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Influence of Efflux Transporters on the Accumulation and Efflux of Four Quinolones (Ciprofloxacin, Levofloxacin, Garenoxacin, and Moxifloxacin) in J774 Macrophages

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Ciprofloxacin is subject to efflux from J774 macrophages through a multidrug resistance-related protein-like transporter (J. M. Michot, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens, Antimicrob. Agents Chemother. 48:2673–2682, 2004). Here, we compare ciprofloxacin to levofloxacin, garenoxacin, and moxifloxacin for transport. At 4 mg/liter, an apparent steady state in accumulation was reached after 30 to 60 min for all quinolones but to quite different levels (approximately 3, 5, 10, and 16 fold). Accumulation of ciprofloxacin was increased (to about 16 to 20 fold) by ATP depletion, increase in extracellular concentration, and the addition of probenecid, gemfibrozil, or MK571 (but not verapamil or GF120918). These treatments did not affect the accumulation of moxifloxacin. Levofloxacin and garenoxacin showed an intermediate behavior. Efflux of ciprofloxacin was slowed down by probenecid (half-life, 7.2 versus 1.6 min). Moxifloxacin efflux was faster and unaffected by probenecid (half-lifes, 0.27 versus 0.33 min). Efflux of levofloxacin and garenoxacin was modestly decreased by probenecid (1.5 and 2.1 fold). Accumulation of ^{14}C-labeled ciprofloxacin was increased by unlabeled ciprofloxacin and moxifloxacin, but moxifloxacin was two times less potent. Accumulation of moxifloxacin at 4°C was almost identical to that at 37°C, whereas that of ciprofloxacin was minimal (levofloxacin and garenoxacin showed intermediate behaviors). Cells subjected to thermal shock (56°C; 10 min) accumulated all quinolones at a similar level (16 to 23 fold). We conclude that moxifloxacin is apparently not subject to efflux from J774 macrophages, even though it can interact with the ciprofloxacin transporter. Levofloxacin and garenoxacin are partially effluxed. Data suggest that efflux plays an important role in the differential accumulation of quinolones by J774 macrophages.

Fluoroquinolones have long been known to accumulate in phagocytic cells (8), but quite significant differences among closely related derivatives have been observed (3, 7, 13) which have so far not received satisfactory explanation. One factor that can modulate antibiotic accumulation in eucaryotic cells is their differential recognition by active efflux transporters (see reference 28 and the references cited therein). Fluoroquinolones are recognized by several eucaryotic multidrug transporters, most notably by two main members of the ATP-binding cassette superfamily, namely the multidrug resistance-related proteins (MRP) and the P-glycoprotein (28). In J774 macrophages, norfloxacin has been shown to be subject to efflux by a probenecid- and gemfibrozil-inhibitable transporter (2), which has been tentatively identified as a member of the MRP family (16). In this context, we have now examined the accumulation and efflux of levofloxacin and moxifloxacin in J774 macrophages in comparison with ciprofloxacin. These quinolones were chosen on the basis of their increasingly lipophilic character and potential clinical interest. We extended the study to include garenoxacin (25), as a typical member of the new class of desfluoroquinolones, to gain more information on the potential structure-activity relationships governing quinolone accumulation and efflux in macrophages. The data show that moxifloxacin is not subject to significant MRP-mediated efflux, which explains its higher cellular level of accumulation. Levofloxacin and garenoxacin display an intermediate behavior.

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MATERIALS AND METHODS

Cell culture, cell antibiotic accumulation and efflux, and assessment of cell intactness. Unless otherwise stated, all experiments were performed with J774 macrophages, following exactly the methods and conditions reported previously (16). For short-term kinetic studies, bicarbonate-free media were prepared and buffered with 5 mM phosphate at pH 7.4, and experiments were conducted in the open air. Intactness of cells (assessed by the release of lactate dehydrogenase, a cytosolic enzyme) was satisfactory in all conditions used (<10% release) (16).

Assay of cell-associated quinolones. Assays were done routinely by fluorimetry, except (i) for some experiments where ^{14}C-labeled garenoxacin had to be used due to the lack of sensitivity of the fluorimetric assay of this quinolone, (ii) for the experiments comparing the influence of ciprofloxaxin and moxifloxacin on ciprofloxacin accumulation (for which ^{14}C-labeled ciprofloxacin was used), and (iii) for confirming the efflux kinetics of moxifloxacin (for which ^{14}C-labeled moxifloxacin was used). The fluorimetric assay of ciprofloxacin has been described in detail (16), and only minor adaptations were needed for the other quinolones. Excitation and emission wavelengths were set at 275 and 450 nm, 298 and 500 nmn, 292 and 414 nm, and 298 and 504 nm, and the lowest limits of detection and linearities were 5 μg/liter (R^2, >0.99 to 200 μg/liter), 10 μg/liter (R^2, >0.99 to 200 μg/liter), 100 μg/liter (R^2, >0.99 to 1,300 μg/liter), and 25 μg/liter (R^2, >0.99 to 350 μg/liter) for ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin, respectively. We checked that the presence of probenecid added in large excess to each of the quinolones studied did not interfere with their assay. For assay of radiolabeled garenoxacin, moxifloxacin, and ciprofloxacin, cells were collected in water, and samples were sonicated to homogeneity. Standards of radiolabeled garenoxacin, moxifloxacin, and ciprofloxacin were run

in parallel. We checked in preliminary experiments that radiochemical and fluorescence assays gave consistent cell accumulation results. For garenoxacin and levofloxacin, for which we did not have historical controls, we checked also that the amount of antibiotic accumulated by cells, as determined by either fluorescence or radioactivity, corresponded to an equivalent amount of bioactive drug (assayed by a conventional disk diffusion method with *Bacillus subtilis* as test organism and using antibiotic medium 11 adjusted to pH 8).

Calculation of apparent cellular quinolone accumulation. The cell antibiotic content of each sample was expressed by reference to its total protein content measured by the Folin-Ciocalteu/biuret method (14). The latter was then used to compute the corresponding cell volume (3.08 μl/mg cell protein) (16). The level of accumulation of each antibiotic was then expressed as the ratio of its apparent cellular concentration to its known extracellular concentration.

Treatments of the cells. Addition of efflux transporter inhibitors and ATP depletion was performed as described previously (16) with routine checks for cell viability and effective lowering of the cell ATP levels to <10% of the control values (~35 nmol/mg protein).

