International Journal of Antimicrobial Agents 38 (2011) 249–256

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

# 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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ARTICLE INFO

*Article history:*
Received 22 March 2011
Accepted 17 May 2011

*Keywords:*
Ciprofloxacin
Moxifloxacin
Gemifloxacin
Mrp4
*Staphylococcus aureus*
*Listeria monocytogenes*
Macrophages

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. Whilst 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 *Listeria monocytogenes* or *Staphylococcus aureus* (typical cytoplasmic and phagolysosomal organisms, respectively), gemifloxacin was equipotent to moxifloxacin and ciprofloxacin in concentration-dependent experiments if data are normalised based on the minimum inhibitory concentration (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 three fluoroquinolones, with approximately two-thirds of the cell-associated drug recovered in the soluble fraction (cytosol). These data suggest that 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. Whilst these observations do not decrease the intrinsic value of fluoroquinolones for the treatment of intracellular infections, they indicate that ranking fluoroquinolones based on cell accumulation data without measuring the corresponding intracellular activity may lead to incorrect conclusions regarding their real potential.



## 1. Introduction

Fluoroquinolone antibiotics are important in the current therapeutic arsenal because of their broad spectrum, highly bactericidal activity and favourable pharmacokinetic properties [1]. Their wide tissue distribution allows them to reach 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 in relation to the intracellular forms of *Listeria monocytogenes* in J774 macrophages in experiments where the drug's 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 polymorphonuclear leukocytes and is active against intracellular bacteria [5,6]. This prompted us to compare it with other fluoroquinolones for cellular pharmacokinetics and activity in an established model of murine J774 macrophages [7]. Ciprofloxacin and moxifloxacin, when needed, were used as comparators as these antibiotics show low and high 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.

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doi:10.1016/j.ijantimicag.2011.05.011

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 *Staphylococcus aureus*, localised in the cytosol and in phagolysosomes, respectively.

## 2. Materials and methods

### 2.1. Antibiotics and main reagents

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

### 2.2. Cell lines

Murine J774 macrophages (wild-type cells [9]) and their ciprofloxacin-resistant derivatives overexpressing the 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 achieved as previously described [9].

### 2.3. Determination of cellular accumulation of fluoroquinolones

A previously described protocol was used [9,14]. Cell-associated fluoroquinolones were assayed by fluorimetry (see [10] for ciprofloxacin and moxifloxacin; for gemifloxacin, the conditions were $\lambda_{ex.} = 270$ nm, $\lambda_{em} = 402$ 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* oxidase for mitochondria, *N*-acetyl-β-hexosaminidase for lysosomes, and lactate dehydrogenase for cytosol [7]).

### 2.5. Bacterial strains and susceptibility testing

*Listeria monocytogenes* strain EGD and *S. aureus* strain ATCC 25923 were used. Minimum inhibitory concentration (MIC) determinations were made according to the Clinical and Laboratory Standards Institute (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 ($E_{min}$ and $E_{max}$, respectively, in $\log_{10}$ units) and (ii) the relative potencies ($EC_{50}$) and static concentrations ($C_s$). This type of analysis and its usefulness for comparing antibiotics and the response of different bacteria have been described in detail in previous publications [14,17–19]. As discussed previously [20], the large dilution of samples before spreading on agar plates for colony-forming unit (CFU) counting ensures the absence of a carry-over effect.

### 2.7. Curve fitting and statistical analyses

Curve fitting analyses were done using GraphPad Prism® 4.03 (GraphPad Software Inc., San Diego, CA). Statistical analyses were made with the same software for comparing concentration–response functions, and with GraphPad InStat® v3.06 (GraphPad Software Inc.) 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. Fig. 1A shows that (i) gemifloxacin accumulated to a larger extent than the other two fluoroquinolones both in 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]; and (iv) the level of accumulation of ciprofloxacin was similar to that of moxifloxacin in THP-1 cells and was 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. Fig. 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 approximately two-fold slower than that of ciprofloxacin, including in the very initial period (see inset); (iii) was only partial, with ca. 25% of the accumulated drug remaining cell-associated in an apparent stable fashion after 30 min of incubation in drug-free medium compared with negligible amounts for ciprofloxacin.

