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Identification of the Efflux Transporter of the Fluoroquinolone Antibiotic Ciprofloxacin in Murine Macrophages: Studies with Ciprofloxacin-Resistant Cells[▽]

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Ciprofloxacin, the most widely used totally synthetic antibiotic, is subject to active efflux mediated by a MRP-like transporter in wild-type murine J774 macrophages. To identify the transporter among the seven potential Mrps, we used cells made resistant to ciprofloxacin obtained by long-term exposure to increasing drug concentrations (these cells show less ciprofloxacin accumulation and provide a protected niche for ciprofloxacin-sensitive intracellular *Listeria monocytogenes*). In the present paper, we first show that ciprofloxacin-resistant cells display a faster efflux of ciprofloxacin which is inhibited by gemfibrozil (an unspecific MRP inhibitor). Elacridar, at a concentration known to inhibit P-glycoprotein and breast cancer resistance protein (BCRP), only slightly increased ciprofloxacin accumulation, with no difference between resistant and wild-type cells. Analysis at the mRNA (real-time PCR) and protein (Western blotting) levels revealed an overexpression of Mrp2 and Mrp4. *Mrp4* transcripts, however, were overwhelmingly predominant (45% [wild-type cells] to 95% [ciprofloxacin-resistant cells] of all *Mrp* transcripts tested [*Mrp1* to *Mrp7*]). Silencing of *Mrp2* and *Mrp4* with specific small interfering RNAs showed that only Mrp4 is involved in ciprofloxacin transport in both ciprofloxacin-resistant and wild-type cells. The study therefore identifies Mrp4 as the most likely transporter of ciprofloxacin in murine macrophages but leaves open a possible common upregulation mechanism for both Mrp4 and Mrp2 upon chronic exposure of eukaryotic cells to this widely used antibiotic.

Fluoroquinolones are substrates of several prokaryotic and eukaryotic efflux transporters (2, 37). Efflux from bacteria is now recognized as a significant mechanism of resistance to fluoroquinolones and a potential cause of therapeutic failures (14). In eukaryotic cells, it is considered an important factor in determining their pharmacokinetics, including tissue accumulation (38). Concentrating on ciprofloxacin and murine J774 macrophages, we and others noted the presence of a MRP (multidrug resistance-associated protein)-like (Mrp refers specifically to mouse transporters) efflux transporter for this antibiotic (7, 25). MRP proteins belong to the large C subfamily of ABC (ATP-binding-cassette) transporters, among which at least eight (MRP1 to MRP6 [ABCC1 to ABCC6] and MRP7-MRP8 [ABCC10-ABCC11]) are able to transport organic anions (10). To further identify the ciprofloxacin transporter among the different MRP candidates, we undertook to obtain J774 macrophages resistant to concentrations of ciprofloxacin causing toxicity to wild-type cells. This was achieved by long-term culture in the presence of progressively increasing drug concentrations, a procedure successfully used to select cells with efflux-mediated resistance to anticancer agents (11) (while fluoroquinolones act as antibiotics by impairing bacterial topoisomerases at low concentrations, they inhibit the eukaryotic topoisomerases II and kill mammalian cells at large concentrations). The phenotype of these cells has been described in a previous publication (24). They display a markedly reduced accumulation of ciprofloxacin, which could be brought almost to control levels by ATP depletion or addition of typical inhibitors of MRP efflux transporters, such as probenecid or MK571. Additional studies comparing other quinolones in both wild-type and ciprofloxacin-resistant cells showed that moxifloxacin was not affected by this efflux transporter in both cell types, while levofloxacin and garenoxacin showed an intermediate behavior (23, 24). Beyond displaying a markedly reduced accumulation of ciprofloxacin, these ciprofloxacin-resistant cells provide a protected environment against ciprofloxacin for *Listeria monocytogenes* (24). Moreover, we recently showed that this eukaryotic transporter can cooperate with a prokaryotic ciprofloxacin transporter to make intracellular *L. monocytogenes* still more resistant to the drug (20). In the present study, we now identify the ciprofloxacin efflux transporter as most likely being Mrp4.

MATERIALS AND METHODS

Materials. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Ciprofloxacin (98% purity) was kindly provided by Bayer HealthCare A.G., Leverkusen, Germany. Elacridar (GF120918) was the generous gift of Glaxo Wellcome Research and Development (Laboratoire Glaxo Wellcome, Les Ulis, France). Gemfibrozil and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell lines and culture conditions. J774 mouse macrophage-like cells were cultured and maintained as already described (25). J774 macrophages resistant to ciprofloxacin and their revertants were obtained as described earlier (24). In

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TABLE 1. Primers used for real-time PCR studies of *Mrp* genes[a]

Gene	Primer sequence (5′–3′)		Annealing temp (°C)	Amplicon size (bp)
	Forward	Reverse		
Mrp1	CGATCAAGAGTGGCGAAGG	AGGTGATGCCATTCAGTGTG	56.4	93
Mrp2	TGTTGGGCTTATGGTTCTCC	CGAATGCTGTTCACTTGCTC	61.5	189
Mrp3	CCTGATCCAGAACCTACTCG	GCAGTCCGTAGCCTCAAG	52.7	198
Mrp4	TGCTCCTCGTCGTAAGTGTG	TGGAGGGAGGACGATAAATG	55.0	187
Mrp5	GCTGTTTGCTCTCGTGTGG	TCCCTGTCACTTCTGTACCC	63.5	152
Mrp6	GGATTGACAGCAGAAGAGG	GCAGAGGAAGAGGAACAGG	55.0	120
Mrp7	GGCTGAGAAGGAACAAGTGG	AGCAGAGAGACGAGGATGG	61.5	216

