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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*

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Antibiotic efflux pumps expressed in eukaryotic cells can decrease the intracellular accumulation of the corresponding drugs and therefore impair their activity against intracellular bacteria. We have investigated whether verapamil (an inhibitor of P-glycoprotein) and gemfibrozil (an inhibitor of multidrug resistance proteins (MRP) and other organic anion transporters), can modulate the intracellular activity of azithromycin and ciprofloxacin against *Listeria monocytogenes* and *Staphylococcus aureus* in J774 macrophages. In parallel, we have measured the cell accumulation and subcellular distribution of both drugs. Antibiotics were used at equipotent extracellular concentrations (from 0.5 × to 10 × MIC) to allow for pharmacological comparisons. Azithromycin was bacteriostatic against *L. monocytogenes* and slightly bactericidal against *S. aureus*. Verapamil did not improve the maximal activity of azithromycin but allowed it to reach a similar effect at extracellular concentrations about seven-fold lower in both models. Azithromycin was predominantly localized in cell granules (66%), the remainder being in the cytosol and in the 'nuclei/unbroken cells' fraction. Verapamil increased the cellular accumulation of azithromycin by almost 2.4-fold without modifying its subcellular distribution. Ciprofloxacin displayed a strong concentration-dependent bactericidal activity in both models. Gemfibrozil increased ciprofloxacin activity almost 2.5-fold against *L. monocytogenes*, but not against *S. aureus*. Ciprofloxacin was predominantly (65%) distributed in the cytosol. Gemfibrozil increased ciprofloxacin total accumulation by ~2.4-fold, but the excess was only found in the cytosol. Inhibition of efflux pumps may be a useful strategy to improve antibiotic efficacy against intracellular bacteria when increased accumulation can be obtained in the compartment where bacteria sojourn.

Keywords: transporters, intracellular, accumulation, verapamil, gemfibrozil

Introduction

Treatment of intracellular infections requires the use of antibiotics capable of (i) penetrating eukaryotic cells at a sufficiently high rate and (ii) reaching a concentration allowing them to cope with the development of the bacteria in the infected compartment(s).[1] Macrolides and fluoroquinolones are well known to accumulate rapidly in phagocytes,[2,3] and both classes of antibiotics were shown to be active in a series of models of intracellular infections, such as those caused by *Staphylococcus aureus* and *Listeria monocytogenes*.[4–6] Little is known, however, about the intracellular pharmacodynamic properties of these antibiotics and, in particular, how and to what extent their intracellular accumulation directly and specifically relates to their activity. We recently showed that the intracellular accumulation of macrolides and fluoroquinolones is modulated in J774 macrophages by the activity of drug efflux pumps. Thus, verapamil, ciclosporin and GF 120918 (known inhibitors of P-glycoprotein), increase the accumulation of azithromycin,[7] whereas gemfibrozil

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or probenecid [inhibitors of multidrug resistance proteins (MRP) and other transporters of organic anions], increase the accumulation of ciprofloxacin.[8,9] In the present study, verapamil and gemfibrozil were used to modulate the cellular accumulation of azithromycin and ciprofloxacin in J774 macrophages. We have then examined the variations in activity of these drugs towards intracellular *S. aureus* and *L. monocytogenes*. These models were chosen based on the observations that (i) azithromycin localizes predominantly in lysosomes[3] whereas ciprofloxacin is found in the cytosol (B. Scorneaux, unpublished data; see also data in this paper and Carlier *et al.*[2] for other fluoroquinolones), and (ii) *S. aureus* develops in phagolysosomes (C. Seral, F. Van Bambeke & P. M. Tulkens, unpublished results) whereas *L. monocytogenes* has access to, and multiplies in, the cytosol.[10]

Materials and methods

Materials

Azithromycin (dihydrate salt; potency 94.4%) was obtained from Pfizer Inc. (Groton, CT, USA) as a laboratory sample for microbiological investigations. Unlabelled ciprofloxacin (potency 85%) and ^{14}C-labelled ciprofloxacin (6.96 MBq/mg) were kindly donated by Bayer AG (Wuppertal, Germany). Gentamicin was obtained from GlaxoSmithKline-Belgium as Geomycin (the commercial form for clinical usage in Belgium), on behalf of Schering-Plough Corp. Verapamil was supplied by Fluka Chemie (Buchs, Switzerland) and gemfibrozil by Sigma–Aldrich Chemie (Steinheim, Germany). Cell culture media and serum were from Gibco Biocult (Paisley, Scotland, UK). All other reagents were obtained from E. Merck AG (Darmstadt, Germany).

