Université catholique de Louvain

Louvain Drug Research Institute

Research group: Pharmacologie cellulaire et moléculaire

CHARACTERIZATION OF THE ACTIVE EFFLUX OF FLUOROQUINOLONES IN EUKARYOTIC CELLS

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2012

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Thèse présentée en vue de l'obtention du grade de Docteur en Sciences Pharmaceutiques et biomédicales

« Je suis le maître de mon destin,
Je suis le capitaine de mon âme. »

William Ernest HENLEY,
Invictus

Remerciements

Me voilà à la fin d'une incroyable aventure, et il est temps de remercier toutes les personnes ayant contribué, de près ou de loin, à ce travail.

Je voudrais commencer par remercier ma promotrice, Françoise Van Bambeke, pour son accueil, son soutien quotidien, son dynamisme et ses idées. Tous ses conseils ont contribué à la bonne qualité de ce travail. Je tiens par la même occasion à remercier Béatrice Marquez qui m'a encadrée et conseillée durant plus de deux ans. Et je remercie le Professeur Paul Tulkens pour l'intérêt et le dévouement (« scout, toujours prêt ») portés à ce travail, ainsi que Marie-Paule Mingeot-Leclercq pour m'avoir accueilli dans son laboratoire.

Mes remerciements vont également au Dr. Wetzstein (Bayer Healthcare, Germany) qui m'a fourni une multitude de fluoroquinolones qui ont été le point de départ d'une grande aventure… Aventure qui a donné naissance à une collaboration avec Martine Prévost et Julien Dupont (ULB) que je remercie chaleureusement pour s'être penchés sur une idée un peu farfelue de modélisation.

Je remercie sincèrement les membres de mon jury, les professeurs Véronique Préat, Nathalie Delzenne, René Rezsohazy, Patrick Augustijns, Axel Dalhoff et Bernard Gallez.

Au terme de ces quatres ans, il va de soi que je remercie tous les membres de l'unité FACM. Un très grand merci à Marie-Claire Cambier pour les « litres » de cellules fournis tout au long de ma thèse. Merci à Martial, Charlotte et Virginie pour l'aide technique apportée lors des expériences d'accumulation, d'efflux, etc… Et un immense merci à Ahalieyah qui a porté une attention toute particulière à une histoire de toxicité, et qui a choisi ce sujet pour son mémoire.
Lors de cette aventure, beaucoup de mes collègues sont devenus mes amis, et je les remercie tous pour m'avoir si bien entourée. En particulier, je remercie Julien, Aurélie, Laetitia et Ahalieyah qui m'ont apporté leur soutien lors d'une certaine période plutôt difficile en 2011, et je remercie également Sandrine qui m'a montré où se situait le commissariat de police !! Je voudrais dire merci à tous les belges qui m'ont appris leur « language » afin que je puisse me fondre dans leur univers.
Mes remerciements vont enfin à ceux qui m'ont si gentilment aidée à « décompresser » : je pense ici à Jupp, Olivier, Jérôme, Philippe et Fred pour les matchs endiablés de badminton, et à Olivier (encore), Guillaume, Greg, Xavier, Alice et Ana pour m'avoir fait découvrir l'escalade.

Mes remerciements s'adressent également à ma famille, mes parents, ma sœur, mon frère et ma grand-mère. Vous avez su résister, et rester éveillés lors de mes longues tirades qui avaient pour but de vous expliquer mon « obscur » sujet de thèse.

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1. FLUOROQUINOLONE ANTIBIOTICS

Works of pioneering scientists such as Koch, Pasteur and Lister defined the role of microbes in the causation of disease, and rapidly led to works of Ehrlich and others in the field of antimicrobial chemotherapy. Most of antibiotics discovered at this period were natural compounds made by microorganisms, such as Penicillin, a β-lactam antibiotic discovered by Fleming in 1928 and isolated from *Penicillium notatum* fungi. Work on penicillin led Fleming, Chain and Florey to get the Nobel Prize in 1945. This first anti-infective agent was really useful during the Second World War in the 1940s and is still used now, but shows only anti Gram positive properties. Waksman also got the Nobel Prize for his work on streptomycin, another natural antibacterial agent discovered and isolated from *Streptomyces griseus* in 1946, and which showed anti Gram negative properties. In order to enlarge the antimicrobial armamentarium, scientists are constantly trying to discover and develop new molecules which could have a broader spectrum and which would be more effective.

In the early 1960s, Lesher and colleagues discovered the naphthyridine agent nalidixic acid (as a by-product of chloroquine synthesis, an antimalarial agent) that has anti Gram negative properties, and which was the parent molecule of the totally synthetic new class of antibiotics: fluoroquinolones. But the poor serum and tissue concentration and limited spectrum of activity restricted the use of this product to urinary tract infections. To improve this class of antibiotics, structural modifications were made to the naphthyridone nucleus which gave birth to the next generations of fluoroquinolones in the 1980s. The better understanding of structure-function relationships at the beginning of the 21st century helped industries to develop better fluoroquinolones in terms of both spectrum of antimicrobial cover and pharmacokinetics. The development of this class of antibiotics targets a broader spectrum of bacteria, a better activity, a lower toxicity and a lower susceptibility to resistance mechanisms.

