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RESEARCH PAPER

Pharmacological Characterization of 7-(4-(Piperazin-1-yl)) Ciprofloxacin Derivatives: Antibacterial Activity, Cellular Accumulation, Susceptibility to Efflux Transporters, and Intracellular Activity

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ABSTRACT

Purpose To evaluate pharmacological properties (antibacterial activity; accumulation in phagocytic cells; activity against intracellular bacteria; susceptibility to fluoroquinolone efflux transporters) of ciprofloxacin derivatives modified at C-7 of the piperazine ring.

Methods N-acetyl- (**1**), N-benzoyl- (**2**), N-ethyl- (**3**), and N-benzyl- (**4**) ciprofloxacin were synthesized. MICs against *Escherichia coli* and *Staphylococcus aureus* were determined following CLSI guidelines. Cellular accumulation, subcellular distribution, and intracellular activity (towards *S. aureus* and *Listeria monocytogenes*) were determined in J774 mouse macrophages. Efflux in bacteria (NorA [*S. aureus*], Lde [*L. monocytogenes*]) and in macrophages (Mrp4) was assessed using the corresponding inhibitors reserpine and gemfibrozil, respectively.

Results All derivatives were active, though less than ciprofloxacin. **2** and **3** accumulated 2–3 fold more than ciprofloxacin in mouse macrophages but remained substrates for efflux by Mrp4. **4** was insensitive to NorA and Lde, accumulated approx 50-fold more than ciprofloxacin in macrophages, was barely affected by Mrp4, localized in the soluble fraction of cells, and was equipotent to ciprofloxacin against intracellular bacteria.

Conclusions Benzyl substitution at C7 markedly affects the pharmacological profile of ciprofloxacin with respect to recognition by efflux transporters and cellular accumulation. N-benzyl-ciprofloxacin may serve as basis for designing molecules with higher intrinsic activity while remaining poorly susceptible to efflux.

KEY WORDS antibacterial activity · drug accumulation · fluoroquinolones · lipophilicity · Mrp4

ABBREVIATIONS

ABC	ATP-Binding Cassette
ATCC	American Type Culture Collection
BCRP	Breast Cancer resistance Protein
CLSI	Clinical and laboratory Standards Institute
DCM	Dichloromethane
DIEA	Diisopropyl ethyl amine
MIC	Minimal Inhibitory Concentration
MRP	Multidrug-related Resistance Protein (human)
Mrp	Multidrug-related resistance protein (murine)

INTRODUCTION

Ciprofloxacin (1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid) is a potent and widely used antibiotic from the fluoroquinolone family. It shows a broad spectrum of activity covering wild-type Gram-positive and Gram-negative organisms, with clinical indications being however restricted today to infections caused by Gram-negative pathogens due to emergence of resistance and availability of newer molecules displaying higher intrinsic activity on Gram-positive bacteria (1). Fluoroquinolone

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pharmacological targets are bacterial DNA gyrase and topoisomerase IV, two key enzymes for DNA replication (see for review (1)). They accumulate rapidly into eukaryotic cells and are active against intracellular bacteria (2,3). Their cellular accumulation, however, is partially defeated by active efflux mechanisms involving the multidrug transporters from the ATP-binding cassette (ABC) family (4,5). Ciprofloxacin is substrate of the mouse and human BCRP in polarized cells (6) and of the Mrp4 transporter in murine macrophages (7) and its human homologue MRP4 (8,9). We have demonstrated that the activity of ciprofloxacin against intracellular *Listeria monocytogenes* is markedly decreased in macrophages overexpressing the Mrp4 transporter (10). Moreover, Mrp4 can cooperate with the bacterial fluoroquinolone efflux pump of *Listeria monocytogenes* (Lde), making the intracellular forms of this bacteria almost totally resistant to ciprofloxacin (11). Modulating the recognition of ciprofloxacin by efflux transporters may thus help optimizing its pharmacological profile. Conversely, we have also shown that fluoroquinolones characterized by a high level of cellular accumulation in macrophages are not necessarily more potent against intracellular bacteria than those accumulating to lower level (12), suggesting that cellular bioavailability rather than mere accumulation is the key factor limiting the cellular activity for this class of drugs. To reconcile these two observations, we are lacking a comparative study examining the relationship between recognition by efflux transporters, cellular accumulation, and intracellular activity for homogeneous series of fluoroquinolones.

It has been suggested that the introduction of hydrophobic substituents in position C-7 of fluoroquinolones may reduce their susceptibility to efflux in Gram-positive organisms (13). Starting from ciprofloxacin, our aim was therefore to examine how the nature of the side chain on the C-7 piperazine ring could influence its pharmacological profile with focus on efflux by both prokaryotic and eukaryotic transporters, and its antimicrobial activity, including against intracellular pathogens. A variety of substitutions has already been described in that position which can accommodate a great diversity in structures and the corresponding derivatives often retain antibacterial activity (14) because the fluoroquinolone key pharmacophore is maintained. They also show less susceptibility to bacterial efflux transporters and are even inhibitors of these (15). We opted here for substituting the piperazine nitrogen at position C-7 by acetyl- (**1**), benzoyl- (**2**), ethyl- (**3**) (also known as enrofloxacin, used in veterinary medicine (16)), or benzyl- (**4**), in order to modulate both the lipophilicity and the basicity of the amine function (see Fig. 1). Of interest in the context of the present work, these compounds were shown to keep antibacterial activity (16–18). We demonstrate here that N-benzyl ciprofloxacin (**4**), which exhibits very high accumulation levels in macrophages, is not affected by Mrp4 and some bacterial efflux pumps, but is not more potent than ciprofloxacin in intracellular infection models.

MATERIALS AND METHODS

Chemistry

All reagents were of analytical grade and obtained from Aldrich-Fluka, Acros or ROCC. Ciprofloxacin HCl (potency: 85%) was received from Bayer A.G (Wuppertal, Germany) as microbiological standard. Solutions were made with solvents of HPLC grade. Water for the HPLC analyses was generated by "reverse-osmosis" using Milli-Q-Water (Millipore Co, Bedford, MA) and filtered through 0.22 μm polytetrafluoroethylene filter. NMR spectra were recorded in $CDCl_3$ or CD_3OD at room temperature using two Bruker (Billerica, MA) spectrometers, depending on the resolution needed (Avance II 300 for ^{1}H [300 MHz] and ^{19}F [282 MHz] spectra and AM-500 spectrometer for ^{1}H [500 MHz] and ^{13}C [125 MHz] spectra). Chemical shifts δ are reported in ppm are calibrated on the solvent signal at 7.26 ppm for $CHCl_3$. ^{13}C NMR spectra were obtained with broadband proton decoupling. Figure 1 shows the atom numbering used for the description of NMR data. Low resolution mass spectra were acquired by using a Thermo Finnigan LCQ spectrometer in positive-APCI mode (Atmospheric Pressure Chemical Ionisation). High-resolution mass spectrometry (HRMS) analyses in the ESI mode (Electro Spray Ionization) were performed at University College of London (UK). Melting points were recorded with a calibrated Büchi Melting Point B-540 apparatus. UV spectra were recorded on a UV–vis–NIR Varian-Cary spectrophotometer. Thin-Layer Chromatography (TLC) analyses were performed on aluminium plates coated with silica gel 60F254 from Merck and visualized with UV (254 nm). Column chromatographies were performed on silica gel Merck 60 (40–63 μm). Synthesis and characterization of ciprofloxacin derivatives are presented in details in the Supplementary Material (S1). The purity of the products (as checked by HPLC) was higher than 95%.