Materials. Unlabeled antibiotics were obtained as microbiological standards from their corresponding manufacturers as follows: ciprofloxacin (potency, 85%) and moxifloxacin (potency, 91%) from Bayer A.G., Leverkusen, Germany; levofloxacin (potency, 95%) from Aventis Pharma, Antony, France; and garenoxacin (potency, 79%) from Bristol Myers Squibb, New Brunswick, CT. 3-^{14}C-labeled garenoxacin (0.80 MBq/mg; radiochemical purity, 98.3%) was donated by Bristol Myers Squibb and ^{14}C-labeled ciprofloxacin (6.96 MBq/mg; radiochemical purity, 98.0%, labeled atoms on two adjacent carbons of the piperazine substituent in position 7) and 3-^{14}C-labeled moxifloxacin (2.94 MBq/mg; radiochemical puritym 98.8%) was donated by Bayer AG. Verapamil and 2-D-deoxyglucose were supplied by Fluka Chemie, Buchs, Switzerland; probenecid and gemfibrozil were supplied by Sigma-Aldrich Chemie, Steinheim, Germany; and MK571 (3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid), was supplied by Alexis Corporation, San Diego, CA. GF120918 was kindly donated by Glaxo Wellcome Research and Development, Laboratoire Glaxo Wellcome, Les Ulis, France. Cell culture media and serum were from Gibco Invitrogen Corporation (Paisley, Scotland). All other reagents were from E. Merck AG (Darmstadt, Germany).

Statistical analyses. Curve-fitting analyses (including calculations of regression parameters, 95% confidence intervals (95% CI), and significance of slope deviations from zero were made with GraphPad Prism version 4.00 for Windows; other statistical analyses were made with GraphPad InStat version 3.00 for Windows (GraphPad Software, San Diego Calif.).

RESULTS

Kinetics of accumulation of quinolones. In the first series of experiments, the kinetics of accumulation of the four quinolones were examined using a fixed concentration of 5 mg/liter. Figure 1 shows that all four drugs accumulated quickly in cells reaching an apparent steady-state level after 30 to 60 min. These levels, however, were markedly different among quinolones, ciprofloxacin reaching the lowest value (around 3 fold), followed by levofloxacin (about 5 fold) and garenoxacin (about 10 fold), and moxifloxacin reaching the highest value (about 16 fold). Because this type of experiment did not allow us to measure and compare the early phases of uptake, short-term kinetics studies concentrating on the first 5 min of accumulation were run independently. These experiments were made at two different extracellular concentrations (5 mg/liter and 17 mg/liter), since we knew from previous studies (16) that the extent of ciprofloxacin accumulation is influenced by its concentration. Results for ciprofloxacin and moxifloxacin are presented in Fig. 2. They show that the uptake of quinolones largely proceeded at a similar fractional rate within the first minute of the experiment. In absolute value, however, fluxes were about five- to sixfold higher for moxifloxacin than for ciprofloxacin at the same extracellular concentration. After this first phase, a slower uptake process became apparent, especially for ciprofloxacin, as suggested from the data shown in Fig. 2. Also of interest was the fact that the capacity of the cells to concentrate ciprofloxacin at 5 min was larger at an extracellular concentration of 17 mg/liter than at 5 mg/liter, whereas no significant difference (and actually a trend to a decrease) was seen for moxifloxacin. Similar experiments were made with levofloxacin and garenoxacin and showed (i) that these quinolones also displayed a fast first phase of influx, followed by a slower phase, and (ii) that the accumulation of levofloxacin at 5 min, but not that of garenoxacin, was greater at 17 mg/liter than at 5 mg/liter.

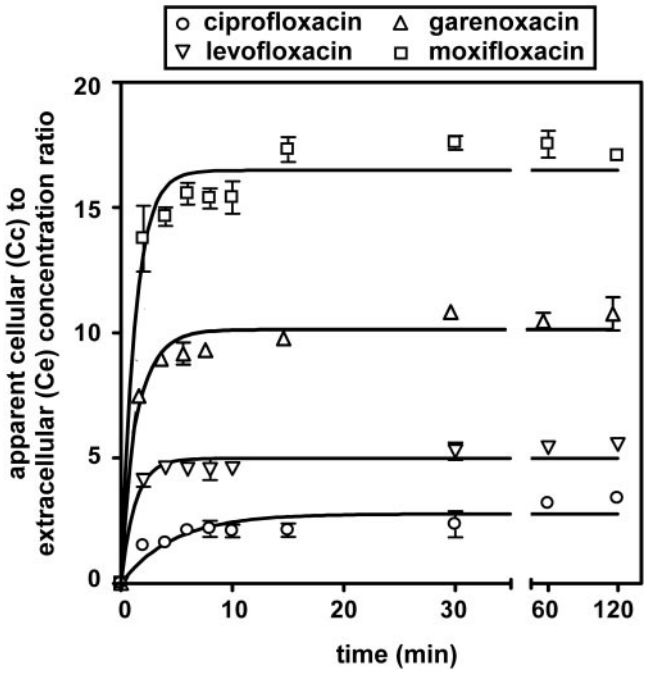

FIG. 1. Comparative kinetics of the accumulation of the four quinolones at an extracellular concentration of 5 mg/liter and at 37°C in J774 murine macrophages (circles, ciprofloxacin; inverted triangles, levofloxacin; triangles, garenoxacin; squares, moxifloxacin). All data are means ± standard deviation (SD) of three experiments (for symbols without bars, the SD is smaller than the symbol size). Results are expressed as the ratio of the apparent cellular concentration of each quinolone to its extracellular concentration. Data obtained for each drug were fitted to one-phase exponential association equations; regression parameters are as follows: ciprofloxacin, $R^2 = 0.817$; $y_{max} = 2.775$ [95% CI, 2.259 to 3.289]; levofloxacin, $R^2 = 0.950$; $y_{max} = 4.987$ [95% CI, 4.600 to 5.373]; garenoxacin, $R^2 = 0.983$; $y_{max} = 9.50$ [95% CI, 9.95 to 10.06]; moxifloxacin, $R^2 = 0.967$; $y_{max} = 16.48$ [95% CI, 15.61 to 17.35]).

Influence of extracellular concentration and of ATP depletion. Our previous experiments (16) had shown that the accumulation of ciprofloxacin at equilibrium (defined by the ratio of its apparent cellular to its extracellular concentration) was influenced not only by the drug extracellular concentration (as hinted from the data shown in Fig. 2) but also by ATP depletion of the cells. These conditions were therefore systematically explored here for the three other quinolones in comparison with ciprofloxacin. Figure 3 shows that, as anticipated, raising the extracellular concentration of ciprofloxacin from 2 to 200 mg/liter caused its accumulation in control cells to increase about fivefold, with a change becoming noticeable from an extracellular concentration of approximately 15 mg/liter. In ATP-depleted cells, the accumulation of ciprofloxacin