Bacteria

S. aureus (ATCC 25923) and *L. monocytogenes* (haemolysin-producing strain EGD serotype 1/2a) were used for all the experiments, with MICs determined in broth by standard methods.

Cell infection and assessment of intracellular activity of antibiotics

We used the general methods described for *L. monocytogenes*[6] (with adaptation for adherent cells) and for *S. aureus* (C. Seral, F. Van Bambeke & P. M. Tulkens, unpublished results). In brief, J774 mouse macrophages were cultivated in RPMI medium supplemented with 10% fetal calf serum. The lack of toxic effect exerted by verapamil or gemfibrozil (at the concentrations used) was checked by measuring the release of lactate dehydrogenase[7] (release <10% in excess of control values). Infection with *L. monocytogenes* was obtained using untreated bacteria at an initial bacteria/macrophage ratio of 5 and with incubation carried out for 1 h. Extracellular bacteria were removed by extensive washing and intracellular growth evaluated after 5 h, in the absence of antibiotic (control) or in the presence of azithromycin or ciprofloxacin. Infection by *S. aureus* was made with opsonized bacteria (0.5 h incubation in fresh human serum). Phagocytosis was initiated at a bacteria/macrophage ratio of 0.5 and with incubation carried out for 1 h. Extracellular bacteria were eliminated by incubation for 1 h in the presence of 50 μg/mL gentamicin followed by extensive washing with PBS. Intracellular growth was evaluated after 24 h of incubation in the absence (controls) or in the presence of azithromycin or ciprofloxacin. Extracellular growth of *S. aureus* in control cultures (no antibiotic added) was prevented by addition of 0.5 mg/L gentamicin (1 × MIC) (C. Seral, F. Van Bambeke & P. M. Tulkens, unpublished results). Cells were collected by gentle scraping in distilled water after extensive *in situ* washing with PBS. The number of cell-associated viable bacteria was then measured by plating aliquots on tryptic soy agar (TSA) after vigorous mixing and suitable dilution and by counting the number of colonies after 24 h incubation at 37°C. Activity was defined as the change in the number of cfu recovered from cells at the time considered compared with the value found at time = 0 h (post-phagocytosis). The incubation periods were systematically 5 h for *L. monocytogenes* and 24 h for *S. aureus*. These time periods were selected to obtain an intracellular bacterial growth of ~10-fold in each case without evidence of growth of extracellular bacteria (based on direct microscopic examination and of plating of the culture media and washing fluids). To allow for direct pharmacodynamic comparisons between the two antibiotics under study, all extracellular concentrations were adjusted to multiples of their MIC for the corresponding organisms. All antibiotics were also compared at an extracellular concentration corresponding to the maximal serum concentration achievable in patients during conventional treatment (C_{max}; values taken from literature data[6]).

Antibiotic accumulation and subcellular distribution

Cells were incubated with azithromycin or [^{14}C]ciprofloxacin, collected and homogenized in 0.25 M sucrose, 1 mM EGTA, 3 mM imidazole pH 7.4, as described previously.[11] The homogenate was separated into a nuclear fraction (containing mostly nuclei and unbroken cells) and a cytosolic extract by three successive low-speed centrifugations (2000 rpm, 10 min; 1800 rpm, 10 min; 1600 rpm, 10 min). This cytosolic extract was then separated by high-speed centrifugation (40000 rpm; 30 min) into a sedimentable fraction (henceforth referred to as 'granules fraction' and containing the bulk of the intracellular organelles) and a supernatant (henceforth referred to as 'soluble fraction' and containing soluble proteins and free ribosomes). Details concerning these procedures are given in Renard *et al.*[11] and used the methods described previously for cultured fibroblasts[12]. The

