquinolones were highly effective against the three species of intracellular bacteria evaluated here, but this was not the case for imipenem, especially considering the phagolysosomal *Y. pseudotuberculosis*. Interestingly, the E_{max} values for imipenem were of the same order of magnitude as previously reported for meropenem against other cytosolic (*Listeria monocytogenes*) and phagolysosomal (*Staphylococcus aureus*) bacteria [27]. Among fluoroquinolones, only finafloxacin reduced the cfu below the limit of detection at both neutral and acidic pH, and for extracellular concentrations of only 10xMIC. Such an efficacy seems unique to the bacterial species studied here as it has not been observed for finafloxacin and other fluoroquinolones against several other cytosolic or phagolysosomal bacteria [9;21;28]. Artefacts related to the release of bacteria out of the cells or to the carry-over of antibiotics accumulated in cells can most likely be excluded considering that (a) imipenem demonstrates only modest activity including at high concentrations; (b) the integrity of the pericellular membrane was not compromised; and (c) charcoal was added to the culture plates to adsorb residual antibiotic.

Fourthly, all 3 fluoroquinolones were equipotent against intracellular bacteria at neutral pH (C_s close to their MIC), although demonstrating different levels of cellular accumulation [9;21]. A similar observation was made when comparing other fluoroquinolones with variable accumulation levels against other intracellular bacteria, and has been ascribed to commensurate differences in intracellular bioavailability [28]. Conversely, finafloxacin demonstrated both an increased relative potency (lower C_s) and a higher cellular

accumulation when experiments were conducted at acidic pH rather than at a neutral pH, linking activity to accumulation levels in these conditions.

Lastly, finafloxacin, as other fluoroquinolones [21;28], showed similar intracellular activity against bacterial species localized in different subcellular compartments, although being predominantly recovered in the cell supernate in both uninfected and infected cells. Similar observations have been made earlier for other antibiotics from different pharmacological classes but which, like finafloxacin, show a rapid influx and efflux from THP-1 cells [18;19], suggesting that they all easily diffuse through membranes. Since fluoroquinolones are not irreversibly bound to their bacterial target, we are probably witnessing a dissociation of finafloxacin from its site of action due to the extensive dilution of the samples taking place during the cell homogenization and fractionation procedures. This would also imply that finafloxacin can easily move across both intracellular and bacterial membranes, explaining why it may exert antibacterial activity against bacteria located in different subcellular compartments. This is in contrast to less diffusible drugs such as oritavancin, which enters eukaryotic cells by endocytosis but is thereafter restricted to phagolysosomes, acting easily upon *S. aureus* located within these organelles but not against cytosolic *L. monocytogenes* [22].

There are two recognized limitations from this work. Firstly, for evident safety reasons, we used surrogates of the biothreat pathogens, and could therefore not take into account a possible influence of specific virulence factors in the response from these human pathogens to antibiotics. Secondly, we used a single phagocytic cell type, which does not necessarily represent the repertoire of cells infected *in vivo* by these bacteria, but it was selected as a permissive cell line allowing to assess the antibiotic effect without interference of cell defense mechanisms. Yet, taken globally, our data provide strong support for the further evaluation

of finafloxacin against the intracellular forms of these biothreat pathogens. They also help rationalizing the recently demonstrated efficacy of this fluoroquinolone in animal models of infection with *B. pseudomallei* and *F. tularensis* [29;30].

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Table 1. MIC and extracellular and intracellular pharmacodynamic parameters as calculated from the Hill equation of the concentration-response curves shown in Figures 1 and 2.

Species	pH of medium	Antibiotics											
		Finafloxacin			Levofloxacin			Ciprofloxacin			Imipenem		
		MIC[a]	C_s[b]	E_{max}[c]	MIC	C_s	E_{max}	MIC	C_s	E_{max}	MIC	C_s	E_{max}
EXTRACELLULAR													
B. thailandensis	7.4	4	3.1	-5*	4	2.11	-5*	4	1.4	-5*	0.5	0.2	-5*
	5.5	1	0.9	-5*	32	6.6	-5*	32	14.8	-5*	8	6.5	-5*
Y. pseudotuberculosis	7.4	0.25	0.18	-5*	0.12	0.05	-5*	0.06	0.03	-5*	0.25	0.10	-5*
	5.5	0.12	0.03	-5*	2	0.29	-5*	1	0.41	-5*	8	4.17	-5*
F. philomiragia	7.4	0.06	0.026	-5*	0.03	0.01	-5*	0.015	0.006	-5*	0.25	0.15	-4.8
	5.5	0.008	0.01	-5	0.12	0.02	-5*	0.06	0.01	-5*	32	10	-5*
INTRACELLULAR													
B. thailandensis	7.4	4	1.6	-5*	4	1.8	-5*	4	1	-5*	0.5	0.7	-1.7
	5.5	1	0.7	-5*	32	6.3	-5*	32	3.7	-5*	8	2.2	-1.5
Y. pseudotuberculosis	7.4	0.25	0.15	-5*	0.12	0.06	-4.6	0.06	0.08	-3.3	0.25	1.9	-0.8
	5.5	0.12	0.03	-5*	0.12	0.18	-3.7	1	0.14	-3.6	0.25	2.10	-0.9
F. philomiragia	7.4	0.06	ND[d]	-4*	0.03	ND	-4*	0.015	ND	-4*	0.25	ND	-1.8
	5.5	0.008	ND	-4*	0.12	ND	-4*	0.06	ND	-4*	32	ND	-0.1

[a] MIC: minimal inhibitory concentration in broth at pH 7.4 or 5.5 (mg/L; data from Table 2 reproduced here for sake of easy comparison with C_s values))

[b] C_s: apparent static concentration, *i.e.* the extracellular concentration (mg/L) resulting in no apparent bacterial growth (number of cfu identical to the initial [extracellular] or post-phagocytosis [intracellular] inoculum)

[c] E_{max}: relative maximal efficacy, *i.e.* the inoculum decrease (in $\log_{10}$ units) compared to the initial (extracellular) or post-phagocytosis (intracellular) inoculum as extrapolated for an infinitely large antibiotic concentration. When cfu counts fell below the lowest detection level within the range of extracellular drug concentrations investigated, E_{max} was arbitrarily set at -5 (or -4 for intracellular *F. philomiragia*) and marked with an *.

[d] ND: not determinable (the intracellular inoculum at 24h is lower than the initial inoculum).

Figure 1: Concentration-response curves of antibiotics against extracellular *B. thailandensis*, *Y. pseudotuberculosis*, or *F. philomiragia* in broth at pH 7.4 (panel A) or pH 5.5 (panel B). The graphs show the changes in the cfu count from the initial inoculum per mL following 24 h of incubation at increasing extracellular concentrations expressed in mg/L (left graph of each panel) or in multiples of the MIC at pH 7.4 and pH 5.5, respectively (right graph of each panel). The thick horizontal dotted line highlights a static effect (C_s) and the thin horizontal dotted line, the lowest limit of detection (cfu decrease of 5 $\log_{10}$ units compared to the initial inoculum). The vertical dotted line shows the MIC on the right graphs of each panel. All data are means ± SD (n=3).