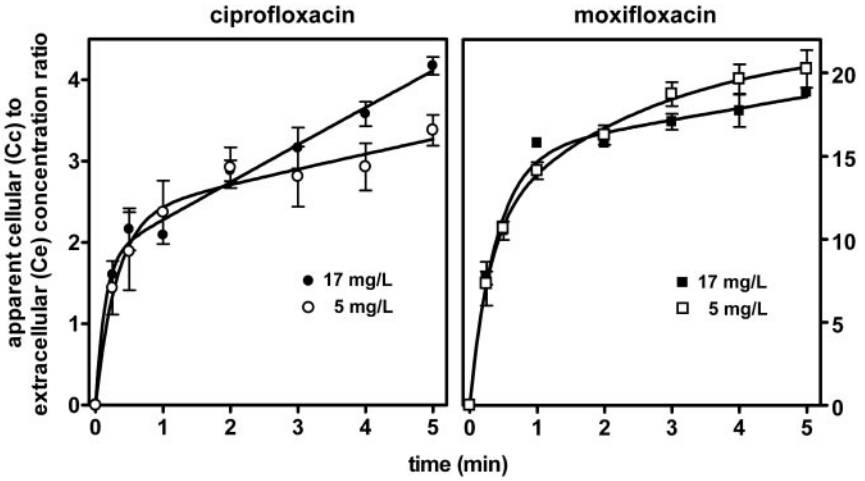

FIG. 2. Comparative short-term kinetics of the accumulation of ciprofloxacin (left) and moxifloxacin (right) at two different extracellular concentrations (open symbols, 5 mg/liter; closed symbols, 17 mg/liter). All data are the mean ± SD of three experiments (for symbols without bars, the SD is smaller than the symbol size). Results are expressed as the ratio of the apparent cellular concentration of each quinolone to its extracellular concentration. Data obtained for each drug were fitted to a two-phase exponential association; regression parameters are as follows: ciprofloxacin, 5 mg/liter and $R^2 = 0.987$; ciprofloxacin, 17 mg/liter and $R^2 = 0.992$; moxifloxacin, 5 mg/liter and $R^2 = 0.999$; moxifloxacin, 17 mg/liter and $R^2 = 0.991$. The parameters best describe the biphasic character of the uptake of these two quinolones.

was already markedly increased at the lowest extracellular concentration tested (2 mg/liter), reaching values approximately 2.5-fold higher than those of control cells). It still increased when the extracellular concentration was raised, but in a less marked fashion than in control cells. At 200 mg/liter, the accumulation levels observed for ATP-depleted and control cells were essentially similar. Levofloxacin also showed an increase in its accumulation in control cells (no ATP depletion) when its extracellular concentration was raised, but this occurred at lower values (with levels of about 75% of the maximal value already observed at an extracellular concentration of 17 mg/liter). No significant influence of the extracellular concentration was noted for garenoxacin or moxifloxacin in control cells (no ATP depletion) throughout the whole range of concentrations investigated. With ATP-depleted cells, increasing the levofloxacin or garenoxacin concentration slightly but nevertheless significantly ($P < 0.02$ for slope deviation from zero) reduced the corresponding accumulation levels, whereas no effect was seen for moxifloxacin.

Influence of preferential MRP and P-glycoprotein inhibitors on quinolone accumulation. In our previous studies (16, 21, 22), we showed that J774 macrophages express at least two antibiotic transporters, one belonging to the family of the MRP and the other identified as the P-glycoprotein. These transporters are responsible for a decreased accumulation of ciprofloxacin and azithromycin, respectively. Preferential inhibitors of each of these transporters were therefore used in the present study to examine their influence on the cellular accumulation of levofloxacin, garenoxacin, and moxifloxacin in comparison with ciprofloxacin. Figure 4 shows that probenecid and gemfibrozil (two inhibitors of organic anion transporters, including MRP) and MK571 (a preferential inhibitor of the MRP transporters) increased the accumulation of ciprofloxacin (about fivefold) as anticipated but had no effect on the accumulation of moxifloxacin. Their effect on the accumulation of levofloxacin and garenoxacin was noticeable and significant but less intense than for ciprofloxacin. As a result, and quite interestingly, the levels of accumulation of ciprofloxacin and garenoxacin became quite similar in the presence of the MRP inhibitors and only slightly lower than that of moxifloxacin. Levofloxacin and garenoxacin accumulation increased in the presence of MRP inhibitors, but to a lesser extent than that of ciprofloxacin. In contrast, GF120918 (a preferential inhibitor of the P-glycoprotein) had no statistically significant effect on the accumulation of the quinolones. Verapamil, which also inhibits the P-glycoprotein but is far less specific, was without significant effect on the accumulation of ciprofloxacin, levofloxacin, or garenoxacin. Quite intriguingly, however, a slight but statistically significant decrease in the accumulation of moxifloxacin was observed. Globally, however, neither GF120918 nor verapamil modified to the ranking of accumulation seen for controls, i.e., ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin; differences between quinolones remained essentially unchanged.

Influence of probenecid on quinolone efflux. Since the previous experiments had disclosed differential effects of MRP inhibitors on the accumulation of quinolones, experiments were conducted with probenecid to examine whether this behavior could be related to differences in drug efflux. For this purpose, we used cells loaded for 2 h with 17 mg/liter of quinolones and examined the drug efflux in short-term kinetic studies (this high concentration of quinolone was needed for sake of sensitivity). Probenecid was added to the treated cells during both the loading time and the efflux period to ensure a maximal inhibition of the transporter. Results for ciprofloxacin and moxifloxacin are presented in Fig. 5. As anticipated, the efflux of ciprofloxacin was markedly slowed down by probenecid (with a apparent half-life increase of about 4.5 fold). But quite surprisingly, we observed that moxifloxacin was released from cells at a much faster rate than ciprofloxacin was, and that probenecid (5 mM) did not influence this behavior (these data, obtained using the fluorimetric assay, were independently con-