activity of lactate dehydrogenase and *N*-acetyl-β-hexosaminidase was assayed in all fractions, as markers of the soluble proteins (cytosol) and of lysosomes, respectively.[11] Azithromycin was assayed by the disc diffusion method using *Micrococcus luteus* ATCC 9341 as test organism[7] and [^{14}C]ciprofloxacin by scintillation counting. The total drug cell content was expressed with reference to the protein content of the unfractionated homogenate, and the apparent cellular to extracellular concentration ratio calculated using a conversion factor of 5 µL of cell volume per mg of cell protein as in previous publications.[2,3,7]

Statistical analyses

Group comparisons (Student's *t*-test) were carried out with Instat Prism (V.3.01) from GraphPad Prism Software, San Diego, CA, USA.

Results

The MIC of azithromycin was 0.5 mg/L for both *S. aureus* and *L. monocytogenes*; that of ciprofloxacin was 0.125 mg/L for *S. aureus* and 1.25 mg/L for *L. monocytogenes*. We checked that the addition of verapamil or gemfibrozil (at a concentration similar to that used in the cell culture model, 20 and 250 µM, respectively) did not modify these values.

Figure 1 shows the activity of azithromycin and ciprofloxacin against intracellular bacteria in macrophages incubated with increasing extracellular concentrations of these antibiotics (expressed as multiples of their respective MIC). Considering the *L. monocytogenes* model first, we see that azithromycin was poorly active against the intracellular form of this bacterium, becoming bacteriostatic only at an extracellular concentration corresponding to 10 × MIC. Addition