[a] Each real-time PCR started with denaturation at 95°C for 5 min and then 40 cycles of amplification (15 s at 95°C followed by 1 min at the annealing temperature). The melting curve analysis was made from 60°C to 95°C, with continuous fluorescence readings. The annealing temperature was 60°C for both housekeeping genes (*Ywhaz* [amplicon size, 141 bp] and *Rpl13A* [amplicon size, 130 bp]).

brief, selection of resistant cells was obtained by cultivating wild-type cells for about 50 passages (approximately 8 months) in the presence of increasing concentrations of ciprofloxacin large enough to induced a selective pressure (from 0.1 mM to 0.2 mM, i.e., 34 to 68 mg/liter) and far above serum levels reached in treated patients (human maximum concentration of drug in serum, 2.4 to 4 mg/liter for oral doses of 500 to 750 mg [Cipro package insert; Bayer HealthCare Pharmaceuticals]). Cells were then maintained in the presence of 0.2 mM ciprofloxacin and used between the 60th and 80th passages. Revertants were obtained by returning resistant cells to ciprofloxacin-free medium for 90 passages (about 9 months) and were used between the 100th and 110th passages.

Accumulation and efflux of ciprofloxacin in J774 macrophages. We followed the procedures described in our previous publications (24, 25) for fluorometric assay of ciprofloxacin (λ_{exc} = 275 nm and λ_{em} = 450 nm; limit of detection, 5 ng/ml, or ~15 nM), with appropriate controls to exclude interference by gemfibrozil and elacridar. For studies with ciprofloxacin-resistant cells, which were cultivated in the continuing presence of ciprofloxacin, cells were rinsed twice in phosphate-buffered saline prior to the start of the experiment to avoid contaminating cell extracts with extracellular drug. All experiments were performed using a ciprofloxacin extracellular concentration of 50 μM (17 mg/liter), which was needed, although it is supratherapeutic, to ensure reliable assays of ciprofloxacin in ciprofloxacin-resistant cells (see references 23 and 25 for further details on dose-effect relationships of ciprofloxacin accumulation in wild-type and ciprofloxacin-resistant cells). The cell ciprofloxacin content of each sample was expressed by reference to its total protein content measured by Lowry's method.

Isolation of RNA and synthesis of cDNA. Total RNA was isolated from J774 cell monolayers by use of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was purified with Turbo DNase (Ambion, Austin, TX), and the concentration was measured with a Qubit fluorimeter, using a Quant-iT RNA assay kit (Invitrogen). cDNA was synthesized using 1 μg of total purified RNA and random hexamer primers (Promega reverse transcription system; Promega Co., Madison, WI).

Real-time PCR. Primer pairs for all *Mrp* (also referred to as *Abcc*) genes investigated were designed using Primer 3 and Autoprime programs (40), taking into account exon-intron boundaries to prevent genomic DNA amplification. The specificity of each pair of primers (Table 1) was first checked in silico and then assessed in PCR experiments, where a single band at the expected size was obtained. Housekeeping genes (*Ywhaz* [encoding phospholipase A2] and *Rpl13a* [encoding ribosomal protein L13a]) were selected among 12 potential candidates from a mouse geNorm normalization kit (PrimerDesign Ltd., Southampton, United Kingdom), using geNorm software (39). Real-time PCR experiments were performed with an iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA), using Sybr green supermix (Bio-Rad) (see Table 1 for amplification protocol details). Standard curves for each gene were generated by using serially diluted solutions of purified PCR products. Melting curves were obtained to confirm the specificity of each product in each sample. Results were analyzed using iCycler iQ software (version 3.1; Bio-Rad), and relative quantification of each *Mrp* gene in resistant or revertant cells versus wild-type cells was made in comparison with the two selected housekeeping genes, based on Pfaffl's equation (29).

Cell crude extracts and membrane preparation. Monolayers from culture dishes were rinsed and scraped, and cells washed twice in ice-cold phosphate-buffered saline. For stripped-membrane preparations, cells were resuspended in 10 mM Tris-HCl, pH 7.4, plus 1 tablet of protease inhibitor cocktail (Complete mini EDTA-free; F. Hoffmann-La Roche Ltd. Diagnostics Division, Basel, Switzerland) per 10 ml of buffer and homogenized with a Dounce B homogenizer (glass-glass, tight pestle, 25 strokes). The homogenates were layered on top of a 50% (wt/vol; 1.5 M) sucrose solution plus 1 mM phenylmethylsulfonyl fluoride and centrifuged in a Beckman SW40 rotor at 280,000 × *g* for 1 h at 4°C. The crude membrane fraction isolated from the interphase buoyant sucrose was diluted to 8 ml with cold 100 mM Na_2CO_3, pH 11.0, loaded on top of a fresh cushion, and ultracentrifuged under the same conditions as those used previously. Alkaline-stripped membranes were washed in 10 mM Tris-HCl, pH 7.4, plus protease inhibitor cocktail and centrifuged in a type 50 Ti rotor at 100,000 × *g* for 20 min at 4°C. Membrane pellets were flash frozen and stored at −80°C for protein solubilization and quantification. Protein samples were solubilized in 7 M urea and quantified using a bicinchoninic acid protein assay (Pierce, Rockford, IL).