We next measured the level of accumulation of gemifloxacin compared with 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). Fig. 2A shows that (i) gemifloxacin accumulation was reduced (but in a non-statistically significant manner) in ciprofloxacin-resistant cells compared with wild-type cells; (ii) ATP depletion increased its accumulation both in 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; and (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 both in wild-type J774 macrophages and in ciprofloxacin-resistant cells over increasing concentrations of

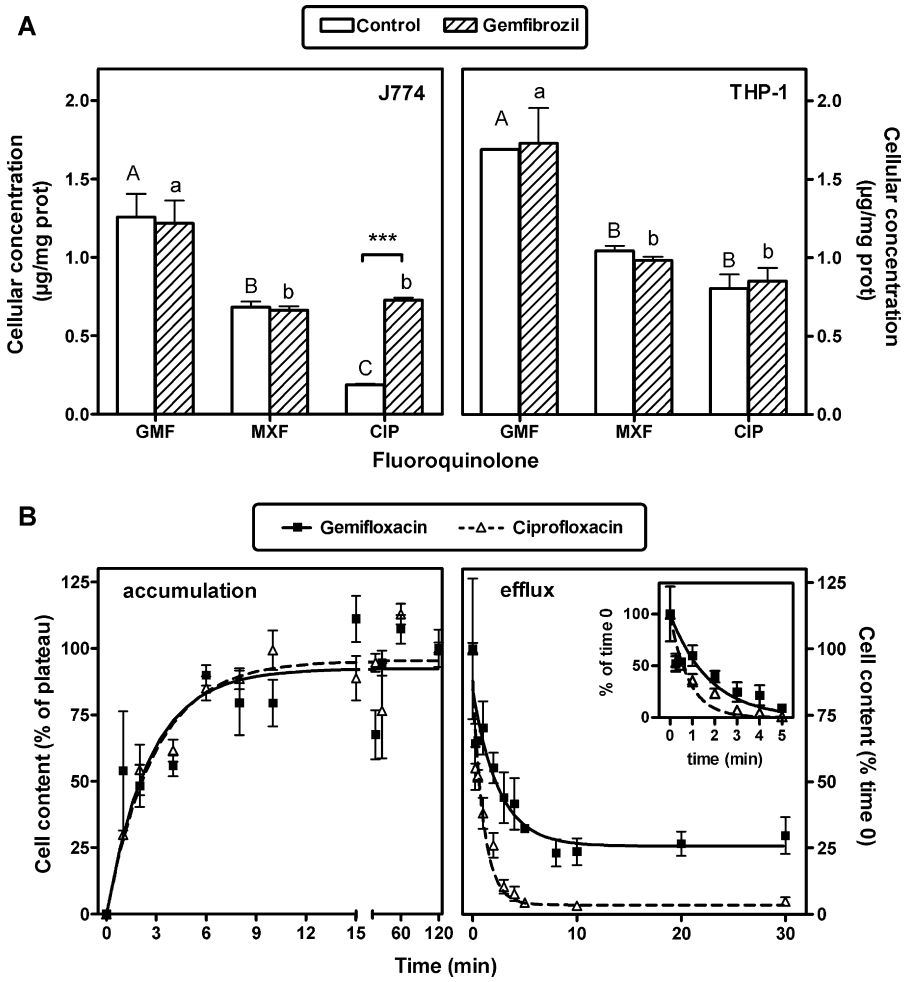


**Fig. 1.** Accumulation and efflux of fluoroquinolones. (A) Accumulation of gemifloxacin (GMF), moxifloxacin (MXF) and ciprofloxacin (CIP) in wild-type J774 mouse macrophages (left) and human THP-1 monocytes (right) incubated for 2 h with 20 mg/L in control conditions or in the presence of the Mrp inhibitor gemfibrozil (500 μM). All values are the mean of three independent determinations ± standard deviation. Statistical analysis (ANOVA): control vs. gemfibrozil, ***$P < 0.001$; comparison between fluoroquinolones, bars with different letters are different from one another ($P < 0.001$; upper case letters, control conditions; lower case letters, + gemfibrozil). (B) Kinetics of accumulation (left) and efflux (right) of gemifloxacin compared with 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 were collected at the times indicated on 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 on the abscissa. Data were used to fit a one-phase exponential association function for influx [$y = y_{max}x(1 - e^{-k_{in}xt})$] and a one-phase exponential decay function for efflux [$y = y_{max}xe^{-k_{out}xt}$ + plateau)] by non-linear regression. Regression parameters for influx: (a) gemifloxacin, $R^2 = 0.780$, $k_{in} = 0.386 \pm 0.123\ min^{-1}$; (b) ciprofloxacin, $R^2 = 0.922$, $k_{in} = 0.348 \pm 0.066\ min^{-1}$. Regression parameters for efflux: (i) main graph, (a) gemifloxacin, $R^2 = 