Cellular accumulation of fluoroquinolones is not predictive of their intracellular activity: studies with gemifloxacin, moxifloxacin and ciprofloxacin in a pharmacokinetic/pharmacodynamic model of uninfected and infected macrophages

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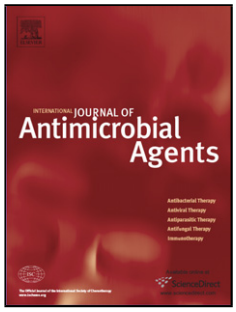

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Cellular accumulation of fluoroquinolones is not predictive of their intracellular activity: studies with gemifloxacin, moxifloxacin, and ciprofloxacin in a pharmacokinetic/pharmacodynamic model of uninfected and infected macrophages.

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Running title: Cellular pharmacokinetics and activity of fluoroquinolones

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ABSTRACT

Fluoroquinolones enter eukaryotic cells but the correlation between cellular accumulation and activity remains poorly established. Gemifloxacin is known to accumulate to a larger extent than most other fluoroquinolones in tissues. Using murine J774 macrophages and human THP-1 monocytes, we show that gemifloxacin accumulates more than ciprofloxacin and even moxifloxacin. While showing indistinguishable kinetics of accumulation in J774 macrophages, gemifloxacin was released at an approximately two-fold slower rate than ciprofloxacin and its release was only partial. Gemifloxacin was also a weaker substrate than ciprofloxacin for the efflux transporter Mrp4 active in J774 macrophages. In cells infected with *L. monocytogenes* or *S. aureus* (typical cytoplasmic and phagolysosomal organisms, respectively), gemifloxacin was equipotent to moxifloxacin and ciprofloxacin in concentration-dependent experiments if data are normalized based on MIC in broth. Thus, larger cellular concentrations of gemifloxacin than of moxifloxacin or ciprofloxacin were needed to obtain a similar target effect. Fractionation studies showed a similar subcellular distribution for all 3 fluoroquinolones, with about 2/3 of the cell-associated drug recovered in the soluble fraction (cytosol). The data suggest that the cellular accumulation of fluoroquinolones is largely a self-defeating process as far as activity is concerned, with the intracellular drug made inactive in proportion to its accumulation level. While these observations do not decrease the intrinsic value of fluoroquinolones for treatment of intracellular infections, they indicate that ranking fluoronolones based on cell accumulation data without measuring the corresponding intracellular activity may lead to incorrect conclusions concerning their real potential.

Keywords: ciprofloxacin, moxifloxacin, gemifloxacin, Mrp4, Staphylococcus aureus, Listeria monocytogenes, macrophages

1. Introduction

Fluoroquinolone antibiotics are important in our current therapeutic arsenal, because of their broad spectrum, highly bactericidal activity, and favourable pharmacokinetic properties [1]. Their wide tissue distribution allows them reaching therapeutic concentrations in deep body compartments as well as in the intracellular milieu, which may be an advantage in the treatment of intracellular infections. Accumulation and activity in cells are usually linked when considering a given fluoroquinolone in a specific cell type, as demonstrated for ciprofloxacin *vis-à-vis* the intracellular forms of *L. monocytogenes* in J774 macrophages in experiments where its cellular concentration was modulated by inhibition or overexpression of the constitutive ciprofloxacin efflux transporter Mrp4 [2;3]. There is, however, a lack of quantitative data comparing distinct fluoroquinolones in this context.

Gemifloxacin [4] accumulates to high levels in human PMN and is active against intracellular bacteria [5;6]. This prompted us to compare it to other fluoroquinolones for cellular pharmacokinetics and activity in an established model of murine J774 macrophages [7]. Ciprofloxacin and moxifloxacin, when needed, were used comparators, as these show a low and large accumulation, respectively, in relation to differential susceptibility to efflux [8-11]. We also examined THP-1 cells, where no active fluoroquinolone efflux has been evidenced so far. We found that gemifloxacin accumulates to higher levels than ciprofloxacin and moxifloxacin in both cell types and that all three drugs have a similar subcellular distribution. Yet, gemifloxacin showed no improved activity against two types of intracellular bacteria, *L. monocytogenes* and *S. aureus*, localized in the cytosol and in phagolysosomes, respectively.

2. Materials and methods

2.1. Antibiotics and main reagents

Gemifloxacin mesylate (LG Life Sciences, Seoul, Korea), and ciprofloxacin-HCl and moxifloxacin-HCl (Bayer HealthCare AG, Leverkusen, Germany) were obtained as microbiological standards (potencies: 79 %, 85 %, and 91 %). Gemfibrozil was from Sigma-Aldrich (St-Louis, MO, USA), human serum from Lonza Ltd (Basel, Switzerland), and cell culture media and sera from Invitrogen Corp. (Carlsbad, CA).

2.2. Cell lines

Murine J774 macrophages (wild-type cells [9]) and their ciprofloxacin-resistant derivatives overexpressing Mrp4 efflux transporter [8;11] were used for most experiments. Human THP-1 cells (ATCC TIB-202, American Tissue Culture Collection, Manassas, VA) [12;13] were used for comparison purposes. ATP depletion was obtained as previously described [9].

2.3. Determination of the cellular accumulation of fluoroquinolones.

We used a previously described protocol [9;14]. Cell -associated fluoroquinolones were assayed by fluorimetry (see [10] for ciprofloxacin and moxifloxacin; for gemifloxacin, the conditions were: $\lambda_{ex.}$ = 270nm; $\lambda_{em.}$ = 402 nm [lowest limit of detection: 50 µg/L; linearity: 0-1.5 mg/L]). The cell drug content was expressed by reference to the total cell protein content [15]. The apparent total cellular concentration was then calculated using a conversion factor of 3.08 µL of cell volume per mg of cell protein [9].

2.4. Cell fractionation studies in J774 cells

The main subcellular organelles were separated by differential centrifugation as previously described [2]. The protein and antibiotic content of each fraction was determined in parallel with the activity of marker enzymes of the main organelles (cytochrome *c*-oxydase for mitochondria; N-acetyl-β-hexosaminidase for lysosomes, and lactate dehydrogenase for cytosol [7]).

2.5. Bacterial strains and susceptibility testings.

We used *L. monocytogenes* strain EGD and *S. aureus* strain ATCC 25923. MIC determinations were made according to CLSI guidelines [16], using Tryptic Soy broth for *L. monocytogenes* [13] and Mueller Hinton broth for *S. aureus* [14].

2.6. Cell infection and assessment of antibiotic intracellular activities.

Cell infection was performed as described previously [2], with pharmacological comparison between drugs and bacteria based on concentration-dependent effects analyses [14], to determine (i) the relative minimal and maximal efficacies (E_{min} / E_{max}, in log_{10} units), and (ii) the relative potencies (EC_{50}) and static concentrations). This type of analysis and its usefulness for comparing antibiotics and the response of different bacteria has been described in details in previous publications [14;17-19]). As discussed previously [20], the large dilution of samples before spreading on agar plates for cfu counting ensures an absence of carry-over effect.