Bacterial Strains and Determination of Minimal Inhibitory Concentrations (MICs)

Escherichia coli ATCC25922 and *Staphylococcus aureus* ATCC25923 (American Type Culture Collection, Manassas, VA) were used as references. The other strains used, their origin and resistance phenotype are shown in Table III (further characterization of Gram-positive strains with respect to susceptibility to moxifloxacin, another fluoroquinolone with higher intrinsic activity, can be found in previous publications (11,19)). MICs were determined in Mueller Hinton broth (MHB) according to the recommendations of the Clinical and Laboratory Standard Institute [CLSI] (20). MICs for *S. aureus* ATCC25923 were also determined in the same medium adjusted to pH 5.5, to mimic the pH of

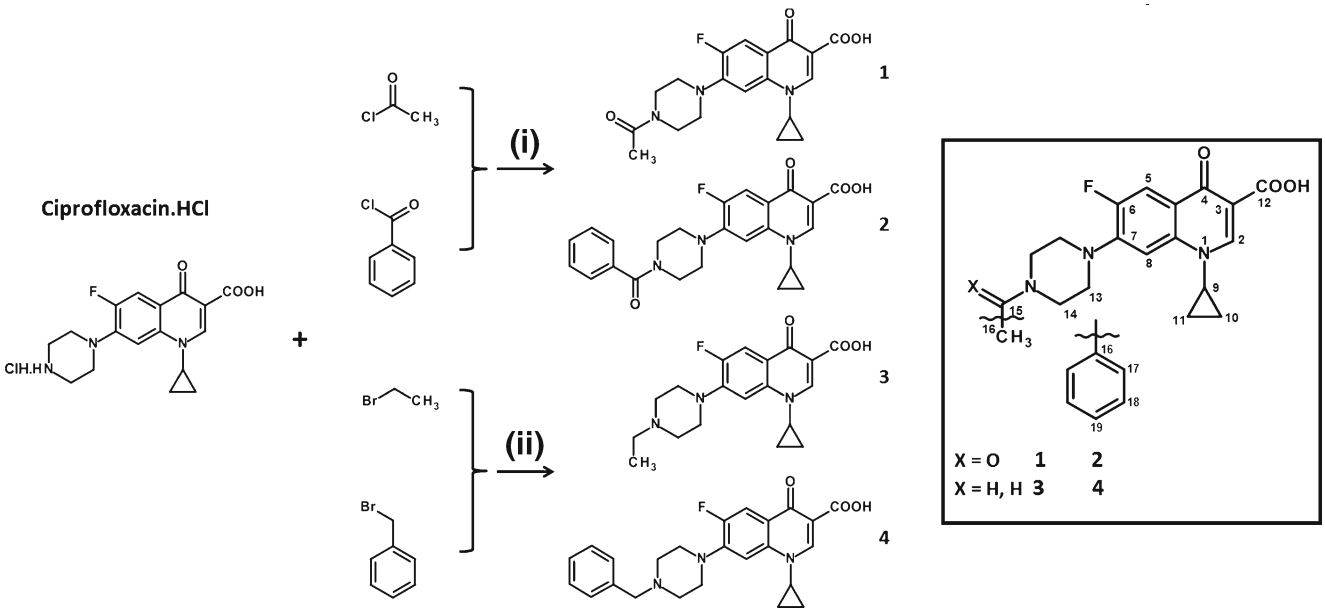

Fig. 1 Preparation of the 7-(4-(piperazin-1-yl)) derivatives of ciprofloxacin. Reagents and conditions: (i) NaOH 2 M, 1,4 Dioxane, 0°C to rt, 2 h; (ii) DIEA, KI (cat), H_2O:CH_3CN (1:1), rt, 12 h. The squared structure on the right illustrates the atom numbering used for NMR description.

the phagolysosomes where this bacterium survives intracellularly (21).

Fluoroquinolone Assay

Fluoroquinolones were assayed by fluorimetry in glycine HCl buffer (pH 3; 0.1 mM) for ciprofloxacin (as previously described (2)) and in Phosphate Buffer Saline (PBS) pH 7.4 for the other molecules, using a LS-30 fluorimeter (Perkin-Elmer, Beaconsfield, United Kingdom). Specific conditions of the assay were as follows: λ_{exc} and λ_{em}=275 and 450 nm for ciprofloxacin; 275 and 432 nm for **1** and **2**; 270 and 406 nm for **3** and **4**. The lower limit of detection and the zone of linearity were 5 to 120 ng/mL for ciprofloxacin, **1**, **2**, and **3**, and 100 to 1000 ng/mL for **4**. The slopes of the calibration curve (fluorescence arbitrary units/[ng/mL]) were 7.9 for ciprofloxacin; 5.6 for **1**, 7.5 for **2**; 3.3 for **3** and 0.6 for **4**.

Ciprofloxacin and **4** were also detected by HPLC-UV with a method adapted from (22). The HPLC system consisted of a Waters (Waters Corp., Milford, MA) Alliance 2695 separation module combined with a Waters 2998 photodiode array detector. Analytical separations were performed with a XTerra® RP18 column (4.6×150 mm, 5 µm) (Waters) equipped with a pre-column. The mobile phase consisted of acetonitrile (CH_3CN) and 0.025 M disodium hydrogen phosphate buffer (PB) (pH 3, adjusted with phosphoric acid). The analysis was carried out at room temperature (25°C) with a sample injection volume of 50 µL using a flow rate of 1 mL/min under isocratic conditions for 3 min (CH_3CN:PB, 20/80 v/v) followed by a linear gradient from 20/80 to 80/20 (CH_3CN:PB v/v) in 12 min. Detection was performed at 275 nm and online UV/Vis absorbance scans were performed.

Fluoroquinolone Accumulation in J774 Mouse Macrophages Cell Lines

J774 mouse macrophages (referred to as wild-type cells) were cultured and maintained as already described (2). J774 macrophages made resistant to high concentrations (0.2 mM) of ciprofloxacin that would be toxic for wild-type cells (10) were obtained by long-term exposure to progressively increasing concentrations of ciprofloxacin followed by maintenance in the continuous presence of 0.2 mM ciprofloxacin. These cells overexpress the Mrp4 efflux pump, as a major mechanism conferring ciprofloxacin resistance (7). They were used for experiments between the 60th and the 80th passage. For accumulation experiments, we followed the procedures described in our previous publications (2,7,10,12) with minor modifications. Cells were incubated for 2 h with drugs at an extracellular concentration of 20 µg/mL in complete RPMI-1640 medium and in a 5% CO_2-95% air atmosphere, except for experiments investigating the influence of pH on drug accumulation, for which cells were incubated in bicarbonate-free RPMI-1640 medium buffered with 5 mM phosphate to the desired pH and placed in a 100% air atmosphere ((2); the pH was checked and its value recorded before and after incubation). When determining fluoroquinolone concentration by fluorimetry, cells were washed three times with ice-cold PBS at the end of the incubation period, scraped with a Teflon policeman, and collected in glycine-HCl 0.1 M (pH 3) for cells incubated with ciprofloxacin, or in PBS (pH 7.4) for cells incubated with ciprofloxacin derivatives. Cells were then left overnight in the dark to release their content in antibiotic and homogenized by vortexing. An aliquot was taken to measure cell protein content by the Folin-Ciocalteu/biuret method

(23). The remaining sample was centrifuged (10 min at 14, 000 rpm) to discard cellular debris; supernatants were collected and assayed for drug content by fluorimetry as described above and the concentration of each sample was expressed by reference to the total protein content. This procedure gave results consistent with those obtained with the previously published method (2,10,24) in which samples were sonicated (2) but markedly improved to signal-noise ratio (by reducing the interference of cell protein in the fluorescence signal). When determining fluoroquinolone concentration by HPLC-UV, samples were obtained as described above but were sonicated. An aliquot was taken for protein assay. The remaining sample was centrifuged and an aliquot of the supernatant was used for HPLC-UV assay described above. The accumulation factor (apparent cellular-to-extracellular concentration ratio) was calculated using a conversion factor of 3.08 μL/mg cell protein, as determined experimentally for J774 macrophages (2).

Fluoroquinolone Cellular Toxicity

Cellular toxicity was assessed by determining the percentage of release of the cytosolic enzyme lactate deshydrogenase (LDH) in the culture medium of cells. To this effect, the enzymatic activity (25) was determined in parallel in cell lysates and in culture medium collected at the end of the accumulation experiments.