Western blot analysis. Membrane fractions or whole-cell extracts were loaded on acrylamide gels without being boiled (to avoid altering membrane protein mobility). After electrophoresis, proteins were electrotransferred to a nitrocellulose membrane (0.45 μm; Bio-Rad) and blocked overnight at 4°C with 5% milk in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 0.05% Tween 20. Membranes were exposed to anti-MRP2 monoclonal antibody (M_2III-5; 250 μg/ml) (Alexis Biochemicals, Lausen, Switzerland), anti-MRP4 monoclonal antibody (M_4I-10; 150 μg/ml) (Alexis Biochemicals), or anti-actin polyclonal antibodies (Sigma-Aldrich), followed by appropriate horseradish peroxidase (HRP)-coupled secondary antibodies (see figure captions for dilutions). Blots were then revealed by chemiluminescence assay (SuperSignal West Pico; Pierce).

Confocal microscopy. Mrp2 and Mrp4 in wild-type and ciprofloxacin-resistant macrophages were detected in cells seeded at a low density, fixed with 0.8% formaldehyde in acetone, and permeabilized with saponin, as previously described (25), using a polyclonal anti-mouse Mrp2 antibody (36) (diluted 1:60) and anti-Mrp4 antibody (M_4I-10; diluted 1:20), with appropriate fluorescein isothiocyanate- or Texas Red-labeled secondary antibodies (from Sigma-Aldrich [diluted 1:300]; Santa Cruz Biotechnology, Santa Cruz, CA [diluted 1:80]; and Unité d'Immunologie Expérimentale, Université Catholique de Louvain, Brussels, Belgium [diluted 1:80]). Actin was stained with tetramethylrhodamine-labeled phalloidin (5 U/ml; Invitrogen). Observations were made with MRC1024 confocal scanning equipment (Bio-Rad) mounted on a Zeiss Axiovert confocal microscope (Zeiss, Oberkochen, Germany), using a λ_{exc} of 495 nm and λ_{em} of 519 nm for fluorescein (green signal) and a λ_{exc} of 578 nm and λ_{em} of 603 nm for Texas Red and tetramethylrhodamine (red signal).

Silencing of *Mrp2* and *Mrp4* genes. Ciprofloxacin-resistant J774 macrophages were subjected to transient silencing experiments with two specific small interfering RNAs (siRNAs), targeting either *Mrp2* or *Mrp4* (Silencer predesigned siRNA [Ambion]; *Mrp2* siRNA 71770, with antisense sequence UGAUGUUA CAAGUAAUCCCtt [targeting exon 28], and siRNA 161716, with antisense sequence UCUCCAAUCGUGUACUGCCtc [targeting exon 10]; and *Mrp4* siRNA 284555, with antisense sequence CAGAAUCUUGGAAAUCUCCtt [targeting exon 8] and siRNA 284556, with antisense sequence ACUGUUAAG GCACAAAACCtg [targeting exon 31]). siRNA negative control 1 (Ambion), whose sequence does not target any endogenous transcript, was used as a negative control. siRNAs diluted in OptiMEM I reduced serum medium (Invitrogen) were mixed with INTERFERin (Polyplus Transfection, Illkirch, France) and incubated for 10 min at room temperature to allow siRNA-liposome complexes to form. Next, 0.2 ml of the siRNA-liposome mixture was added to six-well culture dishes seeded the day before at a density of 3 × 10^5 cells/well and containing fresh RPMI medium (2 ml). Plates were rocked gently and incubated for 24 h at 37°C in a 5% CO_2–95% air atmosphere. After 24 h, the medium was removed and replaced by fresh RPMI medium, and the incubation was continued

for 24 h, at which time *Mrp2* and *Mrp4* levels were measured by real-time PCR, the corresponding protein contents were assessed by Western blot analysis, and cells were used to measure ciprofloxacin accumulation (as described above).

Curve fitting and statistical analysis. Curve-fitting analyses and calculations of regression parameters were made with GraphPad Prism, version 4.03, for Windows, and statistical analysis was done with GraphPad Instat, version 3.06, for Windows (GraphPad Prism Software, San Diego, CA).

RESULTS

Effects of efflux pump inhibitors (gemfibrozil and elacridar) on ciprofloxacin accumulation and efflux. In a first series of experiments, we characterized the phenotype of the ciprofloxacin-resistant J774 macrophages versus that of wild-type J774 macrophages with respect to ciprofloxacin accumulation and efflux in the presence and absence of gemfibrozil, a well-known inhibitor of MRP efflux transporters shown previously to modulate the accumulation and intracellular activity of ciprofloxacin in wild-type J774 macrophages (25, 32). Figure 1A shows that gemfibrozil increased the accumulation of ciprofloxacin (2 h of incubation; 50 μM [17 mg/liter]) in a concentration-dependent fashion for both cell types, eventually reaching similar values at a large concentration (1 mM), but with an about sixfold lower potency (reflected by a commensurate difference in concentration causing an increase of 50% of the maximal value [EC_{50}]) toward ciprofloxacin-resistant versus wild-type cells. Figure 1B shows that the efflux of ciprofloxacin from cells preloaded with the antibiotic (after transfer to a ciprofloxacin-free medium) was much faster in ciprofloxacin-resistant than in wild-type cells in the absence of gemfibrozil (half-lives of 0.09 and 1.75 min, respectively) (left panel). Both cell types, however, released ciprofloxacin at similar, lower rates (half-lives of 4.39 and 4.35 min, respectively) (Fig. 1B, right panel) in the presence of gemfibrozil (used at a concentration of 1 mM to obtain a maximal effect on both cell types [Fig. 1A]).