2.7. Curve fitting and statistical analyses

Curve-fitting analyses were made using GraphPad Prism® version 4.03, GraphPad Software, San Diego, CA, USA. Statistical analyses were made with the same software for comparing concentration-response functions and with GraphPad Instat® version 3.06 (GraphPad Software) for other studies.

3. Results

3.1. Cellular pharmacokinetics

We first compared the cellular accumulation of gemifloxacin with that of ciprofloxacin and moxifloxacin, and examined the influence of gemfibrozil, a broad spectrum inhibitor of anion transporters including the Mrp transporters, on this accumulation. Figure 1A (upper panels) shows that (i) gemifloxacin accumulated to a larger extent than the other two fluoroquinolones in both J774 and THP-1 cells; (ii) the accumulation of gemifloxacin and moxifloxacin was not influenced by gemfibrozil; (iii) in contrast, ciprofloxacin, which accumulated to the lowest extent in J774 macrophages, reached a cellular concentration similar to that of moxifloxacin in these cells in the presence of gemfibrozil, as already observed in the same model [10]; (iv) the level of accumulation of ciprofloxacin was similar to that of moxifloxacin in THP-1 cells and not influenced by the addition of gemfibrozil.

We then compared the kinetics of accumulation and efflux of gemifloxacin with that of ciprofloxacin using J774 macrophages only as this is where the largest difference of accumulation was observed. Figure 1B shows that the two fluoroquinolones could not be distinguished with respect to accumulation kinetics but displayed marked differences for efflux. Thus, gemifloxacin release (i) occurred at the same rate as its uptake (compare k_{in} and k_{out} parameters); (ii) was about 2-fold slower than that of ciprofloxacin, including at the very initial period phase (see inset); (iii) was only partial, with about 25 % of the accumulated drug remaining cell-associated in apparent stable fashion after 30 min incubation in drug-free medium *vs.* negligible amounts for ciprofloxacin.

We next measured the level of accumulation of gemifloxacin compared to that of ciprofloxacin in J774 macrophages overexpressing the ciprofloxacin efflux transporter Mrp4 (ciprofloxacin-resistant cells), using normal conditions and conditions of ATP depletion (which inhibits all ATP-dependent active transporters including Mrp4). Figure 2A (upper

panels) shows that (i) gemifloxacin accumulation was reduced (but in a non-statistically significant manner) in ciprofloxacin-resistant cells compared to wild-type cells; (ii) ATP depletion increased its accumulation in both wild-type and ciprofloxacin-resistant cells, but with a significant difference in the latter cells only; (iii) ciprofloxacin accumulation was significantly reduced in ciprofloxacin-resistant cells, but was markedly increased by ATP depletion, reaching a value similar to that observed in wild-type cells after ATP depletion; (iv) in line with our previous observations [11], ATP depletion markedly increased the accumulation of ciprofloxacin in wild-type cells.

Because the ciprofloxacin efflux transporter is saturable in a 10-200 mg/L range [9], we measured the accumulation of gemifloxacin in both wild-type J774 macrophages and ciprofloxacin-resistant cells over increasing concentrations of gemifloxacin in that range. Figure 2 (lower panel) shows that while gemifloxacin accumulation was not significantly influenced by its extracellular concentration in wild-type cells, there was a significant increase over the range of concentrations investigated for ciprofloxacin-resistant cells. In contrast, and as described earlier [9], ciprofloxacin showed a marked increase in its accumulation over the same concentration range in wild-type cells. For ciprofloxacin-resistant cells, the increase in cell accumulation of ciprofloxacin was much less marked in the range of drug concentrations investigated due to overexpression of the Mrp4 transporter (see [11]).

These results suggest that gemifloxacin could be a poor, albeit still recognized substrate for efflux transport in J774 macrophages if Mrp4 is overexpressed. We, therefore, compared the kinetics of gemifloxacin efflux in ciprofloxacin-resistant *vs.* wild-type cells. While the *plateau* values observed at 30 min remained close from each other, denoting an incomplete release of gemifloxacin in both cases, its rate of efflux was significantly accelerated in ciprofloxacin-resistant cells compared to wild-type cells (k_{out} = 2.393 ± 0.907 *vs.* 0.403 ± 0.122 min^{-1}; $p < 0.001$; see graphical representation in Figure SP1 in the Supplementary Material).

184 *3.2. Intracellular activity*

185 To examine the correlation between cellular accumulation and intracellular activity, we
186 compared all 3 fluoroquinolones in our pharmacological model of intracellular infection
187 [14;17], using J774 macrophages since this is where the largest differences in accumulation
188 levels had been observed. *L. monocytogenes* and *S. aureus* were selected as bacterial
189 targets as they represent a typical cytoplasmic and phagolysosomal organism, respectively.
190 Data presented in Figure 3A (with analysis of the key pharmacological descriptors in Table 1)
191 show that all 3 antibiotics induced essentially a similar response when expressed as a
192 function of equipotent extracellular concentrations (multiples of MIC). Thus, in all cases, a
193 single sigmoid function could be fitted to the individual responses of each antibiotic (see
194 Figure SP2 in the Supplementary Material and the pertinent regression parameters and
195 pharmacological descriptors in Table 1). As no statistically significant difference was
196 observed between the 3 antibiotics, all data were pooled to fit a single function shown in
197 Figure 3A. Thus, for each bacterium, the relative minimal efficacies (E_{min} [growth in the
198 absence of antibiotic]), maximal relative efficacies (E_{max} [maximal antibiotic-related killing]),
199 relative potencies (E_{50}), and static concentrations (C_s; [in multiples of MIC) were not
200 statistically significantly different. We then calculated for each fluoroquinolone which cellular
201 drug concentration would be needed to reach two predefined pharmacodynamic targets
202 (static effect and a 1 or 2 $\log_{10}$ cfu decrease). The results (with the mode of calculation) are
203 presented in Figure 3B and show that the potencies of the drugs with respect to their
204 intracellular targets is in inverse proportion to their respective cellular accumulations.

205

206 *3.3. Subcellular distribution*

207 Lastly, we compared the subcellular distributions of ciprofloxacin, moxifloxacin, and
208 gemifloxacin. Figure 4 shows that all 3 fluoroquinolones shared essentially the same
209 distribution, with about 70 % recovered in the soluble fraction, about 10 % of ciprofloxacin
210 and gemifloxacin and 18 % of moxifloxacin in the nuclei/unbroken cells fraction, and the

211 remaining in the organelles/membranes fraction. As previously described [7], lactate
212 dehydrogenase was mostly recovered in the soluble fraction, and cytochrome oxydase and
213 N-acetyl-β-hexosaminidase, in the granules/membranes fraction, indicating that the
214 fractionation method effectively separated the corresponding subcellular entities with only a
215 very low proportion of unbroken cells left after homogenization.