In this first chapter, we will introduce the fluoroquinolone antibiotics in general and their evolvement from antibacterial agents with a limited spectrum of predominantly anti Gram negative antimicrobial activity and a restricted number of indications, to a class of widely used antibiotics with extensive indications for infections caused by many bacterial pathogens in most body tissues and fluids.

1.1. General properties of fluoroquinolones

1.1.1. Chemical structure

All fluoroquinolones have the same bicyclic nucleus derived from nalidixic acid, and the nomenclature used is always the same as presented in Figure 1. It has been shown that every substituent around the bicyclic structure can vary, except the positions 2, 3 and 4 (otherwise the molecule loses its activity). As they are completely synthetic compounds having a broad spectrum and good bioavailability, fluoroquinolones became one of the most promising class of molecules in contemporary anti-infective chemotherapy (it has been reported that more than 10.000 molecules have been synthesized since nalidixic acid, but only a few of them were clinically approved).

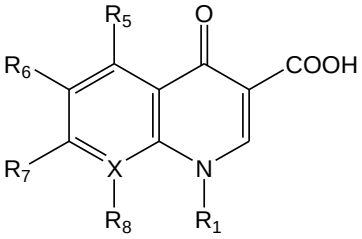

Figure 1. Structure of quinolones. X can be a nitrogen or a carbon atom. If a fluorine atom is at position R_6, it is a fluoroquinolone.

Only 10 years after the discovery of nalidixic acid, fluoroquinolones were developed with the addition of a fluorine atom at position 6 of the bicyclic nucleus, giving birth to flumequine. In the next decades after the flumequine discovery, structural modifications on the pharmacophore at positions 1, 5, 7 and 8 were made and gave rise to other fluoroquinolone antibiotics always with the aim of finding more potent molecules with a broader spectrum (Figure 2).

Based on their structure and antibacterial activity, fluoroquinolones are classified into 4 generations, considering nalidixic acid as the parent compound in the first generation. Flumequine, also a first generation molecule was the first compound to be fluorinated at position 6 (which gave the name “fluoro”quinolone to the whole class), showed a marked increased activity compared to nalidixic acid. These first-generation quinolones have a good oral absorption, but unchanged quinolones and active metabolites accounted for only 10% of the dose ((MCCHESNEY et al. 1964); (Shimizu et al. 1970)) as they were readily metabolized in the body and inactivated.

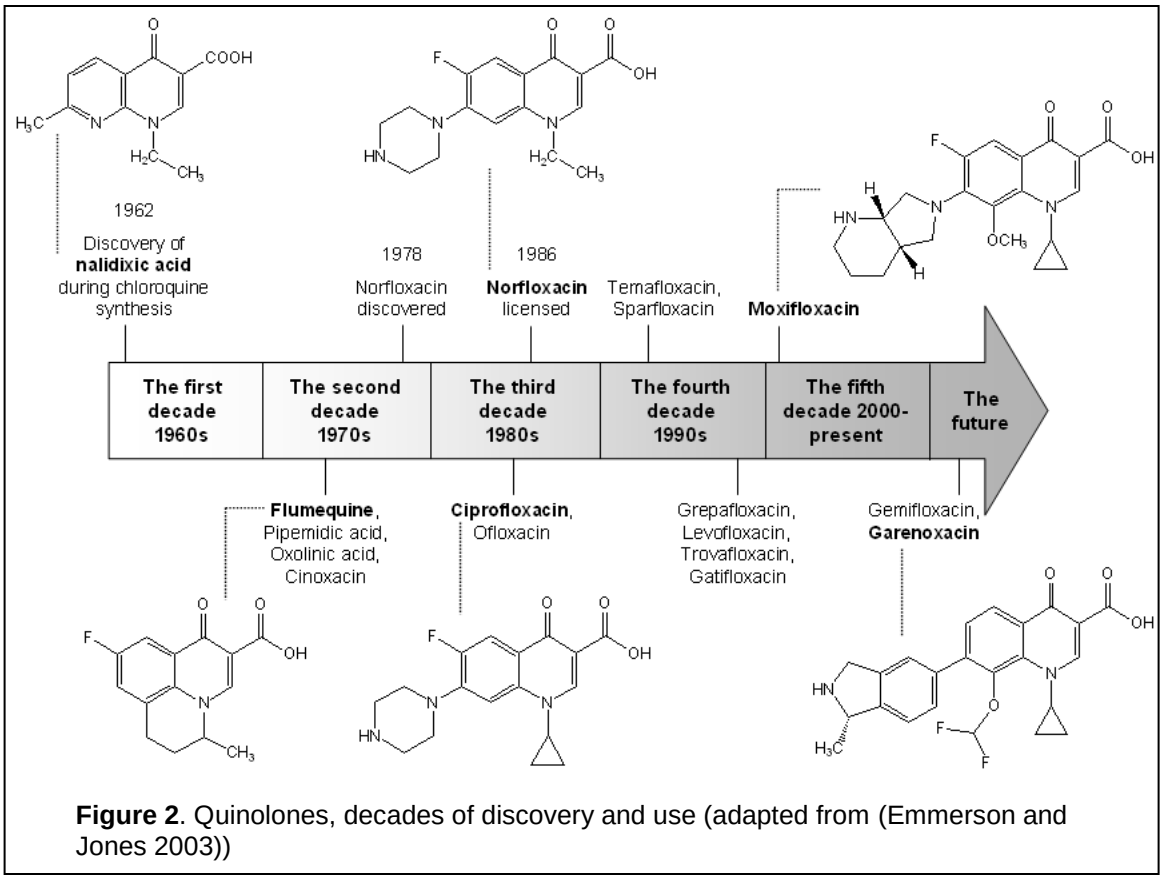

Figure 2. Quinolones, decades of discovery and use (adapted from (Emmerson and Jones 2003))