We also examined the influence of elacridar, a well-known inhibitor of two ABC transporters involved in drug efflux, namely, P-glycoprotein (also referred to as MDR1 or ABCB1) at low concentrations and BCRP (breast cancer-related protein; also referred to as ABCG2) at high concentrations (9). At 1 μM, no significant effect on ciprofloxacin accumulation was noted, whereas at 10 μM (a concentration known to inhibit mouse Bcrp1 [17]), increases of accumulation of 47% ± 14% in wild-type cells and of 48% ± 18% in ciprofloxacin-resistant cells were observed, indicating that the intervention of Bcrp1 could be minor and did not contribute to the resistance phenotype.

Measurement of Mrp mRNA expression. In the next step, we examined in wild-type, ciprofloxacin-resistant, and revertant cells the expression of the mRNAs corresponding to the seven Mrps described for the mouse and having demonstrated transport activities, namely, Mrp1 to -6 (encoded by *Abcc1* to *Abcc6*) and Mrp7 (encoded by *Abcc10*). Figure 2 (upper panel) shows that there was marked (20- to 30-fold) overexpression of both *Mrp2* and *Mrp4* in ciprofloxacin-resistant cells compared to wild-type cells, with a return to much lower levels for *Mrp2* and to control values for *Mrp4* in revertant cells. Expression of the other *Mrp* transcripts was not significantly or only very modestly modified in ciprofloxacin-resistant cells as well as in the revertants. If data are expressed as the abundance of each transcript relative to the total amount of *Mrp* transcripts (Fig. 2, lower panel), it clearly appears that *Mrp4* (i) accounted for