Subcellular Distribution

Subcellular distribution of **4** was assessed in J774 wild-type macrophages as previously described (3). In brief, cells were incubated with **4** (50 mg/L) for 2 h, then washed in 0.25 M sucrose—1 mM EGTA—3 mM imidazole (pH 7.4) and collected by gentle scraping in the same medium. The cells were then homogenized with a Dounce tissue grinder, and the homogenate was separated into a nuclear fraction (containing mostly nuclei and unbroken cells [N fraction]) and a cytosolic extract, by three successive low-speed centrifugations (770, 625, and 500×*g*, 10 min). The resulting cytoplasmic extract was further fractionated into a "granule" fraction (containing the bulk of the cells' organelles and membranes [MLP fraction]) and a final supernatant fraction (containing soluble proteins and free ribosomes [S fraction]) by high speed centrifugation (145,000×*g*) for 30 min (Ti50 rotor; Beckman instruments, Inc., Fullerton, CA). The activity of marker enzymes of the main organelles, namely, cytochrome *c* oxidase (for mitochondria), *N*-acetyl-β-glucosaminidase (for lysosomes), and lactate deshydrogenase (LDH; for cytosol) was assayed in all fractions (26). Phospholipids were extracted and assayed as described previously (27). The amount of **4** in all fractions was measured by fluorimetry following the procedure described above and the drug cell content was expressed with reference to the protein content in each fraction (26).

Cell Infection and Measurement of Intracellular Activity

The intracellular activity of ciprofloxacin and N-benzyl-ciprofloxacin (**4**) was determined toward *S. aureus* ATCC 25923 and *L. monocytogenes* EGD as described previously (3,10,11). In brief, infection was achieved by incubating J774 macrophages with bacteria (human serum-opsonized in the case of *S. aureus*) for 1 h at a bacterium-to-macrophage ratio of 4 and 5 for *S. aureus* and *L. monocytogenes*, respectively. Extracellular bacteria were eliminated by extensive washing in PBS (after a first exposure of cells for 1 h to 50 mg/L gentamicin in culture medium for *S. aureus*). The change in the number of viable intracellular bacteria was measured after 24 h incubation in the presence of antibiotics, using a wide range of extracellular concentrations (typically from 0.01 to 100-fold and 0.001 to 10-fold the MIC in broth for *S. aureus* and *L. monocytogenes*, respectively), followed by washing, collecting and lysing cells, and plating aliquots on tryptic soy agar (TSA) for colony counting (colony forming units [cfu]). Activity was defined as the change in the number of cfu recovered from cells after 24 h of incubation, compared with the post-phagocytosis inoculum. Cell viability was tested by trypan blue exclusion assay to determine the maximal usable drug concentration. Because drugs had to be dissolved in DMSO, we determined that cell viability became compromised at DMSO concentrations >1%. This limited the maximal usable concentration of **4** to 50 μg/mL.

Curve Fittings and Statistical Analysis

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

RESULTS

Chemistry

N-Acetyl-ciprofloxacin (**1**) and N-benzoyl-ciprofloxacin (**2**) were prepared following a protocol adapted from (28). Acylation of the piperazinyl moiety of ciprofloxacin with acetyl and benzoyl chlorides lead to **1** and **2** in 43% and 100% yields, respectively (Fig. 1). N-ethyl-ciprofloxacin, also known as enrofloxacin (**3**), and N-benzyl-ciprofloxacin (**4**) derivatives were prepared from ciprofloxacin by N-alkylation of the 4-piperazin-1-yl moiety with the corresponding alkyl bromides

in 70% and 44% yields respectively. Whereas we obtained high conversions in all cases (as checked by NMR), the difference of yields could be explained by the difficult separation and quantitative recovery of those compounds from ciprofloxacin due to the coexistence of several zwitterionic species (29) and to the π-π stacking interactions between them.

Lipophilicity (logP, logD) and acidity (pKa) of the ciprofloxacin derivatives were calculated using standard *in silico* methods (Table I). Average differences between predicted and experimental values (when available) were about 1.05 log unit, which is considered as a satisfactory agreement (30). Of note, the acylation at the N-4 position of the piperazinyl group of ciprofloxacin results in compounds without a protonable substituent, whereas the alkylation leads to compounds with weaker basic character (lower pKa_2 values) than ciprofloxacin.

Antibacterial Activity Against Wild-Type Strains

MICs were first determined against reference strains considered as representative of Gram-negative species (*E. coli*) and Gram-positive species (*S. aureus*), in comparison with ciprofloxacin (Table II). Against *E. coli*, all derivatives kept antibacterial activity but were less potent than ciprofloxacin, with MICs 2–8 log_2 dilutions higher than those of their parent compound. Against *S. aureus*, **1**, **4** were equipotent to ciprofloxacin (MICs 0–1 dilution higher) while **2**, **3** were slightly less potent (MICs 2–3 dilutions higher). MICs were also determined at pH 5.5 for *S. aureus*, to mimic the conditions prevailing in phagolysosomes where this bacterium sojourns inside the cells (21). For ciprofloxacin, **3** and **4**, MICs were 1–2 dilutions higher in acidic medium, whereas **1** and **2** were more active at acidic pH, their MICs being 3 log_2 dilutions lower than at neutral pH.

Cellular Accumulation of Ciprofloxacin Derivatives

The cellular accumulation of ciprofloxacin derivatives was measured in J774 mouse macrophages incubated with 20 μg/mL of each molecule during 2 h. The left panel of Fig. 2 compares first the apparent accumulation levels in wild-type cells. In control conditions, ciprofloxacin accumulated 4-fold in the cells, which is consistent with our previous data obtained with sonicated samples (2,7,10,24). The accumulation of **1** was lower than that of ciprofloxacin whereas that of the other derivatives was larger but to very different extents, with **4** reaching the exceptional value of 120-fold compared to the extracellular milieu. To determine whether the derivatives were substrates for the efflux transporter Mrp4 that expels ciprofloxacin out of these cells (7), we first used the broad spectrum efflux pump inhibitor gemfibrozil. As also shown in the left panel of Fig. 2, gemfibrozil caused a 4-fold increase in the accumulation of ciprofloxacin (as anticipated), but only a 3-, 1.3- and 2-fold increase for **1**, **2**, and **3**, respectively, and, most conspicuously, no marked effect for **4**. We then compared the accumulation of the derivatives by wild-type macrophages *vs*. macrophages overexpressing the Mrp4 transporter (Mrp4-J774 (7,10)). The right panel of Fig. 2 shows that the accumulation of all molecules was reduced to 30–50% in Mrp4-J774 cells compared to wild-type cells except for **4**, for which accumulation was only reduced of 18%.

Because of the unexpectedly high accumulation level of **4**, we checked whether it would not exert toxicity against J774 cells. To this effect, we determined the release of lactate deshydrogenase but saw only minimal release with no statistically significant difference between ciprofloxacin and **4** for either cell types ($1.3 \pm 0.1\%$ *vs*. $2.1 \pm 0.7\%$ ($n = 3$) in wild-type cells, and $8.0 \pm 4.0\%$ *vs*. $6.6 \pm 1.3\%$ ($n = 3$) in J774-Mrp4 cells for ciprofloxacin and **4**, respectively). We also checked whether the accumulation of **4**, using the fluorimetric method, corresponded to the genuine, unmetabolized molecule. For this purpose, we detected ciprofloxacin and **4** using HPLC-UV and applying a protocol that enabled us to see both compounds in a single run. Standards of ciprofloxacin and **4** prepared in PBS were detected with retention times of 4 and 9 min respectively. In lysates of cells incubated for 2 h with 20 μg/mL of either ciprofloxacin or **4**, a single peak with a retention time of 4 or 9 min, respectively, was detected (see Fig. S1 [chromatograms and UV spectra] in Supplementary Material; a minor signal appeared at 16 min, but already

Table I Calculated Physicochemical Properties of Ciprofloxacin Derivatives[a]

Compounds	R	M (g/mol)	pKa_1[b]	pKa_2[b]	Clog P[b]	Clog $D_{pH=5.5}$[b]	Clog $D_{pH=7.4}$[b]
CIP	H	331.34	5.76[1]	8.68[1]	1.5535	−1.54	−1.39[2]
1	$COCH_3$	373.38	6.06		1.1634	1.06	−0.19
2	COC_6H_5	434.45	6.06		3.0176	2.91	1.66
3	C_2H_5	359.39	5.69[3]	6.68[3]	2.2933[4]	0.96	0.58
4	$CH_2C_6H_5$	421.46	5.70	6.74	3.6610	2.26	1.94

[a] Experimental values from literature: [1] : 6.08/8.58 (29), 6.15/8.66 (39) and 6.18/8.76 (40); [2] : −1.13 ± 0.03 (29); [3] : 6.12/7.68 (29); [4] : 4.70 (41)

[b] *in silico* calculations using ChemAxon's Marvin (www.chemaxon.com/marvin). The calculated log P (Clog P) are the arithmetic average of three methods of calculations: Viswanadhan's fragmentation (42), Klopman's fragmentation (43) and PHYSPROP© database

Table II Minimum inhibitory concentration (MIC) against wild-type bacterial strains of the ciprofloxacin derivatives and the reference drug ciprofloxacin, as determined at pH 7.4 and 5.5 (for *S. aureus*)

Compound	MIC (μg/mL)		
	E. coli	*S. aureus*	
	pH 7.4	pH 7.4	pH 5.5
CIP	0.008	0.25	1
1	0.5	0.5	0.063
2	2[a]	1	0.125
3	0.031	2[a]	4[b]
4	0.25	0.25	0.5

[a] MIC ≥ to the clinically resistant breakpoint of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org) for ciprofloxacin (> 1 mg/L) but still below the resistant MIC interpretative criterion of the Clinical and Laboratory Sandard Institute (CLSI; http://www.clsi.org) (≥ 4 mg/L)

[b] MIC above both the EUCAST clinically resistance breakpoint and the CLSI resistant MIC interpretative criterion

present in the standard solution, and corresponding therefore most likely to an impurity present in ciprofloxacin powder).