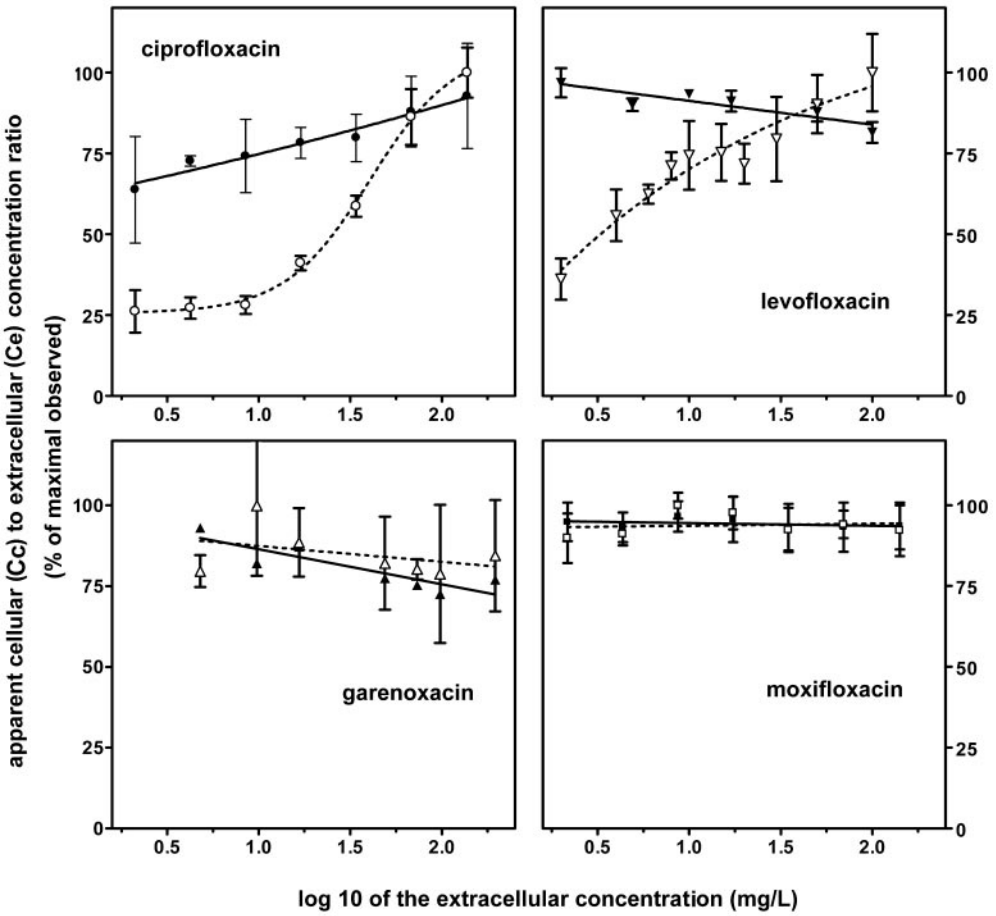

FIG. 3. Influence of the extracellular concentration of quinolones on their accumulation at equilibrium (2 h) in J774 murine macrophages in control conditions (open symbols) or under conditions of ATP depletion (closed symbols). All data are expressed as the percentages of the maximal ratio of apparent cellular to extracellular concentration observed for the corresponding drug in these experiments (ciprofloxacin, 23.05 ± 0.71; levofloxacin, 9.33 ± 0.45; garenoxacin, 15.00 ± 0.50; moxifloxacin, 16.73 ± 0.31) and are shown as means ± the 95% confidence interval (for three experiments). For ciprofloxacin and levofloxacin (controls), data were fitted to a variable-slope sigmoidal dose-response equation. For all other conditions, the figure shows straight lines corresponding to arbitrary linear regression equations.

firmed by using ^{14}C-labeled moxifloxacin). We then extended those studies to levofloxacin and garenoxacin and saw (i) that their rate of release from control cells was in the same range as that of ciprofloxacin (half-lives [95% CI]: 2.13 [1.87 to 2.46] and 1.64 [1.27 to 2.31] versus 1.62 [1.18 to 2.59]) and (ii) that probenecid (5 mM) slowed down their efflux slightly less than ciprofloxacin (half-lives [95% CI]: 3.28 [2.88 to 3.81] and 3.43 [2.31 to 3.49] versus 7.20 [4.83 to 14.20]).

Comparison of influx and efflux kinetic parameters in control cells. Table 1 shows the half-lives of initial uptake and of efflux derived from the experiments illustrated in Fig. 2 and 5 for ciprofloxacin and moxifloxacin in the absence of probenecid. It is interesting (i) that the initial absolute rate of intake of moxifloxacin was strictly proportional to its extracellular concentration, whereas that of ciprofloxacin showed some degree of enhancement in its uptake upon increase in its extracellular concentration; (ii) that the absolute rate of influx of moxifloxacin was about 3- to 4-fold faster than that of ciprofloxacin; (iii) that the efflux of moxifloxacin was almost 7.7-fold faster than that of ciprofloxacin; and (iv) that the rates of influx and efflux of moxifloxacin were similar.

Influence of moxifloxacin on the accumulation of ciprofloxacin. In these experiments, we wished to specifically address the question of a potential interaction of moxifloxacin with the ciprofloxacin efflux transporter. This was assessed by examining to what extent moxifloxacin could increase the cellular accumulation of ciprofloxacin as does ciprofloxacin itself (Fig. 3), an effect that we interpreted as a self-induced impairment of its efflux (see reference 16 for discussion). For this purpose, cells were incubated with a fixed concentration of ^{14}C-labeled ciprofloxacin in the presence of either (i) increasing amounts of unlabeled ciprofloxacin (range, 5 to 195 mg/liter [15.1 to 596 μM]; the second range is close to the solubility limit of ciprofloxacin in the medium) or (ii) a fixed concentration of 5 mg/liter of unlabeled ciprofloxacin plus increasing concentrations of moxifloxacin (5 to 700 mg/liter [12.5 to 1,746 μM]). Results presented in Fig. 6 show that moxifloxacin could increase the accumulation of ^{14}C-labeled ciprofloxacin but was about globally two times less potent than ciprofloxacin itself (based on equimolar comparisons).

Accumulation at 4°C and after transient exposure of the cells to 56°C. Early investigations had revealed that certain

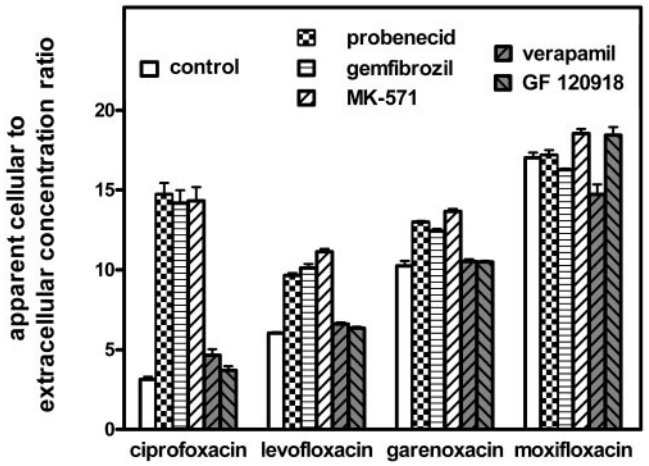

FIG. 4. Influence of MRP inhibitors (probenecid [5 mM], gemfibrozil [500 μM], MK571 [100 μM]) and P-glycoprotein inhibitors (verapamil [100 μM] and GF120918 [2 μM]) on the accumulation of quinolones. Cells were incubated for 2 h in the presence of 5 mg/liter of each quinolone in the absence (control) or in the presence of the corresponding inhibitors. All values are the means of three independent determinations ± SD. Statistical analysis (one-way analysis of variance): $P < 0.01$ for ciprofloxacin, levofloxacin, or garenoxacin alone versus these antibiotics in the presence of probenecid, gemfibrozil, or MK571, respectively; $P < 0.05$ for moxifloxacin alone versus moxifloxacin in the presence of verapamil. All differences between each antibiotic alone and the same antibiotic in the presence of other inhibitors are not significant ($P > 0.05$).

quinolones are accumulated to some extent by macrophages even when these are maintained at 4°C (3, 13) and that ciprofloxacin accumulation is enhanced in cells subjected to transient heat shock (9). This intriguing behavior was systematically reexamined here for all four quinolones studied. As shown in Fig. 7 and concentrating first on the comparison between 37°C and 4°C, it clearly appeared that cells maintained at 4°C in the presence of ciprofloxacin accumulated much less drug than at 37°C (2 h in both cases). Percentagewise, the difference was much smaller for cells incubated with moxifloxacin. In absolute values, however, it appears that incubation at 4°C reduced the accumulation of all four drugs by about the same amount. Levofloxacin and garenoxacin showed an intermediate behavior. With cells exposed transiently to 56°C (10 min) and then incubated with the quinolones at 37°C (2 h), all four quinolones showed a high accumulation level (approximately 15-fold over the extracellular concentration for ciprofloxacin and levofloxacin and up to about 22-fold for garenoxacin and moxifloxacin). Interestingly enough, this common level of accumulation (15 to 23 fold) was of the order of magnitude of that observed with ATP-depleted cells or in cells exposed to high drug concentrations (Fig. 3) or with cells exposed to preferential MRP inhibitors (Fig. 4). If cells were maintained at 56°C for 20 min, accumulation of quinolones dropped to values around twofold, but cells examined by phase-contrast microscopy showed manifest signs of loss of integrity (data not shown).