0.897$, $k_{out} = 0.403 \pm 0.122\ min^{-1}$, plateau = 25.71 ± 4.63; (b) ciprofloxacin, $R^2 = 0.949$, $k_{out} = 0.949 \pm 0.204\ min^{-1}$, plateau = 3.56 ± 3.24; (ii) inset: data for the initial stage of efflux (0–5 min) and corrected for differences in plateau reached after 10 min, (a) gemifloxacin, $R^2 = 0.658$, $k_{out} = 0.571 \pm 0.138\ min^{-1}$; (b) ciprofloxacin, $R^2 = 0.909$, $k_{out} = 1.216 \pm 0.209\ min^{-1}$. 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 Fig. 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, $P < 0.001$.

gemifloxacin in that range. Fig. 2B shows that whilst 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 previously [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 owing to overexpression of the Mrp4 transporter (see [11]).

These results suggest that gemifloxacin could be a poor, albeit still recognised, 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. Whilst the plateau values observed at 30 min remained close to each other, denoting an incomplete release of gemifloxacin in both cases, its rate of efflux was significantly accelerated in ciprofloxacin-resistant cells compared with wild-type cells ($k_{out} = 2.393 \pm 0.907\ min^{-1}$ vs. $0.403 \pm 0.122\ min^{-1}$; $P < 0.001$) (see graphical representation in Supplementary Fig. 1).

### 3.2. Intracellular activity

To examine the correlation between cellular accumulation and intracellular activity, we compared all three fluoroquinolones in a pharmacological model of intracellular infection [14,17] using J774 macrophages as host cells since this is where the largest differences in accumulation levels had been observed. *Listeria monocytogenes* and *S. aureus* were selected as bacterial targets as they represent a typical cytoplasmic and phagolysosomal organism, respectively. Data presented in Fig. 3A (with analysis of the key pharmacological descriptors in Table 1) show that all three antibiotics induced essentially a similar response when expressed as a function of equipotent