The second generation of fluoroquinolones showed a real breakthrough with the combination of fluorination at position 6 and a piperazine ring at position 7 (Appelbaum and Hunter 2000). Indeed, compounds as norfloxacin show a better activity and a broader spectrum (including Gram positive as well as Gram negative bacteria), and the introduction of the piperazinyl group at position 7 increased the tissue penetration. Replacement of the ethyl side chain at position 1 by a cyclopropyl group improves the activity and gave rise to ciprofloxacin, a fluoroquinolone still used nowadays. But the emergence of resistance to this group of antibiotics became a main problem, and fluoroquinolone research aimed at decreasing the rate of emergence of resistance, as well as improving activity against pneumococci, ciprofloxacin-resistant strains, and Gram negative bacteria including pseudomonal strains.

This led to the development of the third generation of fluoroquinolones which includes moxifloxacin, a useful molecule in the treatment of respiratory tract infections caused by *Streptococcus pneumoniae* for example. As a whole class, fluoroquinolones are generally well-tolerated with a little number of side-effects, but some molecules led to

serious adverse effects, and thus development of new compounds with better tolerability was of interest.

With garenoxacin, one of the fourth-generation fluoroquinolones, the optimisation of all substituents has allowed the removal of the fluorine atom at position 6 which could decrease side effects as genotoxicity (Domagala and Hagen 2003).

1.1.2. Antibacterial activity and mode of action

As fluoroquinolones are amphiphilic molecules, they can pass easily through the membrane of Gram positive bacteria by passive diffusion, whereas their entry in Gram negative bacteria needs proteins called porins crossing the outer membrane and through which lot of little molecules as fluoroquinolones can pass (Neves et al. 2005).

In 1964, it has been shown that nalidixic acid selectively inhibits DNA synthesis of bacteria (GOSS et al. 1964), suggesting that the mechanism of action of quinolones is different from that of other antibacterial drugs. Indeed, since the discovery of DNA gyrase (Gellert et al. 1976), it has been proven that fluoroquinolones have a unique mechanism of action resulting in inhibition of bacterial type II topoisomerases ((Gellert et al. 1977); (Sugino et al. 1977)), enzymes that are able to control the DNA topology. As these enzymes are required for cell growth and division, antibiotics will induce cell death, and therefore are so-called bactericidal. Indeed, these enzymes are essential in the processes of replication and transcription in eukaryotic and prokariotic cells as they are able to supercoil or to relax DNA molecules.

1.1.2.1. Type II topoisomerases

Type II topoisomerases are different from type I topoisomerases in two main points: type II enzymes catalyze DNA topological changes via a double-strand breakage-and-rejoining mechanism and ATP is necessary for their activity, whereas type I enzymes catalyse DNA topological changes via a single-stranded breakage-and-rejoining mechanism and do not need ATP (Hsieh 1990). The first type II topoisomerase to be discovered in 1976 by Gellert and colleagues (Gellert et al. 1976) was DNA gyrase in *Escherichia coli.* This enzyme is a heterotetramer made of two subunits encoded by *gyrA* and *gyrB* (Levine et al. 1998), and is able to introduce negative superhelical turns into DNA and can also relax superhelical DNA, albeit less efficiently (Huang 1996).

Topoisomerase IV, also discovered in *E. coli*, is a heterotetramer made of two subunits encoded by *parC* and *parE* genes, and has a strong activity of DNA decatenation which relax DNA molecules (Drlica and Zhao 1997). Figure 3 shows type II topoisomerase activity.