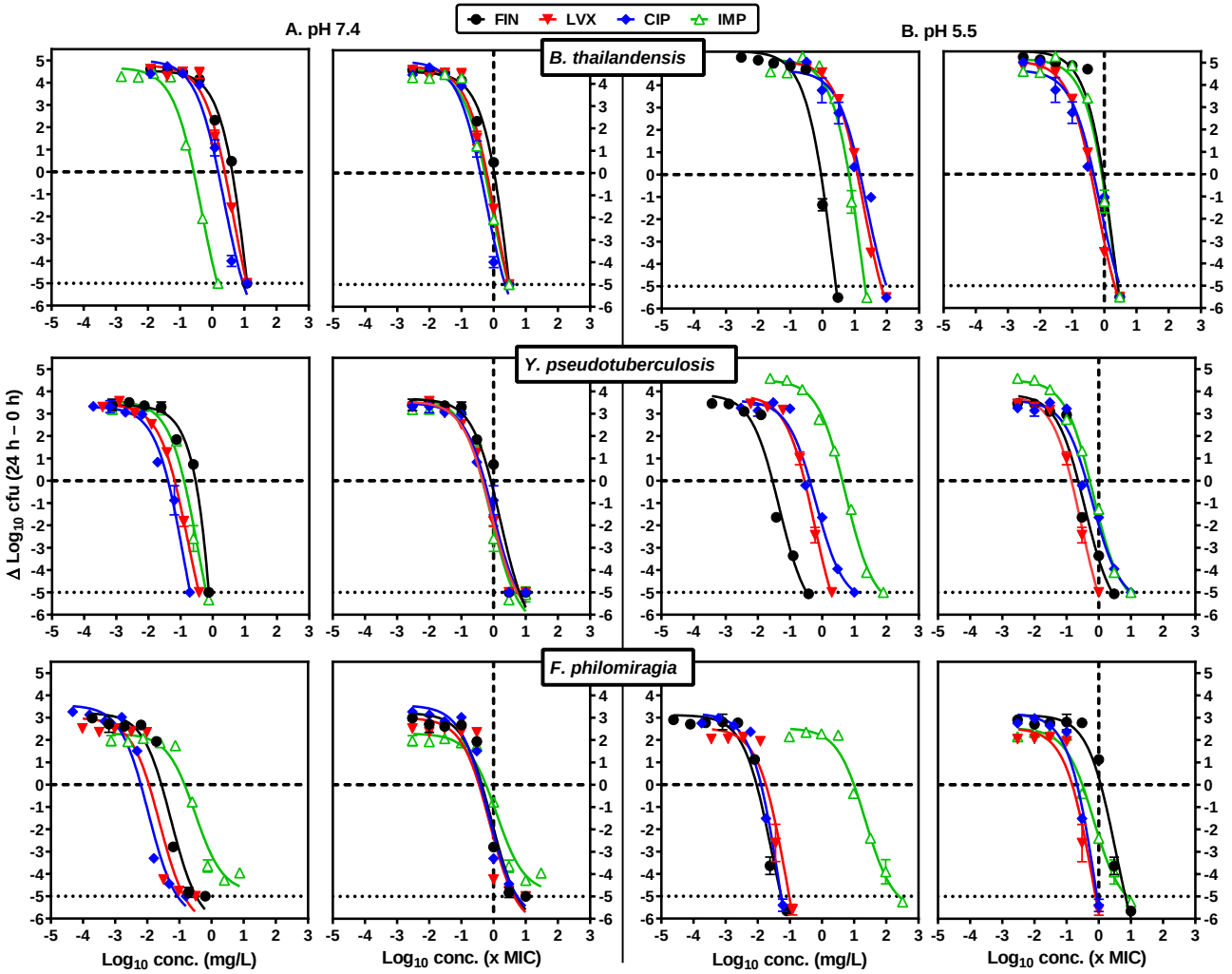

FIN
LVX
CIP
IMP
A. pH 7.4
B. pH 5.5
B. thailandensis
Y. pseudotuberculosis
F. philomiragia
Δ Log10 cfu (24 h – 0 h)
Log10 conc. (mg/L)
Log10 conc. (x MIC)
Log10 conc. (mg/L)
Log10 conc. (x MIC)

Figure 2: Concentration-response curves of antibiotics against intracellular *B. thailandensis* (top), *Y. pseudotuberculosis* (medium), and *F. philomiragia* (bottom) in a model of THP-1 monocytes incubated in a medium at pH 7.4 (panel A) or pH 5.5 (panel B). The graphs show the changes in the cfu counts from the initial inoculum per mg of cell protein following 24 h of incubation at increasing extracellular concentrations expressed in mg/L (left graphs of each panel) or in multiples of the MIC at pH 7.4, respectively (right graph of each panel). The thick horizontal dotted line highlights a static effect (C_s) and the thin horizontal dotted line the lowest limit of detection (cfu decrease of 5 [or 4 for *F. philomiragia*] $\log_{10}$ units compared to the initial inoculum). The vertical dotted line shows the MIC (on the right graphs of each panel). All data are means ± SEM (triplicates from 2 experiments) for *B. thailandensis* and *Y. pseudotuberculosis* and mean ± SD (triplicates from 1 experiment) for *F. philomiragia.*

