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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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## Accepted Manuscript

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

5  
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29

30 **ABSTRACT**

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

52

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

55

56

57 **1. Introduction**

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

68  
69 Gemifloxacin [4] accumulates to high levels in human PMN and is active against intracellular  
70 bacteria [5;6]. This prompted us to compare it to other fluoroquinolones for cellular  
71 pharmacokinetics and activity in an established model of murine J774 macrophages [7].  
72 Ciprofloxacin and moxifloxacin, **when needed**, were used comparators, as these show a low  
73 and large accumulation, respectively, in relation to differential susceptibility to efflux [8-11].  
74 We also examined THP-1 cells, where no active fluoroquinolone efflux has been evidenced  
75 so far. We found that gemifloxacin accumulates to higher levels than ciprofloxacin and  
76 moxifloxacin in both cell types and that all three drugs have a similar subcellular distribution.  
77 Yet, gemifloxacin showed no improved activity against two types of intracellular bacteria,  
78 *L. monocytogenes* and *S. aureus*, localized in the cytosol and in phagolysosomes,  
79 respectively.

80

## 81 2. Materials and methods

### 82 2.1. Antibiotics and main reagents

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

88

### 89 2.2. Cell lines

90 Murine J774 macrophages (wild-type cells [9]) and their ciprofloxacin-resistant derivatives  
91 overexpressing Mrp4 efflux transporter [8;11] were used for most experiments. Human THP-  
92 1 cells (ATCC TIB-202, American Tissue Culture Collection, Manassas, VA) [12;13] were  
93 used for comparison purposes. ATP depletion was obtained as previously described [9].

94

### 95 2.3. Determination of the cellular accumulation of fluoroquinolones.

96 We used a previously described protocol [9;14]. Cell -associated fluoroquinolones were  
97 assayed by fluorimetry (see [10] for ciprofloxacin and moxifloxacin; for gemifloxacin, the  
98 conditions were:  $\lambda_{\text{ex.}} = 270\text{nm}$ ;  $\lambda_{\text{em.}} = 402\text{ nm}$  [lowest limit of detection: 50  $\mu\text{g/L}$ ; linearity: 0-  
99 1.5 mg/L]). The cell drug content was expressed by reference to the total cell protein content  
100 [15]. The apparent total cellular concentration was then calculated using a conversion factor  
101 of 3.08  $\mu\text{L}$  of cell volume per mg of cell protein [9].

102

### 103 2.4. Cell fractionation studies in J774 cells

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

109 *2.5. Bacterial strains and susceptibility testings.*

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

113

114 *2.6. Cell infection and assessment of antibiotic intracellular activities.*

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

123

124 *2.7. Curve fitting and statistical analyses*

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

129

### 130 3. Results

#### 131 3.1. Cellular pharmacokinetics

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

142  
143 We then compared the kinetics of accumulation and efflux of gemifloxacin with that of  
144 ciprofloxacin using J774 macrophages only as this is where the largest difference of  
145 accumulation was observed. Figure 1B shows that the two fluoroquinolones could not be  
146 distinguished with respect to accumulation kinetics but displayed marked differences for  
147 efflux. Thus, gemifloxacin release (i) occurred at the same rate as its uptake (compare  $k_{in}$   
148 and  $k_{out}$  parameters); (ii) was about 2-fold slower than that of ciprofloxacin, including at the  
149 very initial period phase (see inset); (iii) was only partial, with about 25 % of the accumulated  
150 drug remaining cell-associated in apparent stable fashion after 30 min incubation in drug-free  
151 medium vs. negligible amounts for ciprofloxacin.

152  
153 We next measured the level of accumulation of gemifloxacin compared to that of  
154 ciprofloxacin in J774 macrophages overexpressing the ciprofloxacin efflux transporter Mrp4  
155 (ciprofloxacin-resistant cells), using normal conditions and conditions of ATP depletion  
156 (which inhibits all ATP-dependent active transporters including Mrp4). Figure 2A (upper



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

165  
166 Because the ciprofloxacin efflux transporter is saturable in a 10-200 mg/L range [9], we  
167 measured the accumulation of gemifloxacin in both wild-type J774 macrophages and  
168 ciprofloxacin-resistant cells over increasing concentrations of gemifloxacin in that range.  
169 Figure 2 (lower panel) shows that while gemifloxacin accumulation was not significantly  
170 influenced by its extracellular concentration in wild-type cells, there was a significant increase  
171 over the range of concentrations investigated for ciprofloxacin-resistant cells. In contrast,  
172 and as described earlier [9], ciprofloxacin showed a marked increase in its accumulation over  
173 the same concentration range in wild-type cells. For ciprofloxacin-resistant cells, the  
174 increase in cell accumulation of ciprofloxacin was much less marked in the range of drug  
175 concentrations investigated due to overexpression of the Mrp4 transporter (see [11]).