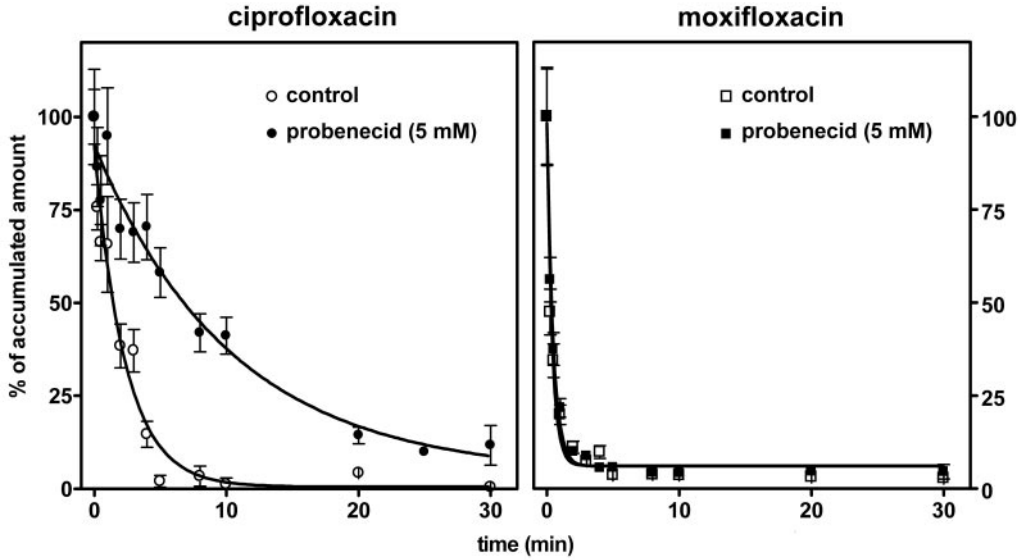

FIG. 5. Influence of probenecid on the efflux of ciprofloxacin and moxifloxacin from J774 macrophages. Control (open symbols), cells were loaded by incubation at 37°C for 2 h in the presence of 17 mg/liter of the corresponding quinolone and then transferred to a quinolone-free medium to measure efflux (controls). Probenecid-treated cells are shown by closed symbols. Loading and efflux studies were performed in the presence of 5 mM probenecid; we showed in a previous report (16) that probenecid does not alter the kinetic parameters of the influx of ciprofloxacin. Data are expressed as a percentage of the amount accumulated by the cells at the end of the loading phase (apparent cellular to extracellular concentration ratios: ciprofloxacin, 3.92 ± 0.29, ciprofloxacin plus probenecid, 15.83 ± 0.2.03; moxifloxacin, 17.09 ± 2.26; moxifloxacin plus probenecid, 17.94 ± 2.30). They were fitted to one-phase exponential decay curves (goodness of fit [R^2] and half-lives: ciprofloxacin, 0.967 and 1.62 ± 0.25 min; ciprofloxacin plus probenecid, 0.963 and 7.20 ± 0.59 min; moxifloxacin, 0.980 and 0.27 ± 0.03 min; moxifloxacin plus probenecid, 0.993 and 0.33 ± 0.22 min).

TABLE 1. Kinetics parameters for influx and efflux of ciprofloxacin and moxifloxacin in control cells, calculated from the regression analyses of the data presented in Fig. 2 (influx) and Fig. 5 (efflux)

Quinolone	Extracellular concn (mg/liter)	Influx		Efflux	
		$t_{1/2}$ of initial phase (min)[a]	Initial influx rate (pmol/mg prot/s)[b]	$t_{1/2}$ (min)[c]	Initial efflux rate (pmol/mg prot/s)[b]
Ciprofloxacin	5	0.21 ± 0.08	4.8 ± 0.7		
	17	0.09 ± 0.04	20.2 ± 5.5	1.62 ± 0.25	10.2 ± 1.6
Moxifloxacin	5	0.18 ± 0.04	19.9 ± 1.4		
	17	0.26 ± 0.08	66.7 ± 3.4	0.27 ± 0.03	78.9 ± 5.7

[a] Curve fitting by two-phase exponential association; values are given for the first, rapid phase of uptake.
[b] Calculated from the difference in cell associated amount between $t = 0$ s and $t = 15$ s in experiments shown in Fig. 2 and Fig. 5.
[c] Curve fitting by one-phase exponential decay.

DISCUSSION

The data presented in this paper, together with those of previous publications (3, 5, 7, 13), show that quinolones are accumulated by macrophages but to quite different extents. Many studies also report that quinolones accumulated by cells are active against intracellular bacteria sojourning in different subcellular compartments (see reference 4 for a recent review). This implies that part of the cell-associated quinolones must be truly intracellular and therefore able to cross the pericellular membrane to some extent. In this context, differences in apparent cellular accumulation could result from differences in influx, susceptibilities to cellular sequestration, or efflux. We show here that moxifloxacin (i) is accumulated more than ciprofloxacin, (ii) is not apparently subject to efflux by the MRP-like ciprofloxacin transporter (based on marked differential effects of ATP depletion and addition of MRP inhibitors), and (iii) is not subject to efflux through the P-glycoprotein transporter either (based on the lack of effect of the preferential P-glycoprotein inhibitors which increase the accumulation of azithromycin in these cells) (21, 22). These data, therefore, suggest that the differences in accumulation seen between ciprofloxacin and moxifloxacin may actually result from their differential susceptibility to efflux by the ciprofloxacin transporter.

