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Modulation of the expression of ABC transporters in murine (J774) macrophages exposed to large concentrations of the fluoroquinolone antibiotic moxifloxacin

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ABSTRACT

Long-term exposure to pharmacological agents can select for cells that overexpress efflux transporters. We previously showed that mouse J774 macrophages cultivated for a prolonged period of time with toxic concentrations of the fluoroquinolone ciprofloxacin overexpress the efflux transporter Mrp4 and display a reduced accumulation of this antibiotic, but no change in the accumulation of moxifloxacin, a closely related molecule (Antimicrob. Agents Chemother. [2006] 50, 1689–1695 and [2009] 53, 2410–2416). Because of this striking difference between the two fluoroquinolones, we have now examined the modifications in the expression of ABC efflux transporters induced by the prolonged exposure of J774 macrophages to high concentrations of moxifloxacin. The resulting cell line showed (i) no difference in the accumulation of moxifloxacin but an increased accumulation and decreased efflux of ciprofloxacin; (ii) an overexpression of the multidrug transporters Abcb1a (P-gp), Abcc2 (Mrp2) and Abcg2 (Bcrp1), and a decreased expression of Abcc4 (Mrp4). While P-gp and Bcrp1 were functional, they did not modify the cellular accumulation of fluoroquinolones. The data show that exposing cells to high concentrations of a drug that is not affected by active efflux can trigger a pleiotropic response leading to a modulation in the expression of several transporters. These changes, however, are not sufficient to protect cells against the toxicity that fluoroquinolones may exert at large concentrations. They could also cause unanticipated drug interactions in vivo, should the drug exposure grossly exceed what is anticipated from its current registered use.

1. Introduction

Exposure of eukaryotic cells to drugs can trigger modifications in the expression of mechanisms susceptible to favor their elimination. In hepatocytes, this results in a global activation of the phases I, II, and III of drug elimination and transport, and is most often related to the transient induction of the transcriptional regulation of the corresponding genes by nuclear receptors (Fardel et al., 2001; Klaassen and Aleksunes, 2010; Scotto, 2003; Xu et al., 2005). In cancer cells, overexpression of efflux transporters is one of the best known mechanisms of resistance to chemotherapy (Baguley, 2010; Eckford and Sharom, 2009; Gillet et al., 2007). This resistance can be reproduced *in vitro* by exposing cells for prolonged periods of time to increasing concentrations of anticancer agents and overexpression of drug transporters can result in this case from a multitude of mechanisms that are often slowly acquired and poorly reversible (Gottesman et al., 1998; Scotto, 2003; Turk et al., 2009). Such stepwise selection approach can be applied to many other drugs to identify the transporter(s) responsible for their efflux, provided they can exert sufficient toxicity at the initial concentration used to trigger selection of a resistance mechanism.

Using this procedure, we were able to identify, in J774 macrophages, the efflux transporter for the fluoroquinolone antibiotic ciprofloxacin (see Fig. SP1 for structure) as being Mrp4[3] (Abcc4), a member of the C subfamily of ATP binding cassette

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[3] According to common conventions, genes and proteins of murine origin are written in low-case letters after the first initial, while genes and proteins of human or non-specific origin are written in all-uppercase letters.

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(ABC) transporters, which is expressed at high level even in wild-type macrophages (Marquez et al., 2009; Michot et al., 2006). Overexpression of this protein effectively reduces the cellular concentration of ciprofloxacin in cells exposed to increasing concentrations of this drug, providing a simple and straightforward resistance mechanism. However, not all fluoroquinolones are subjected to efflux due to apparently minor but probably critical differences in their structures (Michot et al., 2005; Vallet et al., 2011). Specifically, moxifloxacin (see Fig. SP1 for structure), is insensitive to ATP-energized efflux in J774 macrophages, and accumulates to a similar level in wild-type or in ciprofloxacin-selected macrophages (Michot et al., 2005, 2006). We have now used J774 macrophages to examine how cells would respond to stepwise exposure to increasing concentrations of a drug in absence of detectable basal efflux. Much to our surprise, we observed major changes in the expression of several ABC transporters that did not affect the accumulation or efflux of moxifloxacin itself, but caused a dramatic increase in ciprofloxacin accumulation, associated to a slowed-down efflux. This phenotype is thus strikingly different from that observed in ciprofloxacin-selected cells (Michot et al., 2006).

2. Materials and methods

2.1. Chemicals

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Moxifloxacin HCl and ciprofloxacin HCl (potency: 90.9% and 85%, respectively) were received from Bayer Schering Pharma AG (Berlin, Germany) as microbiological standards. Fumitremorgin C (FTC) was kindly provided by Dr. R. Robey (NIH, Bethesda, MD). MK-571 was purchased from Enzo Life Sciences (Farmingdale, NY), and other chemical products, from Sigma–Aldrich (St. Louis, MO).

2.2. Cell lines and culture conditions

J774 mouse macrophages (referred to as wild-type cells) were cultured and maintained as already described (Michot et al., 2005). Moxifloxacin-selected cells were obtained by serial culture in media containing increasing moxifloxacin concentrations (37 mg/l [0.1 mM] for 3 months [10 passages], 55 mg/l [0.15 mM] for 3 months [10 passages], and 74 mg/l [0.2 mM] for 12 months [50 passages]), following the general procedure used for selecting cells by ciprofloxacin (Michot et al., 2006). Cells were maintained thereafter in the continuous presence of 0.2 mM moxifloxacin and used for experiments between the 60th and the 80th passage.

2.3. Assessment of cell membrane intactness and mitochondrial metabolism

Trypan blue exclusion assay was used to detect alteration of membrane intactness. Cells detached by trypsinization and pelleted by low speed centrifugation (10 min, 100 × *g*), were incubated with 0.2% Trypan blue in phosphate buffered saline (PBS) for 2 min and counted for stained and unstained cells. Mitochondrial metabolism was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay. In brief, cells were incubated for 48 h with ciprofloxacin or moxifloxacin and rinsed with PBS. MTT was added at a concentration of 0.5 mg/ml to each well. After 1 h incubation at 37 °C, DMSO was added to dissolve the formazan crystals and the absorbance was measured at 570 nm.

2.4. Accumulation and efflux of fluoroquinolones

Experiments were performed as previously described for wild-type and ciprofloxacin-selected cells (Marquez et al., 2009; Michot et al., 2005, 2006), except that moxifloxacin was removed from the culture medium of the moxifloxacin-selected cells for the last 48 h to eliminate cell-associated drug. Fluoroquinolones were assayed by fluorimetry (Michot et al., 2005). ATP-depletion was obtained by pre-incubating cells for 20 min in a medium containing 60 mM 2-D-deoxyglucose and 5 mM NaN_3, and maintaining them in the same medium during the whole experiment, as previously described (Michot et al., 2004). The cell drug content was expressed by reference to the total protein content measured by the Lowry's method (Lowry et al., 1951).