176  
177 These results suggest that gemifloxacin could be a poor, albeit still recognized substrate for  
178 efflux transport in J774 macrophages if Mrp4 is overexpressed. We, therefore, compared the  
179 kinetics of gemifloxacin efflux in ciprofloxacin-resistant vs. wild-type cells. While the *plateau*  
180 values observed at 30 min remained close from each other, denoting an incomplete release  
181 of gemifloxacin in both cases, its rate of efflux was significantly accelerated in ciprofloxacin-  
182 resistant cells compared to wild-type cells ( $k_{out} = 2.393 \pm 0.907$  vs.  $0.403 \pm 0.122$  min<sup>-1</sup>;  
183  $p < 0.001$ ; 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- $\beta$ -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

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217 **4. Discussion**

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

236

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

240

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

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

250  
251 Considering efflux, Mrp4 has been proposed as the main transporter responsible for the  
252 lower accumulation of ciprofloxacin in J774 macrophages compared to levofloxacin,  
253 garenoxacin, and moxifloxacin. These fluoroquinolones, indeed, reach a similar level of  
254 accumulation when Mrp4 is made inactive by ATP depletion or addition of gemfibrozil [10].  
255 Moreover, ciprofloxacin accumulation is significantly increased by silencing the gene coding  
256 for Mrp4 [8]. The present data show that this conclusion cannot be generalized to all  
257 fluoroquinolones and all situations. Thus gemifloxacin not only accumulates more than  
258 moxifloxacin in J774 macrophages under conditions of ATP depletion or in the presence of  
259 gemfibrozil, but also in THP-1 macrophages in which no gemfibrozil-inhibited efflux can be  
260 demonstrated. Another compelling reason to disregard efflux as being the main cause for  
261 the differential accumulation of gemifloxacin vs. ciprofloxacin and moxifloxacin is that  
262 gemifloxacin actually seems a weak but nevertheless effective substrate of Mrp4 in J774  
263 macrophages, whereas we know that moxifloxacin is not. Thus, globally and in contrast to  
264 what we proposed for moxifloxacin, the higher cellular concentration of gemifloxacin  
265 compared to other fluoroquinolones must find an explanation beyond considerations of influx  
266 and efflux rates only.

267  
268 Considering intracellular trapping, a model has been presented [33] that relates  
269 fluoroquinolone accumulation in eukaryotic cells to their trapping under a protonated form in  
270 lysosomes due to the acid pH ( $\sim 5.4$ ) prevailing therein. This, however, is unlikely because  
271 fluoroquinolones are not weak bases but zwitterionic compounds. Moreover, differences in  
272 accumulation of drugs in acidic membrane-bounded compartments should result from

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

283  
284 Actually, a more likely explanation for the larger cellular accumulation of gemifloxacin  
285 compared to moxifloxacin and ciprofloxacin could be its tighter binding to still undefined  
286 cellular constituents such as soluble proteins. This hypothesis would account for the  
287 pharmacokinetic and subcellular distribution data presented here, including (i) the lower  
288 efflux rate of gemifloxacin compared to ciprofloxacin (which, however, may also result from  
289 the less efficient recognition of gemifloxacin by the Mrp4 efflux transporter, both mechanisms  
290 being not mutually exclusive) and, (ii) its incomplete release upon transfer of the cells to  
291 drug-free medium. It is also consistent with the larger serum protein binding of gemifloxacin  
292 (55-73 %) compared to moxifloxacin (39-52 %) and ciprofloxacin (30 % only) [38;39].

293  
294 Determining the molecular nature of the intracellular binding sites for fluoroquinolones still  
295 require further investigations, but the mechanism proposed provides a rational explanation  
296 for the main critical observation made here, namely that all 3 fluoroquinolones are equipotent  
297 against intracellular bacteria in spite of their differences in cellular accumulation. We show,  
298 indeed, that it is the MIC of each drug that drives its intracellular potency (as defined by the  
299  $C_s$  and  $EC_{50}$  pharmacological descriptors) since all 3 fluoroquinolones show superimposable  
300 concentration-effects relationships once the data are normalized on basis of multiples of the

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

315

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

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326

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336 **Ethical approval:** Not applicable