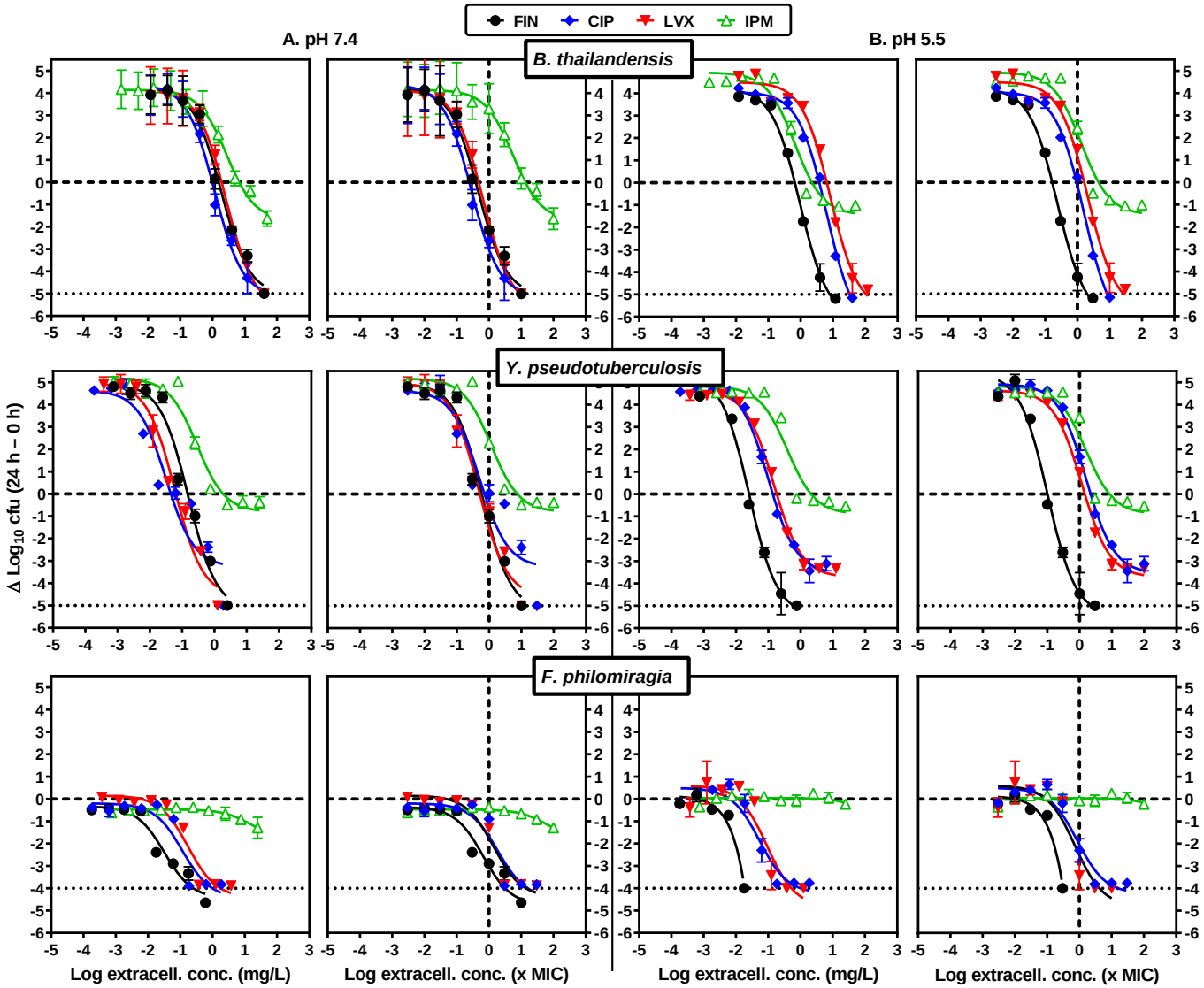

FIN
CIP
LVX
IPM
A. pH 7.4
B. pH 5.5
B. thailandensis
Y. pseudotuberculosis
F. philomiragia
Δ Log10 cfu (24 h – 0 h)
Log extracell. conc. (mg/L)
Log extracell. conc. (x MIC)
Log extracell. conc. (mg/L)
Log extracell. conc. (x MIC)

Figure 3: Cellular pharmacokinetics of finafloxacin in THP-1 cells.

Upper panels: The kinetics of accumulation and efflux of finafloxacin in cells incubated at pH 7.4 or 5.5 with an extracellular concentration of 8 mg/L. Efflux was measured from cells that had been incubated with the drugs for 120 minutes. The data were used to fit one phase exponential association or decay functions with the following calculated rate constants: k_{in} = 0.13 min^{-1} at neutral pH and was too rapid to be calculated at acidic pH; k_{out} = 0.18 min^{-1} at neutral pH and 0.04 min^{-1} at acidic pH.

Lower panels: The influence of the pH of the medium on the apparent cellular accumulation of finafloxacin following 2 h of incubation with an extracellular concentration of 8 mg/L (left) and the influence of the extracellular concentration of finafloxacin on its cellular accumulation in cells incubated for 2 hat neutral or acidic pH (right).

All data are expressed as the apparent cellular accumulation (i.e. the ratio between the cellular and extracellular concentrations); they are mean ± SD (n=3).

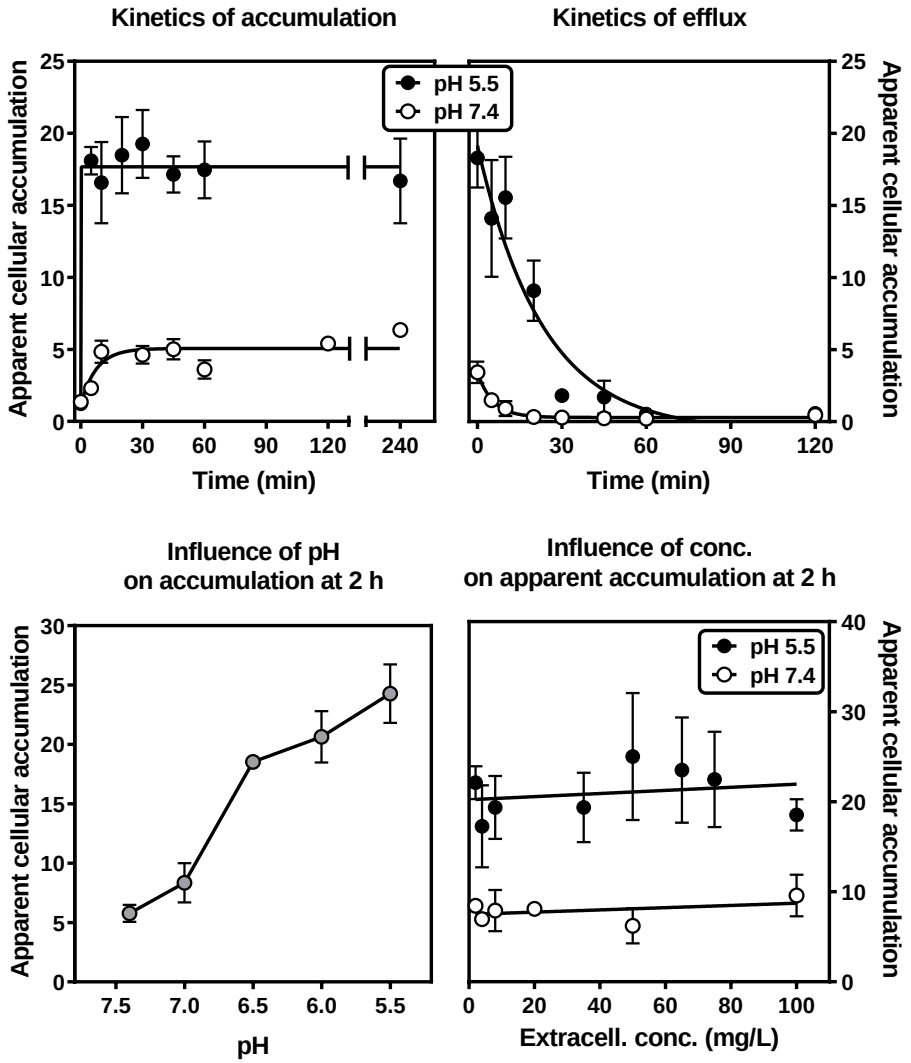
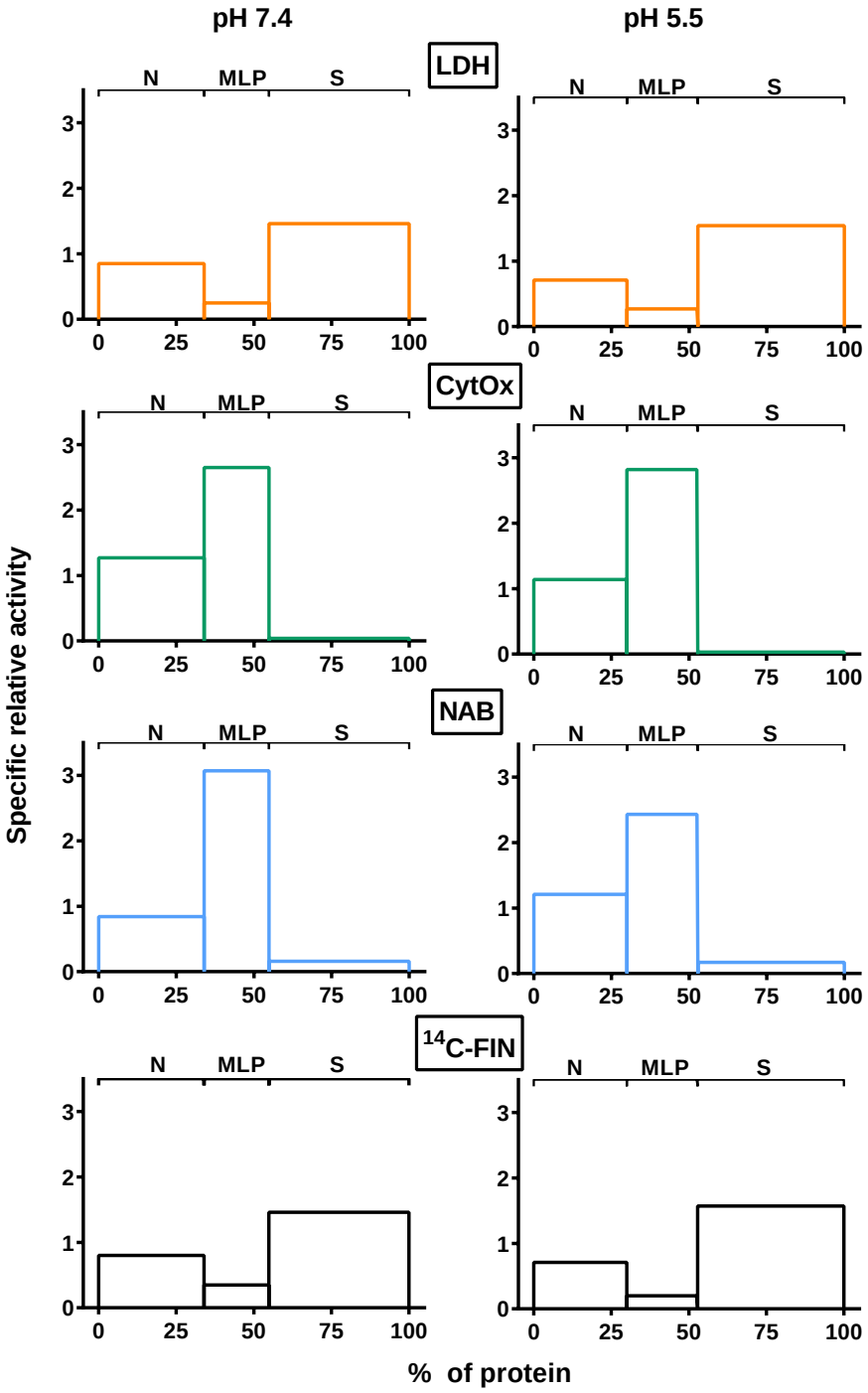