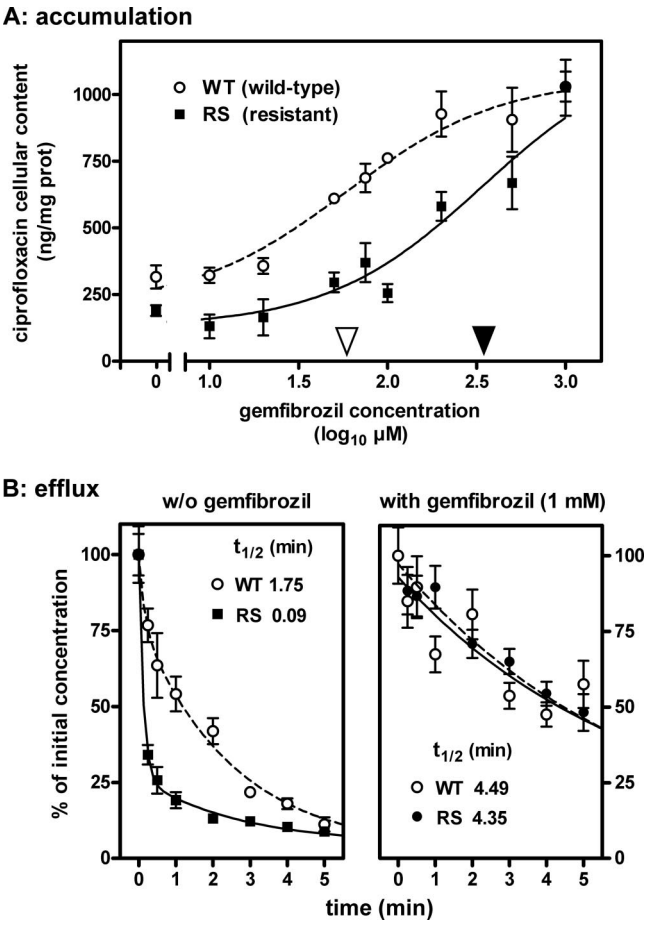

FIG. 1. Influence of gemfibrozil on the accumulation and efflux of ciprofloxacin in wild-type and ciprofloxacin-resistant J774 macrophages. Data are the means of three independent measurements ± standard deviations (SD) (when not visible, the SD bar is smaller than the symbol). (A) Cells were incubated with 50 μM ciprofloxacin for 2 h at 37°C in the presence of gemfibrozil at concentrations ranging from 0 to 1 mM. Regression parameters (nonlinear, sigmoidal dose-response curve [Hill's coefficient = 1]) for wild-type and ciprofloxacin-resistant cells were as follows: R^2 = 0.975 and 0.956, respectively; and EC_{50} (μM [95% confidence interval]; shown by the open and closed triangles on the abscissa) = 58.1 (30.1 to 112.4) and 338.7 (142.6 to 804.4), respectively. (B) Cells were incubated for 2 h at 37°C with 50 μM ciprofloxacin alone for wild-type cells and with 50 μM ciprofloxacin plus 200 μM gemfibrozil for ciprofloxacin-resistant cells (to reach cell contents allowing for sufficiently accurate measurements during efflux from both cell types; actual initial values, 338 ± 23 and 474 ± 44 ng/mg protein for wild-type and ciprofloxacin-resistant cells, respectively), transferred to ciprofloxacin-free medium in the absence (left) or presence of 1 mM gemfibrozil (right), and reincubated for up to 30 min at 37°C (for the sake of clarity, the graphs show only the data recorded during the first 5 min, but all data points were used for analysis). Results are expressed as percentages of the ciprofloxacin cell content observed before transfer to ciprofloxacin-free medium. Data were best fitted to a two-phase exponential decay function, with the first phase covering more than 70% of the analyzed time span. Regression parameters for wild-type and ciprofloxacin-resistant cells in the absence of gemfibrozil and for wild-type and ciprofloxacin-resistant cells in the presence of gemfibrozil were as follows: R^2 = 0.975, 0.998, 0.990, and 0.945, respectively; and initial half-lives (minutes [95% confidence interval]) = 1.75 (1.31 to 2.64), 0.09 (0.07 to 0.11), 4.49 (3.71 to 5.68), and 4.35 (2.90 to 8.68), respectively (the second phase is not visible on the graphs, except for ciprofloxacin-resistant cells in the absence of gemfibrozil, for which the first phase is sufficiently rapid).

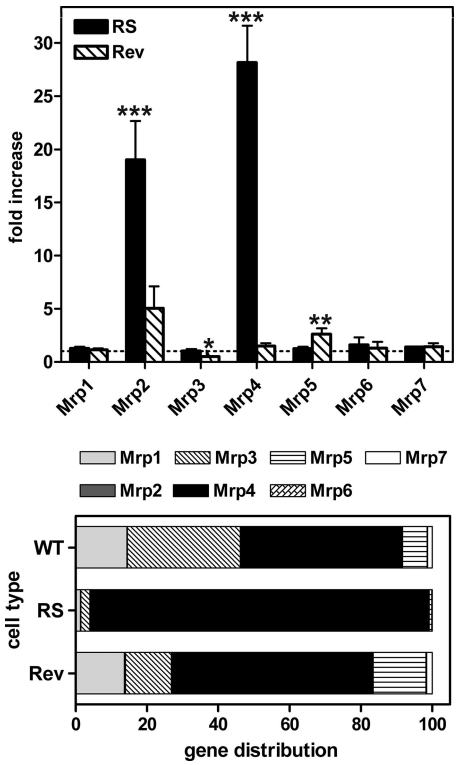

FIG. 2. Quantification of mRNA transcripts of Mrps 1 to 7 in ciprofloxacin-resistant (RS) and revertant (Rev) J774 macrophages in comparison with wild-type cells (WT). (Top) Increase in expression over that in WT cells (set arbitrarily at 1 [dotted line]). Values of all samples were normalized using *Ywhaz* and *Rpl13A* as housekeeping genes. Ratios shown are means ± SD ($n = 3$). Statistical analysis was done by one-way analysis of variance with the Dunnett multiple-comparison test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (compared to values in wild-type cells). (Bottom) mRNA transcripts (copy number determined by real-time PCR) expressed as percentages of the total number of *Mrp* transcripts detected, starting from 1 μg purified total RNA for each cell type. Data were obtained from the mean value for the number of copies calculated for each *Mrp* in each cell type. Note that the boxes corresponding to *Mrp2* and *Mrp6* are not visible because the values are smaller than the corresponding surrounding lines.

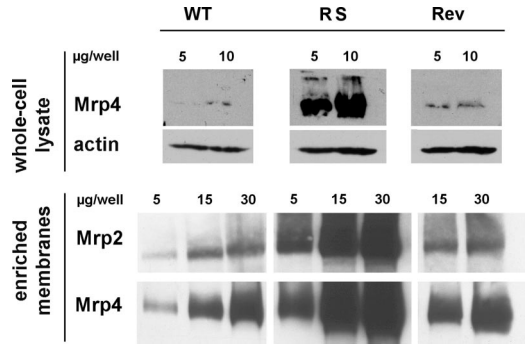

FIG. 3. Western blots of proteins prepared from wild-type (WT), ciprofloxacin-resistant (RS), and revertant (Rev) J774 macrophages. Gels were loaded with the amounts of protein indicated. (Top) Whole-cell lysates, with revelation with anti-Mrp4 (upper row; 1:1,000) or anti-actin (lower row; 1:600) antibodies, followed by anti-immunoglobulin G (anti-IgG) HRP-labeled antibodies (1:1,500). (Bottom) Membrane proteins, with revelation with anti-Mrp4 antibody (upper row; 1:2,000) or anti-Mrp2 antibody (lower row; 1:200), followed by anti-IgG HRP-labeled antibody (1:1,500). Note that enriched membrane samples did not contain actin.

about 45% of this total in wild-type cells, (ii) increased to 95% of the total in ciprofloxacin-resistant cells, and (iii) decreased to 56% of the total in revertant cells. Interestingly, the *Mrp2* expression level was only 0.08% of total *Mrp* transcripts in wild-type cells, so its 20-fold increase in ciprofloxacin-resistant cells still corresponded to a very minor amount (0.34%) of the mRNA coding for *Mrps*.