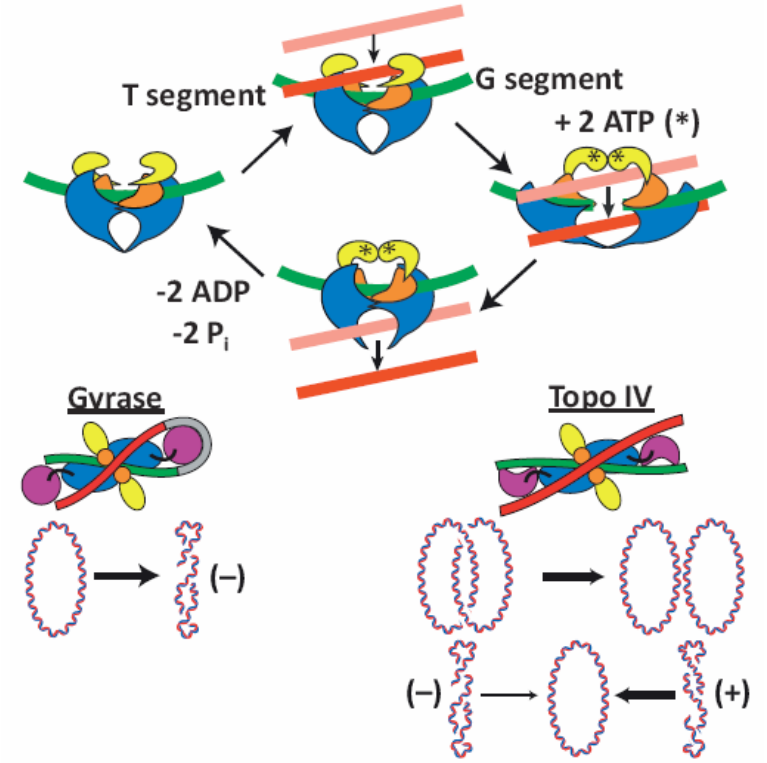

Figure 3. Core type II topoisomerase strand passage mechanism. (Upper, clockwise from top) Type II topoisomerase (blue, salmon and yellow) binds gate segment DNA (green) and subsequently captures a transfer segment DNA (pink to red denoting movement of the DNA) followed by the binding of two ATP molecules, which close the N-terminal gate (yellow). This is followed by double-strand cleavage of the gate strand and passage of the transfer strand across the cleaved DNA and through the enzyme. The transfer strand and products of ATP hydrolysis are then released as the enzyme resets for another enzymatic cycle. This core strand passage mechanism is coupled with substrate specificity to achieve different topological activities. Gyrase (lower left, blue, salmon and yellow with purple GyrA C-Terminal Domain (CTD)) wraps DNA around its GyrA CTD, resulting in the formation of a left-handed DNA crossing, which is converted into a negative supercoil after passage of the transfer segment (red) through the gate segment (green). Topo IV (lower right, with purple ParC CTD) unlinks catenated DNA molecules and relaxes positive supercoils more efficiently than negative supercoils. (Figure and comments from (Neuman 2010))

1.1.2.2. Inhibition of type II topoisomerase activity by fluoroquinolones

Fluoroquinolones are known to inhibit DNA gyrase and topoisomerase IV by forming a ternary complex between the DNA, the enzyme and the antibiotic, as presented in figure 4. Fluoroquinolones will inhibit topoisomerase activity when these enzymes are covalently linked to the cleaved DNA. Stacked fluoroquinolones intercalate in a pocket made during the local breakage of the double stranded DNA (Siegmund et al. 2005). They interact with DNA and the GyrA subunit of the DNA gyrase, or the ParC subunit of the topoisomerase IV, stabilizing the DNA-enzyme cleavage complex, and leading to the blockage of the replication machinery progression, DNA lesions formation, and finally to bacterial death ((Hooper 1998); (Hooper 2001b)). The main target in Gram positive bacteria is topoisomerase IV, whereas in Gram negative bacteria it is DNA gyrase.

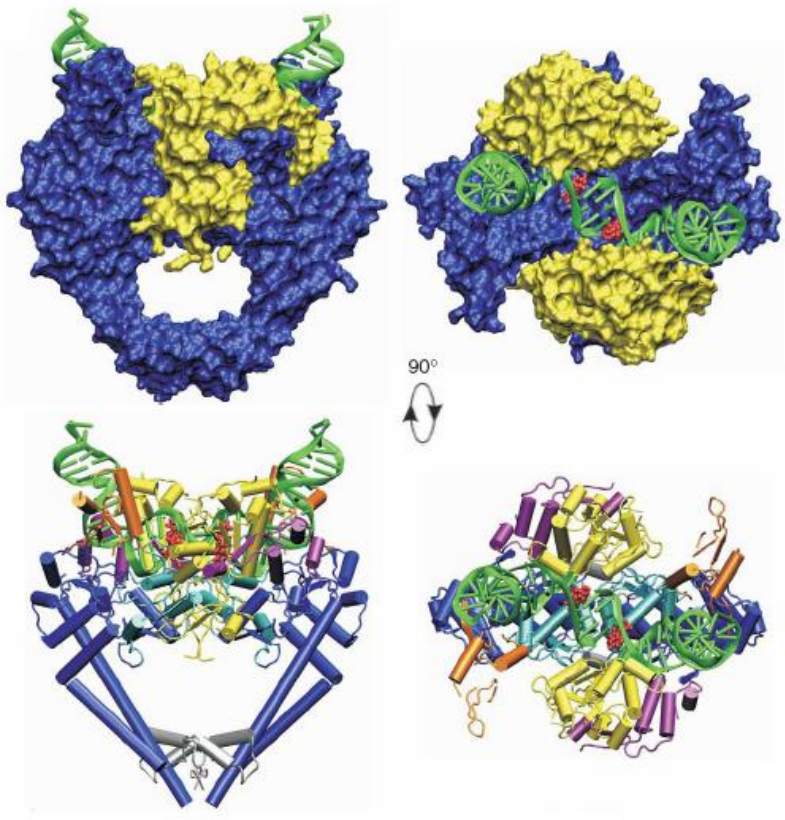

Figure 4. Structure of the enzyme-quinolone-DNA cleavage complex of *S. pneumoniae* topo IV. Front and top views of the topo IV ParC (blue) and ParE (yellow) proteins complexed with the G-segment DNA (green) and a quinolone drug moxifloxacin (red), shown both in surface (above) and detailed cartoon (below) representations. (Figure and comments from (Laponogov et al. 2009))