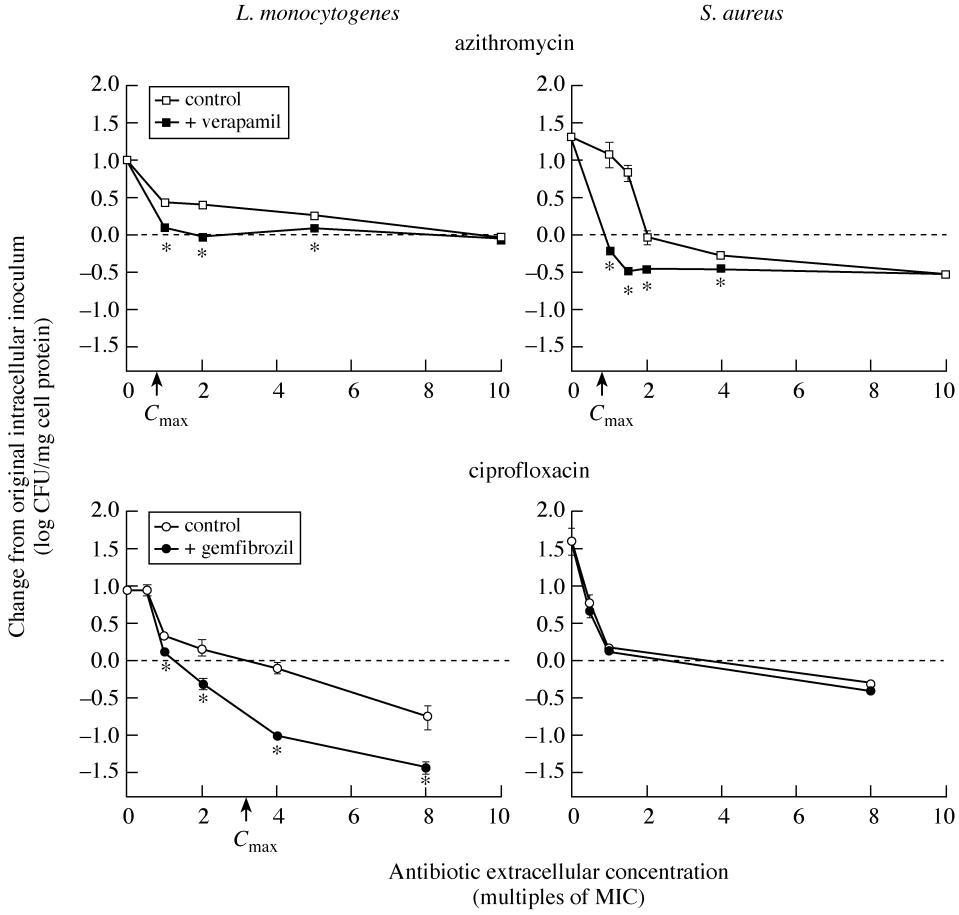

Figure 1. Influence of verapamil 20 µM and gemfibrozil 250 µM on the activity of azithromycin and ciprofloxacin on intracellular *L. monocytogenes* or *S. aureus*. The ordinate shows the changes in cfu (as log cfu/mg cell protein) from the initial inoculum at the end of the incubation with the antibiotics (5 h for *L. monocytogenes*; 24 h for *S. aureus*; these periods of time were selected to obtain an intracellular bacterial growth of about 1 log in the absence of antibiotic). The dotted line corresponds to a bacteriostatic effect. The abscissa shows the extracellular concentration of antibiotic used {as multiple of the corresponding MIC [azithromycin: 0.5 mg/L for both bacteria; ciprofloxacin, 1.25 (*L. monocytogenes*) and 0.125 mg/L (*S. aureus*)]}. The corresponding maximal serum peak level that can be reached in the serum of patients undergoing a conventional treatment is shown by an arrow marked C_{max} (for ciprofloxacin and *S. aureus*, this value is higher than the maximal multiple of MIC examined). Each data point is the mean ± S.D. of three independent determinations. The asterisks show the statistically significant differences between inhibitor-treated and control cells (paired points; $P < 0.01$).

of verapamil allowed for the same levels of activity to be obtained at extracellular concentrations of about 1–2 × MIC. No increase in activity, however, was noted at higher extracellular concentrations demonstrating that azithromycin was only bacteriostatic in this model. Addition of gemfibrozil did not affect azithromycin activity towards *L. monocytogenes* (data not shown). In contrast to azithromycin, ciprofloxacin showed a strong concentration-dependent activity towards intracellular *L. monocytogenes* over the whole range of concentrations examined, being bacteriostatic at an extracellular concentration equivalent to its MIC and becoming markedly bactericidal at higher concentrations. As shown in Figure 1, addition of gemfibrozil made the drug more effective at all concentrations examined, decreasing by about two-fold the extracellular concentration needed to obtain a bacteriostatic effect while increasing the bactericidal effect observed at higher concentrations in comparison with controls. No effect of verapamil was seen on ciprofloxacin activity for *L. monocytogenes* (data not shown).

Moving now to the *S. aureus* model, we observed that azithromycin and ciprofloxacin were both, globally-speaking, poorly effective. Actually, azithromycin was ineffective up to an extracellular concentration of about 1 × MIC, and abruptly became bacteriostatic when its extracellular concentration was raised to about twice this value. Yet, further increase in activity was very minimal, and only a slight reduction in the inoculum over the post-phagocytosis (0.5 log) value was seen at 10 × MIC. Addition of verapamil shifted the effect–concentration curve of azithromycin to the left, causing the drug to achieve a similar antibacterial effect at an extracellular concentration corresponding to about 1.5 × MIC, but with no further gain at higher extracellular concentrations. No effect was noted of the presence of gemfibrozil on the intracellular activity of azithromycin against intracellular *S. aureus* (data not shown). The activity of ciprofloxacin against intracellular *S. aureus* was also quite poor, with a bacteriostatic activity obtained at an extracellular concentration corresponding to its MIC, but with only a slight trend towards a bactericidal activity at 8 × MIC. As shown in Figure 1, no significant effect of gemfibrozil was noted on ciprofloxacin activity against *S. aureus* at all concentrations studied. No effect of verapamil was seen either (data not shown). Figure 1 also shows the values of extracellular concentrations corresponding to the C_{max} of the two antibiotics (i.e. the maximal serum level observed in patients undergoing a conventional treatment with these antibiotics). For azithromycin, a bacteriostatic effect could only be observed at this value for cells incubated with verapamil in both models. For ciprofloxacin, the addition of gemfibrozil allowed the drug to become bactericidal at its C_{max} value against *L. monocytogenes* (as compared with a mere bacteriostatic effect in the absence of gemfibrozil). For *S. aureus*, all concentrations tested were below the C_{max}.