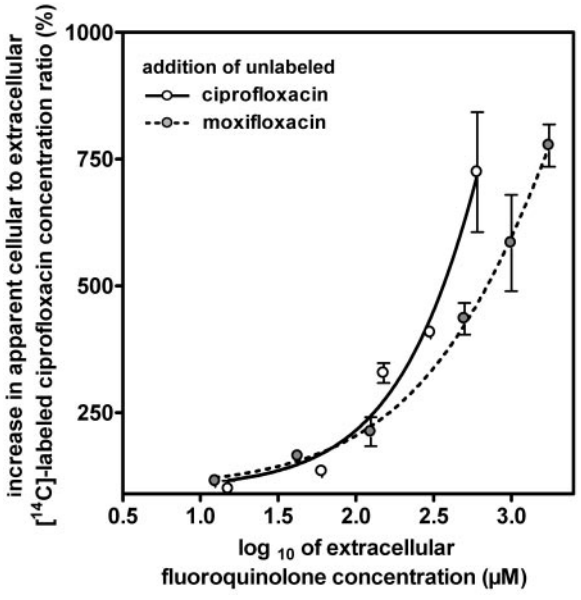

FIG. 6. Influence of the addition of unlabeled ciprofloxacin on the accumulation of ^{14}C-labeled ciprofloxacin. Cells were maintained for 2 h at 37°C with a constant concentration of 940 nCi (34.8 kBq) of ^{14}C-labeled ciprofloxacin per ml (corresponding to a weight concentration of 5 mg/liter and a molar concentration of 15.1 μM). Open symbols and the solid line indicate that the medium contained increasing amounts of unlabeled ciprofloxacin, starting from a concentration of 0 mg/liter (the first point on the graph) to a maximum of 195 mg/liter (590 μM, close to the limit of solubility in the culture medium). Grey symbols and the dotted line indicate that the medium contained 5 mg/liter of unlabeled ciprofloxacin plus increasing amounts of moxifloxacin, starting from a concentration of 5 mg/liter (12.5 μM). Data were fitted to sigmoidal dose-response curves ($R^2 = 0.979$ and 0.997, respectively) according to a previously described model of the cooperative effect of ciprofloxacin on ciprofloxacin accumulation by J774 macrophages (16).

In a first analysis, our data seem compatible with a model in which (i) ciprofloxacin penetrates J774 macrophages by passive diffusion through membrane bilayers (as also suggested by others) (19, 24) and binds loosely to as-yet-unidentified intracellular constituents, while being simultaneously subject to active efflux, and (ii) moxifloxacin simply avoids recognition by the transporter and therefore reaches maximal accumulation. Levofloxacin and garenoxacin would be partially subject to efflux by ciprofloxacin transporter and therefore would consistently show an intermediate behavior. The ranking of susceptibility to transport to which this model would arrive (ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin) is actually quite similar to what has been concluded from a study of the bacterial transporter NorA (a member of the major facilitator superfamily) (27). For this transporter, recognition was indeed shown not to depend so much on the hydrophobic character of the quinolones studied but on the bulkiness of the C-7 and the bulkiness and hydrophobicity of the C-8 substituents (26). As shown in Fig. 8, it is interesting to see that moxifloxacin and garenoxacin, which are the least effluxed by J774 macrophages, possess both substituents, which are absent from ciprofloxacin. Levofloxacin does not possess a bulky C-7 but displays a C-8 substituent. The latter, however, is not fully mobile and may not be bulky enough. Further studies may address directly this question by running systematic structure-activity relationships with homogenous series of derivatives. A direct comparison of the quinolone-binding sites of NorA and of the J774 ciprofloxacin transporter could also be very instructive.

This first model, however, is not entirely compatible with

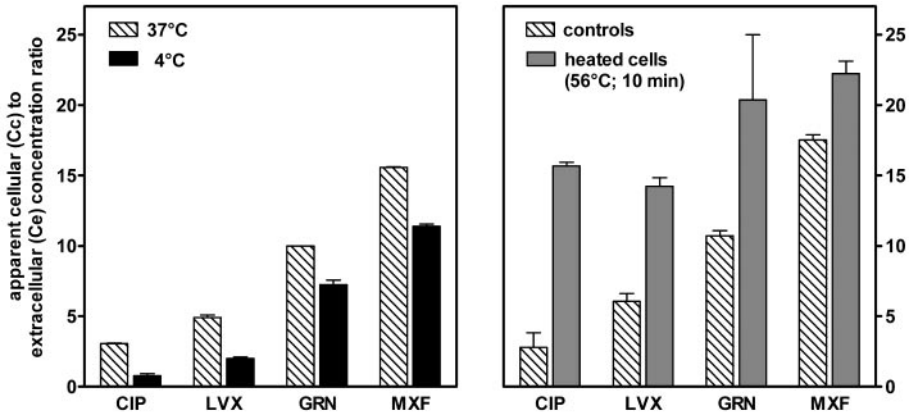

FIG. 7. Cellular accumulation of quinolones after 2 h incubation at an extracellular drug concentration of 5 mg/liter. (Left) Hatched bars, controls (incubated at 37°C); black bars, cells were incubated at 4°C. (Right) Hatched bars, controls (incubated at 37°C); grey bars, cells were exposed to 56°C for 10 min in the absence of drug, cooled to 37°C, and then incubated at 37°C in the presence of drug for 2 h. All values are the means of three independent determinations ± SD.

several observations presented here. We see indeed that moxifloxacin is able to increase the accumulation of ciprofloxacin, even though it is less effective than ciprofloxacin itself. Based on all data assembled so far, we interpret this effect as demonstrating an impairment of ciprofloxacin efflux by moxifloxacin, which implies that moxifloxacin can be recognized by the ciprofloxacin efflux transporter (we unfortunately could not directly document the impairment of ciprofloxacin efflux in the presence of moxifloxacin because of an insufficient supply of labeled ciprofloxacin). We need, therefore, to envisage a second model in which moxifloxacin would interact with the ciprofloxacin transporter and be partially extruded from cells. However, moxifloxacin would then be able to immediately penetrate again in cells, thanks to its greater lipophilicity. This is what has been observed in cells overexpressing MRP transporters and challenged with a series of anthracyclines of increasing lipophilicities: a larger cellular accumulation was seen for derivatives with the faster influx rate (15). This would explain the apparently puzzling data presented in Fig. 5, where we see that moxifloxacin leaked out of cells faster than ciprofloxacin and yet accumulated to a larger extent (as shown in Table 1, the absolute initial rates of influx and efflux of moxifloxacin, expressed as molar amounts per milligram of cell protein, were indeed quite similar and about fourfold higher than those of ciprofloxacin at a comparable extracellular concentration). This model is, actually, quite similar to what has also been proposed for the P-glycoprotein. For this transporter, indeed, there is ample evidence that this protein will not reduce the cellular concentration of amphiphilic substrates if their transbilayer movement is faster than their turnover through the protein (10). When applied to the quinolones, the model is also in agreement with the observation that the transmembrane flux of these drugs is determined by their lipophilicity (1, 11, 24). The faster transmembrane movement of moxifloxacin could also be facilitated by the fact that the higher pK_a value of its protonable substituent in position 7 (4a,7a-octahydro-6H-pyrrolo[3,4-b]pyridine) will increase the proportion of the molecule being zwitterionic at neutral pH in comparison with the other quinolones studied here. We know, indeed, that it is the zwitterionic form of quinolones that is the most diffusible (12).