216

4. Discussion

Gemifloxacin, approved for clinical use in over 27 countries [21], is characterized by very low MICs against Gram-positive bacteria [22;23] related to the presence of an oximinomethyl group [4] in its C7 side-chain and by a high tissular accumulation [24]. Human PK/PD studies show that gemifloxacin achieves higher AUC/MIC ratios in epithelial lining fluid and alveolar macrophages than other currently used fluoroquinolones, suggesting an advantage in terms of availability and efficacy at the site of infection [25;26]. The present study, however, shows that the higher accumulation of gemifloxacin in J774 macrophages (i) is not associated with differences in influx rates compared to a fluoroquinolone with lower accumulation (ciprofloxacin); (ii) does not preclude and cannot be explained by differences in active efflux transport (in comparison with moxifloxacin); (iii) does not lead to a higher intracellular activity. This goes against commonly accepted pharmacokinetic and pharmacodynamic concepts that tend to link accumulation and lack of efflux on the one hand, and accumulation and activity on the other hand. Our model may be questioned, but it is important to note that it reproduces (i) with respect to pharmacokinetics, what is observed in human alveolar macrophages where the concentrations of ciprofloxacin, moxifloxacin, and gemifloxacin are respectively 2-5 x, 20-40 x, and 90 x higher than serum levels [26-28], and (ii) with respect to intracellular activity what has been observed in human polymorphonuclear leucocytes infected by *S. aureus* [5].

Mechanistically, differences in accumulation of drugs in cells and tissues usually result from commensurate differences in influx or efflux rates, or from differential trapping by intracellular organelles or constituents.

Considering influx first, faster drug accumulation is usually related to a higher lipophilicity (which is supposed to facilitate transmembrane diffusion) or from the activity of transporters. This does not seem to apply to gemifloxacin, as this fluoroquinolone (i) is not globally more lipophilic than ciprofloxacin (see Table SP1 for experimental and calculated log P and log D

values), (ii) is probably not the substrate of a specific influx transporter when compared to ciprofloxacin (same rate accumulation constants). Non-specific influx transporter(s) observed in PMN and human monocytes [29-31] can probably be dismissed here as these belong to the Solute Carrier Organic Anion (SLCO) family [32] that is inhibited by gemfibrozil, which was not the case here.

Considering efflux, Mrp4 has been proposed as the main transporter responsible for the lower accumulation of ciprofloxacin in J774 macrophages compared to levofloxacin, garenoxacin, and moxifloxacin. These fluoroquinolones, indeed, reach a similar level of accumulation when Mrp4 is made inactive by ATP depletion or addition of gemfibrozil [10]. Moreover, ciprofloxacin accumulation is significantly increased by silencing the gene coding for Mrp4 [8]. The present data show that this conclusion cannot be generalized to all fluoroquinolones and all situations. Thus gemifloxacin not only accumulates more than moxifloxacin in J774 macrophages under conditions of ATP depletion or in the presence of gemfibrozil, but also in THP-1 macrophages in which no gemfibrozil-inhibited efflux can be demonstrated. Another compelling reason to disregard efflux as being the main cause for the differential accumulation of gemifloxacin vs. ciprofloxacin and moxifloxacin is that gemifloxacin actually seems a weak but nevertheless effective substrate of Mrp4 in J774 macrophages, whereas we know that moxifloxacin is not. Thus, globally and in contrast to what we proposed for moxifloxacin, the higher cellular concentration of gemifloxacin compared to other fluoroquinolones must find an explanation beyond considerations of influx and efflux rates only.

Considering intracellular trapping, a model has been presented [33] that relates fluoroquinolone accumulation in eukaryotic cells to their trapping under a protonated form in lysosomes due to the acid pH (~ 5.4) prevailing therein. This, however, is unlikely because fluoroquinolones are not weak bases but zwitterionic compounds. Moreover, differences in accumulation of drugs in acidic membrane-bounded compartments should result from

commensurate differences in the number and/or the pK_a of their basic functions (see [34]), which is not the case for the 3 fluoroquinolones studied here (see individual basic pK_a values in Table SP1). More factually, cell fractionation studies show a predominant association of the cell-associated fluoroquinolones with the cytosol rather than with lysosomes, in line with the results of previous studies with ciprofloxacin [2;35] (studies using the same technique have shown that macrolides are predominantly associated with lysosomes in J774 macrophages [2;36;37]). Lastly, experimental studies have shown a lack of effect of monensin (a H^+ ionophore that collapses the cytosolic-lysosomal Δ pH) on ciprofloxacin accumulation under conditions in which it drastically reduces the accumulation of azithromycin in J774 macrophages [9].

Actually, a more likely explanation for the larger cellular accumulation of gemifloxacin compared to moxifloxacin and ciprofloxacin could be its tighter binding to still undefined cellular constituents such as soluble proteins. This hypothesis would account for the pharmacokinetic and subcellular distribution data presented here, including (i) the lower efflux rate of gemifloxacin compared to ciprofloxacin (which, however, may also result from the less efficient recognition of gemifloxacin by the Mrp4 efflux transporter, both mechanisms being not mutually exclusive) and, (ii) its incomplete release upon transfer of the cells to drug-free medium. It is also consistent with the larger serum protein binding of gemifloxacin (55-73 %) compared to moxifloxacin (39-52 %) and ciprofloxacin (30 % only) [38;39].

Determining the molecular nature of the intracellular binding sites for fluoroquinolones still require further investigations, but the mechanism proposed provides a rational explanation for the main critical observation made here, namely that all 3 fluoroquinolones are equipotent against intracellular bacteria in spite of their differences in cellular accumulation. We show, indeed, that it is the MIC of each drug that drives its intracellular potency (as defined by the C_s and EC_{50} pharmacological descriptors) since all 3 fluoroquinolones show superimposable concentration-effects relationships once the data are normalized on basis of multiples of the

MIC. MICs are measured in broth where little protein binding takes place, which means that their values must essentially be interpreted as corresponding to free drug levels [40]. Intracellularly, a static effect (C_s) for gemifloxacin was obtained for an extracellular concentration corresponding to its MIC, although its intracellular concentration is much larger. It is, therefore, tempting to speculate that only a fraction of the total intracellular gemifloxacin is available for activity, corresponding essentially to its free form. Moxifloxacin should show an intermediate behaviour with intracellular activity also driven by its MIC (measured in broth), which is what we observe. Thus, the larger cellular accumulation of some fluoroquinolones, taking gemifloxacin an example, would essentially be a self-defeating process as far as activity is concerned (assuming all comparisons are made on basis of the MIC), leading to a larger concentration of bound drugs with, however, no or little difference in their free forms. This confirms and extends previous work that showed that the intracellular activity of fluoroquinolones was weaker and not in proportion to what could be anticipated from the level of their cellular accumulation [13;41-43].