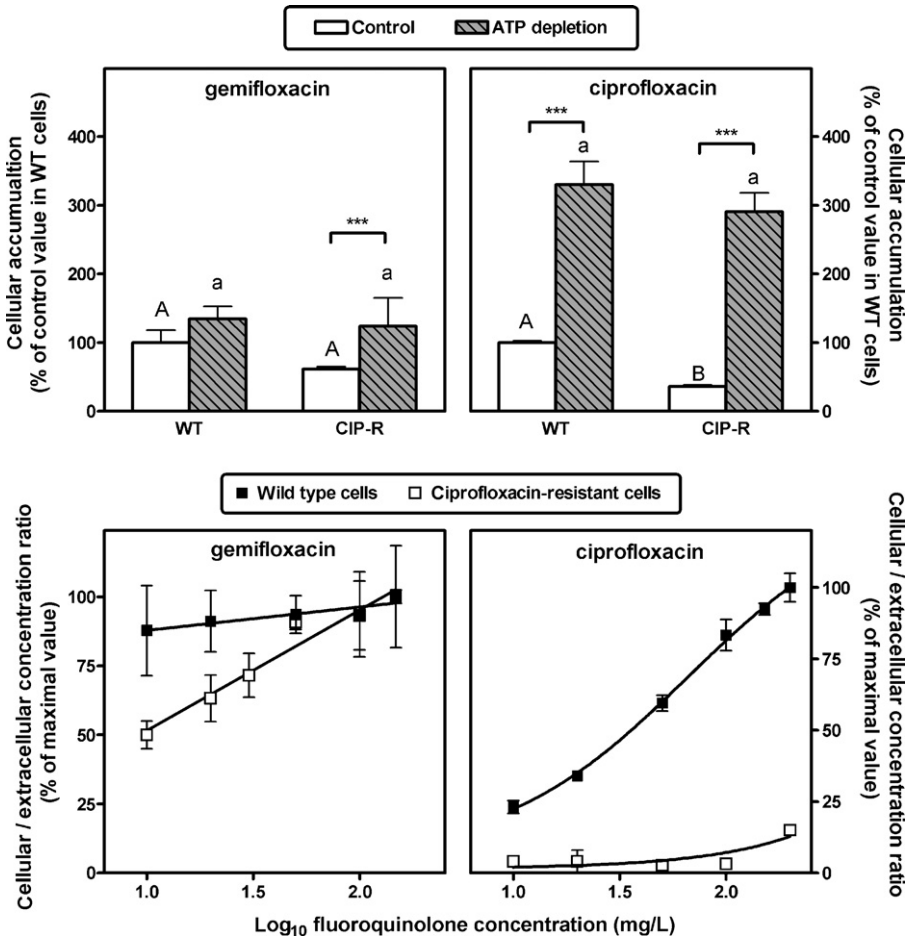


**Fig. 2.** Cellular accumulation of gemifloxacin compared with ciprofloxacin in wild-type (WT) or ciprofloxacin-resistant (CIP-R) J774 mouse macrophages (see [11] for the corresponding data for moxifloxacin). (A) Cells were incubated for 2 h in control conditions or ATP-depleted with a fixed concentration (20 mg/L) of gemifloxacin (left) or ciprofloxacin (right) with WT or CIP-R cells. Data are expressed as percentage of the value measured in WT cells in control conditions for each fluoroquinolone. All values are the means of three independent determinations ± standard deviation (S.D.). 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) Influence of the extracellular concentration of gemifloxacin (left) and ciprofloxacin (right) on their cellular-to-extracellular concentration ratio in WT or CIP-R J774 mouse macrophages measured after 2 h of incubation. The cellular concentration was expressed as μg per mg protein. Data are expressed as percentage of the highest value observed in WT cells for each fluoroquinolone. All values are the means of three independent determinations ± S.D.

extracellular concentrations (multiples of the MIC). Thus, in all cases, a single sigmoid function could be fitted to the individual responses of each antibiotic (see Supplementary Fig. 2 and the pertinent regression parameters and pharmacological descriptors in Table 1). As no statistically significant difference was observed between the three sets of experiments with respect to relative minimal efficacies ($E_{min}$; growth in the absence of antibiotic), maximal relative efficacies ($E_{max}$; maximal antibiotic-related killing), relative potencies ($EC_{50}$) and static concentrations ($C_s$; in multiples of the MIC), all data were pooled to fit a single function shown in Fig. 3A. We then calculated for each fluoroquinolone which cellular drug concentration would be needed to reach two predefined pharmacodynamic targets (static effect and a 1 or 2 $\log_{10}$ CFU decrease). The results (with the mode of calculation) are presented in Fig. 3B and show that the potencies of the drugs with respect to their intracellular targets were in inverse proportion to their respective cellular accumulations.

### 3.3. Subcellular distribution

Lastly, we compared the subcellular distributions of ciprofloxacin, moxifloxacin and gemifloxacin. Fig. 4 shows that all three fluoroquinolones shared essentially the same distribution, with ca. 70% recovered in the soluble fraction, ca. 10% of ciprofloxacin and gemifloxacin and 18% of moxifloxacin in the nuclei/unbroken cells fraction, and the remainder in the organelles/membranes fraction. As previously described [7], lactate dehydrogenase was mostly recovered in the soluble fraction, and cytochrome oxidase and *N*-acetyl-β-hexosaminidase in the granules/membranes fraction, indicating that the fractionation method effectively separated the corresponding subcellular entities with only a very low proportion of unbroken cells left after homogenisation.