2.5. Genomic characterization of efflux transporters (real-time PCR and TaqMan Low Density Array [TLDA])

Abcc4/Mrp4 mRNA levels were determined as previously described using real-time PCR experiments with SYBR Green detection and normalization with *Ywhaz* and *Rpl13a* (Marquez et al., 2009). The complete transcriptional profile of 47 murine ABC transporters was performed using TaqMan® Low Density custom Array on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For these experiments, total RNA was extracted from J774 mouse macrophage samples with TRIzol (Invitrogen) and treated with Turbo DNase (Ambion, Austin, TX) followed by RNA cleanup with RNeasy Mini Columns (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. Purified RNA concentration was measured with a Qubit fluorimeter, using the Quant-iT RNA BR assay kit (Invitrogen). High-capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to synthesize cDNA, starting from 2 or 3 μg of total purified RNA in a final volume of 50 μl following the manufacturer's instructions.

To identify stable genes to use for housekeeping in our study (Huggett et al., 2005), we first performed real-time PCR using the TaqMan® Mouse Endogenous Control Arrays (Applied Biosystems). 200 ng of cDNA and 50 μl of TaqMan® Universal Master Mix were mixed in a final volume of 100 μl and each sample was used to load one port of the microfluidic card. 16 potential housekeeping genes were tested in triplicate for each sample. The most stable genes were then selected by analyzing the results with GeNorm and Normfinder functions in the Genex 4.3.8 software (MultiD, Göteborg, Sweden).

Gene expression quantification of the murine ABC transporters family was determined using custom-designed TaqMan® Array cards format 96a (Supplemental Table SP1), pre-loaded with the TaqMan® assay for 47 murine ABC transporters and the 10 most stable housekeeping genes that have been selected previously. 600 ng of cDNA and 100 μl of TaqMan® Universal Master Mix were mixed in a final volume of 200 μl and used to load 2 sample ports. Technical replicates and biological replicates were performed for each biological sample. Real-time q-PCR amplifications were carried out (10 min at 94.5 °C followed by 40 cycles of 30 s at 97 °C and 1 min at 59.7 °C). Thermal cycling and fluorescence detection was performed with ABI SDS 2.3 and RQ manager Software. The thresholds and baselines were set manually and cycle threshold (Ct) values were extracted. The optimal Ct value cut-off was limited to 35 cycles. According to Genex software analyses, the best normalization was obtained with *Gapdh* and *Gusb*. The geometric mean of these two genes was then used to normalize gene expression levels of ABC transporter. Analysis of gene expression values was performed using relative quantification (Pfaffl, 2001), which determines target gene expression relative to housekeeping genes expression levels and relative to the wild-type J774 control sample. Variations in gene expression observed for *Abcb1a*, *Abcc2* and *Abcg2* were also validated in microplate experiments, using different TaqMan® Gene Expression Assays. 25 ng of cDNA were mixed with 10 μl of TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) and the target gene assay (ABC transporter or housekeeping gene) in a final volume of 20 μl. Each sample was measured in triplicate with the following parameters: 95 °C for 20 s and 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Gene expression analysis was performed as described previously for TaqMan® Arrays experiments. mRNA levels are expressed as a variation ratio between the resistant (sample) and the wild-type (sample calibrator) cell lines, using *Gapdh* and *Gusb* as housekeeping (reference) genes, according to: $RQ = 2^{((Ct\ sample\ calibrator,\ target\ gene - Ct\ sample,\ target\ gene) - (Ct\ sample\ calibrator,\ reference\ gene - Ct\ sample,\ referencegene))}$.

2.6. Western blot analysis of efflux transporters

Western-blots were performed on cell crude extracts for Abcc2/Mrp2 and *Abcc4/Mrp4*, and on membrane-enriched preparations for Abcb1a/P-gp and Abcg2/Bcrp1, using the NuPAGE electrophoresis system (Invitrogen) as previously described (Marquez et al., 2009). Crude extracts consisted of cells collected in ice-cold PBS and pelleted by low speed centrifugation, resuspended in 10 mM Tris–HCl pH 7.4 and the subjected to heating for 10 min at 70 °C and to sonication, with gross debris eliminated by centrifugation for 30 min at 14,000 rpm (20,000 × *g*). Membrane-enriched preparations were prepared exactly as described previously (Marquez et al., 2009). The protein content of both preparations was measured using the bicinchoninic acid protein assay (Bradford assay; Pierce BCA Reagents, Pierce, Rockford, IL). Appropriate quantities of proteins were mixed to 4X NuPAGE LDS Sample buffer and 10X NuPAGE reducing agent, then heated for 10 min at 70 °C. Samples were loaded on acrylamide gels (NuPAGE 10% Bis-Tris Gel, Invitrogen). After migration, proteins were electro-transferred onto a PVDF membrane (0.45 μm, Pierce), which was blocked by a 2 h incubation with 5% defatted milk in Tris–buffered saline (TBS; 20 mM Tris–HCl, 500 mM NaCl pH 7.5) containing 0.05% tween-20. Membranes were then incubated overnight with the primary antibodies M_2III-5 (Alexis Biochemicals, Lausen, Switzerland), M_4I-10 (Alexis Biochemicals), C219 (Signet, Covance Inc, Princeton, NJ), or BXP-53 (SantaCruz Biotechnology, Santa Cruz, CA) to detect Mrp2 (Abcc2), Mrp4 (Abcc4), P-gp (Abcb1), and Bcrp1 (Abcg2), respectively (see figure caption for dilutions) and exposed to appropriate horseradish peroxidase-coupled secondary antibodies [diluted 1/300] for 5 h. Anti-actin (Sigma–Aldrich) or anti-prohibitin H-80 (SantaCruz Biotechnology) polyclonal antibodies were used as loading control and treated the same way. Blots were revealed by chemiluminescence (SuperSignal West Pico, Pierce).

2.7. Phenotypic characterization of efflux transporters

The activity of Mrp4 was measured using ciprofloxacin as substrate and gemfibrozil and MK571 as broad spectrum and specific inhibitors, as described previously (Michot et al., 2004). The activity of P-gp and Bcrp1 was measured with rhodamine