1.1.2.3. Spectrum of activity and structure-activity relationship of fluoroquinolones

Certain molecular determinants are essential for the bactericidal activity of fluoroquinolones (Van Bambeke et al. 2005): as shown in Figure 5, the pyridone cycle with the amine function at position 1 substituted with an aliphatic chain or a cycle is necessary for the stacking of molecules ; the carboxylic acid at position 3 and the exocyclic oxygen at position 4 are thought to bind the enzyme and the DNA. Both of these characteristics are needed to the activity of this class of antibiotics (Sharma et al. 2009).

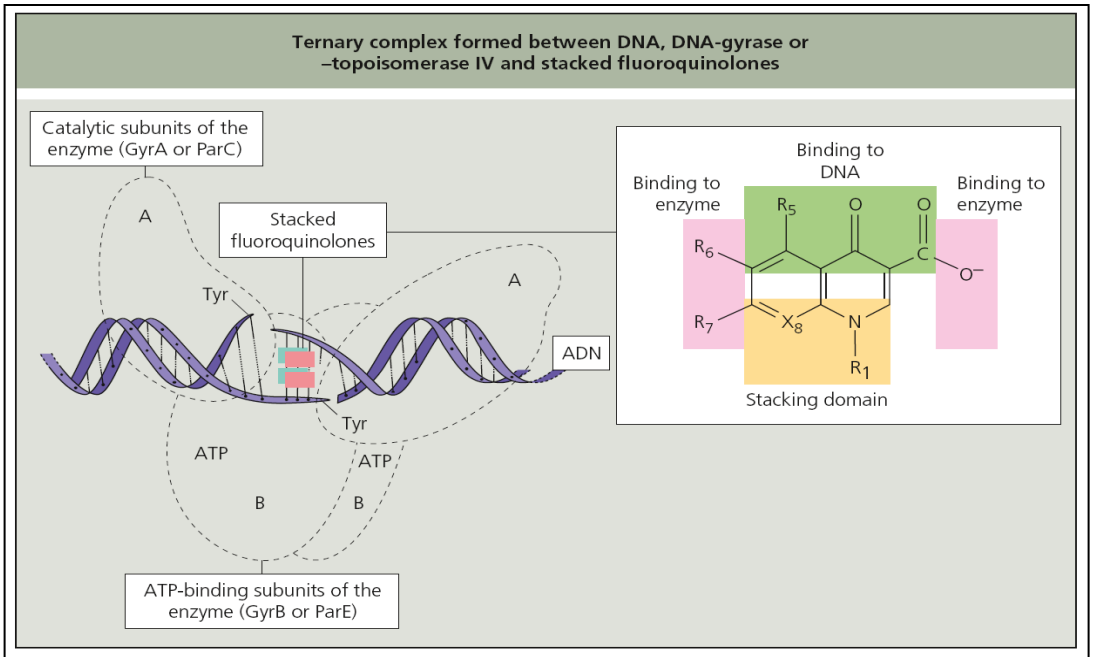

Figure 5. Schematic organisation of the ternary complex antibiotic-DNA-topoisomerase (DNA gyrase or topoisomerase IV) (figure from (Van Bambeke et al. 2010)).

Other substituents in the quinolone ring have been studied, and modifications of these at positions 5, 6, 7 and 8 can change the potency or the spectrum of activity of fluoroquinolones. First, the addition of a fluorine atom at position 6 has considerably increased the activity (such as a cyclopropyl group at position 1) (Peterson 2001). But this fluorine atom is considered to play a role in photosentitization side effect (which will be discussed later). The optimization of other substituents on the quinolone ring have led to the remove of this fluorine atom in the fourth generation of fluoroquinolones which have still good activity. The position 7 is considered to be one that directly interacts with

enzymes (DNA gyrase or topoisomerase IV), and the optimal substituents at this position have been found to be groups that contain a 5- or 6-membered nitrogen heterocycle such as amino-pyrrolidines and piperazines. Piperazine ring was found to target Gram negative bacteria, while an amino-pyrrolidine group targets Gram positive bacteria (Appelbaum and Hunter 2000), and alkylation of these heterocycles enhances the activity against Gram-positive bacteria ((Piddock et al. 1998); (Domagala 1994)). Position 8, as position 5, is considered to affect the overall molecular stearic configuration (Llorente et al. 1996), and therefore affect target affinity. Halogen substituents as Cl, methyl or methoxy increase the activity against anaerobes, as well as naphtyridones (with nitrogen in the place of the carbon in the ring).

1.1.3. Pharmacokinetic and pharmacodynamic parameters of fluoroquinolones

As Leslie Z. Benet said: “Pharmacokinetics may be simply defined as what the body does to the drug, and pharmacodynamics which may be defined as what the drug does to the body”. In these terms, pharmacokinetic (PK) parameters include absorption, distribution, metabolism and excretion of a drug, and pharmacodynamic (PD) is represented by the activity of the antibiotic (Minimum Inhibitory Concentration (MIC) against the bacterium in question) versus time and concentration.