## 4. Discussion

Gemifloxacin, approved for clinical use in over 27 countries [21], is characterised 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 tissue accumulation [24]. Human pharmacokinetic/pharmacodynamic studies show that gemifloxacin achieves higher area under the concentration–time curve (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]. However, the present study shows that the

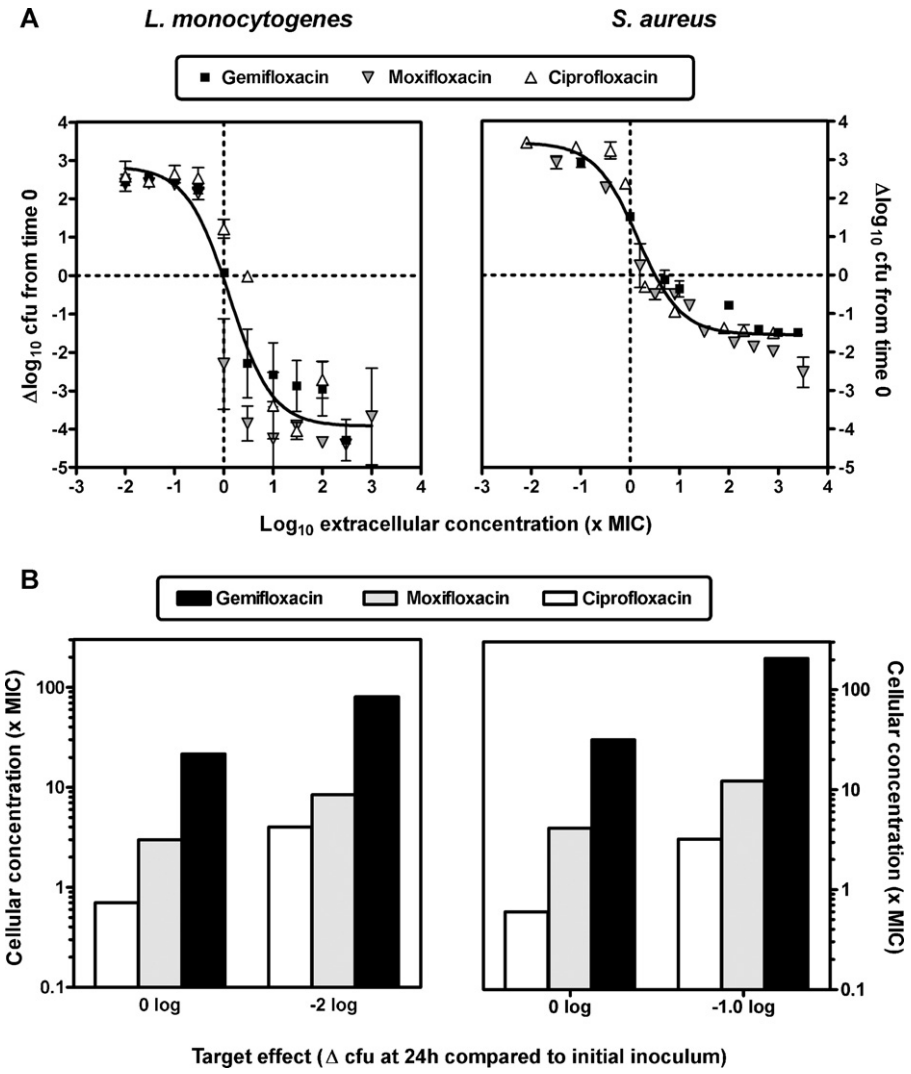


**Fig. 3.** Concentration–response of the activities of gemifloxacin, moxifloxacin and ciprofloxacin against phagocytosed *Listeria monocytogenes* EGD (left) and *Staphylococcus aureus* ATCC 25923 (right) in wild-type J774 macrophages. (A) After phagocytosis and elimination of extracellular bacteria, cells were incubated for 24 h with increasing concentrations of antibiotic (total drug) covering a minimum inhibitory concentration (MIC) range of ca. 0.01× to ca. 1000× MIC [MICs were 1 mg/L and 0.125 mg/L (ciprofloxacin), 0.5 mg/L and 0.03 mg/L (moxifloxacin) and 0.5 mg/L and 0.008 mg/L (gemifloxacin) for *L. monocytogenes* and *S. aureus*, respectively]. The graphs show the change in the number of colony-forming units (CFU) (log scale) per mg of cell protein compared with 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 Supplementary Fig. 2 for individual regression curves). The pertinent regression parameters and numerical values of the four key pharmacological descriptors ($E_{min}$, $E_{max}$, $EC_{50}$ and $C_s$) are shown in Table 1 for each drug–bacteria combination. (B) 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) and 2 (*L. monocytogenes*) or 1 (*S. aureus*) $\log_{10}$ CFU decrease compared with 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 interpolation) and (ii) using the data of Fig. 2 (lower panel; wild-type cells) to calculate the corresponding apparent total cellular concentrations of gemifloxacin and ciprofloxacin (for moxifloxacin, the accumulation data published in [10] was used) 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].

higher accumulation of gemifloxacin in J774 macrophages (i) is not associated with differences in influx rates compared with a fluoroquinolone with lower accumulation (ciprofloxacin); (ii) does not preclude and cannot be explained by differences in active efflux transport (in comparison with moxifloxacin); and (iii) does not lead to 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×, 20–40× and 90× higher than serum levels [26–28] and (ii) with