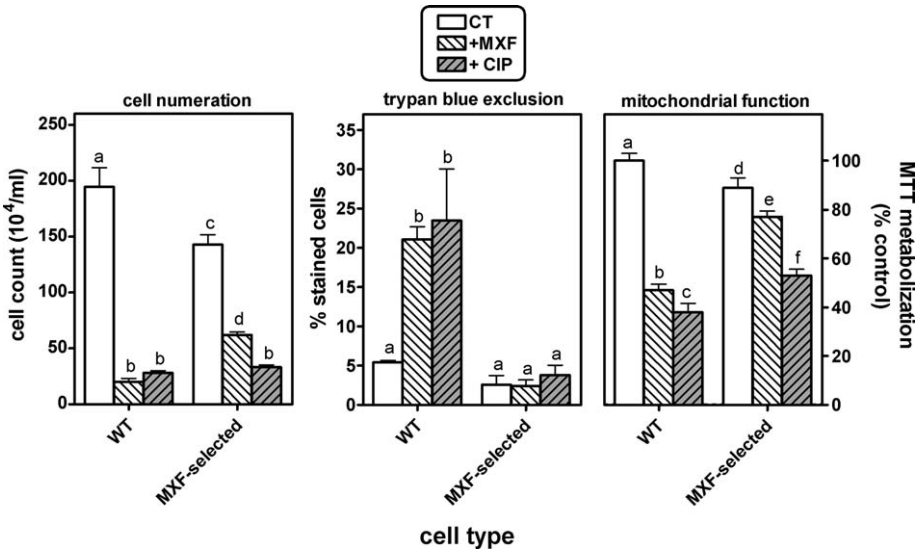

Fig. 1. Effect of fluoroquinolones on cell proliferation, viability and mitochondrial metabolism in wild-type (WT) or moxifloxacin-selected (MXF-selected) cells. Cells were plated for 48 h in drug-free medium (open bars) or in medium containing 0.2 mM moxifloxacin (left-diagonally hatched bars with white background) or 0.2 mM ciprofloxacin (right diagonally hatched bars with grey background). Left and middle panels: cells were incubated with trypan blue and counted to determine the total no. of cells per ml (left panel) or the % of trypan blue stained cells (middle panel). Right panel: cells were incubated with MTT and formazan absorbance was calculated in % of the control value recorded in WT-cells in control conditions. Data are means ± SD ($n = 3$; bars with different letters are significantly different from each another [one-way ANOVA; Tukey post hoc test $p < 0.05$]).

123 and Bodipy-prazosin, respectively, in the absence or in the presence of specific efflux pumps inhibitors (verapamil, for P-glycoprotein and fumitremorgin C for BCRP). Cells were incubated with the substrate, washed 3 times with ice-cold PBS, and collected in 500 μl NaOH 0.3 M (thereafter neutralized with 500 μl HCl 0.3 M) for rhodamine 123 (Takara et al., 2003), or in 500 μl water for BODIPY-prazosin. Samples were then subjected to sonication and the cell-associated fluorophore assayed using a Fluorocount microplate Fluorometer (Packard Instrument Company, Meriden, CT) (λ_{ex} = 485 nm; λ_{em} = 530 nm).

2.8. Sequencing of Mrp4

The *Mrp4* Open Reading Frame (ORF) was sequenced using thirteen primer pairs (Supplemental Table SP2). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), and sequenced using the Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification by precipitation with sodium acetate 3 M pH 4.6 and ethanol, sequencing products were resuspended in formamide and analyzed with a capillary electrophoresis system (ABI PRISM® 3100 Genetic Analyzer; Applied Biosystems). Sequencing results were analyzed by BLAST with the *Mrp4* cDNA sequence from Genebank accession number NM_001033336.

2.9. Curve-fitting and statistical analysis

Curve-fitting analyses were made using GraphPad Prism® version 4.03, GraphPad Software (San Diego, CA). Statistical analyses were made with GraphPad Instat version 3.06 (GraphPad Software).

3. Results

3.1. Influence of moxifloxacin and ciprofloxacin on cell proliferation, viability, and mitochondrial metabolism in wild-type and moxifloxacin-selected cells

As a preliminary step in this work, we examined to what extent cells having gone through the process of selection by moxifloxacin showed a distinct pattern of growth, viability, and maintenance of mitochondrial metabolism compared to wild-type cells. To this effect, both cell types were incubated for 48 h with 0.2 mM moxifloxacin (highest concentration used for selection) or 0.2 mM ciprofloxacin (used as a comparator), and then counted and subjected to Trypan blue and MTT assays (Fig. 1). Cell numeration (left panel) showed that moxifloxacin-selected cells grew somewhat more slowly than wild-type cells in drug-free medium and that both moxifloxacin and ciprofloxacin markedly reduced this growth rate in both cell types, suggesting that toxic effects still occur. Cell viability (middle panel), which was markedly reduced in wild-type cells when exposed to 0.2 mM moxifloxacin or ciprofloxacin, remained, however, unchanged (<5% dead cells) in moxifloxacin-selected cells subjected to the same treatment. Mitochondrial metabolism (MTT assay; right panel), which was markedly impaired by both fluoroquinolones in wild-type cells, was only modestly reduced by moxifloxacin in moxifloxacin-selected cells while remaining inhibitable by ciprofloxacin. Taken together, these data suggest that moxifloxacin-selected cells remain partially susceptible to the toxicity of fluoroquinolones and cannot be considered as fully resistant to the drug used for their selection, in contrast to what was observed for cells selected with ciprofloxacin (Michot et al., 2006).

3.2. Accumulation and efflux of moxifloxacin and ciprofloxacin in wild-type and moxifloxacin-selected cells

In a first approach, we compared the cellular accumulation of moxifloxacin and ciprofloxacin at equilibrium (120 min (Michot et al., 2005)) in moxifloxacin-selected cells vs. wild-type cells in control medium and after addition of gemfibrozil or MK-571 (used as broad spectrum and specific inhibitors of Mrp efflux transporters, respectively (Marquez et al., 2009; Michot et al., 2005)). As shown in Fig. 2, moxifloxacin accumulated to similar levels in both cell types, and this accumulation was not affected by the presence of the Mrp4 inhibitors. In contrast, ciprofloxacin (i) accumulated about 3-times less than moxifloxacin in wild-type cells, as previously described (Michot et al., 2005); (ii) showed an increased accumulation in moxifloxacin-selected cells incubated in control medium, and (iii) reached the same, or a slightly higher level of accumulation than moxifloxacin, in the presence of gemfibrozil or of MK-571 in both cell types.

We then examined in details the kinetics of influx and efflux of moxifloxacin and ciprofloxacin in wild-type and moxifloxacin-selected cells. The data are illustrated in Fig. 3, with the corresponding half-lives displayed in Table 1. For moxifloxacin (upper panels), influx and efflux were very fast with similar rates and no significant difference between cell types. For ciprofloxacin