Our results obtained with cells maintained at 4°C also need to be critically examined. Transporter-mediated efflux, indeed, is likely to be impaired at that low temperature, since it is a strictly energy-dependent process, as observed here with ATP-depleted cells and in other models of MRP-driven efflux of quinolones (17). A higher lipophilicity is probably the main reason for the apparently larger accumulation of garenoxacin and moxifloxacin. Conversely, moxifloxacin and garenoxacin, having higher log P and log D values than levofloxacin and ciprofloxacin, are expected to more easily interact and bind to cell membranes. This process is not temperature dependent, as has been shown with J774 macrophages with basic, lipophilic derivatives of ampicillin (6). In this situation, however, the molecules associated to cells are unlikely to be intracellular, and the similarity of accumulation levels seen at 4°C and 37°C for moxifloxacin and garenoxacin should be viewed as coincidental. More detailed studies using membrane models and membranes isolated from various cell types are, however, needed before more definitive conclusions can be drawn. It is important to remember that the behavior of quinolones at 4°C with respect to cell accumulation seems to be cell dependent (see, for instance reference 18 for divergent results with polymorphonuclear neutrophils, in which moxifloxacin accumulation is reduced to 20% of control values at 4°C).

Considering our results obtained with cells exposed to thermal shock (10 min at 56°C), we know from previous studies that the accumulation of quinolones by macrophages is unaffected or even enhanced by loss of cell viability (9, 18). The new finding made here is that thermal shock largely abolishes the differences in accumulation observed between quinolones. One tentative interpretation about this intriguing phenomenon is that thermal shock caused thermal denaturation or unfolding of the transporter protein(s), resulting in its inactivation. This would let cells accumulate a maximal amount of those quinolones that are normally effluxed and reveal what their maximal accumulation level could be. It is indeed striking that heat-shocked cells accumulate each of the quinolones to almost the

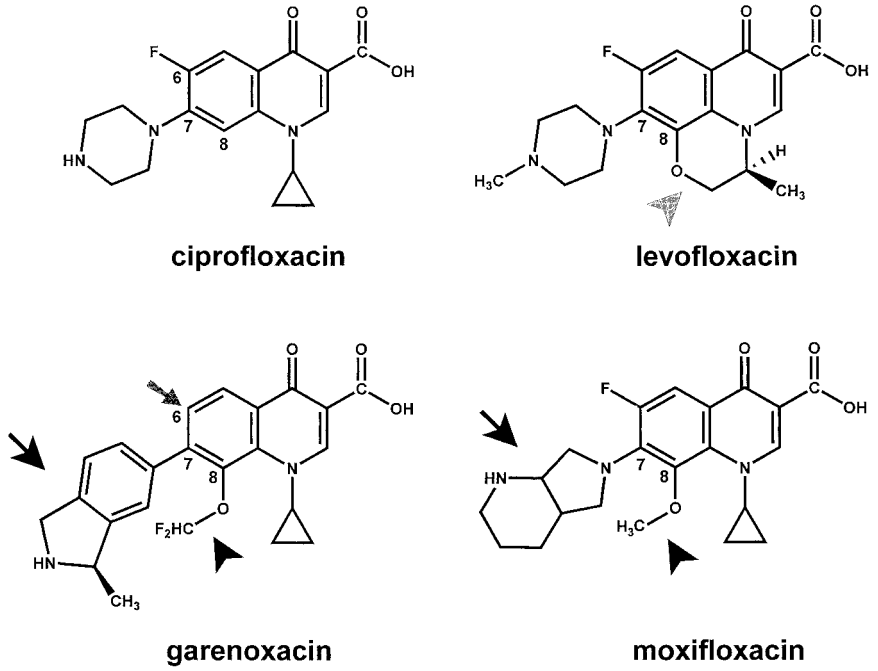

FIG. 8. Structural formula of the quinolones used in the present study. Atoms are numbered counterclockwise starting from the heteroatom (N) in the main byclic quinolone ring, discounting those with no potential substituents. The figure emphasizes the following. (i) Both garenoxacin (also known as T 3811 or BMS-284756) and moxifloxacin possess bulky substituents in position 7 (indicated by the large black arrows; garenoxacin, 2,3-dihydro-1-methyl-1H-isoindole; moxifloxacin, 4a,7a-octahydro-6H-pyrrolo[3,4-b]pyridine) that confer a more hydrophobic character to these molecules than ciprofloxacin or levofloxacin (for which the corresponding substituent is a piperazine or a 4-methyl-piperazine, respectively). (ii) Both garenoxacin and moxifloxacin possess free rotating substituents in position 8 (indicated by the black arrowheads; garenoxacin, difluoromethoxy; moxifloxacin, methoxy) which contribute to an increase in lipophilicity. In levofloxacin, the substituent in position 8 is part of a more rigid structure (morpholino) that bears some degree of similarity with a methoxy. (iii) Garenoxacin is a desfluoroquinolone (having no F substituent in position 6 [grey arrow]). The reported log P (octanol:water partition coefficient) and log $D_{pH=7}$ (octanol/water partition coefficient at pH 7; log $D = \log P - \log[1 + 10^{(-\text{charge} * (pH-pKa))}]$) values, two accepted measures of the hydrophobic character of organic molecules, are 1.31 ± 0.81 and −1.20, 1.49 ± 0.79 and −1.35, 1.62 ± 0.93 and −0.90, and 1.98 ± 0.82 and −0.53 for ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin, respectively. The pKa values of the protonable function of the C7 substituent of the four quinolones are 8.76 ± 0.25 (ciprofloxacin), 6.8 ± 0.3 (levofloxacin), 8.4 ± 0.4 (garenoxacin), and 10.8 ± 0.4 (moxifloxacin). This will result in larger proportion of moxifloxacin (and a lower proportion of levofloxacin) under a zwitterionic form at neutral pH. All log P, log D, and pKa data, calculated with the Advanced Chemistry Development software Solaris V4.67, were obtained from Sci Finder Scholar 2004, American Chemical Society, Washington, D.C.

same level as seen with ATP-depleted cells or cells exposed to preferential MRP inhibitors. This hypothesis will need to be substantiated by specific studies concentrating on direct measurements of the inactivation of the ciprofloxacin transporter, and the identification of the cell-binding sites for quinolones in heat-shocked and ATP-depleted cells.

The data presented here have also implications for drug development and evaluation. The fact that the cellular accumulation of ciprofloxacin is suboptimal at 4 mg/liter (a clinically meaningful concentration for most quinolones) suggests possible improvements through the development of transport inhibitors. These may allow an enhanced activity towards intracellular bacteria, which we know to be directly related to the level of drug accumulation (5, 23). This approach has been successfully followed with conventional MRP inhibitors (19–21) but more specific ones are probably needed in this context. A limitation, however, could be imposed by the potential toxicities associated with higher cellular and tissular accumulation. Conversely, it may be possible to better screen for derivatives with low or no susceptibility to transport. It is interesting, in this context, to see that while neither garenoxacin nor moxifloxacin are effluxed whatever the extracellular concentration used, levofloxacin is transported but only at low concentrations. It is therefore possible that large doses of levofloxacin, typically creating concentrations of 6 to 7 mg/liter or more at which efflux is largely impaired (Fig. 3) will result in a greater-than-anticipated efficacy against intracellular bacteria. It must be remembered, however, that J774 macrophages are only one type of phagocytic cells. Polymorphonuclear neutrophils, for instance, show no influence of probenecid on the accumulation of levofloxacin (29), which is a clear indication that they may behave differently.