Fluoroquinolone antibacterials show an excellent bioavailability (70 to 90% of the administered dose) particularly due to the high absorption localized preferentially at the duodeno-jejunum level, where they are usually absorbed by passive diffusion (Rabbaa et al. 1997). In general, the serum peak is rapidly reached (about 1 or 2 hours after the absorption) but is low because of the high tissular diffusion. Indeed, fluoroquinolones can penetrate into tissues and mammalian cells extremely well, and this is an interesting property to treat intracellular infections. The drug distribution is different following the tissue: it has been shown that they largely accumulate in the liver, the kidneys and the prostate, but they are less accumulated in the cerebrospinal fluid or in the lung ((Fischman et al. 1996); (Fischman et al. 1998) ; (Tamai et al. 2000)). The protein binding is generally weak and the distribution volume is high. Their elimination half-lives are situated between 2 to 30 hours, but most of used fluoroquinolones have an elimination half-life between 3 to 7 hours (Bergogne-Berezin 2002). They are differently eliminated following the molecule: they are usually eliminated by the kidneys in the urine, but they could be eliminated in the bile (Chow et al. 2001).

The efficacy of antibiotics can be predicted by a PK/PD model and three of these models have been identified ((Schentag et al. 2001); and see Figure 6): the ratio of maximum serum concentration (C_{max}) to the MIC (C_{max}/MIC), the ratio of the area under the plasma concentration (AUC) versus MIC (AUC/MIC), and the duration of the dosing interval that plasma concentrations exceed the MIC (T>MIC). Fluoroquinolones efficacy can be predicted by an AUC/MIC model, that means that these antibiotics exhibit a concentration-dependent bactericidal activity (the bacterial population decreases when the antibiotic dose is increased (Lode et al. 1998)). The value of the AUC/MIC ratio is different for one fluoroquinolone to another and following the pathogen targeted: for example, the value of the AUC/MIC ratio to eradicate *Pseudomonas aeruginosa* with ciprofloxacin is 125 (Forrest et al. 1993), but with levofloxacin it is between 87 and 110 (Drusano et al. 2004). These PK/PD models are clearly a good tool to elaborate an antimicrobial regimen that has higher efficacy, but also that can suppress the emergence of drug resistant strains. For fluoroquinolones, the C_{max}/MIC is an important parameter to control the emergence of the resistance, and therefore must be taken into account in a fluoroquinolone treatment ((Madaras-Kelly and Demasters 2000); (Preston et al. 1998)).

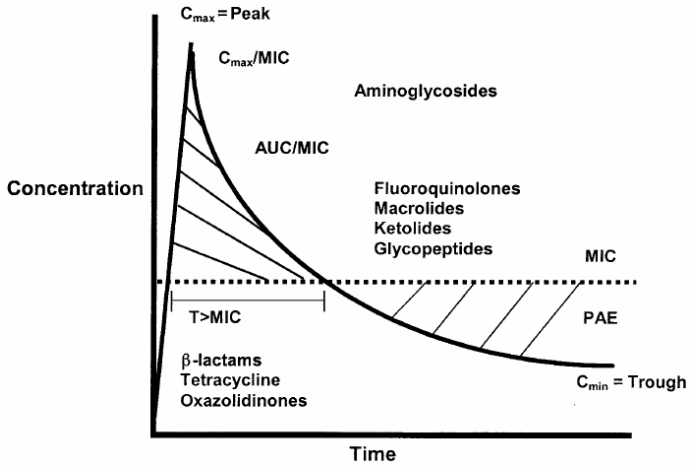

Figure 6. Pharmacodynamic parameters found to be important in describing the efficacy of different antibiotics. AUC, area under the concentration time curve; Cmax, maximum concentration; Cmin, minimum concentration; MIC, minimum inhibitory concentration; PAE, postantibiotic effect; T, time. (From (Rybak 2006))

1.1.4. Resistance mechanisms in bacteria

With increasing utilization of fluoroquinolones to fight against infectious diseases, emerging resistance for these agents is a growing concern. Resistance to this class of agents occurs by two main processes ((Jacoby 2005); (Ruiz 2003)): the first one, caused by mutations in the target enzymes, lowers the affinity of the drug for the topoisomerase-DNA complex, and the second one, caused by overexpression of efflux pumps in Gram

positives or Gram negatives or by underexpression of porines in Gram negatives, decreases the intrabacterial concentration of the drug. Two other, more recent mechanisms of resistance to fluoroquinolones have been found out, they are mediated by plasmids, these encode either a *qnr* gene that produces a protein that lowers gyrase binding to DNA, or a N-acetylase that inactivates quinolones with a piperazinyl group at position 7. All these four mechanisms of acquired resistance to fluoroquinolones are consistent among currently available drugs of this class and are expected to be similar for new and developing agents as well (except for efflux because of the variable recognition of the antibiotic by the protein following the substituent).