Further Characterization of 4 Biological Properties

As N-benzyl-ciprofloxacin (**4**) showed acceptable antibacterial activity against both Gram-positive and Gram-negative reference strains, and a very high intracellular accumulation in macrophages with no apparent susceptibility to efflux by Mrp4 and no cellular toxicity, we further characterized this compound by evaluating its activity against resistant bacteria, the effect of pH on its accumulation, its subcellular localization, and its activity against intracellular bacteria.

MICs of 4 Against Resistant Bacteria

MICs of **4** were measured against a series of strains with resistance mechanisms for ciprofloxacin (Table III). These include strains overexpressing efflux systems conferring resistance to fluoroquinolones (*S. aureus* overexpressing NorA, *L. monocytogenes* overexpressing Lde, and *Enterobacter aerogenes* overexpressing AcrAB-TolC) and *S. aureus* strains (MRSA) with high level of resistance to fluoroquinolones (due to target mutations). Considering first the strains resistant by efflux, we observed that, in sharp contrast with ciprofloxacin, **4** activity was not affected by the overexpression of fluoroquinolone efflux systems in Gram-positive strains, with MICs as low as for the parent susceptible strains and no effect of reserpine on these MICs. However, **4** activity was impaired by the overexpression of the broad spectrum efflux system AcrAB-TolC in the Gram-negative *E. aerogenes*, to a similar extent than that of ciprofloxacin (MIC 5 $\log_2$ dilutions higher). Considering then *S. aureus* strains with target mutations (SA124, SA036, and N41120032), MICs of **4** were high, suggesting full cross-resistance with ciprofloxacin.

Influence of pH on the Accumulation of 4 in J774 Macrophages

Accumulation of **4** and ciprofloxacin was measured in wild-type macrophages incubated during 2 h with 20 μg/mL of drug in buffered media adjusted to specific pH values ranging from 5 to 8 (Fig. 3). Accumulation of ciprofloxacin was the highest at pH 6 but was decreased at lower or higher pH. Accumulation of **4** followed the same pattern, except that it was almost similar at pH 6 and 7. Of note, **4** accumulation levels were 10 to 17-fold higher than those of ciprofloxacin over the pH range investigated. In addition, we calculated the proportion of microspecies for each molecule (lower panels of Fig. 3). The change in **4**

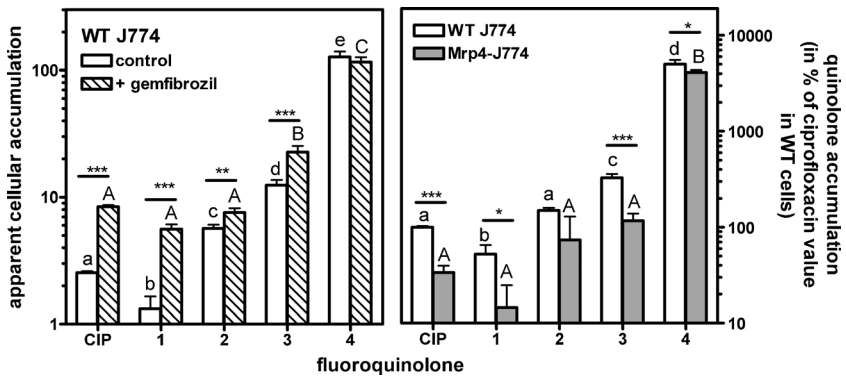

Fig. 2 Cellular accumulation of ciprofloxacin derivatives in macrophages. Wild-type J774 (WT cells) and Mrp4-overexpressing (Mrp4 cells) macrophages were incubated with the indicated molecules at 20 μg/mL for 2 h, in absence (control) or presence (+ gemfibrozil) of gemfibrozil 500 μM. Drug cellular contents, calculated in ng/mg protein, are expressed as apparent cellular accumulation (see "Methods" for accumulation factor calculation) (left panel), or as percentages of the cellular content of ciprofloxacin in wild-type cells (right panel). All values are the means of three independent determinations ± SD. Statistical analysis: ANOVA with Tukey post-hoc test for comparison of molecules in a same condition (*bars* with different letters are different from one another); Student *t*-test to compare control *vs*. gemfibrozil (left) or WT *vs* Mrp4-J774 cells (right): ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Table III Minimum Inhibitory Concentration (MIC) of Ciprofloxacin (CIP) and **4** Against Wild-type and Resistant Bacteria, in Absence (−R) or in Presence of Reserpine at 20 μg/mL (+R) (see Table II for EUCAST Clinically Resistant Breakpoint and CLSI Resistant MIC Interpretative Criteria)

Bacterial species	Strain	Origin	Resistance	MIC (μg/mL)			
				CIP		**4**	
				− R	+ R	− R	+ R
Resistance by efflux							
S. aureus	ATCC 25923	ATCC	Wild-type strain	0.25	0.125	0.25	0.25
	SA1	*In vitro* mutant of ATCC 25923 (44)	NorA overexpression	8	2	0.25	0.25
L. monocytogenes	EGD	Reference strain	Wild-type strain	4	2	4	4
	CLIP21369	Clinical isolate (34)	Lde overexpression	16	2	2	2
E. aerogenes	ATCC 13048	ATCC	Wild-type strain	0.031–0.063	nd	0.5	nd
	CM64	*In vitro* mutant of ATCC 13048 (45)	AcrAB-TolC overexpression	0.5	nd	8	nd
Resistance by target mutation							
S. aureus	SA124	Clinical isolate[a]	MRSA SCCmec II, mutations in *gyrA* (S84L), *gyrB* (R404L) and *grlA* (S80F)	16	8	16	16
	SA036	Clinical isolate[a]	MRSA SCCmec II mutations in *gyrA* (S84L), *gyrB* (E84K)	32	8	16	16
	N41120032	Clinical isolate[b]	Hospitally-acquired MRSA	64	64	16	32

[a] Penn State Milton S. Hershey Medical Center, Hershey, PA; [b] cliniques universitaires de l'UCL à Mont Godinne, Yvoir, Belgium; nd: not determined (reserpine is an efflux pump inhibitor in Gram-positive bacteria only)

accumulation was almost parallel to the proportion of zwitterionic form at each considered pH, but this was not the case for ciprofloxacin, the zwitterionic form being more abundant at pH 7.