Detection of Mrp4 and Mrp2 by Western blot analysis. Analysis of Mrp4 in whole-cell lysate (Fig. 3, upper panel) showed a markedly increased amount of the protein in ciprofloxacin-resistant cells compared to that in wild-type cells, and for revertant cells, there was a considerable decrease of the signal, which still remained somewhat more marked than that in wild-type cells. The analysis was then repeated using enriched membrane protein samples for both Mrp2 and Mrp4 (Fig. 3, lower panel), showing an increase in the signals of both proteins in resistant cells, together with a marked reduction in revertant cells. The subcellular localization of Mrp2 and Mrp4 was then examined by confocal laser scanning microscopy (using anti-Mrp2 and anti-Mrp4 antibodies and appropriate fluorescein-labeled secondary antibodies) and compared with that of actin (detected with rhodamine-labeled phalloidin, allowing us to visualize the cell cytoskeleton and to determine the cell shape). Figure 4 shows the following for both Mrp2 and Mrp4: (i) only a faint staining in wild-type cells and (ii) a much more clearly visible signal in ciprofloxacin-resistant cells, with a localization at the cell periphery. The two proteins, however, colocalized only partially.

Gene silencing experiments. To directly assess the respective involvement of Mrp2 and Mrp4 in ciprofloxacin transport in ciprofloxacin-resistant cells, we performed transient gene si-

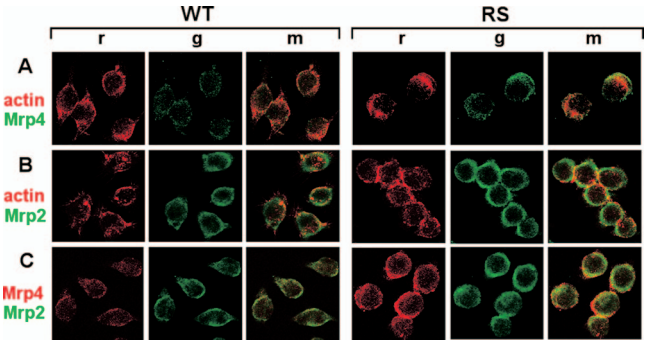

FIG. 4. Confocal microscopy of permeabilized J774 macrophages. WT, wild type; RS, ciprofloxacin resistant. (A) Cells stained with rhodamine-labeled phalloidin (to label actin [red channel]) and monoclonal rat anti-Mrp4 antibody (followed by fluorescein isothiocyanate-labeled anti-rat IgG antibodies [green channel]). (B) Cells stained with rhodamine-labeled phalloidin (to label actin [red channel]) and polyclonal rabbit anti-Mrp2 antibodies (followed by fluorescein isothiocyanate-labeled anti-rabbit IgG antibodies [green channel]). (C) Cells stained with monoclonal rat anti-Mrp4 antibody (followed by Texas Red-labeled polyclonal anti-rat IgG antibodies [red channel]) and polyclonal rabbit anti-Mrp2 antibodies (followed by fluorescein isothiocyanate-labeled anti-rabbit IgG antibodies [green channel]). Note that there is no staining for actin in this panel. r, red channel only; g, green channel only; m, merged images.