respect to intracellular activity, what has been observed in human polymorphonuclear leukocytes 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, a faster drug accumulation is usually related to a higher lipophilicity (which is supposed to facilitate transmembrane diffusion) or due to the activity of transporters. This does not seem to apply to gemifloxacin, as this fluoroquinolone (i) is not globally more lipophilic than ciprofloxacin (see Supplementary Table 1 for experimental and calculated log *P* and log *D* values) and (ii) is probably not the substrate of a specific influx transporter when compared with ciprofloxacin (same rate accu-

**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 Fig. 3A). The first three rows show the analysis for each individual antibiotic and the last row shows the analysis made for all pooled data.

| Antibiotic | *Listeria monocytogenes* | | | | | *Staphylococcus 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 three fluoroquinolones | 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 |

MIC, minimum inhibitory concentration; CFU, colony-forming units.
*Statistical analysis*: the raw data obtained for each individual antibiotic, and the corresponding Hill functions, compared using one-way analysis of variance (ANOVA) (parametric) and Kruskal–Wallis (non-parametric) tests were 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 and moxifloxacin vs. gemifloxacin) using unpaired *t*-test and showed no significant difference for any comparison ($P > 0.18$).
[a] By use of all data for antibiotic concentrations ranging from ca. 0.01× to ca. 1000× MIC [ciprofloxacin 0.01–100 mg/L (*L. monocytogenes*) and 0.001–100 mg/L (*S. aureus*), moxifloxacin 0.005–30 mg/L (*L. monocytogenes*) and 0.001–100 mg/L (*S. aureus*) and gemifloxacin 0.005–150 mg/L (*L. monocytogenes*) and 0.0008–20 mg/L (*S. aureus*)].
[b] Relative minimal efficacy: change in CFU (in $\log_{10}$ units) at 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 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 24 h half way between $E_{min}$ and $E_{max}$.
[e] Static concentration: concentration (in multiples of MIC) resulting in no apparent bacterial growth (no change in CFU from the initial post-phagocytosis inoculum) as determined by graphical interpolation [MIC values are 1 mg/L and 0.125 mg/L (ciprofloxacin), 0.5 mg/L and 0.03 mg/L (moxifloxacin) and 0.5 mg/L and 0.008 mg/L (gemifloxacin) for *L. monocytogenes* and *S. aureus*, respectively].