Subcellular Localization of 4

J774 macrophages were incubated for 2 h with **4** at 50 μg/mL (to allow its reliable detection in the subcellular fractions, but

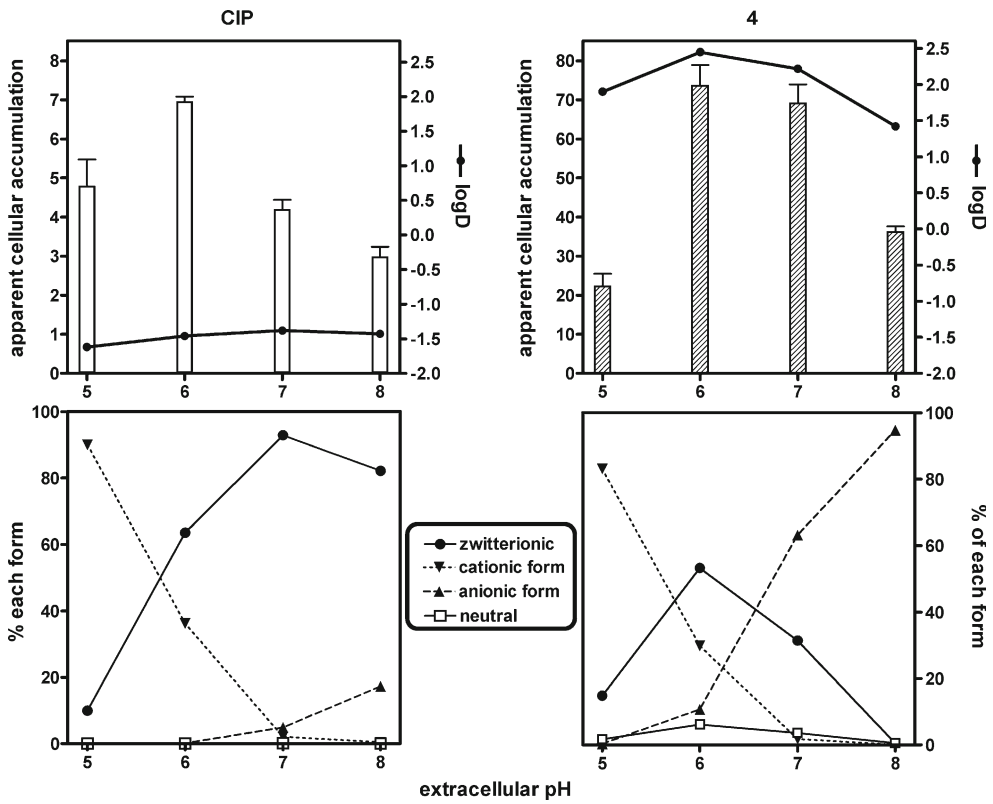

Fig. 3 Influence of pH on ciprofloxacin and **4** accumulation in wild-type J774 macrophages. Wild-type J774 macrophages were incubated with ciprofloxacin (left panel) or **4** (right panel) at 20 μg/mL for 2 h, in buffered media adjusted to specific pH values. All values are the means of three independent determinations ± SD. Upper panel: drug cellular contents, calculated in ng/mg protein, are expressed as apparent cellular accumulation (see "Methods" for accumulation factor calculation); logD values were calculated with ChemAxon's Marvin (www.chemaxon.com/marvin). Lower panel: percentages of the different forms of ciprofloxacin and **4** at the pH considered are calculated with ChemAxon's Marvin (similar figures were obtained if calculation were made using the formula proposed by Furet *et al*. (46)).

this did not affect the cellular accumulation of **4**, which was 95-fold in this experiment). The homogenate was then fractionated in an unbroken cell/nucleus fraction and a cytoplasmic extract that was further separated into a "granules fraction" (containing the cell organelles) and a final supernatant (Fig. 4). The quality of the separation was assessed by showing that the cytosol enzyme lactate deshydrogenase distributed in the cytosol, while the lysosomal enzyme *N*-acetyl-β-glucosaminidase and the mitochondrial enzyme cytochrome *c* oxidase were mainly recovered in the granular fraction. Phospholipids were also found in this fraction, showing that it contained the bulk of the membranes. 95% of **4** was found in the soluble fraction, as previously shown for ciprofloxacin (3), excluding a stable association with subcellular organelles or a tight binding to membranes to explain its high cellular accumulation.

Intracellular Activity of 4

As **4** accumulates to high levels in macrophages, we assessed its antibacterial activity towards intracellular forms of *S. aureus*, which gains access and multiplies in phagolysosomes, and *L. monocytogenes*, which reaches and thrives into cytosol after phagocytosis (3). Wild-type macrophages were infected by susceptible *S. aureus* or *L. monocytogenes* strains and antibacterial activities of ciprofloxacin and **4** evaluated after 24 h of incubation. Both molecules display modest and similar activities against intracellular *S. aureus* (Fig. 5, left panel), with only a bacteriostatic effect at an extracellular concentration corresponding to their respective MIC, and a modest reduction of the inoculum (between 1 and 1.5 log) at higher extracellular concentrations (up to 100-fold the MIC). In contrast, both ciprofloxacin and **4** were much more active against intracellular *L. monocytogenes* (Fig. 5, right panel), reaching respectively a 4 and a 2.5 $\log_{10}$ decrease in cfu at 10× their MIC (the mere examination of the concentration-effect curves suggest that a much lower E_{max} value could be reached at higher concentrations, but these could not be tested on a comparative fashion because of the limited solubility of **4** in media containing 1% DMSO (higher DMSO concentrations would have affected cell viability)).

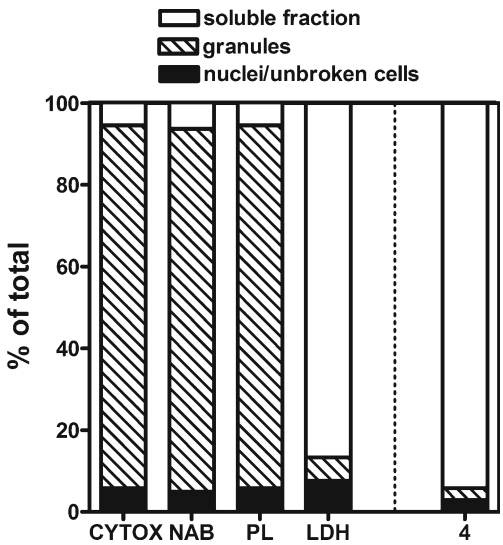

Fig. 4 Subcellular distribution of **4** in wild-type macrophages. Wild-type J774 macrophages were incubated for 2 h with **4** at 50 mg/L (needed to allow subsequent assays). The graph shows the drug content in the nuclear, granules, and soluble fractions expressed as a percentage of the total drug content recovered, and the distribution of enzymatic activities (cytochrome *c* oxidase [CYTOX], marker of mitochondria; *N*-acetyl-β-glucosaminidase [NAB], marker of lysosomes; and lactate dehydrogenase [LDH], marker of the soluble proteins) and of phospholipids (PL) among the three fractions.

DISCUSSION

This study shows that N-acylation or N-alkylation at the N-4 position of the piperazine ring of ciprofloxacin reduces but does not entirely suppress the antimicrobial activity while markedly modulating the susceptibility of the derivatives to efflux transporters of both Gram-positive bacteria and of murine macrophages. N-benzyl substituted ciprofloxacin showed the most important changes associated with a drastic increase in its level of cellular accumulation.

These modifications of key pharmacological properties changes could be related to changes in molecular weight and bulkiness of the side chain, as well as in lipophilicity and basicity of the ionisable function of piperazine compared to ciprofloxacin.

With respect to activity, we confirm that these derivatives retain antibacterial activity against both Gram-negative and Gram-positive bacteria (with MICs lower or close to the current clinical breakpoints of ciprofloxacin) except for **3**, which, like ciprofloxacin, is less active against *S. aureus* than *E. coli*. Of note, acidic pH favours the antibacterial activity of **1** and **2** against *S. aureus*. This could be related to their acidic character compared to ciprofloxacin, **3**, and **4** that are zwitterionic compounds. At acidic pH, **1** and **2** will be mostly present under their uncharged form, which can favour their diffusion through the bacterial membrane, as already observed for sulfanilyl fluoroquinolones (31), or for delafloxacin (a fluoroquinolone under development for which the lowering of the MICs towards *S. aureus* at acid pH has been correlated with its higher accumulation in the bacteria (19)). Conversely, when tested against strains highly resistant to ciprofloxacin, including strains harboring specific mutations in the genes coding for subunits of topoisomerases (see Table III), **4** was inactive, which indicates that the changes made in the structure did not protect against cross-resistance when mediated by target mutations. This confirms and expand on a previous observation that this compound was inactive against uncharacterized fluoroquinolone-resistant strains (18).