Figure 2 shows the influence of the efflux pump inhibitors on the cellular accumulation and subcellular distribution of azithromycin and ciprofloxacin in uninfected cells after 24 h of incubation with these antibiotics. The figure also shows the distribution of *N*-acetyl-β-hexosaminidase (used as a marker of lysosomes) and of lactate dehydrogenase (used as a marker of soluble proteins). No change in the distribution of these markers was seen, demonstrating that neither verapamil nor gemfibrozil grossly affected the biophysical properties and integrity of lysosomes or the distribution of soluble proteins. As anticipated from the results of previous studies,[3,7] azithromycin was accumulated to a large extent by cells, and predominantly localized in the 'granules fraction'. Verapamil increased the cellular accumulation of azithromycin by about 2.4-fold, but without marked change in its subcellular distribution. These experiments needed the use of large extracellular concentrations of azithromycin (50 mg/L) for detection in the subcellular fractions. Yet, verapamil had a similar effect on azithromycin total accumulation in cells exposed to only 5 mg/L antibiotic (data not shown). Gemfibrozil (250 μM) was without effect on azithromycin accumulation (whether tested at 5 or 50 mg/L; data not shown). In contrast to azithromycin, ciprofloxacin was mostly found in the soluble fraction of control cells, as observed in previous studies (B. Scorneaux, unpublished data). Addition of gemfibrozil increased the total drug accumulation also by 2.4-fold. In contrast with what was seen with azithromycin, however, most of the ciprofloxacin accumulated in excess was found in the soluble fraction. Verapamil was without effect on ciprofloxacin accumulation (data not shown). These experiments combining drug accumulation and drug distribution studies were not carried out on infected cells. Yet, in independent experiments, we checked that infection did not affect the antibiotic accumulation whether in control conditions (no inhibitor added) or in cells exposed to verapamil or gemfibrozil.

Discussion

The pharmacodynamic properties of macrolide and fluoroquinolone antibiotics are now well established from animal and *in vitro* models.[13–15] These studies, however, have mainly if not exclusively examined extracellular forms of infections in which bacteria are readily accessible to drugs. Intracellular infection represents a more complex situation in which compartmentalization (of drugs and bacteria), and local physicochemical conditions can profoundly modulate the intrinsic pharmacodynamic properties of the drugs and, consequently, their overall chemotherapeutic efficacy. Using a model of *Listeria*-infected macrophages, we showed before that the cellular accumulation of fluoroquinolones and of azithromycin does not lead to a proportionally larger activity against intracellular bacteria (even though the activity of fluoroquinolones is concentration dependent both in broth and