Figure 4: Subcellular distribution of marker enzymes (lactate dehydrogenase [LDH]: cytosol; cytochrome c-oxidase [cytox]: mitochondria; N-acetyl-β-hexosaminidase [NAB]: lysosomes) and of [^{14}C]-finafloxacin [^{14}C-FIN] in homogenates of THP-1 monocytes incubated with 8 mg/L [^{14}C]-finafloxacin in culture media at pH 7.4 (left) or 5.5 (right) for 30 min prior to collection. The homogenates were separated into 3 fractions by centrifugation at increasing centrifugal fields and indicated by letters on the top of the graph (main cytological content: N, nuclei and unbroken cells; MLP, granules [mitochondria, lysosomes, peroxysomes]; S: final supernatant). These are represented by blocks ordered in the same sequence over the abscissa where they span a length proportional to their relative protein content (in % of total cell protein). The height of each block (ordinate) gives the relative activity (for marker enzymes) or specific content (for [^{14}C]-finafloxacin), i.e. the percentage recovered in the fraction divided by the percentage of total cell protein of the same fraction. The height of each block indicates therefore, the enrichment (if > 1) or impoverishment (if < 1) of each constituent in the fraction on a protein basis compared to an unfractionated homogenate, whilst its surface is proportional to the content or activity recovered in the fraction (the total surface of each diagram is = 1).


pH 7.4
pH 5.5
LDH
CytOx
NAB
14C-FIN
N
MLP
S
Specific relative activity
% of protein
0
25
50
75
100
1
2
3

Influence of pH on the activity of finafloxacin against extracellular and intracellular *Burkholderia thailandensis, Yersinia pseudotuberculosis*, and *Francisella philomiragia* and on its cellular pharmacokinetics in THP-1 monocytes

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Supplementary material

Table S1. MICs of antibiotics against *B. thailandensis*, *Y. pseudotuberculosis* and *F. philomiragia* in CA-MHB at pH 7.4, 6.5 and 5.5. MICs were determined at least 3 times in independent experiments.

	MIC (mg/L)								
	Strain								
	B. thailandensis			***Y. pseudotuberculosis***			***F. philomiragia***		
	pH								
Antibiotic	**7.4**	**6.5**	**5.5**	**7.4**	**6.5**	**5.5**	**7.4**	**6.5**	**5.5**
Gentamicin	128	512	>512	1	2	4	0.25	0.5	4
Finafloxacin	4	2	1	0.25	0.12	0.12	0.06	0.03	0.008
Levofloxacin	4	8	32	0.12	0.25	2	0.03	0.03	0.12
Ciprofloxacin	4	8	32	0.06	0.25	1	0.015	0.015	0.06
Imipenem	0.5	1	8	0.25	0.5	8	0.25	2	32

Table S2: Calculated physicochemical properties of the fluoroquinolones[a] [see 1-3]

Property			Fluoroquinolones[b]				
			CIP	**LVX**	**DFX**	**FIN**	**descyano-FIN**
% microspecies [c,d,e]							
	At pH 7.4	Positive $^{+}H_2N$….COOH	1				
		Zwitterionic $^{+}H_2N$….COO^{-}	**95**	17		**63**	**65**
		Negative (HN)….COO^{-}	4	**83**	**99**	37	34
		Neutral (HN) …COOH			1		
	At pH 5.5	Positive $^{+}H_2N$….COOH	**54**	40		8	**53**
		Zwitterionic $^{+}H_2N$….COO^{-}	**46**	**54**		**92**	**46**
		Negative (HN)….COO^{-}		3	**54**		
		Neutral (HN) …COOH		3	**46**		
Isoelectric point [f]			7.4	6.2	2.05	6.1	6.77
pK_as [g]			5.56/8.77	5.35/6.72	5.43/-	4.33/7.63	5.56/7.68
logD [h]							
	At pH 7.4		-0.9	-0.5	0.6	-1.3	-0.7
	At pH 5.5		-1.3	-0.3	2.0	-1.2	-0.9