In conclusion, the present work documents that (i) recording the cellular accumulation of fluoroquinolones does not allow to predict their intracellular activity; (ii) a higher cellular accumulation may depend from other parameters than influx and efflux rates and/or the activity of specific transporters. This calls for both more mechanistic studies and more comprehensive structure-activity analyses where these two important elements of the pharmacological properties of fluoroquinolones will be examined in a systematic fashion.

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Table 1: Pertinent regression parameters [a] (with confidence intervals [CI]) and statistical analyses of data from experiments examining the concentration-response activities of ciprofloxacin, moxifloxacin and gemifloxacin (shown in Figure 3A). The 3 first rows show the analysis for each individual antibiotic and the last row the analysis made for all pooled data.

antibiotic	*L. monocytogenes*					*S. aureus*				
	R^2	E_{min} [b] (CI)	E_{max} [c] (CI)	EC_{50} [d] (CI)	C_s [e]	R^2	E_{min} [b] (CI)	E_{max} [c] (CI)	EC_{50} [d] (CI)	C_s [e]
ciprofloxacin	0.95	2.82 (1.83 to 3.81)	-3.96 (-5.44 to -2.48)	2.94 (1.00 to 8.66)	0.49	0.96	3.80 (2.92 to 4.68)	-1.60 (-2.26 to -0.94)	1.36 (0.64 to 2.89)	3.1
moxifloxacin	0.95	2.84 (2.05 to 3.63)	-4.48 (-5.21 to -3.56)	1.30 (0.62 to 2.74)	0.47	0.95	2.98 (2.11 to 3.86)	-1.85 (-2.28 to -1.43)	1.62 (0.78 to 3.38)	2.6
gemifloxacin	0.97	3.03 (1.79 to 4.27)	-3.55 (-4.28 to -2.82)	0.65 (0.27 to 1.59)	0.74	0.98	3.07 (2.32 to 3.83)	-1.30 (-1.64 to -0.97)	2.02 (1.01 to 4.03)	4.8
all 3	0.91	3.44 (2.95 to 3.94)	-3.92 (-4.53 to -3.32)	1.44 (0.95 to 2.19)	0.93	0.94	3.44 (2.95 to 3.94)	-1.55 (-1.81 to -1.29)	1.44 (0.95 to 2.19)	3.2

[a] by use of all data for antibiotic concentrations ranging from approx. 0.01 to approx. 1,000 x MIC (ciprofloxacin: 0.01 to 100 mg/L [*L. monocytogenes*] and 0.001 to 100 mg/L [*S. aureus*]; moxifloxacin: 0.005 to 30 mg/L [*L. monocytogenes*] and 0.001 to 100 mg/L [*S. aureus*]; gemifloxacin: 0.005 to 150 mg/L. [*L. monocytogenes*] and 0.0008 to 20 mg/L [*S. aureus*]).

[b] relative minimal efficacy: change in cfu (in $\log_{10}$ units) at time 24 h from the initial, post-phagocytosis inoculum, as extrapolated for an infinitely low antibiotic concentration

[c] relative maximal efficacy: change in cfu (in $\log_{10}$ units) at time 24 h from the initial, post-phagocytosis inoculum, as extrapolated for an infinitely large antibiotic concentration

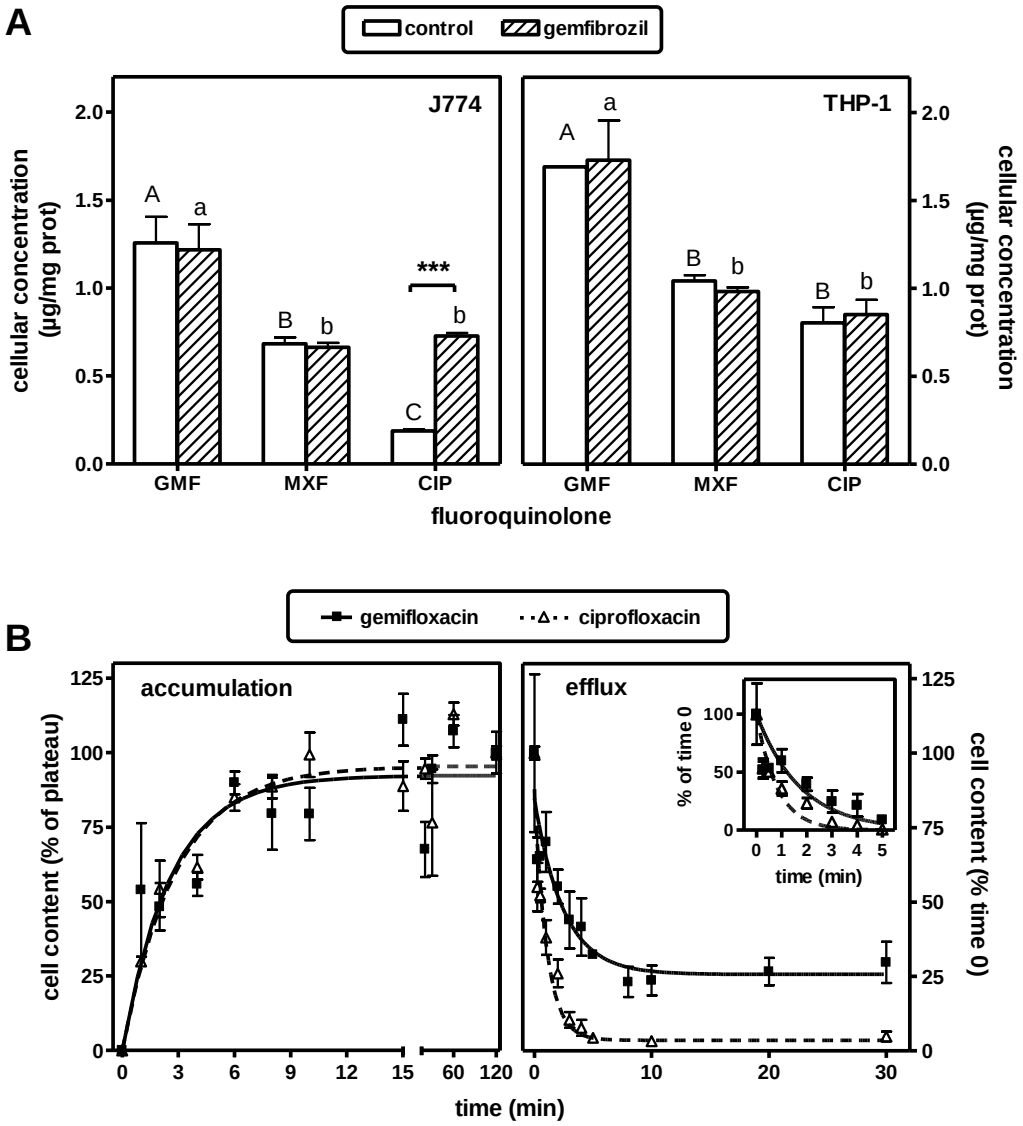
[d] relative potency: extracellular concentration (in multiple of MIC) yielding a change in cfu at time 24 h half way between E_{min} and E_{max}

[e] static concentration: concentration (in multiples of MIC) resulting no apparent bacterial growth (no change in cfu) from the initial, post-phagocytosis inoculum), as determined by graphical intrapolation (MIC values (mg/L) are : 1 and 0.125 (ciprofloxacin), 0.5 and 0.03 (moxifloxacin), and 0.5 and 0.008 (gemifloxacin) for *L. monocytogenes* and *S. aureus* respectively.