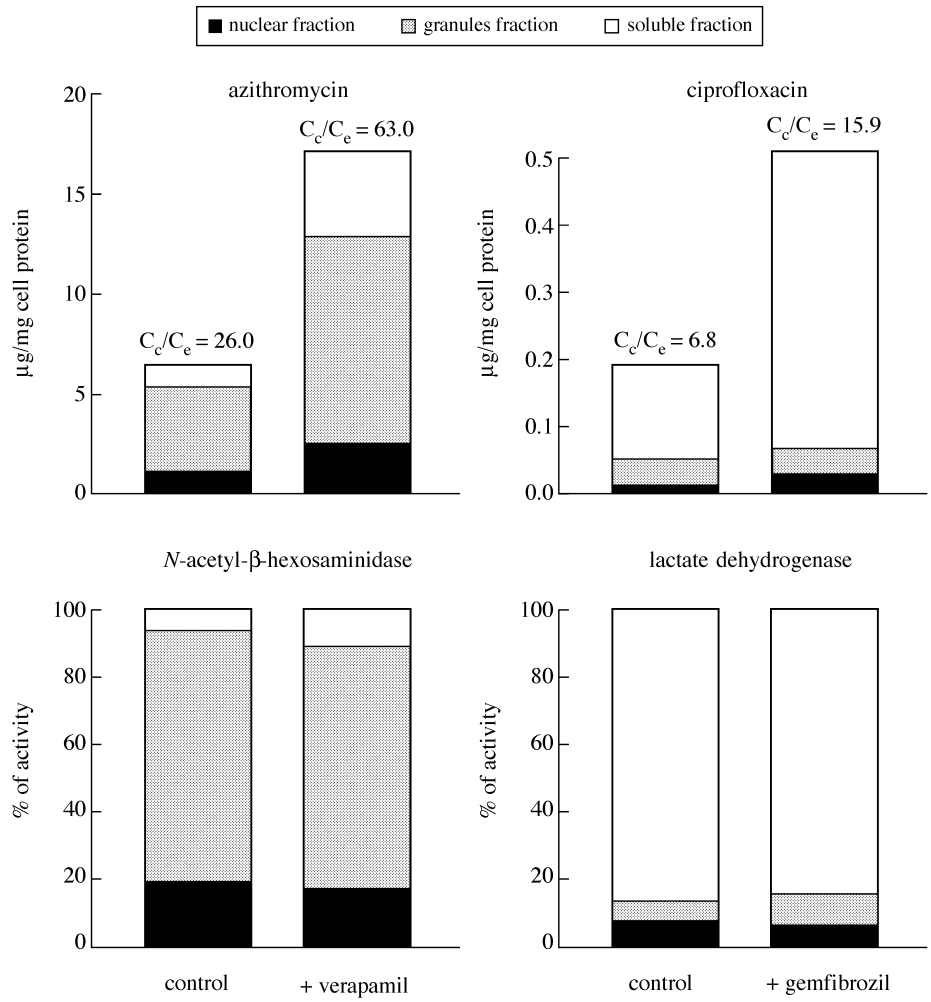

Figure 2. Influence of verapamil (20 µM) and gemfibrozil (250 µM) on the accumulation and subcellular distribution of azithromycin (50 mg/L) and ciprofloxacin (5 mg/L), respectively. Cells were incubated for 24 h with the drugs. The upper panel shows the antibiotic content in the nuclear, granules, and soluble fractions expressed as µg/mg of protein in the unfractionated homogenate. Figures at the top of each bar correspond to the apparent cellular accumulation of the antibiotic (cellular to extracellular concentration ratio [C_c/C_e], calculated using a conversion factor of 5 µL of cell volume per mg of cell protein). The lower panel shows the distribution of *N*-acetyl-β-hexosaminidase (marker of lysosomes) and of lactate dehydrogenase (marker of the soluble proteins). Both enzymes were assayed for each experiment, but only one set of data is shown. Each assay was made in triplicate with variations <5%. The accumulation ratios of azithromycin and of ciprofloxacin observed in this experiment, and the increases brought about by the efflux pump inhibitors were similar to those observed in unfractionated cells [azithromycin accumulation ratios: 25.4 ± 1.5 in control, and 70.0 ± 7.1 in verapamil-treated cells; ciprofloxacin accumulation ratios: 4.6 ± 0.8 in controls, and 11.2 ± 1.3 in gemfibrozil-treated cells (n = 3)]. These values are also consistent with those observed in previous work with J774 macrophages.[2,3,7,9]

towards intracellular bacteria).[6] Similar conclusions have been reached concerning *S. aureus* (C. Seral, F. Van Bambeke & P. M. Tulkens, unpublished results). Yet, these studies did not allow direct examination of whether this loss of activity was due to a change in intrinsic pharmacodynamic properties (such as a loss of concentration dependency for fluoroquinolones) or resulted merely from an apparent unavailability of the intracellular drug to its bacterial target.