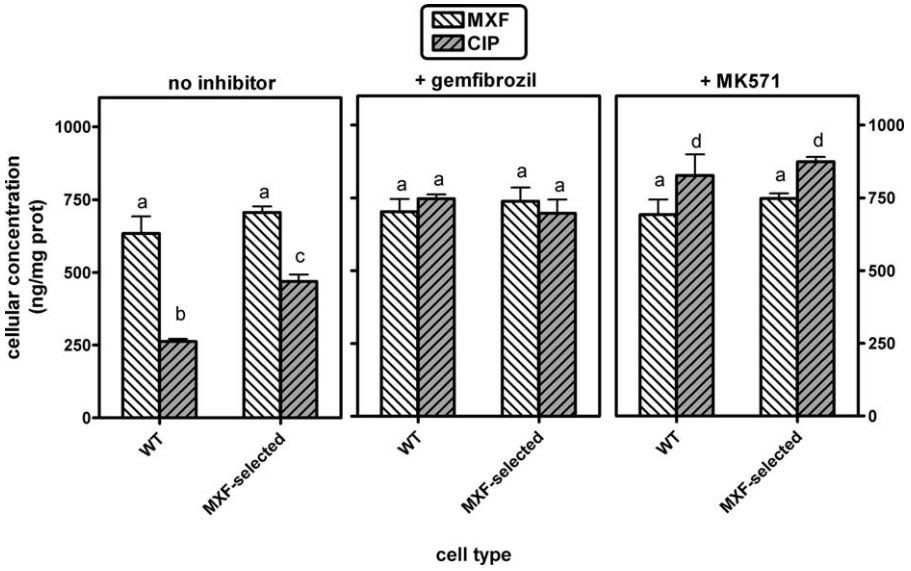

Fig. 2. Cellular concentration of moxifloxacin (MXF; left-diagonally hatched bars with white background) compared to ciprofloxacin (CIP; right diagonally hatched bars with grey background) in wild-type (WT) or moxifloxacin-selected (MXF-selected) cells after 2 h incubation at an extracellular concentration of 50 μM. Left panel; control conditions; middle and right panel: in the presence of 500 μM gemfibrozil (middle panel) or 300 μM MK-571 (right panel). Data are means ± SD ($n = 3$; bars with different letters are different from one another [one-way ANOVA; Tukey post hoc test $p < 0.05$]).

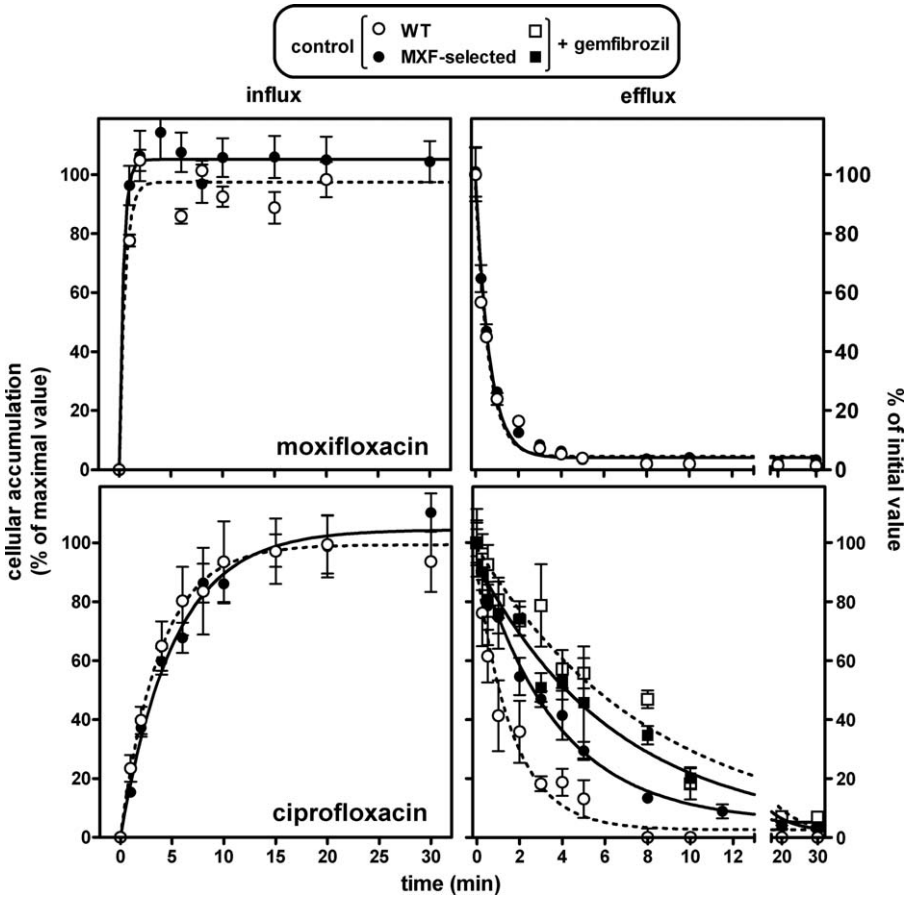

Fig. 3. Kinetics of accumulation and efflux of moxifloxacin (top) and ciprofloxacin (bottom) in wild-type (WT) and moxifloxacin-selected (MXF-selected) macrophages. Cells were incubated in the presence 50 μM fluoroquinolone for 120 min (graph cut at 30 min), washed and re-incubated in antibiotic free medium for 30 min. When present, gemfibrozil was added during both uptake and efflux at a concentration of 500 μM. Data are means ± SD ($n = 3$).

(lower panels), influx was about 5–8 times slower than that of moxifloxacin but not statistically different between cell types. In contrast, ciprofloxacin efflux was slowed down in moxifloxacin-selected cells vs. wild-type cells. Moreover, as compared to influx rate, efflux of ciprofloxacin was about 2-fold faster in wild-type cells but occurred at a similar rate in moxifloxacin-selected cells. Gemfibrozil slowed down ciprofloxacin efflux in wild-type cells as well as in moxifloxacin-selected cells, though to a lesser extent. In

Table 1
Half-lives of influx or efflux of fluoroquinolones[a] in wild-type or moxifloxacin-selected cells.

Antibiotic	Condition	Half-life (min)[b]	
		Wild-type cells	Moxifloxacin-selected cells
Moxifloxacin	Influx; control	0.36 (0.21–1.31) A, a	0.27 (0.19–0.53) A, a
	Efflux; control	0.41 (0.33–0.56) A, a	0.44 (0.39–0.51) A, a
Ciprofloxacin	Influx; control	2.65 (1.40–2.98) B, a	3.57 (3.10–4.20) B, a
	Efflux; control	1.25 (0.89–2.11) C, a	3.41 (2.56–5.12) B, b
	Efflux; +gemfibrozil 500 μM[c]	5.64 (3.98–9.69) D, a	4.54 (3.47–6.57) B, D, a

[a] Extracellular concentration: 50 μM. For influx, the antibiotic was present during the whole period of accumulation. For efflux, cells were incubated with the antibiotic for 2 h and then transferred to antibiotic-free medium.
[b] Accumulation and efflux were followed for up to 30 min and the data used to fit a one-phase exponential association (influx) or decay (efflux) function ($R^2 > 0.98$); values are given as means (95% confidence interval). Statistical analysis (one-way ANOVA) was made using rate constants (k[0.693/half-life]). Values with different upper case and lower case letters are different from each other in the corresponding column or row, respectively ($p < 0.05$).
[c] Present during both pre-incubation and efflux (gemfibrozil was without significant effect on the rate of accumulation kinetics of ciprofloxacin).

contrast, gemfibrozil did not significantly influence ciprofloxacin influx in both cell types (not shown).

Taken together, these pharmacokinetic studies globally show that moxifloxacin-selected cells have essentially an unchanged ability to accumulate or expel this drug whereas they show an increased accumulation of ciprofloxacin that seems associated with a decreased efflux that, however, still remains inhibitable by gemfibrozil.