Concerning the prokaryotic efflux transporters, we see that whereas ciprofloxacin is clearly a substrate of the *S. aureus*

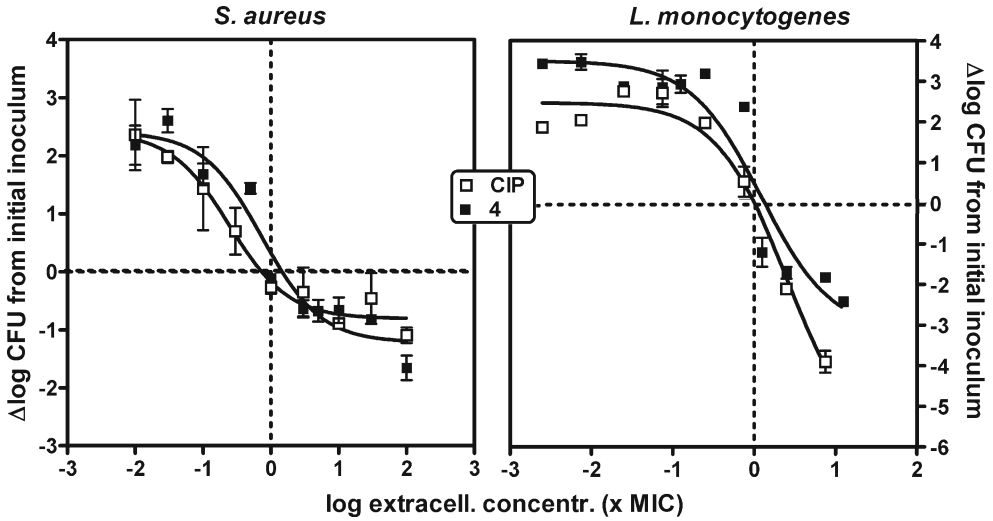

Fig. 5 Intracellular activity of ciprofloxacin (CIP) and **4** in J774 macrophages infected by *S. aureus* ATCC 25923 (left panel) and *L. monocytogenes* EGD (right panel). The ordinate shows the change in cfu (as log cfu/mg cell protein) from the initial inoculum at the end of the incubation time (24 h). The abscissa shows the extracellular concentration of quinolone used, as log of multiple of the corresponding MIC (vertical dotted line; *S. aureus*: 0.25 μg/mL; *L. monocytogenes*: 4 μg/mL, for both ciprofloxacin and **4**). The horizontal dotted line corresponds to a bacteriostatic effect (no apparent change from the original inoculum). Each data point is the mean ± SD of three independent determinations.

NorA, the *L. monocytogenes* Lde and the *E. aerogenes* AcrAB-TolC transporters (as shown by its higher MICs in strains overexpressing these transporters, and by the effect of reserpine for the two Gram-positive bacteria), **4** is only substrate of AcrAB-TolC. This observation is consistent with literature data suggesting that bulky substituents (rather than global lipophilicity) are important for avoiding recognition by the bacterial pump NorA of *S. aureus*. In a study comparing 40 fluoroquinolones in wild-type and NorA-overexpressing *S. aureus* strains, the molecules with the bulkier substituents at the C-7 and C-8 positions and more hydrophobic substituent at the C-8 position were the least affected by NorA activity, whereas there was no correlation with whole molecule hydrophobicity (as assessed by c*LogP*) (32). Another study also concluded that the quinolone structure *per se* was more important than hydrophobicity for recognition by NorA (33). Yet, the fact that **4** is also globally more lipophilic than ciprofloxacin does not allow differentiate between a role of specific substituents *vs*. global lipophilicity in recognition by these transporters. Interestingly, however, since ciprofloxacin is also a good substrate of Mrp4 in eukaryotic cells, whereas **4** is apparently not affected by either type of transporters, our data suggests common features for drug recognition between Mrp4, NorA and Lde, even though there is no homology between these transporters as they belong to different families (primary transporter of the ABC family for Mrp4, secondary transporters of the Major Facilitator Superfamily for NorA and Lde (34,35)). This, however, does not extend to AcrAB-TolC (the multidrug transporters from the Resistance-Nodulation-Division family found in Gram-negative bacteria, which expel many antibiotics from different classes (36)).

Moving now to the accumulation by murine macrophages, we observed major differences amongst derivatives and with ciprofloxacin that could be analyzed in terms of molecular weight, lipophilicity, diffusibility, or susceptibility to active efflux. Differences in molecular weight did not really correlate with variations in accumulation and can probably be dismissed. There is also no clear correlation between lipophilicity of the molecules, even in the absence of active efflux mediated by Mrp4 (such as in the presence of gemfibrozil), since ciprofloxacin and **2** accumulate at similar levels (around 8-fold) despite their great difference in lipophilicity, whereas **3**, which is less lipophilic than **2**, accumulates nearly three-times more (around 25-fold). The fact that **4**, as other fluoroquinolones (3,37), is mostly associated (> 90%) with the soluble fraction of macrophages (cytosol) and not with cell membranes (recovered mostly in the "granules fraction"), also rules out that its huge accumulation could be related to a stable association within lipid bilayers. With respect to diffusibility, it is generally assumed that only globally uncharged forms of fluoroquinolones diffuse through lipid bilayers (38). Being mostly zwitterionic at neutral pH, ciprofloxacin could, therefore, diffuse both inward and outward from macrophages (its accumulation being higher at pH 6 because acidity negatively affects the activity of its efflux transporter (2)). In contrast, **4**, which is present under both zwitterionic and anionic forms at pH 7, could perhaps diffuse into cells under its zwitterionic form but be thereafter trapped under its anionic form, displacing the equilibrium and allowing for further accumulation of this compound. Our data, however, provide direct evidence that differences in susceptibilities to efflux could also be a key determinant in the differences in accumulation observed. In J774 cells, the multidrug efflux transporter Mrp4 actively contributes to lower the

cellular concentration of its substrates, like ciprofloxacin (7,10). Based on the effect of gemfibrozil, a broad-spectrum inhibitor of Mrp transporters, and on the difference in accumulation levels between wild-type cells and cells overexpressing Mrp4, we can conclude that **1** and **3** are substrates for Mrp4, whereas **2** is less and **4** only minimally affected. Thus, smaller substituents at the N-4 piperazinyl position, such as acetyl or ethyl, seem to favour recognition and efflux by Mrp4, and bulkier substituents (benzoyl and benzyl) to impair recognition and/or transport. In our previous studies with wild-type cells and cells overexpressing Mrp4 (7,10,24), we hypothesised that the accumulation level of fluoroquinolones in J774 macrophages was directly related with their lack of susceptibility to efflux by the Mrp4 transporter, with a highly expelled molecule such as ciprofloxacin accumulating moderately into these cells whereas an apparently poorly affected molecule such as moxifloxacin accumulating to a 3- to 4-fold larger extent, and molecules with an intermediate behaviour regarding efflux, such as levofloxacin and garenoxacin, showing an intermediate level of accumulation (24). Here, it seems, at first glance, that there is no direct link between accumulation level and efflux by Mrp4. Thus, comparing **2** and **3**, we showed that **3**, which is clearly affected by the pump, accumulates in wild-type cells to a level that is not much lower than that of moxifloxacin (an apparent non-Mrp4 substrate), but almost 2-fold higher than that of **2**, which seems to be a poor substrate. However, the very large accumulation of **4** is consistent with this molecule being not affected by Mrp4. It must, however, be stressed that an apparent lack of transport (as detected by the methods used here) does not necessarily mean an absence of recognition by the transporter, but may also result from a saturation of the transporter as previously discussed for moxifloxacin (24). Altogether, the data, therefore, suggest that efflux may play a non-negligible role in the modulation of the accumulation of fluoroquinolones by macrophages, but that other molecular properties, not yet clearly recognized, may be as or even more important. A potential limitation of our study, however, resides in the fact that Mrp4 is the main transporter expressed in our cell model (7), preventing us at this stage from extrapolating these conclusions to other efflux systems.

Whatever the reasons for the huge accumulation of **4**, it is noteworthy to observe that it does not confer a higher potency as compared to ciprofloxacin to eradicate intracellular infections caused by *S. aureus* or *L. monocytogenes* in J744 macrophages. Likewise, **1** was found equipotent to ciprofloxacin against intracellular *Bartonella henselae* in a model of endothelial cells (17). Again, this is somehow similar to what we described recently for gemifloxacin (37), which, despite its high level of accumulation in J774 macrophages (around 40-fold), is equipotent to ciprofloxacin in intracellular infection models. It was interpreted as denoting a poor intracellular bioavailability for these molecules, but what limits it remains so far unknown.