[a] calculated using Chemicalize™ (Instant Cheminformatics Solutions; ChemAxon Ltd, Budapest, Hungary; available from https://chemicalize.com [accessed on 10 Oct 2018] , which allows direct comparisons between molecules within a given chemical family

[b] see Figure S1 for the corresponding chemical structures. CIP: ciprofloxacin; LVX: levofloxacin; DFX: delafloxacin; FIN: finafloxacin [(-)-8-cyano-1-cyclopropyl-6-fluoro-7-[(4a*S*,7a*S*)-hexahydropyrrolo[3,4-*b*]-1,4-oxazin-6(2*H*)-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylc acid hydrochloride]; descyano-FIN: descyanofinafloxacin [1-cyclopropyl-6-fluoro-7-[(4a*S*,7a*S*)-hexahydropyrrolo[3,4-*b*]-1,4-oxazin-6(2*H*)-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylc acid hydrochloride]

[c] (non) ionized species in solution at the specified pH

[d] NH or NH_2^+ refer to the amino function in position 7 (absent in delafloxacin); and COO^- or COOH to the carboxylic acid function in position 3

[e] value in bold is the most abundant form for each drug at the considered pH.

[f] pH at which the molecule is electrically neutral

[g] acidic/basic dissociation constants

[h] distribution coefficient (partition coefficient at the specified pH)

Figure S1. Relationship between the OD $_{620\ nm}$ and the log_{10} CFU/mL of the bacterial suspension for the three bacterial species in CA-MHB

Bacteria (at an $OD_{620\ nm}$ of 0.1) were inoculated in CA-MHB, and incubated shaking (130 rpm) for 10-12 h. Aliquots were taken every hour and used to determine optical density and cfu count. Correlation curves plotting $OD_{620\ nm}$ and cfus/mL were then used to adjust the inocula in subsequent experiments.

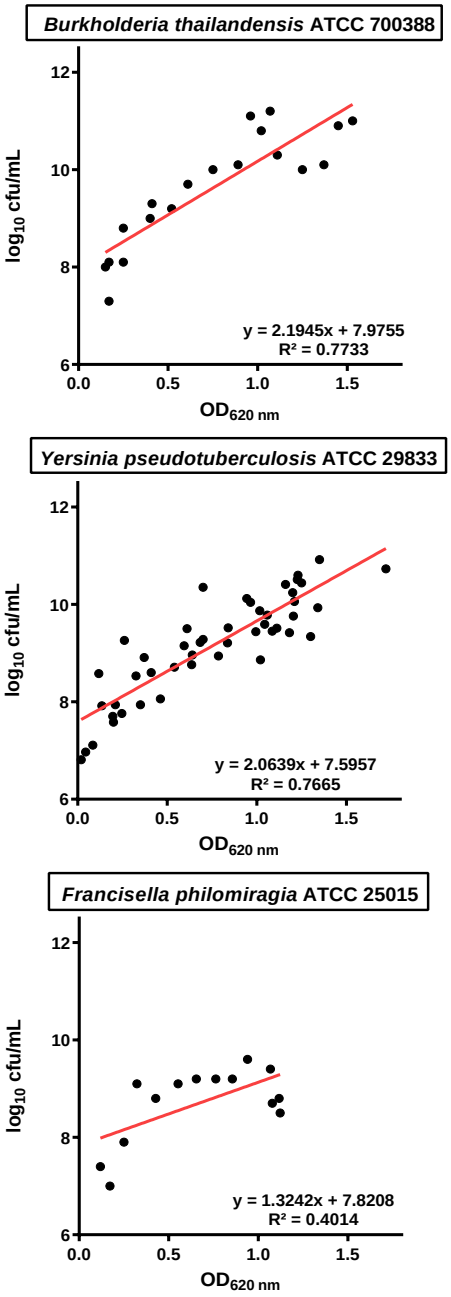
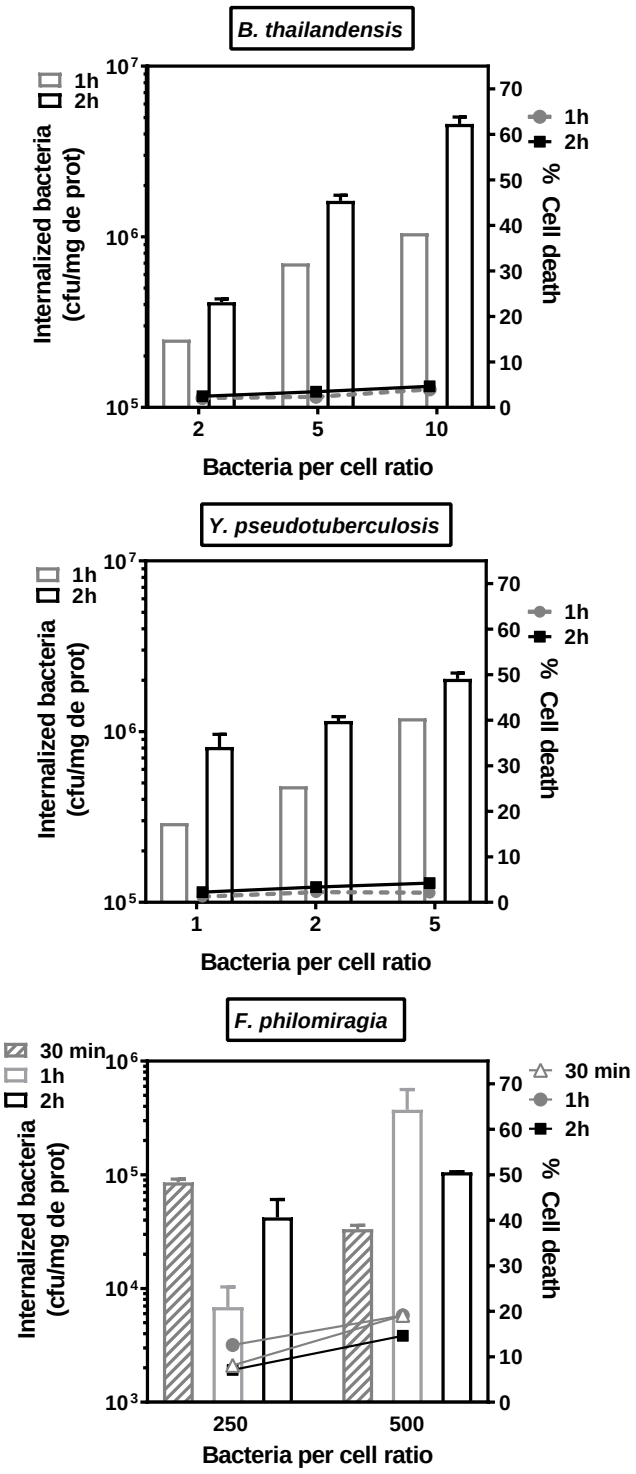


Figure S2: Development of the intracellular models:
The internalization of *B. thailandensis* (top), *Y. pseudo-tuberculosis* (middle), and *F. philomiragia* (bottom) following 30 minutes (hatched bars, bottom panel only), 1 h (open bars with grey border) or 2 h (open bars with black border) of phagocytosis at increasing bacteria-to-cell ratios (left axis) and the percentage mortality of THP-1 cells as determined at the end of the phagocytosis period (right axis; gray line and symbols: 1h phagocytosis; black line and symbols: 2h phagocytosis). All data are means ± SD (n=3).