**Fig. 4.** Subcellular distribution of gemifloxacin (GMF), moxifloxacin (MXF) and ciprofloxacin (CIP) in J774 mouse macrophages incubated for 2 h with 50 mg/L of each drug. The upper panel shows the antibiotic content in the nuclei/unbroken cells, organelles and soluble fraction expressed as a percentage of the total recovered amount (each bar corresponds to a separate experiment). The lower panel shows the distribution of lactate dehydrogenase (LDH; marker of the cytosol), cytochrome *c* oxidase (CYTOX; marker of mitochondria) and *N*-acetyl-β-hexosaminidase (NAB; marker of lysosomes) as the mean values (±standard deviation) of the three experiments (corresponding to each of the individual experiment shown in the upper panel).

mulation constants). Non-specific influx transporter(s) observed in polymorphonuclear leukocytes and human monocytes [29–31] can probably be dismissed here as those 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 with levofloxacin, garenoxacin and moxifloxacin. Indeed, these fluoroquinolones reach a similar level of accumulation when Mrp4 is made inactive by ATP depletion or inhibited by the 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 generalised 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 appears to be 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 with 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 owing 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 p$K_a$ of their basic functions (see [34]), which is not the case for the three fluoroquinolones studied here (see individual basic p$K_a$ values in Supplementary Table 1). 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, which are weak bases, are predominantly associated with lysosomes in J774 macrophages [2,36,37]). Lastly, experimental studies have shown a lack of effect of monensin (an $H^+$ ionophore that collapses the cytosolic–lysosomal $\Delta$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 with 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 with 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 with moxifloxacin (39–52%) and ciprofloxacin (30% only) [38,39].

Determining the molecular nature of the intracellular binding sites for fluoroquinolones still requires further investigation, but the mechanism proposed provides a rational explanation for the main critical observation made here, namely that all three fluoroquinolones are equipotent against intracellular bacteria despite their differences in cellular accumulation. Indeed, we show 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 three fluoroquinolones show superimposable concentration–effect relationships once the data are normalised on the 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 higher. 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 (as measured in broth), which is what we observe. Thus, the larger cellular accumulation of some fluoroquinolones, taking gemifloxacin as 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 drug with, however, no or little difference in their free form. This confirms and extends previous work showing 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 prediction of their intracellular activity and (ii) higher cellular accumulation may depend on 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.

## Acknowledgments

The authors are grateful to M.C. Cambier, C. Misson and M. Vergauwen for dedicated technical assistance. They also thank Oscient Pharmaceuticals Corp. and Bayer HealthCare for the kind gift of gemifloxacin and of moxifloxacin and ciprofloxacin, respectively.

*Funding*: CMV is Boursier of the Belgian Fonds pour la Recherche dans l'Industrie et l'Agriculture (FRIA); BM was postdoctoral fellow of the First post-doctoral programme of the Belgian Région wallonne; SL and FVB are Chargé de Recherches and Maître de Recherches, respectively, of the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS). This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale (grants no. 3.4.597.06 and 3.4.583.08) and the Belgian Fonds de la Recherche Scientifique (grant no. 1.5.195.07).

*Competing interests*: None declared.

*Ethical approval*: Not required.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2011.05.011.