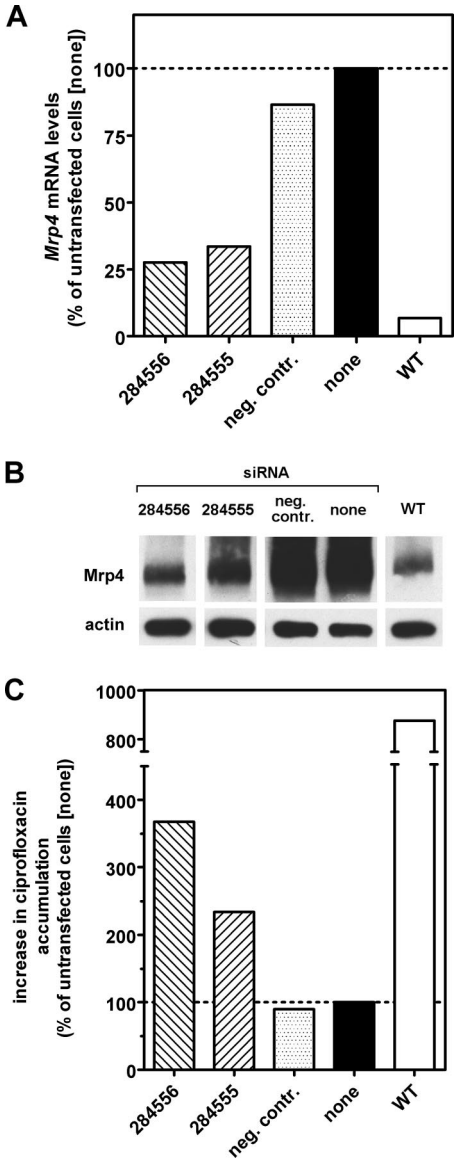

FIG. 5. Effect of siRNAs on expression of *Mrp4* and accumulation of ciprofloxacin in ciprofloxacin-resistant J774 macrophages. Cells were either (i) transfected with a specific anti-*Mrp4* siRNA (targeting exon 31 [284556] or exon 8 [284555]) or with a nontargeting siRNA (neg. contr.), each at 25 nM, or (ii) left untreated (none). (A) *Mrp4* mRNA level determined by real-time PCR and expressed as a percentage of the level observed in untransfected cells (for comparison, the graph also shows the value observed in wild-type J774 macrophages [WT]). Data are from a typical experiment (with determinations made in duplicate). (B) Western blot analysis of whole-cell extracts of the corresponding cells. The gel was loaded with the same amount of protein for each sample (5 μg/well). (Upper row) Gel was revealed with anti-Mrp4 antibody (1:1,000) followed by the appropriate anti-IgG HRP-labeled antibody (1:666). (Lower row) Gel was revealed with anti-actin antibody (1:1,000) followed by the appropriate anti-IgG HRP-labeled antibody (1:1,500). (C) Accumulation of ciprofloxacin, expressed as the percent increase over that in untransfected cells. Cells were incubated with 50 μM ciprofloxacin for 2 h at 37°C. Data are the means of two independent experiments with measurements made in triplicate.

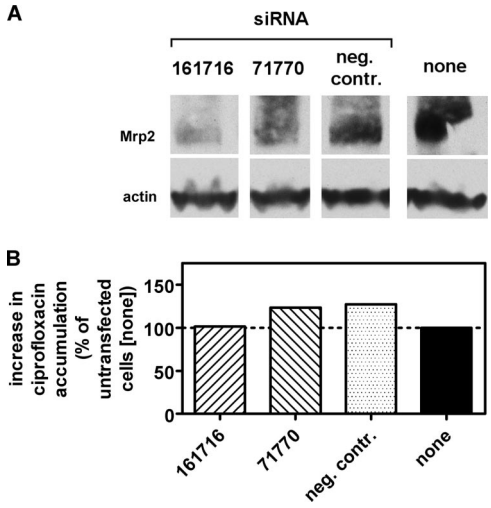

FIG. 6. Effect of siRNAs on cell content of Mrp2 and accumulation of ciprofloxacin in ciprofloxacin-resistant J774 macrophages. Cells were either (i) transfected with a specific anti-*Mrp2* siRNA (targeting exon 10 [161716] or exon 28 [71770]) or with a nontargeting siRNA (neg. contr.), each at 25 nM, or (ii) left untreated (none). (A) Western blot analysis of whole-cell extracts of the corresponding cells. The gel was loaded with the same amount of protein for each sample (45 μg/well). (Upper row) Gel was revealed with anti-Mrp2 antibody (1:200) followed by the appropriate anti-IgG HRP-labeled antibody (1:1,500). (Lower row) Gel was revealed with anti-actin antibody (1:1,000) followed by the appropriate anti-IgG HRP-labeled antibody (1:1,500). (B) Accumulation of ciprofloxacin, expressed as the percent increase over that in untransfected cells. Cells were incubated with 50 μM ciprofloxacin for 2 h at 37°C.

lencing experiments using siRNAs. Two specific siRNAs targeting different exons were selected for each gene, and their influence was examined in comparison with (i) ciprofloxacin-resistant cells transfected with nontargeting siRNA (4), (ii) untreated ciprofloxacin-resistant cells, and (iii) wild-type cells. Figure 5 shows that the two siRNAs targeting *Mrp4* caused (i) a marked reduction of the *Mrp4* mRNA (which, however, still remained more abundant than that in wild-type cells); (ii) a concomitant reduction of the Mrp4 cell content, as detected by Western blot analysis of whole-cell extracts; and (iii) an increase in ciprofloxacin accumulation, which was about fourfold larger for siRNA 284556 than that for untreated ciprofloxacin-resistant cells and reached values of about half those observed for wild-type cells. Transfecting wild-type cells with the same siRNAs caused a decrease of the cell *Mrp4* mRNA content of 40 to 60%, accompanied by an increase of the cell accumulation of ciprofloxacin of about 1.5-fold (data not shown). These experiments were then repeated with siRNAs targeting *Mrp2*. As shown in Fig. 6, there was a decrease in the cell content of Mrp2 (as detected by Western blot analysis) for cells transfected with both siRNAs targeting *Mrp2* but no significant change in the accumulation of ciprofloxacin by these cells.

DISCUSSION

The present study significantly extends our knowledge of the transport of ciprofloxacin in macrophages by identifying Mrp4 as its main, if not exclusive, efflux transporter in J774 cells. This identification stems from three convergent, mutually support-

ive pieces of evidence obtained from studies performed with ciprofloxacin-resistant cells in comparison with their wild-type and revertant counterparts.

First, we showed that the ciprofloxacin resistance phenotype is associated with a decreased accumulation of ciprofloxacin together with a faster drug efflux that can be impaired by the addition of large concentrations of gemfibrozil, a nonspecific inhibitor of anion transporters (32). Gemfibrozil was shown previously to increase ciprofloxacin accumulation and activity in macrophages (24, 25, 32), and its maximal effect on ciprofloxacin accumulation is similar to that of MK571, a specific inhibitor of MRP, in both wild-type and ciprofloxacin-resistant macrophages (24, 25). The use of elacridar as an inhibitor also shows a minor basal role of Bcrp1, which is similar in wild-type and resistant cells: Bcrp1 cannot account for the resistance phenotype of our cells toward ciprofloxacin (BCRP has been described as a transporter of ciprofloxacin in polarized cells [22], but ongoing studies on ABC transporter expression determined by TaqMan low-density array showed no difference in the expression level of *Bcrp1* mRNA in ciprofloxacin-resistant J774 macrophages compared to wild-type cells [E. Jacquet, personal communication]).