337



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476

477

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

antibiotic	<i>L. monocytogenes</i>					<i>S. aureus</i>				
	R <sup>2</sup>	E <sub>min</sub> <sup>b</sup> (CI)	E <sub>max</sub> <sup>c</sup> (CI)	EC <sub>50</sub> <sup>d</sup> (CI)	C <sub>s</sub> <sup>e</sup>	R <sup>2</sup>	E <sub>min</sub> <sup>b</sup> (CI)	E <sub>max</sub> <sup>c</sup> (CI)	EC <sub>50</sub> <sup>d</sup> (CI)	C <sub>s</sub> <sup>e</sup>
<b>ciprofloxacin</b>	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
<b>moxifloxacin</b>	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
<b>gemifloxacin</b>	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
<b>all 3</b>	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

481

482 <sup>a</sup> 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

483 [*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

484 [*S. aureus*]; gemifloxacin: 0.005 to 150 mg/L. [*L. monocytogenes*] and 0.0008 to 20 mg/L [*S. aureus*]).

485 <sup>b</sup> relative minimal efficacy: change in cfu (in log<sub>10</sub> units) at time 24 h from the initial, post-phagocytosis inoculum, as extrapolated for an infinitely  
 486 low antibiotic concentration

487 <sup>c</sup> relative maximal efficacy: change in cfu (in log<sub>10</sub> units) at time 24 h from the initial, post-phagocytosis inoculum, as extrapolated for an  
 488 infinitely large antibiotic concentration

489 <sup>d</sup> relative potency: extracellular concentration (in multiple of MIC) yielding a change in cfu at time 24 h half way between  $E_{\min}$  and  $E_{\max}$

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

493

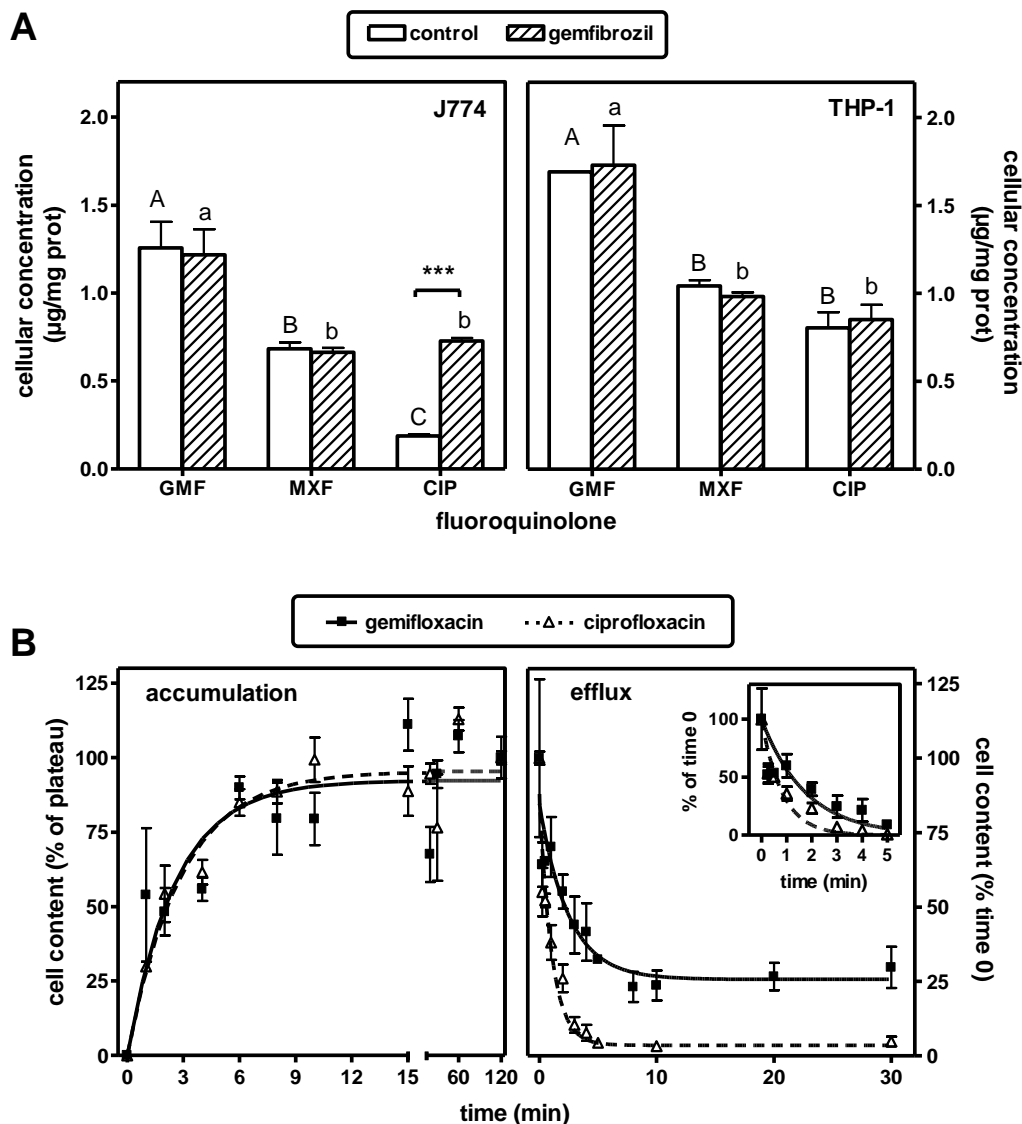
494 Statistical analysis:

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

500



501 Figure 1  
502



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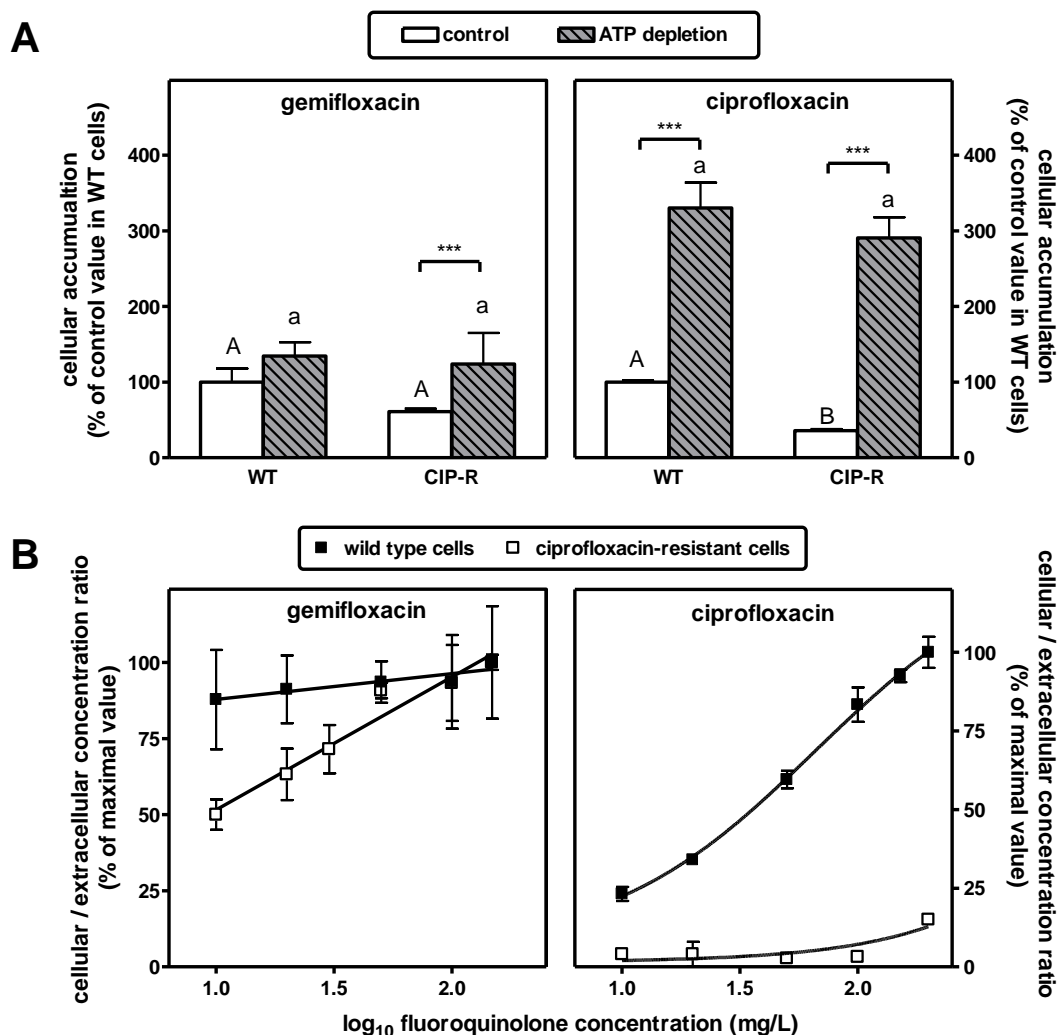
504