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REFERENCES

1. **Bermejo, M., V. Merino, T. M. Garrigues, J. M. Pla Delfina, A. Mulet, P. Vizet, G. Trouiller, and C. Mercier.** 1999. Validation of a biophysical drug absorption model by the PATQSAR system. J. Pharm. Sci. **88:**398–405.
2. **Cao, C. X., S. C. Silverstein, H. C. Neu, and T. H. Steinberg.** 1992. J774 macrophages secrete antibiotics via organic anion transporters. J. Infect. Dis. **165:**322–328.
3. **Carlier, M. B., B. Scorneaux, A. Zenebergh, J. F. Desnottes, and P. M. Tulkens.** 1990. Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages. J. Antimicrob. Chemother. **26** (Suppl. B)**:**27–39.
4. **Carryn, S., H. Chanteux, C. Seral, M. P. Mingeot-Leclercq, F. Van Bambeke, and P. M. Tulkens.** 2003. Intracellular pharmacodynamics of antibiotics. Infect. Dis. Clin. North Am. **17:**615–634.
5. **Carryn, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2002. Comparative intracellular (THP-1 macrophage) and extracellular activities of β-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. Antimicrob. Agents Chemother. **46:**2095–2103.
6. **Chanteux, H., I. Paternotte, M. P. Mingeot-Leclercq, R. Brasseur, E. Sonveaux, and P. M. Tulkens.** 2003. Cell handling, membrane-binding properties, and membrane-penetration modeling approaches of pivampicillin and phthalimidomethylampicillin, two basic esters of ampicillin, in comparison with chloroquine and azithromycin. Pharm. Res. **20:**624–631.
7. **Dorian, M., J. Grellet, and M. C. Saux.** 2001. Uptake of quinolones by in-vitro human monocyte derived macrophages. J. Pharm. Pharmacol. **53:**735–741.
8. **Easmon, C. S., and J. P. Crane.** 1985. Uptake of ciprofloxacin by human neutrophils. J. Antimicrob. Chemother. **16:**67–73.
9. **Easmon, C. S., and J. P. Crane.** 1985. Uptake of ciprofloxacin by macrophages. J. Clin. Pathol. **38:**442–444.
10. **Eytan, G. D., R. Regev, G. Oren, and Y. G. Assaraf.** 1996. The role of passive transbilayer drug movement in multidrug resistance and its modulation. J. Biol. Chem. **271:**12897–12902.
11. **Fresta, M., S. Guccione, A. R. Beccari, P. M. Furneri, and G. Puglisi.** 2002. Combining molecular modeling with experimental methodologies: mechanism of membrane permeation and accumulation of ofloxacin. Bioorg. Med. Chem. **10:**3871–3889.
12. **Furet, Y. X., J. Deshusses, and J. C. Pechere.** 1992. Transport of pefloxacin across the bacterial cytoplasmic membrane in quinolone-susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36:**2506–2511.
13. **Hara, T., H. Takemura, K. Kanemitsu, H. Yamamoto, and J. Shimada.** 2000. Comparative uptake of grepafloxacin and ciprofloxacin by a human monocytic cell line, THP-1. J. Infect. Chemother. **6:**162–167.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
15. **Marbeuf-Gueye, C., D. Ettori, W. Priebe, H. Kozlowski, and A. Garnier-Suillerot.** 1999. Correlation between the kinetics of anthracycline uptake and the resistance factor in cancer cells expressing the multidrug resistance protein or the P-glycoprotein. Biochim. Biophys. Acta **1450:**374–384.
16. **Michot, J. M., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2004. Active efflux of ciprofloxacin from J774 macrophages through an MRP-like transporter. Antimicrob. Agents Chemother. **48:**2673–2682.
17. **Naruhashi, K., I. Tamai, Q. Li, Y. Sai, and A. Tsuji.** 2003. Experimental demonstration of the unstirred water layer effect on drug transport in Caco-2 cells. J. Pharm. Sci. **92:**1502–1508.
18. **Pascual, A., I. Garcia, S. Ballesta, and E. J. Perea.** 1999. Uptake and intracellular activity of moxifloxacin in human neutrophils and tissue-cultured epithelial cells. Antimicrob. Agents Chemother. **43:**12–15.
19. **Rispal, P., J. Grellet, C. Celerier, D. Breilh, M. Dorian, J. L. Pellegrin, M. C. Saux, and B. Leng.** 1996. Comparative uptake of sparfloxacin and ciprofloxacin into human THP 1 monocytic cells. Arzneimittelforschung **46:**316–319.
20. **Rudin, D. E., P. X. Gao, C. X. Cao, H. C. Neu, and S. C. Silverstein.** 1992. Gemfibrozil enhances the listeriacidal effects of fluoroquinolone antibiotics in J774 macrophages. J. Exp. Med. **176:**1439–1447.
21. **Seral, C., S. Carryn, P. M. Tulkens, and F. Van Bambeke.** 2003. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by *Listeria monocytogenes* or *Staphylococcus aureus*. J. Antimicrob. Chemother. **51:**1167–1173.
22. **Seral, C., J. M. Michot, H. Chanteux, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2003. Influence of P-glycoprotein inhibitors on accumulation of macrolides in J774 murine macrophages. Antimicrob. Agents Chemother. **47:**1047–1051.
23. **Seral, C., F. Van Bambeke, and P. M. Tulkens.** 2003. Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. Antimicrob. Agents Chemother. **47:**2283–2292.
24. **Sun, J., Y. Deguchi, J. M. Chen, R. H. Zhang, and K. Morimoto.** 2002. Interactions between quinolone antibiotics and phospholipid membrane for prediction of alveolar macrophage uptake in vitro. Acta Pharmacol. Sin. **23:**430–438.
25. **Takahata, M., J. Mitsuyama, Y. Yamashiro, M. Yonezawa, H. Araki, Y. Todo, S. Minami, Y. Watanabe, and H. Narita.** 1999. In vitro and in vivo antimicrobial activities of T-3811ME, a novel des-F(6)-quinolone. Antimicrob. Agents Chemother. **43:**1077–1084.
26. **Takenouchi, T., F. Tabata, Y. Iwata, H. Hanzawa, M. Sugawara, and S. Ohya.** 1996. Hydrophilicity of quinolones is not an exclusive factor for decreased activity in efflux-mediated resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**1835–1842.
27. **Van Bambeke, F., E. Balzi, and P. M. Tulkens.** 2000. Antibiotic efflux pumps. Biochem. Pharmacol. **60:**457–470.
28. **Van Bambeke, F., J. M. Michot, and P. M. Tulkens.** 2003. Antibiotic efflux pumps in eukaryotic cells: occurrence and impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics. J. Antimicrob. Chemother. **51:**1067–1077.
29. **Vazifeh, D., A. Bryskier, and M. T. Labro.** 1999. Mechanism underlying levofloxacin uptake by human polymorphonuclear neutrophils. Antimicrob. Agents Chemother. **43:**246–252.