1.1.4.1. Target enzymes mutations

These spontaneous mutations in the target sites can occur in both topoisomerase IV and DNA gyrase, and result in reduced drug affinity ((Barnard and Maxwell 2001); (Willmott and Maxwell 1993)). Mutations commonly involve amino acid alterations (Hooper 2001a) near the active binding site of the enzyme, and are found only in one gene encoding for one subunit of the enzymes (in *gyrA* for the DNA gyrase, and in *parC* for the topoisomerase IV). They arise in a stepwise fashion as a result of accumulating mutations: first-step mutations will occur in the preferred drug target enzyme (in *gyrA* for Gram negative, and in *parC* for Gram positive organisms), and are usually sufficient for acquisition of detectable resistance, but second-step mutations can occur in the secondary target enzyme and will further affect quinolone resistance (Gillespie et al. 2002).

Resistance involves amino acid substitutions in the region of the GyrA or ParC subunit, termed the “quinolone-resistance-determining region” (QRDR). This region occurs on the DNA binding surface of the enzyme (Morais Cabral et al. 1997). Alterations in DNA gyrase are clustered between amino acid 67 and 106 in the amino terminus of the subunit A, and the most common substitution is a leucine or tryptophan at the place of a serine 83 which will cause a large increase in resistance (Cattoir et al. 2007). The same alterations can occur in the subunit C of topoisomerase IV. The combination of both alterations results in fluoroquinolone resistance in *S. pneumoniae* ((Piddock 1995); (Taba and Kusano 1998)).

1.1.4.2. Decreased intrabacterial concentration

This kind of resistance mechanism results from either reduced production of outer-membrane proteins or stimulated cell efflux system which leads to active drug expulsion.

It is only in Gram negative bacteria, where porins are needed for the entry of molecules, that a reduced production of outer-membrane proteins can occur, leading to a decreased penetration of the antibiotic. It has been reported that an alteration in the outer-membrane porins (as OmpF porin in *E. coli*) leads to decrease the entry of the drug inside the bacterium, thus decreasing the intracellular concentration of the drug ((Hooper et al. 1986); (Legakis et al. 1989)). Resistance by such a mechanism is of relatively low level, and it often occurs in combination with other resistance mechanisms.

The overexpression of efflux pumps at the bacterial membrane is the second mechanism leading to a decreased intrabaterial concentration of the drug. These proteins are ubiquitous and are probably essential in the physiology of the bacteria (Saier, Jr. et al. 1998), but when overexpressed, they can cause low-to-moderate resistance to fluoroquinolones. Efflux pump-mediated resistance has been observed and described in pneumococci (for PmrA, see (Brenwald et al. 1998); (Gill et al. 1999); and for PatA/PatB, see (Pestova et al. 2002) ; (Brenwald et al. 2003); (Marrer et al. 2006b)), staphylococci (NorA) ((Munoz-Bellido et al. 1999) ; (Aeschlimann et al. 1999)), anaerobes (Miyamae et al. 1998), and Gram negative bacteria as *Pseudomonas aeruginosa* or *E. coli* ((Lomovskaya et al. 1999) ; (Mine et al. 1999) ; (Ziha-Zarifi et al. 1999)). The efflux pumps in bacteria will be developed later in the introduction.

1.1.4.3. Plasmid-mediated resistance

This plasmid-mediated resistance was claimed in 1987 (Munshi et al. 1987), showing a transfer of nalidixic acid resistance from *Shigella dysenteriae* to *E. coli* via a plasmid, but this hypothesis was eliminated as further studies showed that putative donor strains contained *gyrA* mutations (Rahman et al. 1994) and that a plasmid was not directly involved. It was in 1998 that Martinez-Martinez and colleagues (Martinez-Martinez et al. 1998) showed that a plasmid could inscrease resistance to quinolones in *E. coli* not by a reduced quinolone accumulation mechanism, nor by a target enzyme mutation.

The plasmid-mediated quinolone resistance gene found in *E. coli* and *Klebsiella pneumoniae* ((Tran and Jacoby 2002); (Wang et al. 2004)) was named *qnr*, and produces a 219 amino acids protein belonging to the pentapeptide repeat family, members of which are involved in protein-protein interactions. This Qnr protein binds the DNA gyrase by mimic the DNA structure, and will bind the quinolones to form a ternary complex, thus preventing quinolones to inhibit the activity of other enzymes on the real DNA molecule. Because Qnr by itself provides only a level of resistance to quinolones comparable to that provided by a first-step mutation in DNA gyrase, it needs additional resistance mechanisms to display clinically meaningful resistance (Rodriguez-Martinez et al. 2003).

The other mechanism of transferable quinolone resistance reported in 2006 is the enzymatic inactivation of the antimicrobial agent. This resistance mechanism, well known for aminoglycosides (the *aac* genes encoding for aminoglycoside acetyltransferases, enzymes which will inactivate the antimicrobial agent by acetylation at different subsituents of the aminoglycoside), has been reported for fluoroquinolones: the *cr* variant of the *aac(6')-Ib* gene encodes for a ciprofloxacin-modifying enzyme ((Park et al. 2006); (Robicsek et al. 2006)). This aac(6')-Ib-cr variant contains two amino acids changes which are necessary for the enzyme's ability to acetylate fluoroquinolones (Vetting et al. 2008). This enzyme, encoded by a plasmid gene, confers resistance to ciprofloxacin and norfloxacin by N-acetylation of the amino nitrogen on its piperazinyl substituent ((Kim et al. 2009); (Park et al. 2010) ; (de Toro et al. 2010) ; (Wachino et al. 2011)). Up now, no other fluoroquinolones have been reported to be acetylated by this enzyme.