CONCLUSIONS

The present work has demonstrated that addition of a bulky, lipophilic side chain on ciprofloxacin markedly modifies its pharmacological profile, by reducing its susceptibility to efflux (both in Gram-positive bacteria and in eukaryotic cells) and increasing its accumulation in eukaryotic cells, without affecting its subcellular distribution and its intracellular activity. This conspicuous change in cellular accumulation is probably due to a conjunction of factors including diffusibility through the membrane and retention inside the cells, as well as susceptibility to efflux, both being dictated by biophysical parameters (lipophilicity, bulkiness, net charge of the molecule), but none of these parameters alone can explain the differences seen. Moreover, increasing the level of cellular accumulation does not result in an increased intracellular activity, raising questions about the bioavailability of the accumulated drugs. Lower recognition by prokaryotic efflux pumps, however, provide a clear advantage with respect to resistance of bacteria in broth (as demonstrated here) as well as to those multiplying in the cytosol (see (11) for a demonstration of the impact of efflux on the extracellular and intracellular activity on ciprofloxacin towards *L. monocytogenes*). The side chains included in the present analysis may thus serve as a platform for further investigations aimed at finding derivatives with higher intrinsic potency but keeping the advantage of a low susceptibility to efflux.

ACKNOWLEDGMENTS AND DISCLOSURES

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Pharmacological characterization of 7-(4-(piperazin-1-yl)) ciprofloxacin derivatives: antibacterial activity, cellular accumulation, susceptibility to efflux transporters, and intracellular activity

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Supplemental material

S1. Synthesis and characterization of ciprofloxacin derivatives

a) 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-acetyl-piperazin-1-yl)-quinolone-3-carboxylic acid (**1**)

To a solution of 1-cyclopropyl-6-fluoro-1,4-dihydro-7-piperazin-1-yl-4-oxo quinoline-3-carboxylic acid hydrochloride (ciprofloxacin, 50 mg, 0.13 mmol) in 1,4-dioxane (2 mL) and NaOH 2M (0.5 mL) at 0°C, acetyl chloride (12 mg, 0.15 mmol) was added dropwise. The reaction mixture was stirred at room temperature for two hours and HCl 1M (1 mL) was added to reach pH 1. The precipitate was extracted with DCM and the organic phase dried on $MgSO_4$. After solvent evaporation the collected solid was recrystallized from THF at -20°C to give **1** as a bright yellow powder (21 mg, 0.05 mmol) with 43 % yield.

Mp : 270-272°C.

^{1}H NMR (300MHz, $CDCl_3$) δ (ppm): 1.23 (m, 2H, H-11), 1.42 (m, 2H, H-10), 2.19 (s, 3H, H-16), 3.31(m, 2H, H-14), 3.38 (m, 2H, H-14), 3.56 (m, 1H, H-9), 3.74 (m, 2H, H-13), 3.89 (m, 2H, H-13), 7.39 (d, 1H, $^{4}J_{H-F}$ = 7.0 Hz, H-8), 8.07 (d, 1H, $^{3}J_{H-F}$ = 12.9 Hz, H-5), 8.8 (s, 1H, H-2).

^{13}C NMR (125MHz, $CDCl_3$) δ (ppm):177.4 (d, $^{4}J_{C-F}$ =2.5 Hz, C-4), 169.5 (C-15), 167.2 (C-12), 154.0 (d, $^{1}J_{C-F}$ = 250.0 Hz, C-6), 147.9 (C-2), 145.8 (d, $^{2}J_{C-F}$ = 10.6 Hz, C-7), 139.4 (C-8a), 120.6 (d, $^{3}J_{C-F}$ = 7.7 Hz, C-4a), 112.9 (d, $^{2}J_{C-F}$ = 22.5 Hz, C-5), 108.6 (C-3), 105.5 (d, $^{3}J_{C-F}$ = 4.2 Hz, C-8), 50.6 (C-13), 49.8 (C-13), 46.5 (C-14), 41.4 (C-14), 35.7 (C-9), 21.7 (C-16), 8.7 (C-10,11).

^{19}F NMR (282.41MHz, $CDCl_3$) δ (ppm): -121.1.

MS (APCI) m/z 374.2 [M + H^+, 100%].

λ_{max} (10^{-4}M, $CHCl_3$): 283nm (ε_{max}= 10430), 322 (3230), 334 (3290).

b) 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-benzoyl-piperazin-1-yl)-quinolone-3-carboxylic acid (**2**)

To a solution of ciprofloxacin (50 mg, 0.14 mmol) in 1,4-dioxane (2 mL) and NaOH 2M (0.5 mL) at 0°C, benzoyl chloride (31 mg, 0.22 mmol) was added dropwise. The reaction mixture was stirred at room temperature for two hours and HCl 1M (1 mL) was added to reach pH 1. The precipitate was extracted with DCM and the organic phase dried on $MgSO_4$. After solvent evaporation the collected solid was recrystallized from MeOH:iPr_2O (1:2) at -20°C to give **2** (62 mg, 0.14 mmol) with a quantitative yield.

Mp : 317°C (decomposition).

^{1}H NMR (300MHz, $CDCl_3$: CD_3OD (10% v/v)) δ (ppm): 1.19 (m, 2H, H-11), 1.37 (m, 2H, H-10), 3.31(m, 4H, H-14), 3.52(m, 1H, H-9), 3.67(m, 2H, H-13), 3.95(m, 2H, H-13), 7.36(d, 1H, $^4J_{H-F}$ = 7.0 Hz, H-8), 7.40(m, 5H, H-17,18,19), 7.98(d, 1H, $^3J_{H-F}$ = 12.9 Hz, H-5), 8.73(s, 1H, H-2).

^{13}C NMR (125MHz, $CDCl_3$: MeOD (10% v/v)) δ (ppm): 177.1 (C-4), 171.1 (C-15), 167.6 (C-12), 153.7 (d, $^1J_{C-F}$ = 249.9 Hz, C-6), 147.8 (C-2), 145.4 (d, $^2J_{C-F}$ = 10.3 Hz, C-7), 139.1 (C-8a), 134.7(C-16), 130.3 (C-19), 128.7(C-18), 127.0(C-17), 120.3 (d, $^3J_{C-F}$ = 7.8 Hz, C-4a), 112.4 (d, $^2J_{C-F}$ = 23.3 Hz, C-5), 107.6 (C-3), 105.4 (d, $^3J_{C-F}$ = 2.6 Hz, C-8), 50.3 (C-13), 41.9 (C-14), 47.6 (C-13), 35.5 (C-9), 8.1 (C-10,11).

^{19}F NMR (282.41MHz, $CDCl_3$: CD_3OD (10% v/v)) δ (ppm): -121.1.

MS (APCI) m/z 436.2 [M + H^+, 100%].

λ_{max} (10^{-4}M, $CHCl_3$): 280nm (ε_{max}=82414), 318 (18322), 334 (17154).

c) 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-ethyl-piperazin-1-yl)-quinolone-3-carboxylic acid (enrofloxacin) (**3**)

To a solution of ciprofloxacin (100 mg, 0.27 mmol) in CH_3CN:H_2O (1:1, 10 mL) with diisopropylamine (DIEA, 157µL, 0.9 mmol) at 0°C, bromoethane (61µL, 0.82 mmol) and KI (0.1%mol) were added under argon atmosphere. The reaction mixture was stirred at room

temperature for 16 hours and the solvent was evaporated under reduced pressure. The reaction residue was dissolved in 40 mL of DCM and washed under vigorous stirring with 3 mL of aqueous solution adjusted to pH 6.5. The organic layer was dried on $MgSO_4$ and evaporated under reduced pressure to obtain **3** (68 mg, 0.19 mmol) as a white solid with 70% yield.

Mp : 222.4°C.

^{1}H NMR (300MHz, $CDCl_3$) δ (ppm): 1.17 (t, 3H, $^3J_{H-H}$ = 7.2 Hz, H-16), 1.21 (m, 2H, H-11), 1.40 (m, 2H, H-10), 2.55 (q, 2H, $^3J_{H-H}$ = 7.2 Hz, H-15), 2.72 (m, 4H, H-14), 3.40 (m, 4H, H-13), 3.52(m, 1H, H-9), 7.36(d, 1H, $^4J_{H-F}$ = 7.0 Hz, H-8), 7.40(d, 1H, $^4J_{H-F}$ = 7.1 Hz, H-8), 8.04(d, 1H, $^3J_{H-F}$ = 13.1 Hz, H-5), 8.78(s, 1H, H-2).