Detailed protocol : An overnight culture was prepared in 10 mL (for *B. thailandensis* or *Y. pseudotuberculosis*) or 50 mL (for *F. philomiragia*) of CA-MHB. Bacteria were pelleted by centrifugation (4000 rpm, 7 min), resuspended in 10 mL RPMI-1640 supplemented with 10% human serum and incubated for 1h at 37°C, shaking (130 rpm), to allow for opsonisation. Bacteria were pelleted again, resuspended in CA-MHB, added to THP-1 cells (750000 cells/mL) in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) to obtain suitable bacteria:cell ratios, and incubated at 37°C in 5% CO_2 to allow for phagocytosis. Cells were then pelleted by centrifugation (7 min, 1300 rpm), washed with Phosphate Buffer Saline (PBS), and incubated for 1h with gentamicin (16xMIC for *B. thailandensis*; 100xMIC for both *Y. pseudotuberculosis* and *F. philomiragia*) to kill non-internalized bacteria. Cells were again pelleted by centrifugation, washed once with PBS, and resuspended in the same medium.

Data description : The aim when developing these intracellular models is to obtain a sufficiently large intracellular inoculum (approx. 10^6 cfu/mg cell protein) for use in these assays whilst minimizing cytotoxicity related to bacterial virulence. As shown in the figure low inocula yielded sufficient internalization for *B. thailandensis* and *Y. pseudotuberculosis*, but much larger ones (also conferring more cytotoxicity) were required for *F. philomiragia* (which is known to be poorly internalized [4-6]). We finally selected bacteria:cell ratios of 10 for *B. thailandensis*, 5 for *Y. pseudotuberculosis*, and 500 for *F. philomiragia*, with a phagocytosis period of 1 h for all species. This yielded the desired intracellular inoculum with little cytotoxicity for *B. thailandensis* and *Y. pseudotuberculosis* and a lower intracellular inoculum with limited (20%) cytotoxicity for *F. philomiragia.*

Figure S3: comparison of the relative potency (C_s) of antibiotics against bacteria in broth (left; extracellular) or in THP-1 cells (right; intracellular) in media at neutral and acidic pH. C_s values were calculated from the Hill equation of the concentration-response curves shown in Figures 1 and 2 (see also Table 1 for these values). The Y axis in the graphs shows the ratio between the C_s value at neutral pH and at acidic pH for each bacterium and each antibiotic), expressed in log scale. A positive value therefore corresponds to a decrease in C_s upon acidification of the medium (higher relative potency) and a negative value, to an increase in C_s upon acidification of the medium (lower relative potency). ND: not determined because a static effect was not reached against *F. philomiragia* intracellularly.

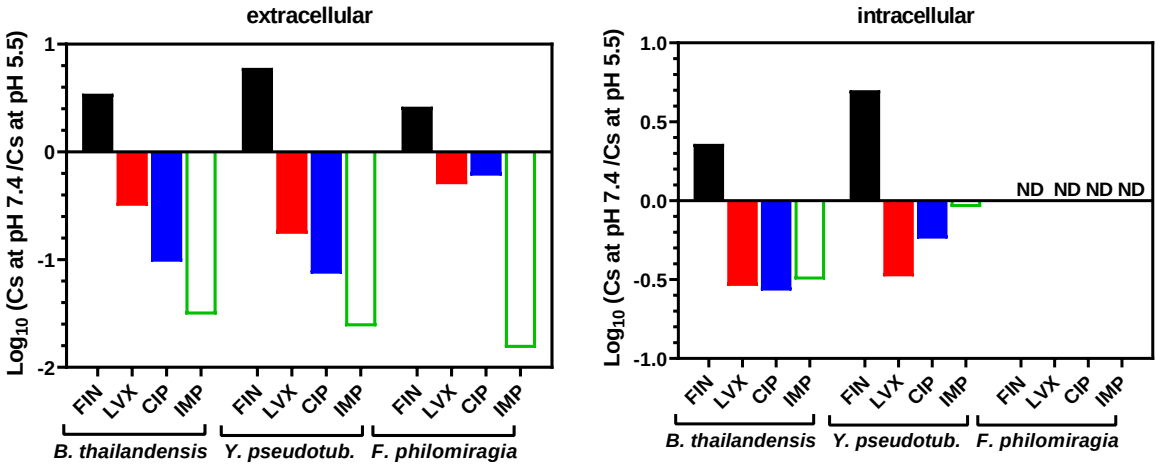
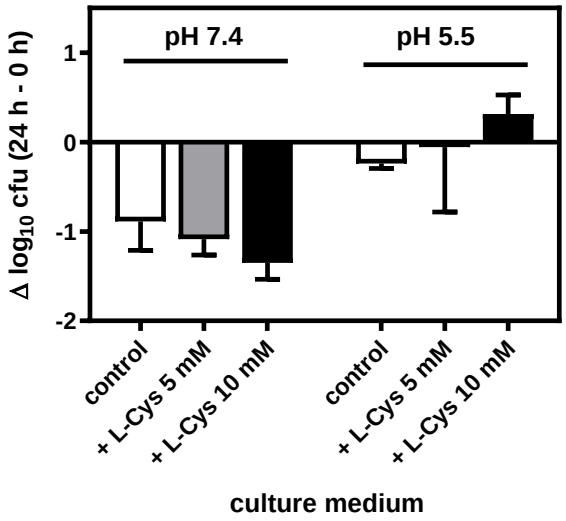


Figure S4: Influence of the addition of L-cysteine into the culture medium on the intracellular growth of *F. philomiragia* ATCC 25015

Infected cells were reincubated for 24 h in the absence (control) or presence of L-cysteine at 5 or 10 mM in media at pH 7.4 or 5.5. The graph below shows the changes in the cfu count from the initial inoculum, per mg of cell protein, following 24 h of incubation. Data are mean ± SD (n=3).