505 Caption to Figure 1: Accumulation and efflux of fluoroquinolones.

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

514 **B** (lower panel): kinetics of accumulation (left) and efflux (right) of gemifloxacin  
 515 **compared** to ciprofloxacin in J774 macrophages (see [10] for efflux of moxifloxacin) .  
 516 For accumulation studies, cells were transferred to medium containing a fixed  
 517 amount of drug (20 mg/L) and collected at the times indicated in the abscissa. For  
 518 efflux, cells were first exposed to the drug for 2 h at a concentration of 20 mg/L,  
 519 gently washed, transferred to drug free medium and collected at the times indicated  
 520 in the abscissa. Data were used to fit a one phase exponential association function  
 521 for influx [  $y = y_{max} x (1 - e^{-k_{in} x t})$  ] and a one phase exponential decay function  
 522 for efflux [  $y = y_{max} x e^{-k_{out} x t} + plateau$  ] by nonlinear regression. Regression  
 523 parameters for influx: (a) gemifloxacin,  $R^2 = 0.780$ ,  $k_{in} = 0.386 \pm 0.123 \text{ min}^{-1}$ ; (b)  
 524 ciprofloxacin,  $R^2 = 0.922$ ,  $k_{in} = 0.348 \pm 0.066 \text{ min}^{-1}$ . Regression parameters for efflux:  
 525 (1) main graph, (a) gemifloxacin,  $R^2 = 0.897$ ,  $k_{out} = 0.403 \pm 0.122 \text{ min}^{-1}$ ,  $plateau =$   
 526  $25.71 \pm 4.63$ ; (b) ciprofloxacin,  $R^2 = 0.949$ ,  $k_{out} = 0.949 \pm 0.204 \text{ min}^{-1}$ ,  $plateau =$   
 527  $3.56 \pm 3.24$ ; (2) Inset: data for the initial stage of efflux (0 – 5 min) and corrected for  
 528 differences in plateau reached after 10 min, (a) gemifloxacin,  $R^2 = 0.658$ ,  
 529  $k_{out} = 0.571 \pm 0.138 \text{ min}^{-1}$ ; (b) ciprofloxacin,  $R^2 = 0.909$ ,  $k_{out} = 1.216 \pm 0.209 \text{ min}^{-1}$ .  
 530 Statistical analysis (paired *t*- test two-tailed): Influx, no significant difference in rate  
 531 constants; absolute values of plateaus of accumulation were different and in line with  
 532 data of Figure 1). Efflux: Main graph, comparison of all values:  $p < 0.001$ , plateaus  
 533 values only:  $p < 0.001$ ,  $k$  values only:  $p < 0.001$ ; Inset: comparison for all values:  
 534  $p = 0.016$ ,  $k_{out}$  values only:  $p < 0.001$ .  
 535