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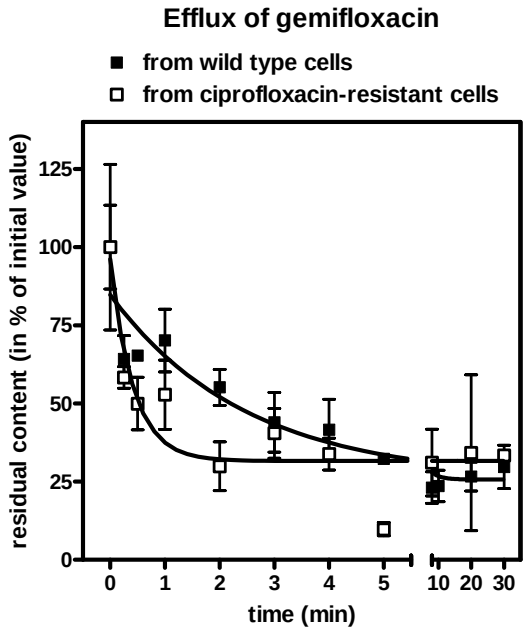
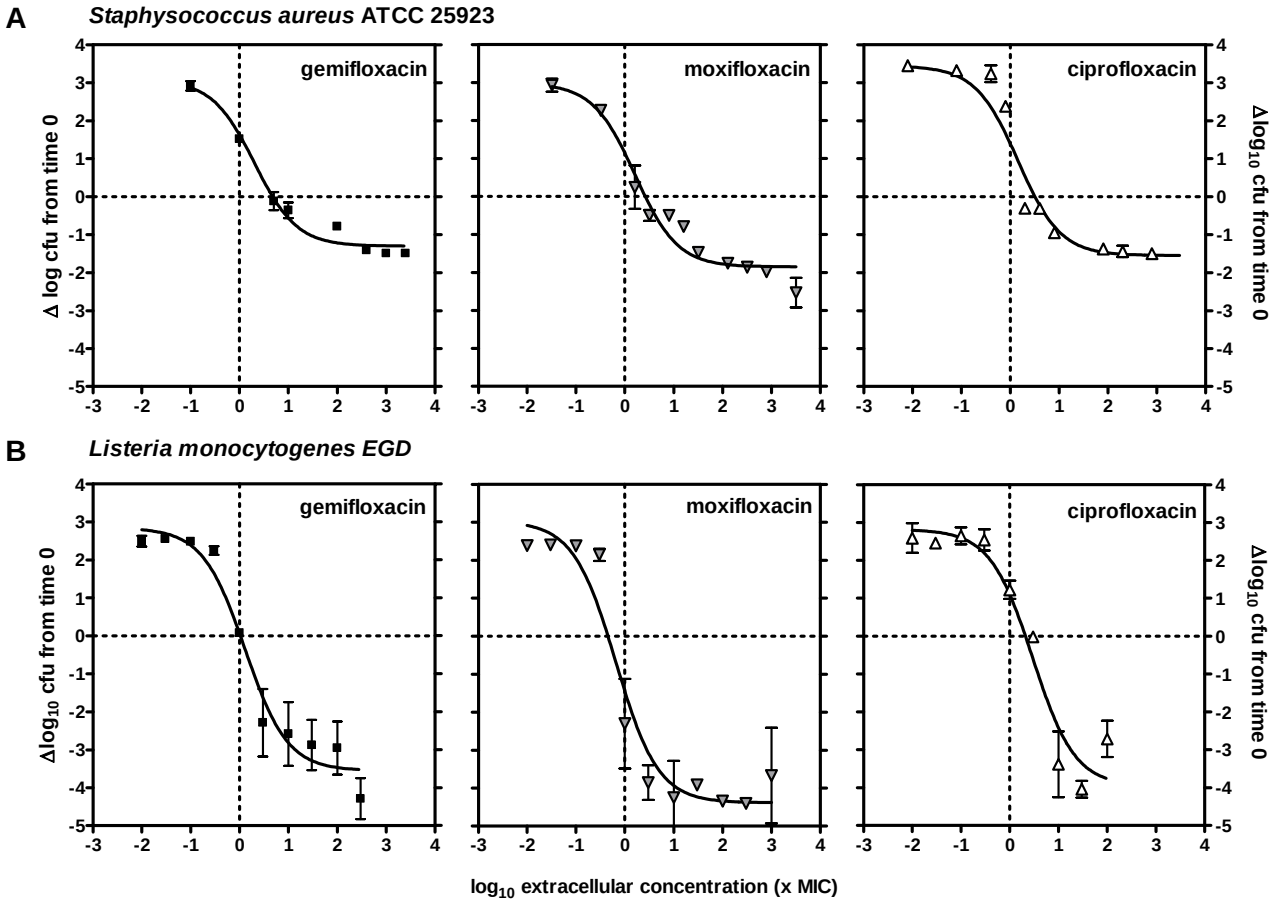
**Vallet *et al.* Intracellular activity of fluoroquinolones - Supplementary Material**

**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 ± 4.63; (b) ciprofloxacin-resistant cells, $R^2 = 0.830$, $k_{out} = 2.39 \pm 0.907$ min$^{-1}$, *plateau* = 31.6 ± 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]<br>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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