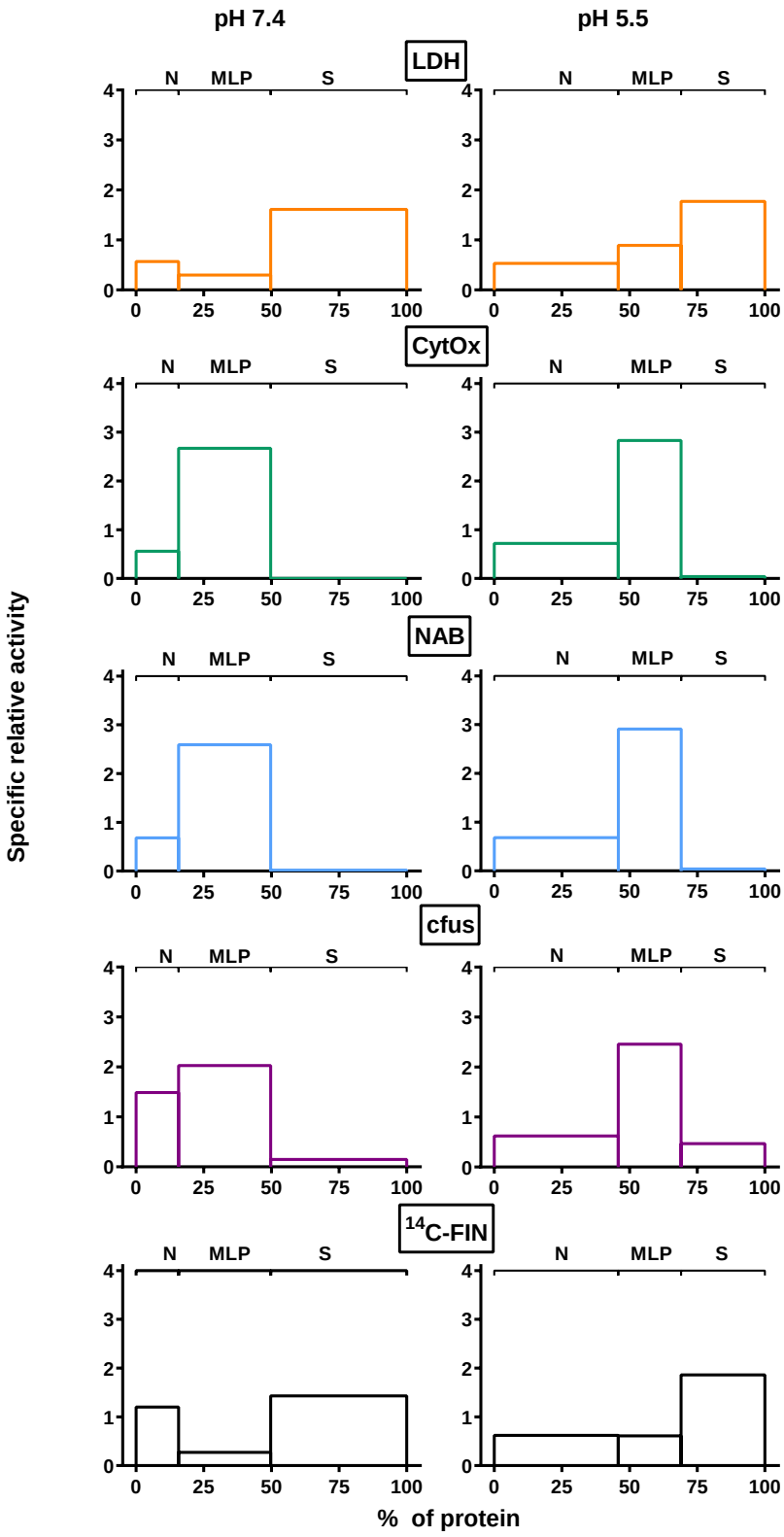


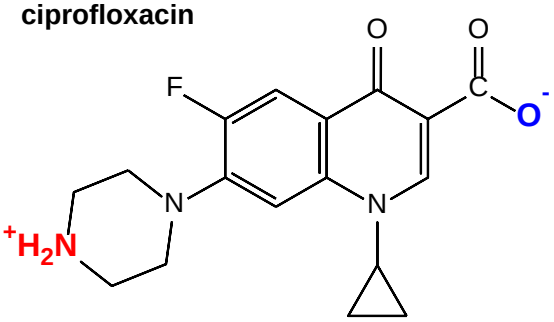
Figure S5: Subcellular distribution of marker enzymes (lactate dehydrogenase [LDH]: cytosol; cytochrome c-oxidase [cytox]: mitochondria; N-acetyl-β-hexosaminidase [NAB]: lysosomes); bacteria (*B. thailandensis* [CFU]) and of [^{14}C]-finafloxacin [^{14}C-FIN] in homogenates of THP-1 monocytes incubated with 2 mg/L [^{14}C]-finafloxacin in culture media at pH 7.4 (left) or 5.5 (right) for 30 min prior to collection.

The homogenates were separated into 3 fractions by centrifugation at increasing centrifugal fields and indicated by letters on the top of the graph (as in Figure 4). These are represented by blocks ordered in the same sequence over the abscissa where they span a length proportional to their relative protein content (in % of total cell protein). The height of each block (ordinate) gives the relative activity (for marker enzymes), cfu number (bacteria) or specific content (for [^{14}C]-finafloxacin), i.e. the percentage recovered in the fraction divided by the percentage of total cell protein of the same fraction. The height of each block indicates therefore the enrichment (if > 1) or impoverishment (if < 1) of each constituent in the fraction on a protein basis compared to an unfractionated homogenate while its surface is proportional to the content or activity recovered in the fraction (the total surface of each diagram is = 1).

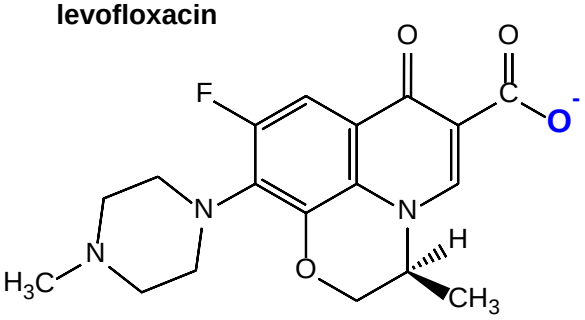
Figure S6: The chemical structures of the fluoroquinolones (the physicochemical properties of which are described in Table S2).