Statistical analysis:

The raw data obtained for each individual antibiotic, and the corresponding Hill functions were compared using one way ANOVA (parametric) and Kuskal-Wallis (non-parametric) tests found to be not significantly different (*L. monocytogenes*: p = 0.420 and 0.152, respectively: *S. aureus*: p = 0.351 and 0.249, respectively). The analysis was then repeated for comparison of antibiotic pairs (ciprofloxacin *vs.* gemifloxacin; ciprofloxacin *vs.* moxifloxacin; moxifloxacin *vs.* gemifloxacin) using unpaired t-test and showed no significant difference for any comparison ($p > 0.18$).

Figure 1

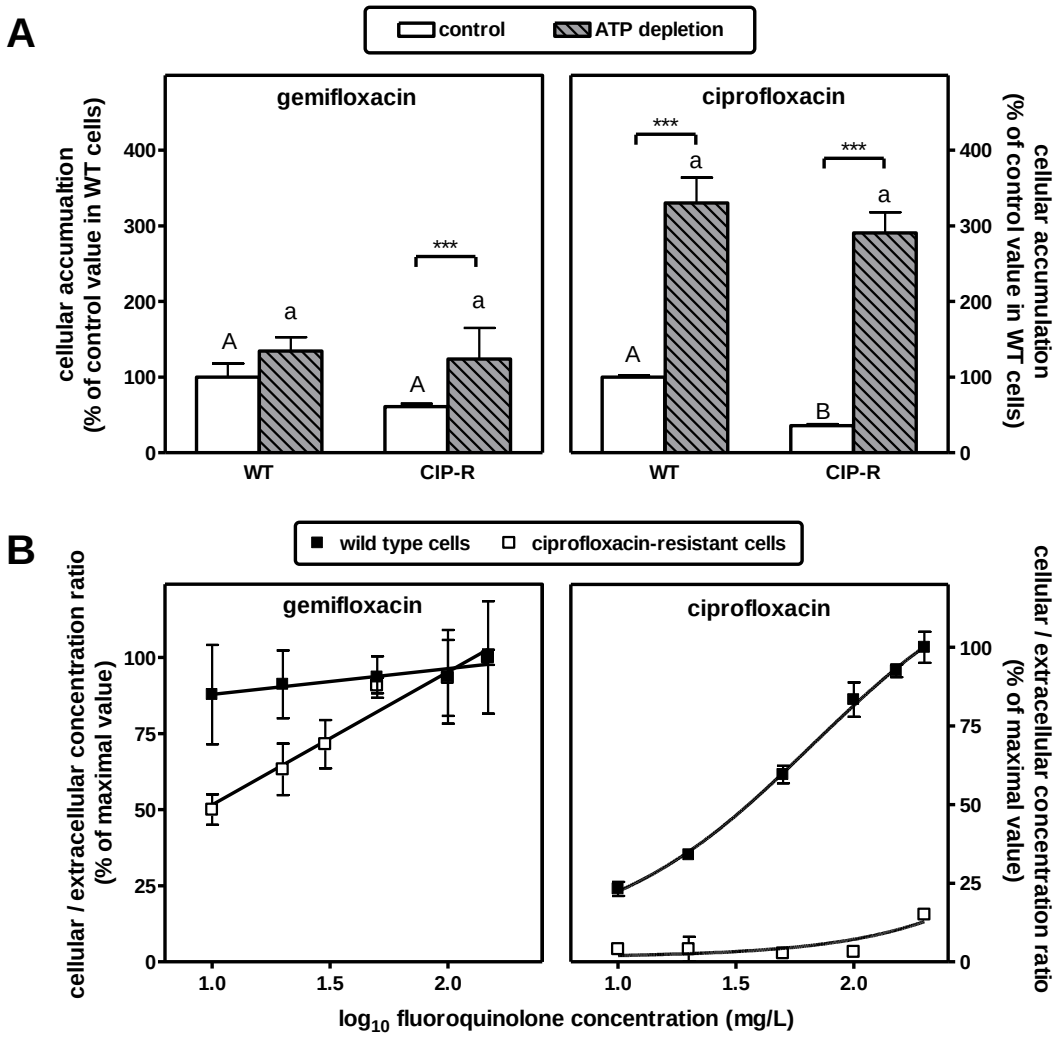


Caption to Figure 1: Accumulation and efflux of fluoroquinolones.

A (upper panel): accumulation of gemifloxacin (GMF), moxifloxacin (MXF), and ciprofloxacin (CIP) in wild-type J774 mouse macrophages (left) and THP-1 human monocytes (right) incubated during 2 h with 20 mg/L in control conditions (open bars) or in the presence of the Mrp inhibitor gemfibrozil (500 µM; hatched bars). All values are the means of 3 independent determinations ± SD. Statistical analysis (ANOVA): control *vs.* gemfibrozil, *** $p < 0.001$; comparison of fluoroquinolones: bars with different letters are different from one another ($p < 0.001$; caps letters, control conditions; small letters, + gemfibrozil).