536 Figure 2



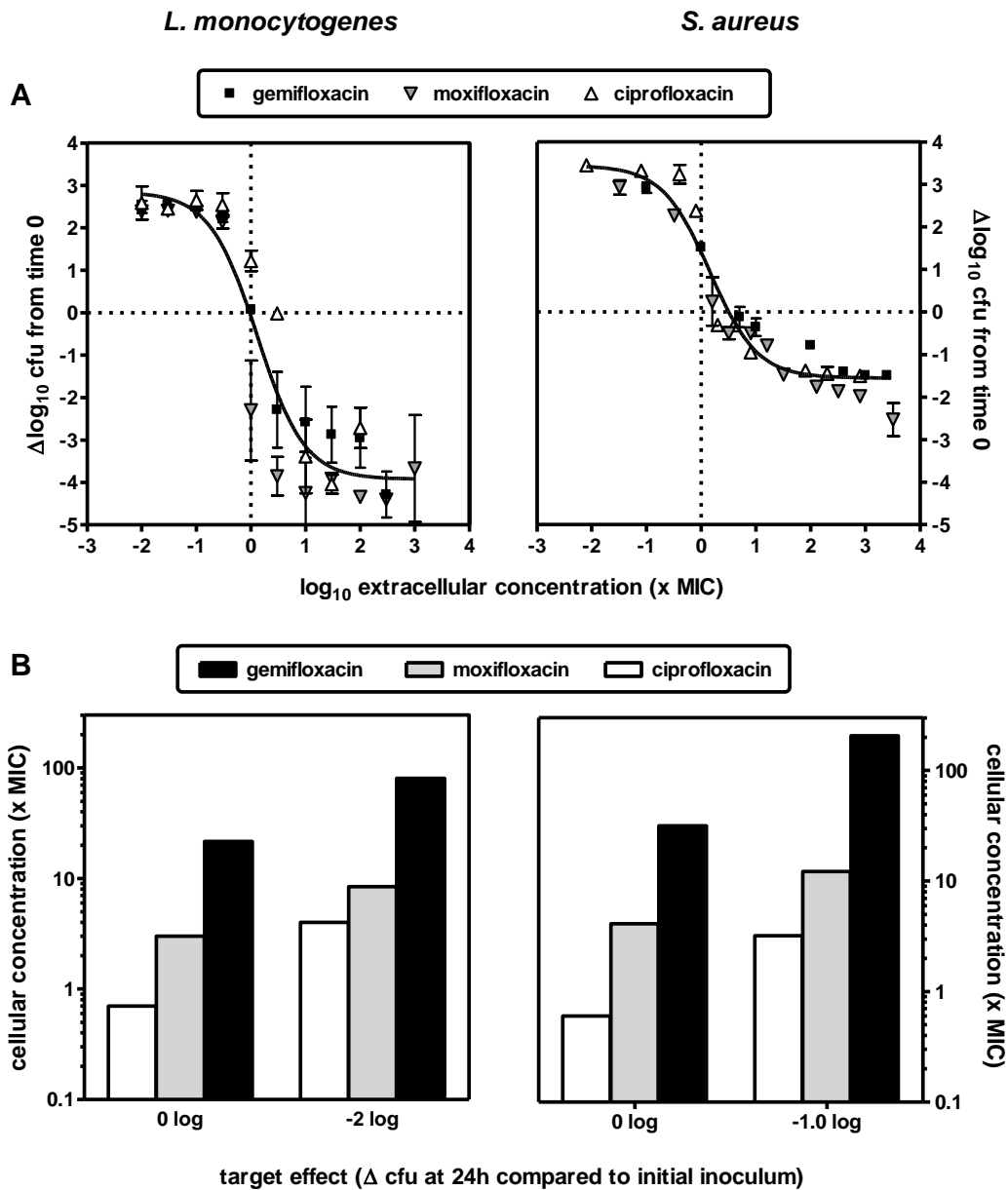
537

538 Caption to Figure 2: Cellular accumulation of gemifloxacin as compared to  
 539 ciprofloxacin in wild-type or ciprofloxacin-resistant J774 mouse macrophages (see  
 540 [11] for the corresponding data with moxifloxacin) **A** (upper panel): cells were  
 541 incubated for 2 h in control conditions (open bars) or in ATP-depleted cells (gray  
 542 hatched bars) with a fixed concentration (20 mg/L) of gemifloxacin (left) or  
 543 ciprofloxacin (right) with wild-type cells (WT) or ciprofloxacin-resistant cells (CIP-R).  
 544 Data are expressed in percentage of the value measured in wild-type cells in control  
 545 conditions for each fluoroquinolone. All values are the means of 3 independent  
 546 determinations  $\pm$  SD. Statistical analysis (ANOVA): control vs. ATP-depletion, \*\*\*  $p$   
 547  $< 0.001$ ; WT vs. CIP-R cells: bars with different letters are different from one another  
 548 ( $p < 0.05$ ; upper case letters, control conditions; lower case letters, ATP-depletion).  
 549 **B** (lower panel): Influence of the extracellular concentration of gemifloxacin (left) and  
 550 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  $\mu\text{g}$  per  $\text{mg}$  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  $\pm$  SD.  
556  
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558 Figure 3  
559



560

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

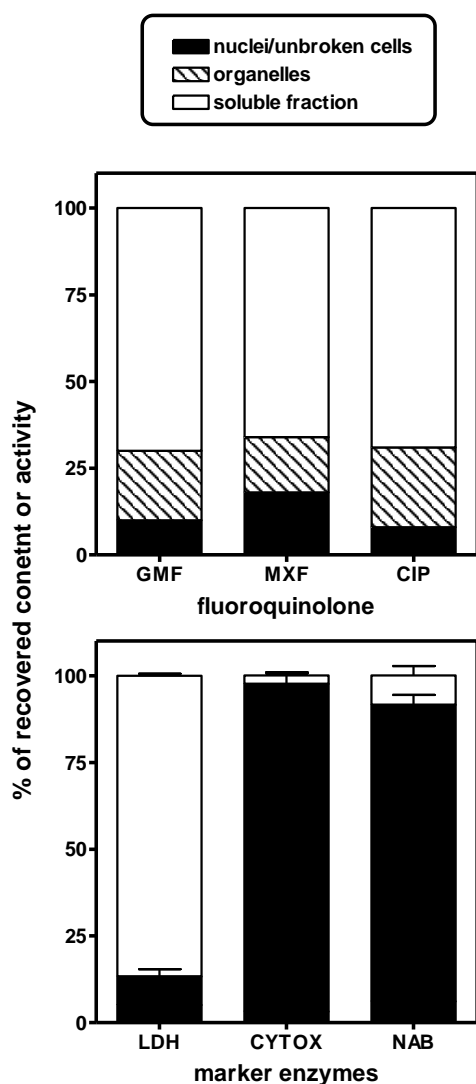
564 **A** (top): after phagocytosis and elimination of the extracellular bacteria, cells were  
565 incubated for 24 h with increasing concentrations of antibiotic (total drug) covering a  
566 ~ 0.01 to ~ 1,000 x MIC range (MIC [mg/L] were 1 and 0.125 [ciprofloxacin], 0.5 and  
567 0.03 [moxifloxacin], and 0.5 and 0.008 [gemifloxacin] for *L. monocytogenes* and  
568 *S. aureus* respectively). The graphs show the change in the number of cfu (log  
569 scale) per mg of cell protein compared to the initial post-phagocytosis inoculum