The data presented here show unambiguously that ciprofloxacin is a concentration-dependent antibiotic towards a cytosolic bacterium (*L. monocytogenes*), and that its intracellular activity can, accordingly, be modulated by changes in its intracellular concentration only, as observed in extracellular media and in broth. This implies that the intracellular drug and the bacteria must come partially into direct contact with one another. Such a contact may take place in the cytosol since (i) *L. monocytogenes* is located in this compartment, and (ii) the data from the fractionation studies demonstrated that ciprofloxacin in most likely present in that compartment also (the results of these studies are entirely consistent with those of previous ones with other fluoroquinolones[2]). Thus, the present study extends and rationalizes the observations of Rudin and co-workers made with norfloxacin and ciprofloxacin. These authors indeed showed that gemfibrozil

enhances the listeriacidal activity of these fluoroquinolones.[16] The present data may also offer a satisfactory explanation for the poor activity of ciprofloxacin towards intracellular *S. aureus*. Indeed, the data indicate that only a minor proportion of the ciprofloxacin will meet these phagosomal bacteria. More importantly, we also see that the increase in total cell concentration of ciprofloxacin (obtained by a decrease in its efflux in the presence of gemfibrozil) does not allow for a commensurate change in the amount of drug associated with the cell granules. This suggests that the constituents of the cytosol which bind ciprofloxacin are saturated under the conditions used here. The consequence should be that gemfibrozil, and other efflux inhibitors, will be unable to increase ciprofloxacin activity against a non-cytosolic bacterium, which is indeed what we observe with *S. aureus*.

As for ciprofloxacin, the intrinsic pharmacodynamic properties of azithromycin observed towards extracellular bacteria (i.e. a concentration-dependent activity within a narrow range of concentrations only, and no or little bactericidal activity) seem also to prevail against intracellular *L. monocytogenes* and *S. aureus* (C. Seral, F. Van Bambeke & P. M. Tulkens, unpublished results).[6] Yet, this activity seems to be considerably nullified considering the exceptionally large accumulation of azithromycin. However, we observe here that verapamil makes azithromycin active at lower extracellular concentrations against both cytosolic (*L. monocytogenes*) and phagosomal (*S. aureus*) bacteria. Although of limited amplitude, because of the intrinsically bacteriostatic activity of azithromycin,[14] this effect develops in parallel with the increase in drug content occurring both in the cytosol and in the cell granules. This can be explained by the mechanism of accumulation of azithromycin, which, like other cationic amphiphiles, diffuses freely through membranes but is trapped under its protonated form in the acidic compartments (i.e. mainly the lysosomes and to a lesser extent the endosomes and related vacuoles of the endocytic system; see data in Reference 3; see also the reviews on this concept in References 17 and 18). The data presented here confirm this behaviour since azithromycin was mainly found in the 'granules fraction' that contains the bulk of lysosomes, as demonstrated by the distribution of the *N*-acetyl-β-hexosaminidase (a typical lysosomal enzyme). Blocking P-glycoprotein at the cell surface should therefore increase azithromycin content in the cytosol (indirect support for this hypothesis is provided by the observation that overexpression of P-glycoprotein decreases the activity of macrolides against *L. monocytogenes*[19]). Yet, we may anticipate that the drug will then quickly re-equilibrate between cytosol and lysosomes, hence the effect seen towards intracellular *S. aureus*.

Beyond their direct pharmacological interest, the conclusions of this study may also lead to potentially useful therapeutic applications. The extracellular concentrations at which we observe an effect of efflux inhibitors on both azithromycin and ciprofloxacin are indeed in the range of those reached in the serum of patients treated with conventional doses of these antibiotics. We may therefore suggest that the activities of azithromycin and ciprofloxacin against intracellular bacteria are actually suboptimal due to basal efflux mechanisms operating in macrophages. Even though verapamil and gemfibrozil are clearly not usable in clinics because of their own pharmacological activities, the data indicate that drugs acting more specifically on antibiotic transporters (such as the specific inhibitors raised against P-glycoprotein and MRP[20,21]) could prove useful in optimizing antimicrobial intracellular therapy.

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