B (lower panel): kinetics of accumulation (left) and efflux (right) of gemifloxacin compared to ciprofloxacin in J774 macrophages (see [10] for efflux of moxifloxacin) . For accumulation studies, cells were transferred to medium containing a fixed amount of drug (20 mg/L) and collected at the times indicated in the abscissa. For efflux, cells were first exposed to the drug for 2 h at a concentration of 20 mg/L, gently washed, transferred to drug free medium and collected at the times indicated in the abscissa. Data were used to fit a one phase exponential association function for influx [$y = y_{max} \; x \; (1 - e^{-k_{in} \; x \; t})$] and a one phase exponential decay function for efflux [$y = y_{max} \; x \; e^{-k_{out} \; x \; t} + plateau)$] by nonlinear regression. Regression parameters for influx: (a) gemifloxacin, R^2 = 0.780, k_{in} = 0.386 ± 0.123 min^{-1}; (b) ciprofloxacin, R^2 = 0.922, k_{in} = 0.348 ± 0.066 min^{-1}. Regression parameters for efflux: (1) main graph, (a) gemifloxacin, R^2 = 0.897, k_{out} = 0.403 ± 0.122 min^{-1}, *plateau* = 25.71 ± 4.63; (b) ciprofloxacin, R^2 = 0.949, k_{out} = 0.949 ± 0.204 min^{-1}, *plateau* = 3.56 ± 3.24); (2) Inset: data for the initial stage of efflux (0 – 5 min) and corrected for differences in plateau reached after 10 min, (a) gemifloxacin, R^2 = 0.658, k_{out} = 0.571 ± 0.138 min^{-1}; (b) ciprofloxacin, R^2 = 0.909, k_{out} = 1.216 ± 0.209 min^{-1}. Statistical analysis (paired *t*- test two-tailed): Influx, no significant difference in rate constants; absolute values of plateaus of accumulation were different and in line with data of Figure 1). Efflux: Main graph, comparison of all values: $p < 0.001$), plateaus values only: $p < 0.001$, k values only: $p < 0.001$; Inset: comparison for all values: $p = 0.016$, k_{out} values only: $p < 0.001$.

Figure 2



Caption to Figure 2: Cellular accumulation of gemifloxacin as compared to ciprofloxacin in wild-type or ciprofloxacin-resistant J774 mouse macrophages (see [11] for the corresponding data with moxifloxacin) **A** (upper panel): cells were incubated for 2 h in control conditions (open bars) or in ATP-depleted cells (gray hatched bars) with a fixed concentration (20 mg/L) of gemifloxacin (left) or ciprofloxacin (right) with wild-type cells (WT) or ciprofloxacin-resistant cells (CIP-R). Data are expressed in percentage of the value measured in wild-type cells in control conditions for each fluoroquinolone. All values are the means of 3 independent determinations ± SD. Statistical analysis (ANOVA): control *vs.* ATP-depletion, *** $p < 0.001$; WT *vs.* CIP-R cells: bars with different letters are different from one another ($p < 0.05$; upper case letters, control conditions; lower case letters, ATP-depletion). **B** (lower panel): Influence of the extracellular concentration of gemifloxacin (left) and ciprofloxacin (right) on their cellular to extracellular concentration ratio in wild-type

551 (closed symbols) or ciprofloxacin-resistant (open symbols) J774 mouse
552 macrophages, measured after 2 h of incubation. The cellular concentration was
553 expressed as µg per mg protein. Data are expressed in percentage of the highest
554 value observed in wild-type cells for each fluoroquinolone. All values are the means
555 of 3 independent determinations ± SD.
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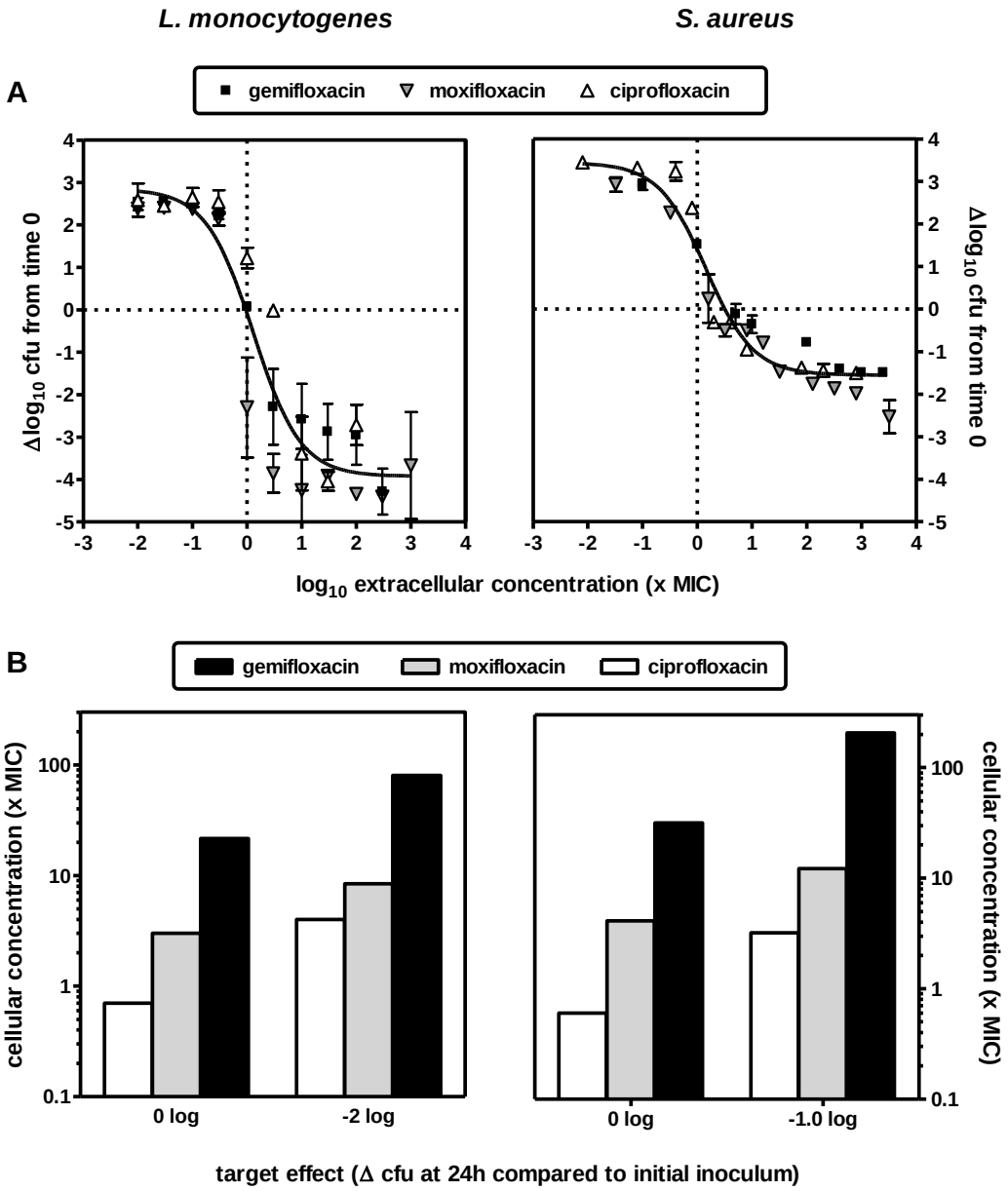
Figure 3



Caption to Figure 3: Concentration-response of the activities of gemifloxacin, moxifloxacin, and ciprofloxacin against phagocytized *L. monocytogenes* EGD (left) and *S. aureus* ATCC25923 (right) in wild-type J774 macrophages.