570 (ordinate) as a function of the extracellular concentration of each drug expressed in  
571 multiple of its MIC (abscissa). In each graph, the horizontal dotted line corresponds  
572 to an apparent static effect and the vertical line to the MIC of the drug. A single  
573 sigmoidal regression has been fit to all data sets (see Figure SP2 for individual  
574 regression curves. The pertinent regression parameters and numerical values of the  
575 4 key pharmacological descriptors ( $E_{\min}$ ,  $E_{\max}$ ;  $EC_{50}$ ,  $C_s$ ) are shown Table 1 for each  
576 drug - bacteria combination.

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

589

590 **Figure 4**  
591



592  
593 Caption for 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-oxidase (CYTOX; marker of mitochondria), and N-acetyl- $\beta$ -  
600 hexosaminidase (NAB; marker of lysosomes) as the mean values ( $\pm$  SD) of the 3  
601 experiments (corresponding to each of the individual experiment shown in the upper  
602 panel).  
603

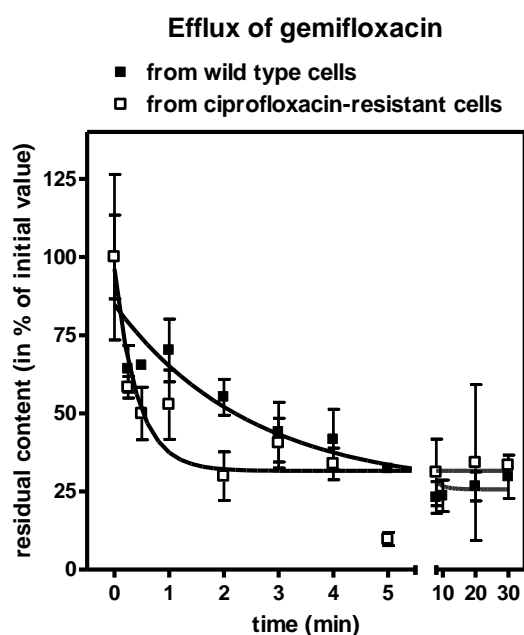
604 Vallet *et al.* Intracellular activity of fluoroquinolones - Supplementary Material