A. Most abundant microspecies at pH 7.4 [a]

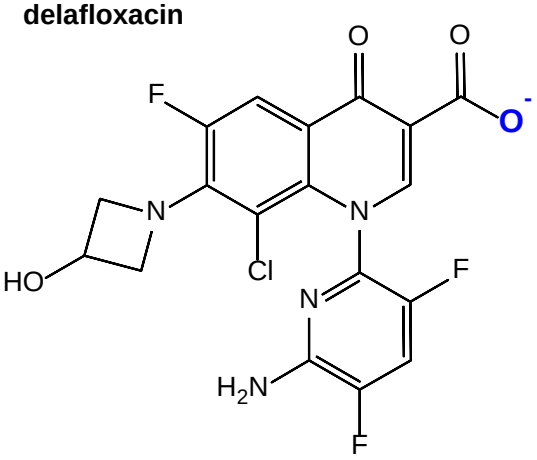
ciprofloxacin



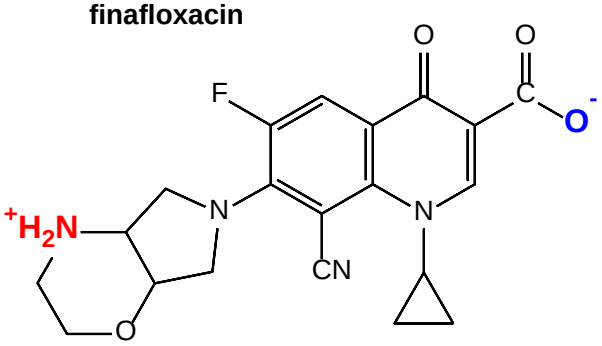
levofloxacin



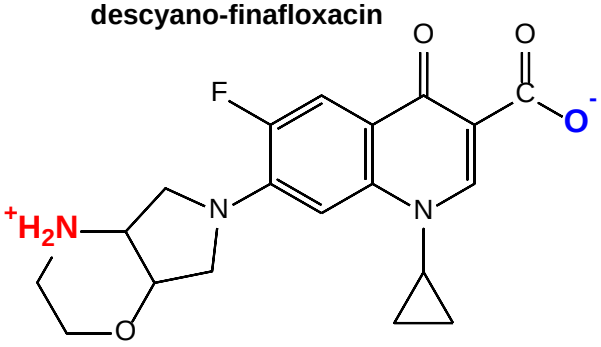
delafloxacin



finafloxacin



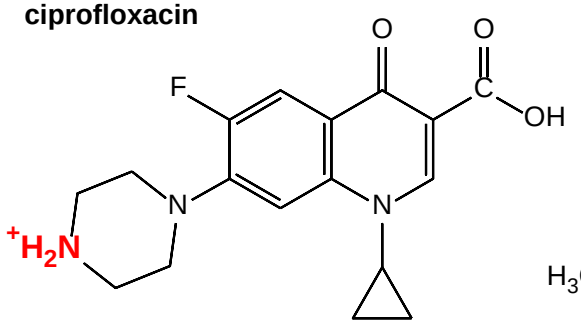
descyano-finafloxacin



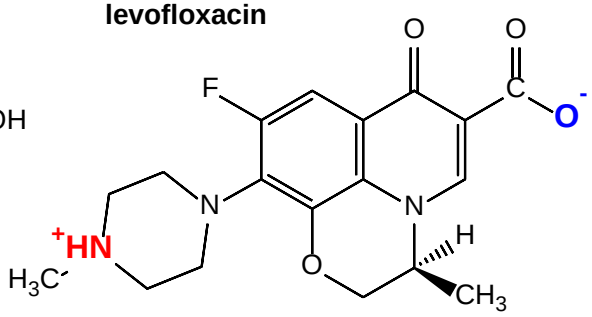
[a] calculated using Chemicalize™ (Instant Cheminformatics Solutions; ChemAxon Ltd, Budapest, Hungary; available from https://chemicalize.com [accessed on 10 Oct 2018]

B. Microspecies ≥ 40% of total at pH 5.5 [a]

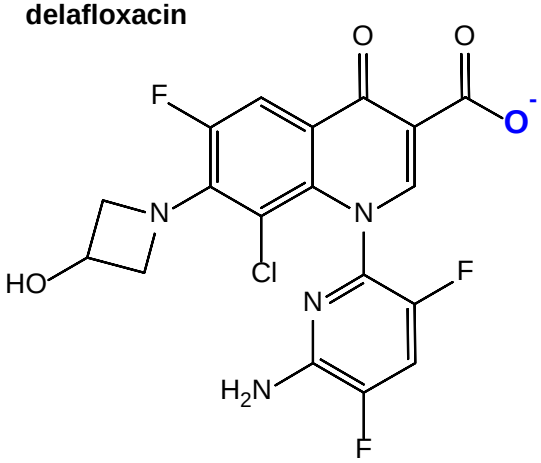
ciprofloxacin



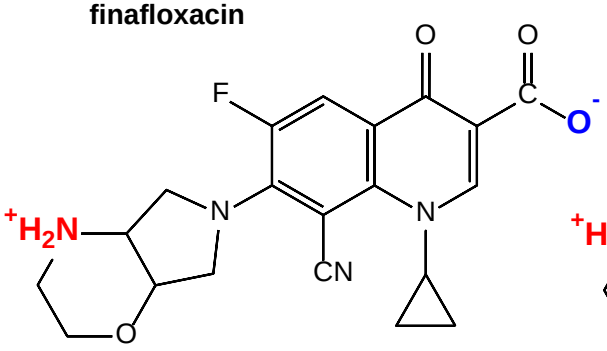
levofloxacin



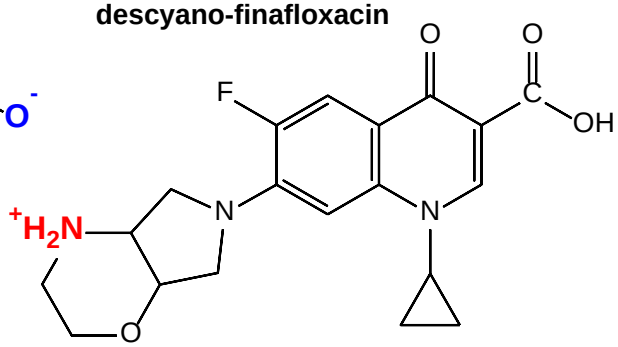
delafloxacin



finafloxacin



descyano-finafloxacin



[a] calculated using Chemicalize™ (Instant Cheminformatics Solutions; ChemAxon Ltd, Budapest, Hungary; available from https://chemicalize.com [accessed on 10 Oct 2018]. For levofloxacin, the proportions of positively-charged and zwitterionic microspecies are 40 and 54%, respectively. For the other fluoroquinolones, the microspecies shown is predominant (See Table S2 for actual values).

Ionization status: As the pK_a of the carboxylic function of finafloxacin is lower than for the other fluoroquinolones (4.33 *vs*. 5.56 and 5.35 for ciprofloxacin and levofloxacin, respectively (see Table S2), the molecule is expected to be essentially zwitterionic at acid pH (92% at pH 5.5) whereas the two other fluoroquinolones are largely positively-charged. We suggest that the nitrile substituent present in position 8 plays a critical role in this respect since (i) calculations show that descyanofinafloxacin loses this predominantly zwitterionic character at acid pH (and

becomes as positively-charged as ciprofloxacin), and (ii) pradofloxacin, another fluoroquinolone carrying a nitrile group in C8, has lower MICs at an acid pH than its C8-H analogue (descyanopradofloxacin) [7]. Experimental determinations of the actual proportions of finafloxacin and ciprofloxacin microspecies at neutral and acidic pH would be worthwhile to be obtained for confirmation of this hypothesis.

References to supplementary material

[1] Anonymous. Chemaxon documentation: pKa calculations. *Chemaxon Ltd, Budapest, Hungary* Budapest, Hungary. 2019. last accessed 15-1-2019.

[2] Szegezdi J, Csizmadia F. Prediction of dissociation constant using microconstants. 27th ACS National Meeting, Anaheim, California, March 28-April 1, 2004.

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[5] Han S, Bishop BM, van Hoek ML. Antimicrobial activity of human beta-defensins and induction by Francisella. Biochem Biophys Res Commun 2008;371:670-674.

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[7] Körber-Irrgang B, Kresken M, Wetzstein HG. pH-dependence of the activity of pradofloxacin and four structural analogs against *Escherichia coli* and *Staphylococcus aureus*. 49th Interescience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2009;E1967.