A (top): after phagocytosis and elimination of the extracellular bacteria, cells were incubated for 24 h with increasing concentrations of antibiotic (total drug) covering a ~ 0.01 to ~ 1,000 x MIC range (MIC [mg/L] were 1 and 0.125 [ciprofloxacin], 0.5 and 0.03 [moxifloxacin], and 0.5 and 0.008 [gemifloxacin] for *L. monocytogenes* and *S. aureus* respectively). The graphs show the change in the number of cfu (log scale) per mg of cell protein compared to the initial post-phagocytosis inoculum

(ordinate) as a function of the extracellular concentration of each drug expressed in multiple of its MIC (abscissa). In each graph, the horizontal dotted line corresponds to an apparent static effect and the vertical line to the MIC of the drug. A single sigmoidal regression has been fit to all data sets (see Figure SP2 for individual regression curves. The pertinent regression parameters and numerical values of the 4 key pharmacological descriptors (E_{min}, E_{max}; EC_{50}, C_s) are shown Table 1 for each drug - bacteria combination.

B (bottom): the ordinates show the calculated cellular concentrations (total drug; in multiples of MIC) needed to achieve two predefined activity levels (targets) shown on the abscissa (static effect [no apparent change in cfu]; 2 (*L. monocytogenes*) or 1 (*S. aureus*) $\log_{10}$ cfu decrease compared to the initial, post-phagocytosis inoculum). The cellular concentrations were calculated by (i) using the concentration-response curves shown in **A** to determine the extracellular concentrations needed to achieve the target effects (graphical intrapolation), and (ii) using the data of Figure 2B (wild type cells) to calculate the corresponding apparent total cellular concentrations of gemifloxacin and ciprofloxacin (for moxifloxacin, we used the accumulation data published in [10]) based on a conversion factor of 3.08 µL of total cell volume per mg protein as determined experimentally for wild type J774 macrophages in previous studies [9].

590 **Figure 4**
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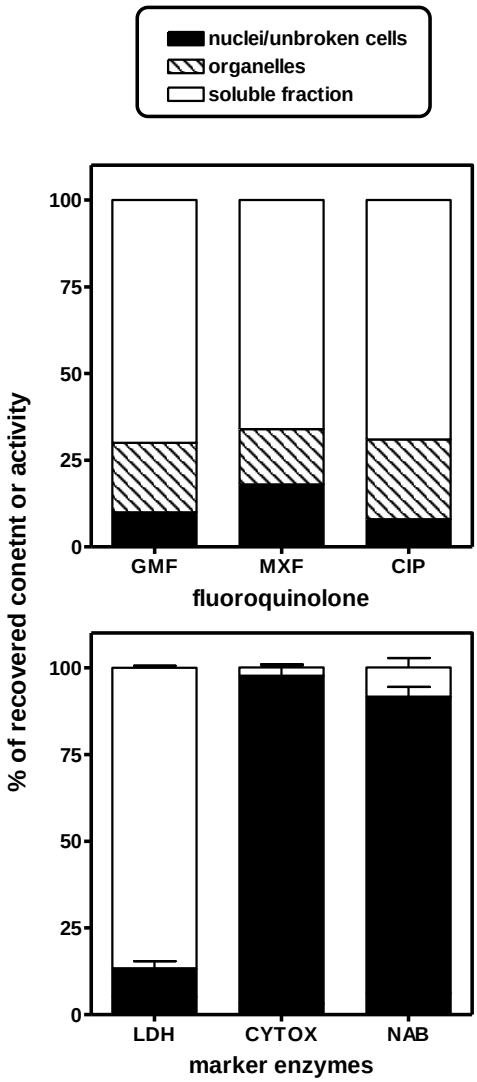
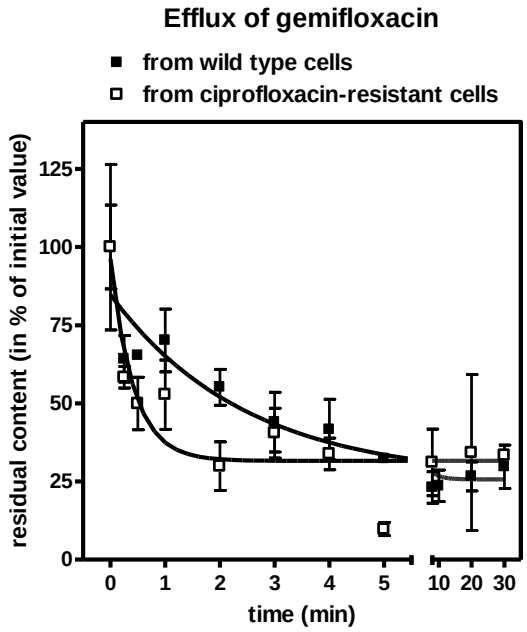


592
593 Caption fo Figure 4: Subcellular distribution of gemifloxacin (GMF), moxifloxacin
594 (MXF), and ciprofloxacin (CIP) in J774 mouse macrophages incubated for 2 h with
595 50 mg/L of each drug. The upper panel shows the antibiotic content in the
596 nuclear/unbroken cells, organelles, and soluble fractions expressed in percentage of
597 the total recovered amount (each bar corresponds to a separate experiment). The
598 lower panel shows the distribution of lactate dehydrogenase (LDH; marker of the
599 cytosol), cytochrome-c-oxydase (CYTOX; marker of mitochondria), and N-acetyl-β-
600 hexosaminidase (NAB; marker of lysosomes) as the mean values (± SD) of the 3
601 experiments (corresponding to each of the individual experiment shown in the upper
602 panel).
603