3.3. Expression of ABC transporters in wild-type and moxifloxacin-selected cells

The data reported so far suggest the stepwise selection process made with moxifloxacin primarily leads to changes in the transport of ciprofloxacin rather than to that of moxifloxacin itself. To gain a broad view of potential changes in ABC transporters induced by chronic exposure to moxifloxacin, TaqMan® Real-Time PCR was used to analyze "*en bloc*" the expression of genes encoding 47 of these transporters (Table 2). For highly expressed transcripts (Ct ≤30 in wild-type cells), we noticed a marked overexpression of *Abcb1a* (one of the two isoforms of the murine P-glycoprotein) and *Abcg2* (also known as Bcrp1), both confirmed by two different probes, as well as of *Abca8b*, and a more modest overexpression of *Abcb2*, *Abcb3*, *Abcb9*. Among transcripts with lower expression (Ct > 30 in wild-type cells), we also observed an overexpression of *Abcc2* (but with discordant responses depending on the probe used) and *Abcc8*. In contrast, *Abca1*, *Abca9* (transcript with low expression), and Abcg1 levels were moderately decreased. *Abcc4* (Mrp4) expression was only slightly decreased. This result was confirmed by SYBR Green real-time PCR, which also detected a slight decrease in the expression of Abcc4 mRNA in moxifloxacin-selected cells compared to wild-type cells (expression ratio: −3.3).

To validate the results of these transcriptomic investigations, we performed a proteomic analysis of the 4 multidrug transporters that show changes in their mRNA level in moxifloxacin-selected cells, namely Abcb1a/P-gp, Abcg2/Bcrp1, and to some extent, *Abcc4/Mrp4* and Abcc2/Mrp2. Western-blots (Fig. 4) were performed on whole cell lysates for Mrp2 and Mrp4, and on membrane proteins for P-gp and Brcp1 (as these were poorly detected in cell lysates). Compared to wild-type cells, moxifloxacin-selected cells showed a marked decrease of Mrp4 level together with a marked increase in P-glycoprotein and a modest increase in Mrp2. Bcrp1 was detected in moxifloxacin-selected cells only. To examine whether the changes in Mrp4 levels were not due to mutations leading to change in amino acid or presence of a premature stop codon, the *Mrp4* ORF was sequenced using as a template cDNA, but no difference

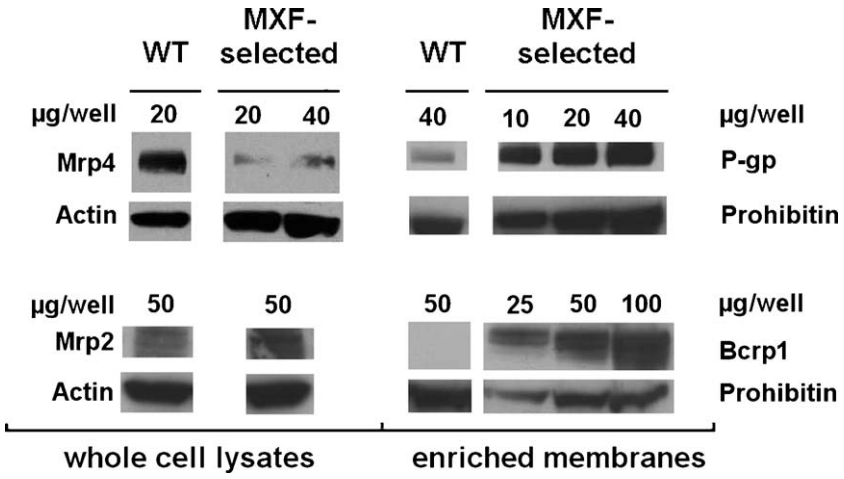

Fig. 4. Western blots of proteins prepared from wild-type (WT) or moxifloxacin-selected (MXF-selected) cells. Gels were loaded with the indicated amounts of proteins. Left: whole cell lysates, revelation with rat anti-Mrp4 (1:2000) or with mouse anti-Mrp2 (1:300), or with rabbit anti-actin (1:1000) antibodies, followed by the appropriate anti-IgG HRP-labeled antibody (1:300). Right: membrane proteins, with revelation with rat anti-Bcrp1 (1:300) or with mouse anti-P-gp (1:300), or with rabbit anti-prohibitin (1:300) antibodies, followed by the appropriate anti-IgG HRP-labeled antibody (1:300).

Table 2
mRNA expression variations of ABC transporters in moxifloxacin-selected cells compared to wild-type macrophages.

Gene	Expression ratio compared to wild-type cells[a]
Abca1	−3.51
Abca2	−1.12
Abca3	−1.56
Abca4	nd
Abca5	2.31
Abca6	nd
Abca7	−1.20
Abca8a	nd
Abca8b	76.20
Abca9	(−10.48)
Abca13	2.02
Abca14	nd
Abca15	nd
*Abcb1a**	76.35
	84.42
Abcb1b	1.72
Abcb2	4.30
Abcb3	3.17
Abcb4	2.48
Abcb6	−1.22
Abcb8	−1.25
Abcb9	9.83
Abcb10	1.43
Abcb11	(1.19)
Abcc1	−1.26
*Abcc2**	(5.09)
	(−1.26)
Abcc3	1.09
Abcc4	−1.82
Abcc5	1.29
Abcc6	nd
Abcc7	nd
Abcc8	(7.74)
Abcc9	nd
Abcc10	−1.40
Abcc12	nd
Abcd1	2.22
Abcd2	1.79
Abcd3	−1.51
Abcd4	−2.03
Abce1	−1.00
Abcf2	1.21
Abcf3	1.11
Abcg1	−4.92
*Abcg2**	108.41
	99.47
Abcg3	nd
Abcg4	(−1.07)

Data are the mean of duplicates from two biological samples. Genes with asterisks were tested with two different probes (Table S2). Values with grey background: Ct < 30 (high expression) and significant variation (>3 or <−3); values with white background: Ct < 30 (high expression but no significant variation); values with white background and in parenthesis: 30 < Ct < 35 (low expression); nd: Ct > 35 (no detectable expression).

[a] See methods for calculations of this ratio; for a better understanding, a decrease in expression, corresponding to 0 < RQ < 1, is noted as a negative value, obtained by: −1/RQ.

was found between the sequence determined for wild-type and moxifloxacin-selected cells.