^{19}F NMR (282.41MHz, $CDCl_3$) δ (ppm): -120.7.

MS (APCI) m/z 360.2 [M + H^+, 56%].

λ_{max} (10^{-4}M, $CHCl_3$): 284nm (ε_{max}=37787), 326 (12765), 334 (12804).

d) <u>1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-benzyl-piperazin-1-yl)-quinolone-3-carboxylic acid (**4**).</u>

To a solution of ciprofloxacin (100 mg, 0.27 mmol) in CH_3CN:H_2O (1:1, 10 mL) with DIEA (157µL, 0.9 mmol) at 0°C, benzyl bromide (49µL, 0.41 mmol) and KI (cat) were added under argon atmosphere. The reaction mixture was stirred at room temperature for 16 hours. After evaporation of the solvent under reduced pressure and lyophilisation of the aqueous phase, the reaction residue was dissolved in 10 mL of DCM and washed under vigorous stirring with 2 mL of saturated $NaHCO_3$ solution for one hour. The organic layer was dried on $MgSO_4$ and evaporated under reduced pressure. The collected solid was dissolved in DCM, precipitated in Et_2O and chromatographied on flash silica with DCM:MeOH:iPrOH (5:0.8:0.1) as eluant to obtain **4** (50 mg, 0.12 mmol) as a yellow solid with 44% yield.

Mp : 242.4°C;

^{1}H NMR (300MHz, $CDCl_3$: CD_3OD (10% v/v)) δ (ppm): 1.19 (m, 2H, H-11), 1.37 (m, 2H, H-10), 2.7 (m, 4H, H-14), 3.37 (m, 4H, H-13), 3.52(m, 1H, H-9), 3.62(2H, H-15), 7.28-7.36 (m, 6H, H-8, 17, 18, 19), 7.97(d, 1H, $^{3}J_{H\text{-}F}$ = 13.1 Hz, H-5), 8.73(s, 1H, H-2).

^{13}C NMR (75MHz, $CDCl_3$) δ (ppm): 177.1 (C-4), 167.1 (C-12), 153.7 (d, $^{1}J_{C\text{-}F}$ = 250.0 Hz, C-6), 147.4 (C-2), 146.0 (d, $^{2}J_{C\text{-}F}$ = 10.3 Hz, C-7), 139.1 (C-8a), 137.7(C-16), 129.2 (C-17), 128.4 (C-18), 127.3 (C-19), 119.7 (C-4a), 112.3 (d, $^{2}J_{C\text{-}F}$ = 23.4 Hz, C-5), 108.1 (C-3), 104.7 (d, $^{3}J_{C\text{-}F}$ = 3.3 Hz, C-8), 62.9 (C-15), 52.7 (C-14), 49.9 (C-13), 49.8 (C-13), 35.3 (C-9), 8.2 (C-10,11).

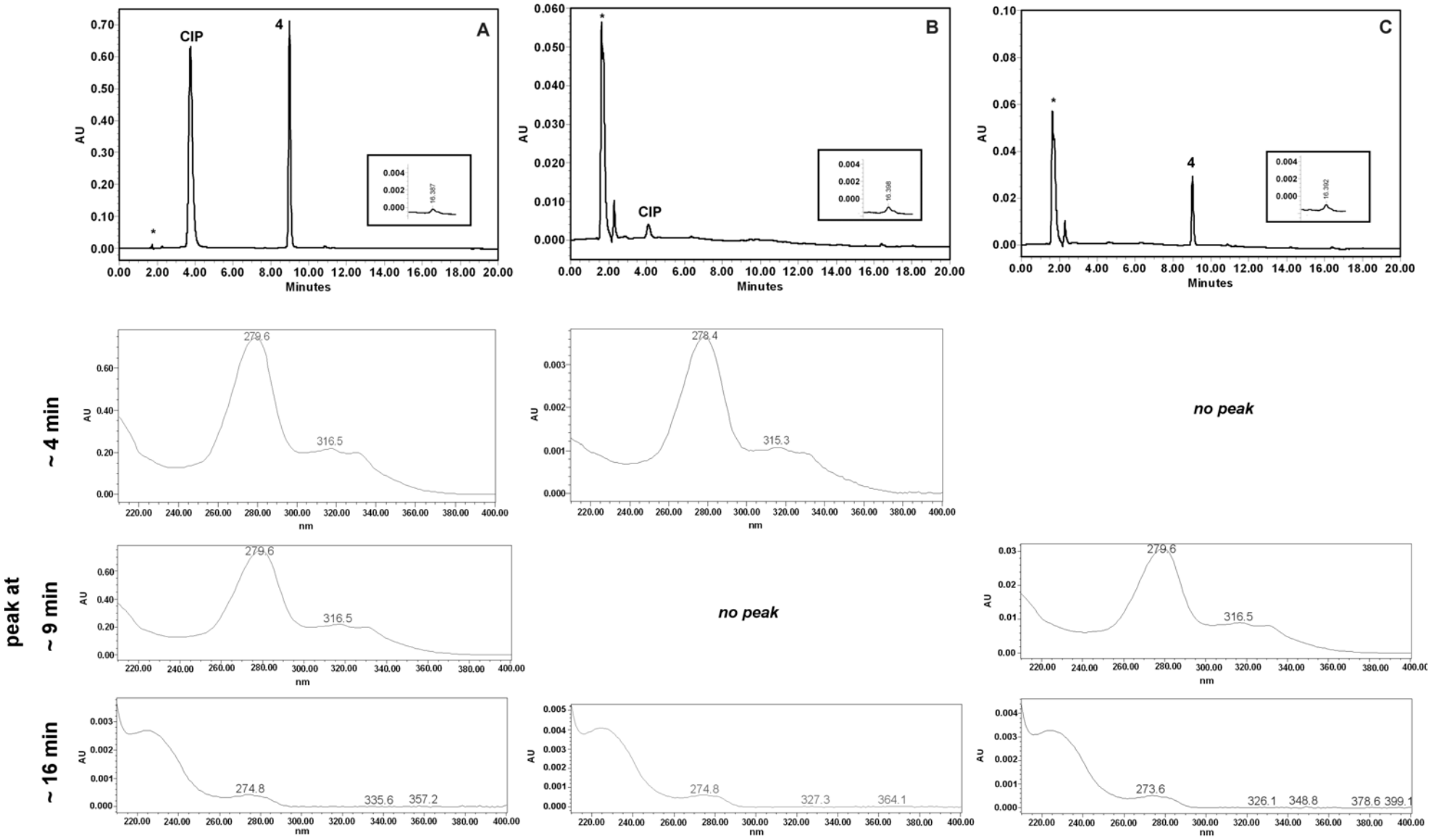
^{19}F NMR (282.41MHz, $CDCl_3$: CD_3OD (10% v/v)) δ (ppm): -120.6.

MS (APCI) m/z 442.5 [M + H+, 100%].

HRMS (ESI) calcd for $C_{24}H_{25}N_3O_3F$ [M + H+]: 422.18799, found 422.18844.

λ_{max} (10^{-4}M, $CHCl_3$): 279nm (ε_{max}= 11901), 285 (13106), 326 (4117), 334 (4123)

Figure S2. HPLC chromatograms and UV spectra of ciprofloxacin and **4**. Upper panel: (A) Chromatogram of standards in PBS: ciprofloxacin (CIP) + **4** (50/50, each molecule at 50 µg/mL); (B and C) chromatograms of cellular samples: cells incubated for 2h with ciprofloxacin (B) or **4** (C) (at 20 µg/mL) were collected in PBS, centrifuged and 50 µl of supernatant were injected. *: mobile phase peak. The inset shows at higher magnification the small peak appearing at 16 minutes. This peak is also present in standard (A), and may therefore not correspond to a metabolite produced in cells. Lower panel: UV spectra for the three peaks detected in HPLC, confirming the identity of compounds.


A
B
C
CIP
4
*
AU
Minutes
peak at
~ 4 min
~ 9 min
~ 16 min
279.6
316.5
278.4
315.3
no peak
274.8
335.6
357.2
327.3
364.1
273.6
326.1
348.8
378.6
399.1
nm