Second, we demonstrated that while both Mrp2 and Mrp4 are overexpressed in ciprofloxacin-resistant cells, Mrp4 is overwhelmingly predominant not only in wild-type cells but, most conspicuously, in ciprofloxacin-resistant cells. Conversely, we were able to rule out an overexpression of all five other Mrps examined, including Mrp1 (overexpressed in probenecid-resistant macrophages [25]; while probenecid is an inhibitor of ciprofloxacin efflux in macrophages [7], we showed previously that probenecid-resistant cells do not exhibit an increased efflux of ciprofloxacin [25]). Mrp8 and Mrp9 were not examined because no homologue of human MRP8 (encoded by *ABCC11*) has been identified in the mouse (35) and Mrp9 (encoded by *Abcc12*) is restricted to the testis and unable to transport any known MRP substrate (28).

Third, we showed that transfection of ciprofloxacin-resistant cells with *Mrp4*-specific siRNAs results in an increase in the accumulation of ciprofloxacin (in parallel with a decrease in the amounts of *Mrp4* mRNA transcripts and of the corresponding protein), while *Mrp2*-specific siRNAs decrease the expression of Mrp2 but do not modify ciprofloxacin accumulation. This may reflect the fact that the expression of Mrp2 is too low, even in ciprofloxacin-resistant cells, to affect ciprofloxacin accumulation or that ciprofloxacin is not a substrate for Mrp2. siRNAs have already been used to reverse multidrug resistance in human cancer cell lines due to overexpression of P-glycoprotein (19), MRP1 (12), MRP2 (21), MRP4 (31), or BCRP (30). The incomplete decreased expression observed here (about 70%) is in line with what is commonly observed with this type of approach for other transporters (12, 21, 41) and could be accounted for by the very large amount of protein present (41) and/or its long half-life (as is the case for other transporters, such as P-glycoprotein [26], MRP1 [1], or MRP2 [16]), as well as the short half-life of the siRNAs.

While the available evidence favors Mrp4 as the only effective transporter of ciprofloxacin in the cells we studied, we have to explain why ciprofloxacin-resistant cells also overexpress Mrp2. This probably results from the method used to select ciprofloxacin-resistant cells (i.e., long-term culture at progressively increasing ciprofloxacin concentrations), which is prone to select for multiple mechanisms of resistance (11). Several examples indeed document the concomitant overexpression of different efflux transporters in drug-resistant cell lines (P-glycoprotein and MRP1 in doxorubicin- or vincristine-resistant cells [5, 8], MRP1 and MRP2 in cisplatin-resistant cells [27], and P-glycoprotein and MRP2 in colchicine-resistant cells [3]). Yet this overexpression of *Mrp2* is most likely unrelated to ciprofloxacin efflux, as it remains slightly elevated in revertant cells (versus wild-type cells) when ciprofloxacin accumulation and *Mrp4* expression have both returned to control values. Further studies will need to examine whether the overexpression of Mrp2 and Mrp4 seen here is coincidental or results from changes at the level of a common regulator, which molecular mechanisms are involved in this overexpression, and the conditions that may trigger this overexpression in pharmacologically pertinent situations. At this stage, we can probably rule out, for instance, that infecting macrophages with *L. monocytogenes* or *Staphylococcus aureus* could affect Mrp4 expression, as the cellular concentration of ciprofloxacin is similar in noninfected or infected cells (34).

The pharmacological and biological significance of our observations also needs to be underscored. MRP4 is indeed able to transport a large variety of molecules involved in cell signaling and of metabolic importance (see references 6 and 33 and references cited therein), such as prostaglandins E_1 and E_2, leukotrienes, and cyclic nucleotides. MRP4 also transports a large array of important drugs, including methotrexate, topotecan, mercaptopurine metabolites, nonsteroidal anti-inflammatory agents, cephalosporins, and the antiviral drug adefovir ([phosphonylmethoxyethyl]adenine). While the transport of these molecules by Mrp4 in macrophages remains to be demonstrated, we may nevertheless draw attention to what may become important but unanticipated drug interactions (as suggested by an ongoing work demonstrating a competition between ciprofloxacin and nonsteroidal anti-inflammatory agents for efflux in macrophages [13]). In this context, it is important to note that a *MRP4* transcript is detected in human macrophages (18) but also in blood cells, hepatocytes, enterocytes, kidney proximal cells, brain capillary endothelial cells, and cells from the choroid plexus, suggesting that it plays a major role in regulating drug absorption, distribution, and elimination (33). Further studies are warranted to examine the role of MRP4 in ciprofloxacin handling by these cells and to determine whether it is as effective in other mammalian species, including humans. Because ciprofloxacin-resistant cells also overexpress Mrp2, we also suspect additional interferences, since this transporter confers resistance to several anticancer drugs (cisplatin, anthracyclines, vinca alkaloids, and methotrexate) and is detected in peripheral blood mononuclear cells (15). The long-term consequences of ciprofloxacin use on drug handling and other functions of phagocytic cells may therefore need to be addressed carefully in relation to efflux. This may now be facilitated by the identification of its most likely and effective transporter in macrophages.

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