3.4. Functionality of P-gp and Bcrp1 in wild-type and moxifloxacin-selected cells

To test for the functionality of Abcb1a/P-gp and Abcg2/Bcrp1 overexpressed in moxifloxacin-selected cells, we measured the accumulation of their respective substrates (rhodamine 123 and BODIPY-prazosin) in comparison with wild-type cells and in the absence and presence of their corresponding inhibitors

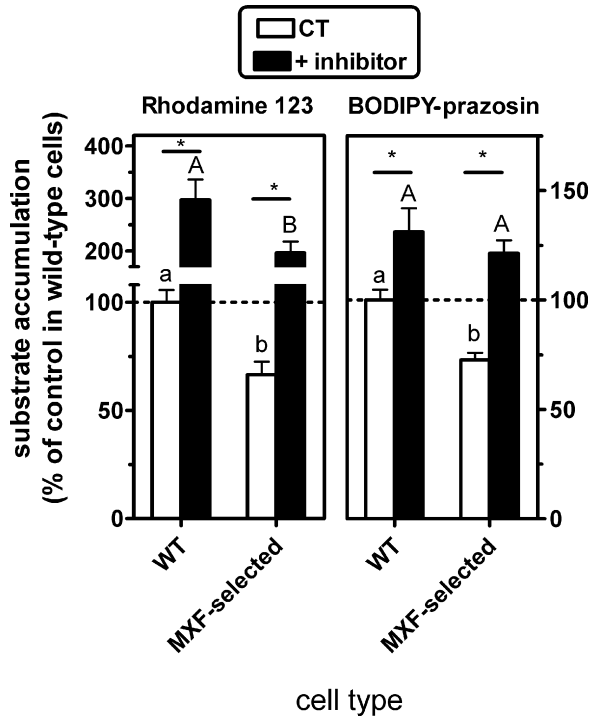

Fig. 5. Cellular accumulation of preferential substrates of P-glycoprotein or Bcrp1 and influence of inhibitors in wild-type (WT) or moxifloxacin-selected (MXF-selected) cells. Left: cells were incubated during 2 h with 10 μM rhodamine 123 in the absence (CT; control) or in the presence of 100 μM verapamil (+inhibitor). Right: cells were incubated during 1 h with 0.1 μM BODIPY-prazosin in the absence (CT; control) or in the presence of 10 μM Fumitremorgin C (+inhibitor). All values are expressed in percentage of the accumulation measured in control conditions for wild-type cells; they are the means of three independent determination ± SD. Statistical analysis (ANOVA, Tukey post hoc test): comparison between conditions: bars with different letters are different from one another (lower case, control conditions; upper case, +inhibitor; $p < 0.05$); comparison between control condition and inhibitor: $^*p < 0.05$.

(verapamil and fumitremorgin C (Fontaine et al., 1996; Robey et al., 2001)). Fig. 5 shows that the accumulations of both rhodamine 123 and BODIPY-prazosin (i) were significantly lower in moxifloxacin-selected cells compared to wild-type cells, and (ii) were increased in the presence of the corresponding inhibitors in both cell types. We then examined whether P-gp and/or Bcrp1 could modulate the accumulation of moxifloxacin or ciprofloxacin. As shown in Fig. 6, there was no significant increase in accumulation of either antibiotic in moxifloxacin-selected cells upon addition of verapamil or fumitremorgin C. Combining these inhibitors with gemfibrozil did not yield additional effect on ciprofloxacin accumulation compared to what has been obtained with gemfibrozil alone. ATP depletion also did not cause any significant change in moxifloxacin accumulation (whatever cell type tested) and did not yield any more increase in accumulation of ciprofloxacin than what was obtained with gemfibrozil.

4. Discussion

The present study demonstrates that the exposure of an eukaryotic cell line to increasing concentrations of moxifloxacin, a drug that is apparently not effluxed by an ABC transporter, can nevertheless cause profound modifications in the expression of several of these transport proteins. These modifications have the potential of affecting the transport of many drugs, and, as documented here, effectively increase the accumulation of another fluoroquinolone, ciprofloxacin. Most conspicuously, these changes do not seem to contribute to the partial resistance of the cells to moxifloxacin itself.

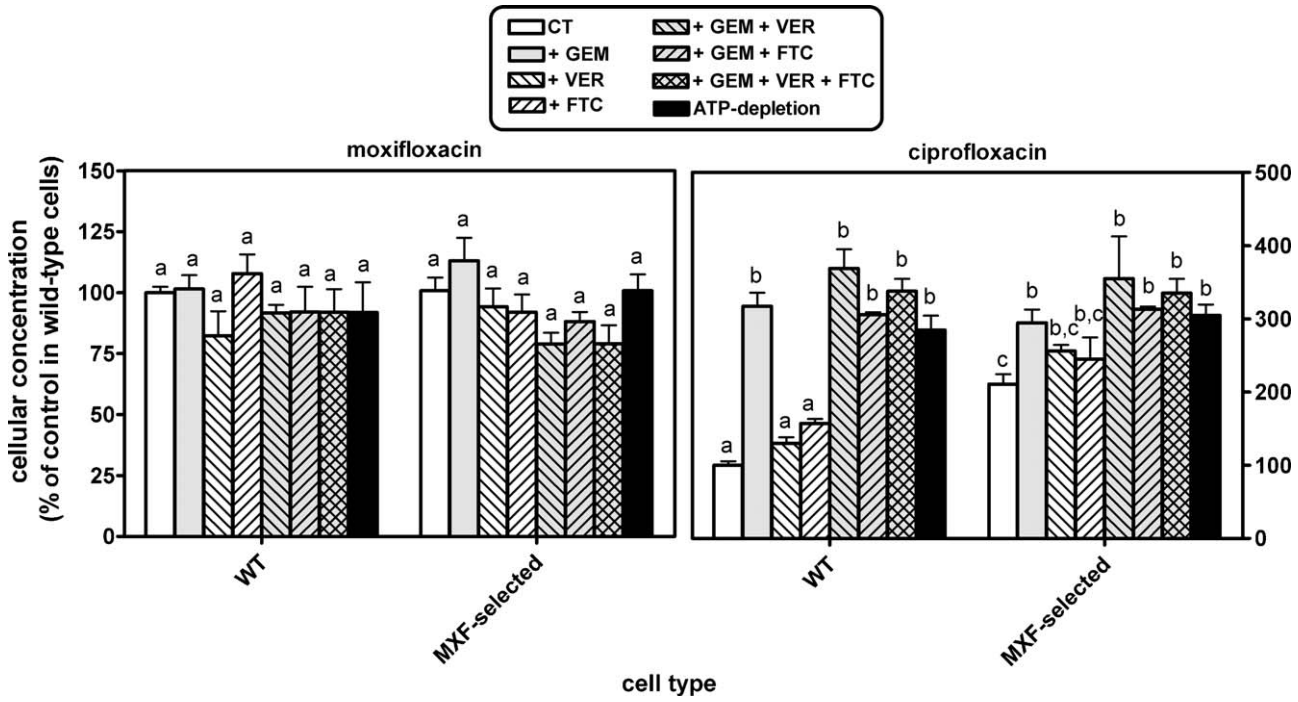

Fig. 6. Influence of preferential inhibitors of efflux transporters on ciprofloxacin and moxifloxacin accumulation. Wild-type (WT) or moxifloxacin-selected (MXF-selected) cells were incubated during 2 h with an extracellular concentration of 50 μM antibiotic alone (control, CT) or in the presence of 500 μM gemfibrozil (+GEM), 100 μM verapamil (+VER), 10 μM fumitremorgin C (+FTC) or combinations thereof, or in ATP-depleted cells. All values are expressed in percentage of the accumulation measured in control conditions for wild-type cells; they are the means of three independent determination ± SD. Statistical analysis (ANOVA, Tukey post hoc test): comparison between conditions: bars with different letters are different from one another ($p < 0.05$).