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606

607

## Figure SP1



608

609 Caption to Figure SP1: Kinetics of efflux of gemifloxacin from wild-type and

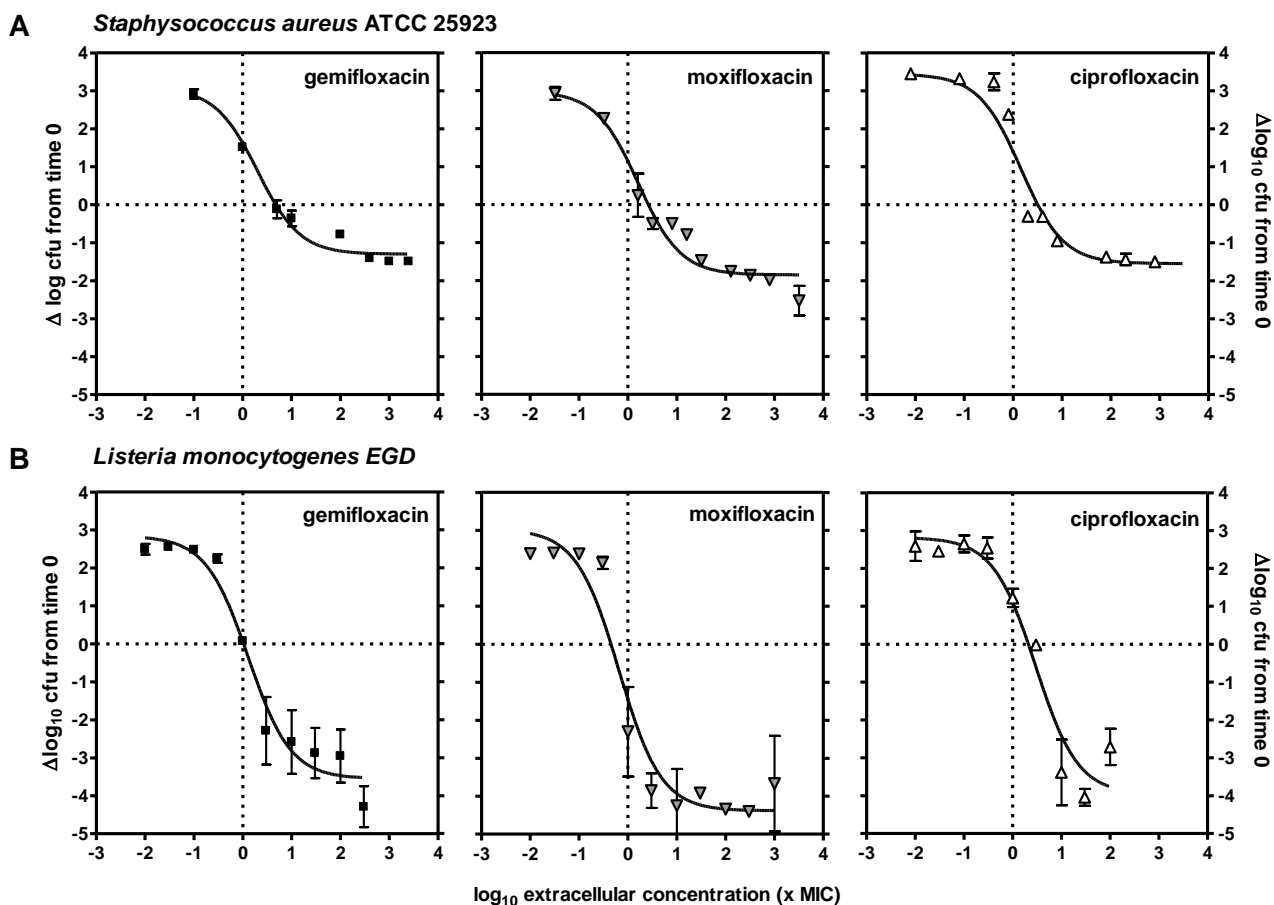
610 ciprofloxacin-resistant J774 cells. Cells were exposed to gemifloxacin (20 mg/L) for

611 2 h and then transferred to antibiotic-free medium for up to 30 min. Regression

612 parameters: (a) wild-type cells (same data as in Figure 2),  $R^2 = 0.896$ ,613  $k_{out} = 0.403 \pm 0.122 \text{ min}^{-1}$ ,  $plateau = 25.71 \pm 4.63$ ; (b) ciprofloxacin-resistant cells,614  $R^2 = 0.830$ ,  $k_{out} = 2.39 \pm 0.907 \text{ min}^{-1}$ ,  $plateau = 31.6 \pm 4.0$ ).

615



616 **Figure SP2**  
617

618  
619 Caption to Figure SP2: Concentration-response of the activities of gemifloxacin,  
620 moxifloxacin, and ciprofloxacin (CIP) against *S. aureus* ATCC25923 (top) and  
621 *L. monocytogenes* EGD (bottom) in wild-type J774 macrophages. Cells were  
622 incubated with increasing concentrations of antibiotic (total drug) for 24 h. Each  
623 graph shows the change in the number of cfu (log scale) per mg of cell protein  
624 compared to the initial post-phagocytosis inoculum (ordinate) as a function of the  
625 extracellular concentration of each drug expressed in multiples of its MIC (abscissa).  
626 In each graph, the horizontal dotted line corresponds to an apparent static effect and  
627 the vertical line to the MIC of the drug. A sigmoidal regression has been fitted to  
628 each set of data (see Table 1 for the pertinent regression parameters and numerical  
629 values of the four key pharmacological descriptors ( $E_{\min}$ ,  $E_{\max}$ ,  $EC_{50}$ ,  $C_s$ )).

630  
631

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

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

637

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

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

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

643 <sup>c</sup> 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  
644 the two phases at a given pH)

645 <sup>d</sup> These values are the arithmetic average of three methods of calculations (Viswanadhan's fragmentation; Klopman's fragmentation; and  
646 PHYSPROP© database [see [https://www.reaxys.com/static/marvin/marvin\\_5\\_3\\_7/help/calculations/partitioning.html](https://www.reaxys.com/static/marvin/marvin_5_3_7/help/calculations/partitioning.html) for details]).

647 <sup>e</sup> value as reported in Drugbank (see <http://www.drugbank.ca> and [2])

648 <sup>f</sup> azithromycin is a dicationic drug

649 <sup>g</sup> 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  
650 values).

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652 References

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