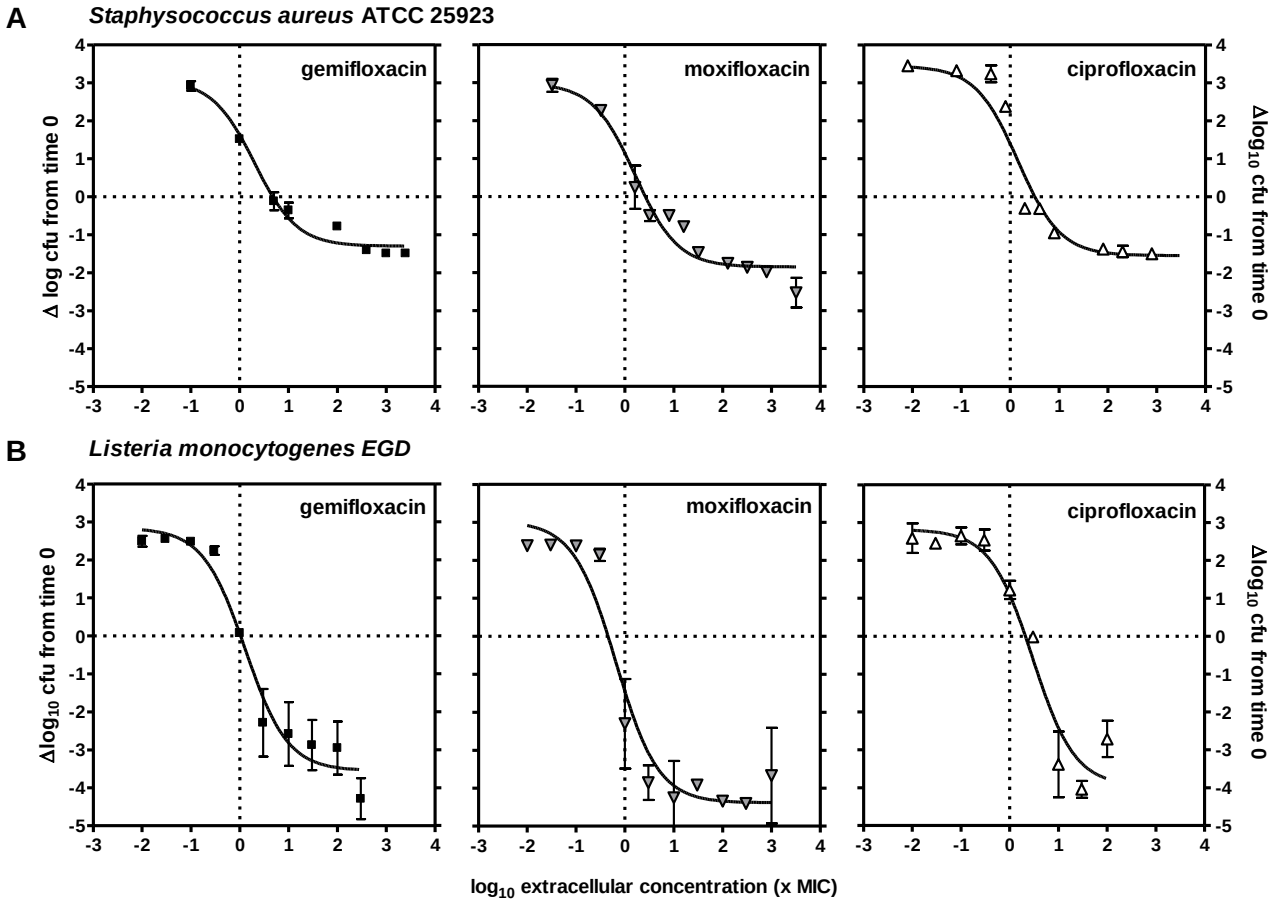
Vallet *et al.* Intracellular activity of fluoroquinolones - Supplementary Material

Figure SP1



Caption to Figure SP1: Kinetics of efflux of gemifloxacin from wild-type and ciprofloxacin-resistant J774 cells. Cells were exposed to gemifloxacin (20 mg/L) for 2 h and then transferred to antibiotic-free medium for up to 30 min. Regression parameters: (a) wild-type cells (same data as in Figure 2), $R^2 = 0.896$, $k_{out} = 0.403 \pm 0.122$ min^{-1}, *plateau* $= 25.71 \pm 4.63$; (b) ciprofloxacin-resistant cells, $R^2 = 0.830$, $k_{out} = 2.39 \pm 0.907$ min^{-1}, *plateau* $= 31.6 \pm 4.0$).

Figure SP2



Caption to Figure SP2: Concentration-response of the activities of gemifloxacin, moxifloxacin, and ciprofloxacin (CIP) against *S. aureus* ATCC25923 (top) and *L. monocytogenes* EGD (bottom) in wild-type J774 macrophages. Cells were incubated with increasing concentrations of antibiotic (total drug) for 24 h. Each graph shows the change in the number of cfu (log scale) per mg of cell protein compared to the initial post-phagocytosis inoculum (ordinate) as a function of the extracellular concentration of each drug expressed in multiples of its MIC (abscissa). In each graph, the horizontal dotted line corresponds to an apparent static effect and the vertical line to the MIC of the drug. A sigmoidal regression has been fitted to each set of data (see Table 1 for the pertinent regression parameters and numerical values of the four key pharmacological descriptors (E_{min}, E_{max}; EC_{50}, C_s).

Table SP1: Physico-chemical properties of fluoroquinolones and azithromycin at physiologically-relevant pHs

The data indicate that (i) ciprofloxacin, moxifloxacin, and gemifloxacin display quite similar biophysical properties although showing distinct cellular accumulation levels (gemifloxacin > moxifloxacin > ciprofloxacin; see Results) that are not correlated to the minor differences seen; (ii) these properties are very different from those of azithromycin, a drug known to accumulate extensively in lysosomes by proton-trapping (see Discussion). The pHs considered are those of the extracellular (7-7.4) and of the lysosomal (5-5.4) milieus, respectively.

Drug	pKa_1 [a] (acidic)	pKa_2 [a] (basic)	species in solution (calculated %) [a]						logP [b]		calculated logD [a,c]	
			pH 7.4			pH 5.4			calculated [a,d]	experimental [e]	pH 7	pH 5
			cationic	zwitterionic	anionic	cationic	zwitterionic	anionic				
ciprofloxacin	5.8	8.7	2	**93**	5	**69**	30	0	1.63'	2.30	-1.38	-1.62
moxifloxacin	5.6	9.4	2	**97**	1	**66**	34	0	1.90	2.90	-1.72	-1.33
gemifloxacin	5.5	9.5	1	**98**	1	**47**	**52**	0	1.04	2.30	-2.54	-1.78
azithromycin	-	8.9 [f] 9.6	**96.98** [g]	0.02	0	**99.97** [g]	0	0	2.44	4.02	-1.99	-4.41

[a] calculated using Reaxys (http://www.reaxys.com) with the ChemAxon's Marvin plug-in calculators (http://www.chemaxon.com/marvin). The actual values of the pK_a of the acidic function may be about 0.5 units higher due to the influence of the vicinal carbonyl function [1].

[b] logP: partition coefficient (log of the ratio of the concentrations of the unionized compound between a non polar [octanol] and a polar [water] phases);

[c] logD: distribution coefficient (log of the ratio of the sum of the concentrations of all forms of the compound [ionized plus un-ionized] in each of thee two phases at a given pH)

[d] These values are the arithmetic average of three methods of calculations (Viswanadhan's fragmentation; Klopman's fragmentation; and PHYSPROP© database [see https://www.reaxys.com/static/marvin/marvin_5_3_7/help/calculations/partitioning.html for details]).

[e] value as reported in Drugbank (see http://www.drugbank.ca and [2]

[f] azithromycin is a dicationic drug

[g] dicationic form (monocationic form: 3 % at pH 7.4 and 0.03 % at pH 5.4; a zwitterionic form is virtually inexistent (< 0.001 %) at these pH values).

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[2] Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J: DrugBank, a comprehensive resource for *in silico* drug discovery and exploration, Nucleic Acids Res. 2006 Jan 1;34 (Database issue): D668-72 [PMID: 16381955]