The transport of fluoroquinolones in eukaryotic cells is highly dependent from the cell type, the species, and the molecule examined (Alvarez et al., 2008). Thus, P-glycoprotein has been shown to transport grepafloxacin, sparfloxacin, danofloxacin, and moxifloxacin (Brillault et al., 2009; Cormet-Boyaka et al., 1998; Lowes and Simmons, 2002; Schrickx and Fink-Gremmels, 2007) and MRP2, grepafloxacin and danofloxacin (Lowes and Simmons, 2002; Schrickx and Fink-Gremmels, 2007) in human epithelial cells. BCRP/Bcrp1 is also involved in grepafloxacin, ciprofloxacin, norfloxacin, ofloxacin, and enrofloxacin transport in human and mice epithelial cells (Ando et al., 2007; Pulido et al., 2006). In the model used here (murine J774 macrophages), Mrp4 is the main transporter of ciprofloxacin, and to a lesser extent, of levofloxacin and gemifloxacin (Marquez et al., 2009; Michot et al., 2005; Vallet et al., 2011) whereas the data reported earlier (and confirmed here) indicate that moxifloxacin is not actively effluxed (no effect of ATP depletion or of the specific or broad spectrum inhibitors used). We actually showed previously (Michot et al., 2005), and further document here, that uptake and efflux of moxifloxacin are much faster than those of ciprofloxacin, probably because this molecule causes less disorder in the packing and ordering of lipid bilayers, as evidenced from model membranes made in comparison with ciprofloxacin (Bensikaddour et al., 2008).

As discussed earlier (Michot et al., 2005), and also observed for other drugs like quinidine and anthracyclines with P-glycoprotein (Eytan et al., 1996; Marbeuf-Gueye et al., 1999), rapid membrane diffusion of a drug can make its active efflux functionally undetectable but does not rule out an interaction of the molecule with the transporter. We, actually, know that moxifloxacin may impair the efflux of ciprofloxacin (Michot et al., 2005). With these considerations in mind, we may tentatively suggest that the drastic reduction in the expression of the Mrp4 ciprofloxacin transporter upon long-term exposure to moxifloxacin could be related to the fact that moxifloxacin can interact with this transporter. Because the effect is observed at the genomic level, however, we also need to postulate some sort of feed-back mechanism. But, actually, such feed-back mechanism could be much broader, as we also see a pleiotropic modification in the expression of several ABC transporters. First, 3 other multidrug transporters shown to play a role in fluoroquinolone transport in other models (Alvarez et al., 2008) are also overexpressed, namely P-gp/Abca1, Bcrp1/Abcg2, and Mrp2/Abcc2. In our model, this did not affect the accumulation of ciprofloxacin probably because Mrp4 is largely predominant in J774 macrophages (Marquez et al., 2009), so that its markedly reduced expression in moxifloxacin-selected cells increases ciprofloxacin accumulation to a level that cannot be compensated by an increase in the expression of the other transporters. But many other ABC transporters were also affected in their expression, which is reminiscent of what can be observed with other toxic drugs. For instance, cells resistant to the P-gp substrate paclitaxel show a modulation of the expression of about 700 genes, among which *ABCB1* was the most upregulated (Yabuki et al., 2007). In the same line, doxorubicin-resistant cells overexpress not only several genes directly implicated in their MDR phenotype (*ABCB1*, *ABCC1* or *ABCG2*) but also many other genes coding for other ABC transporters (*ABCA2*, *ABCC4*, *ABCC3*, *ABCC5*, *ABCB6*, *ABCF3*, or *ABCG1*), depending on the cell line (Gillet et al., 2004). Beside multidrug transporters, many other ABC proteins show a modified expression level in moxifloxacin-selected cells. The potential role of these changes needs to be further explored. It is, nevertheless, tempting to speculate that these are signs of global adaptation of the cells to what seems to be a major toxic stress. The increased expression of *Abcb2* (Tap1) and *Abcb3* (Tap2), involved in antigen presentation and immune response (Herget and Tampe, 2007), of *Abcb9*, a lysosomal ABC transporter associated with antigen processing-like processes (Zhang et al., 2000), of *Abca8b* (encoding a close homolog of the human ABCA8 (Annilo et al., 2003) able to transport leukotriene C4 and estradiol-beta-glucuronide (Tsuruoka et al., 2002), and of *Abcc8*

that codes for a protein (SUR1) acting as a K^+ selective pore (Bryan et al., 2007) may require attention. The reduced expression of *Abca9*, *Abca1*, and *Abcg1*, involved in lipid homeostasis in macrophages (Piehler et al., 2002), and cholesterol and phospholipid transport (Wang et al., 2007) may also be a cause of concern.

We have no simple explanation for all these numerous changes, especially if considering that the toxicity of fluoroquinolones towards eukaryotic cells is thought to be primarily due to their ability to impair topoisomerase activity (at larger concentrations, however, than in prokaryotic cells). There is no evidence for moxifloxacin being more potent than ciprofloxacin in this context (Perrone et al., 2002; Reuveni et al., 2008). Yet, as the method used to select cells is prone to select multifactorial resistance mechanisms (Gottesman et al., 1998), further studies using global approaches to evaluate the proteome or the transcriptome would need to be performed to further characterize the resistance phenotype of moxifloxacin-selected cells. We can neither exclude that the diversity of changes observed arise from the fact that our mode of selection was not clonal, so that the profile of alterations observed could be the resultant of more targeted modifications occurring in individual cells. Although possibly complicating data interpretation, this mode of selection however better reflects what could occur in vivo under drug pressure. At this stage, our data thus clearly illustrate that long-term exposure to close-to-toxic concentrations of drug that is apparently not affected by efflux nevertheless causes complexes changes in the expression of transporters that can trigger drug interactions and other potential undesired effects.

The pharmacological consequences of our observations also need to be discussed. Because of their ability to accumulate inside cells and their bactericidal character, fluoroquinolones are among the most active antibiotics against intracellular infections (see for review (Van Bambeke et al., 2006)). We previously showed that the activity of ciprofloxacin against intracellular *L. monocytogenes* was concentration-dependent, and therefore affected by the level of expression of Mrp4 (drastic reduction of activity in ciprofloxacin-selected J774 macrophages that are characterized by an increased expression of Mrp4 (Michot et al., 2006), but increased activity in wild-type J774 macrophages in the presence of inhibitors of Mrps (Seral et al., 2003)). In a broader context, this means that any circumstance that could affect the cellular accumulation of fluoroquinolones, including a change in the expression of the transporters responsible for their efflux, could also modify their intracellular activity. Yet moxifloxacin should escape this rule and remain equally active, being not affected by the level of expression of efflux transporters.

These effects reported here were obtained for cells exposed to moxifloxacin concentrations typically 5–20-fold larger than those commonly observed in the serum of patients (~1.5 to ~6 mg/l (Stass and Kubitza, 1999)) treated with the approved dose of 400 mg/day for its current indications (Anonymous, 2008), and these treatments are usually of short duration (5–21 days). However, (i) larger concentrations are reached in specific organs such as the urinary tract where concentrations may be 10-fold larger than serum concentrations (Wagenlehner et al., 2008), and (ii) doses up to 800 mg/day and longer treatment durations are being considered (but not registered) for treatment of tuberculosis (Ginsberg, 2010). This may trigger further animal and human investigations to examine whether the present *in vitro* data translate into significant modifications in vivo that could limit the use of moxifloxacin for this indication.

Conflict of interest

None.

Funding source

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2011.09.003.

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Supplementary Material

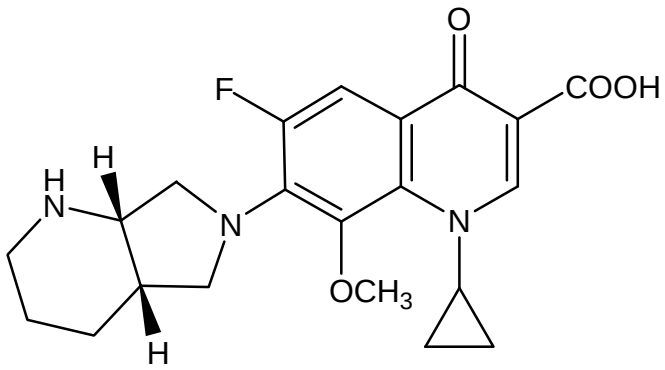
Table SP1: Sequence of the primers used for sequencing of the *Mrp4* ORF, temperature for PCR amplification and size of the corresponding amplicons

Amplicon	Forward primer (5'-3')	Reverse primer (5'-3')	T_a (°C) for PCR reaction	Amplicon size (pb)
4-1	GTGCACACCGAGGTGAAAC	TCGTCGGGGTCATACTTCTC	66	371
4-2	GGGCACTCGAGTAGTTCAGC	GCCAGGCAGGAGATTCCTAT	66	408
4-3	GGTAACCGTCCTCCTCTGG	CACAAACACGTGGCTAGCTG	60	388
4-4	TCGCAAACAAAGTCATCCTG	ACCACGGCTAACAACTCACC	66	355
4-5	AACCCTGCAAGGTCTTTCCT	GCGGATCATCAAGGAGGTAG	66	407
4-6	AGTGGAGGCCAGAAAGCTC	CTGGATGACTGCTGAGACCA	66	392
4-7	CGACACTCAGGAAACGAACC	CTCGCTATGCCAAAAAGGAC	60	406
4-8	CCAGAAATGCGAATGGAAAT	AGCGGAACCAATGGTATGAG	60	370
4-9	TGCTCCTCGTCGTAAGTGTG	GCCCAGCATTCAAAGTCTTC	60	393
4-10	CATCTGCGCCATCTTTGTAA	CCAACCTTTTCCCTGGACTT	60	354
4-11	TGTCAGGAGCTGTTTGATGC	CCCAATTTCGGTTGTCAAGA	60	561
4-12	GGGGAAAATCTGGATCGATAA	TTGCAAGGCACACTAACTGTCT	60	260
4-13	GGATCCAATTTCAGTGTTGGA	CGGGGCTGGTGAATGTAATA	60	397

Table SP2: TaqMan® Gene Expression Assays references

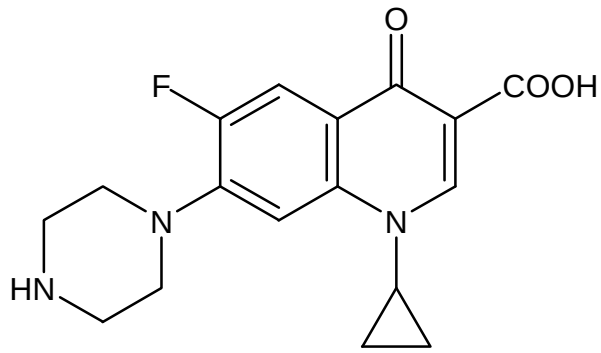
Gene	Assay ID	Gene	Assay ID
Abca1	Mm00442646_m1	Abcc10	Mm00467403_m1
Abca2	Mm00431553_m1	Abcc12	Mm00556685_m1
Abca3	Mm00550501_m1	Abcd1	Mm00431749_m1
Abca4	Mm00492004_m1	Abcd2	Mm00496455_m1
Abca5	Mm00461656_m1	Abcd3	Mm00436150_m1
Abca6	Mm00461636_m1	Abcd4	Mm00436180_m1
Abca7	Mm00497010_m1	Abce1	Mm00649858_m1
Abca8a	Mm00462440_m1	Abcf2	Mm00457400_g1
Abca8b	Mm00457361_m1	Abcf3	Mm00658695_m1
Abca9	Mm00461704_m1	Abcg1	Mm00437390_m1
Abca13	Mm00626523_m1	Abcg2	Mm00496364_m1
Abca14	Mm00509570_m1	Abcg2	Mm01181554_m1
Abca15	Mm00623451_m1	Abcg3	Mm00446072_m1
Abcb1a	Mm00440761_m1	Abcg4	Mm00507250_m1
Abcb1a	Mm01324136_m1	Abcg5	Mm00446249_m1
Abcb1b	Mm00440736_m1	Abcg8	Mm00445970_m1
Abcb2	Mm00443188_m1	18S	Hs99999901_s1
Abcb3	Mm00441668_m1	Aamp	Mm00525080_m1
Abcb4	Mm00435630_m1	Actb	Mm00607939_s1
Abcb6	Mm00470049_m1	B2m	Mm00437762_m1
Abcb8	Mm00472410_m1	Gapdh	Mm99999915_g1
Abcb9	Mm00498197_m1	Gusb	Mm00446953_m1
Abcb10	Mm00497927_m1	Hmbs	Mm00660262_g1
Abcb11	Mm00445168_m1	Hprt1	Mm00446968_m1
Abcc1	Mm00456156_m1	Ipo8	Mm01255158_m1
Abcc2	Mm00496883_m1	Pgk1	Mm00435617_m1
Abcc2	Mm00496899_m1	Polr2a	Mm00839493_m1
Abcc3	Mm00551550_m1	Ppia	Mm02342430_g1
Abcc4	Mm01226381_m1	Rplp2	Mm00782638_s1
Abcc5	Mm00443360_m1	Tbp	Mm00446973_m1
Abcc6	Mm00497685_m1	Tfrc	Mm00441941_m1
Abcc7	Mm00445197_m1	Ubc	Mm01201237_m1
Abcc8	Mm00803450_m1	Ywhaz	Mm01158417_g1
Abcc9	Mm00441638_m1		

Fig. SP1: Structural formulae of moxifloxacin and ciprofloxacin.



Moxifloxacin:

7-[(4aS,7aS)-1,2,3,4,4a,5,7,7a-octahydropyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxoquinoline-3-carboxylic acid



Ciprofloxacin:

1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline- 3-carboxylic acid