1.1.5. Adverse effects and cytotoxicity of fluoroquinolones

1.1.5.1. Adverse effects

With few exceptions, the adverse effects of fluoroquinolones are not too severe when compared to the beneficial features they exhibit. Toxicity is mild at therapeutic doses, and is generally limited to gastrointestinal disturbances such as nausea, vomiting and diarrhea, and also to central nervous system (CNS) reactions including dizziness, convulsions and psychoses ((Christ 1990); (Stahlmann and Lode 1999)). But phototoxicity, cardiotoxicity and tendinitis are also of concern with fluoroquinolones

treatments ((De Sarro and De Sarro 2001); (Bertino, Jr. and Fish 2000); (Van Bambeke et al. 2005); (Katritsis and Camm 2003)).

As we have noted before, more than 10.000 molecules of fluoroquinolone antibiotics have been developed, but just a few of them are currently available on the clinical market. This is mostly due to their potential to engender toxicity, and of course, most of them have been withdrawn or their use has been restricted to some severe infectious diseases. For example, sparfloxacin is associated with high rates of phototoxicity (Martin et al. 1998), as lomefloxacin and fleroxacin, and is able to increase the QTc interval and lead to Torsades de pointes (Lipsky et al. 1999), as grepafloxacin (Owens, Jr. 2004). Pefloxacin has been shown to engender tendinopathy (Olcay et al. 2011), but this phenomenon is dose-dependent. Trovafloxacin has been withdrawn because of a high liver toxicity ((Liguori et al. 2008); (Shaw et al. 2009)), but this fluoroquinolone is an exception in the class, and it has been shown that fluoroquinolone antibiotics lead to lower hepatotoxicity than macrolides or tetracyclines ((Van Bambeke and Tulkens 2009) ; (Andrade and Tulkens 2011)).

As a class, the fluoroquinolones are generally well tolerated and safe, and the incidence of adverse events varies significantly depending on the physicochemical structure of specific agents. Van Bambeke and colleagues in 2005, and De Sarro A and De Sarro G in 2001 show that halogen groups at position 8 of the fluoroquinolone core lead to phototoxicity; chondrotoxicity and quinolone-induced cartilage lesion could be explained by magnesium-chelating properties probably due to the carboxylate function and the exoxyclic oxygen at positions 3 and 4; and finally the subsituent at position 7 is thought to bind to the GABA (gamma-aminobutyric acid) receptor in the brain and so to cause the CNS side effects.

1.1.5.2. Cytotoxicity

As we have seen in the chapter dedicated to pharmacokinetics and pharmacodynamics, fluoroquinolones are able to accumulate in cells at variable levels following the molecule. This property can lead to cytotoxic effects. Some studies have shown that fluoroquinolones at therapeutic concentrations could affect the nucleotides synthesis, lymphocytes growth and the the cell cycle progression ((Forsgren et al. 1987a); (Forsgren et al. 1987b)). Larger concentrations of ciprofloxacin can inhibit the eukaryotic cell proliferation and induce apoptosis in a dose-dependent manner ((Pessina

et al. 2007); (Somekh et al. 1989)). It has been shown that carcinoma cells incubated with ciprofloxacin showed an increased expression of the pro-apoptotic protein Bax (Aranha et al. 2000), and a decrease of the mitochondrial membrane potential with an increased activity of caspases 3, 8 and 9 involved in the apoptotic processes (Herold et al. 2002).

In addition, fluoroquinolones can induce genotoxicity which leads to chromosomal aberrations and the formation of micronucleus ((Curry et al. 1996); (Itoh et al. 2006); (Mukherjee et al. 1993)). These clastogenic effects lead to the contraindications of fluoroquinolones in pregnant women because micronucleus appears to be chromatidic fragments in the cytoplasm able to be transmitted to daughter cells.

All the cytotoxic effects of fluoroquinolones seem to be connected to their ability to interact with topoisomerases. Fluoroquinolone targets in bacteria are type II topoisomerases, and this kind of enzymes is also present in eukaryotic cells. The point is that fluoroquinolones have 1000 times less affinity for eukaryotic topoisomerases than for their prokaryotic homologues. But as those antibiotics are able to accumulate in large amount in cells (much more than in the serum), they can probably have effects on eukaryotic topoisomerases as it has been shown in the literature ((Reuveni et al. 2008); (Fabian et al. 2006); (Robinson et al. 1991) ; (Elsea et al. 1993)).

1.2. Three fluoroquinolones in details

Three fluoroquinolones are of particular interest in the context of this thesis: ciprofloxacin, moxifloxacin and gemifloxacin (a second-, third- and fourth-generation fluoroquinolone respectively) (structures are presented in Figure 7). In this chapter, we will underline the biological and physicochemical properties of each of these three main fluoroquinolone antibiotics which have led to their wide clinical use, and compare their pharmacokinetic parameters (summerized in the Table 1 from (Aminimanizani et al. 2001)), their spectrum of activity (see table 2 for MICs of the three fluoroquinolones against common pathogens), and finally their susceptibility to resistance mechanisms.

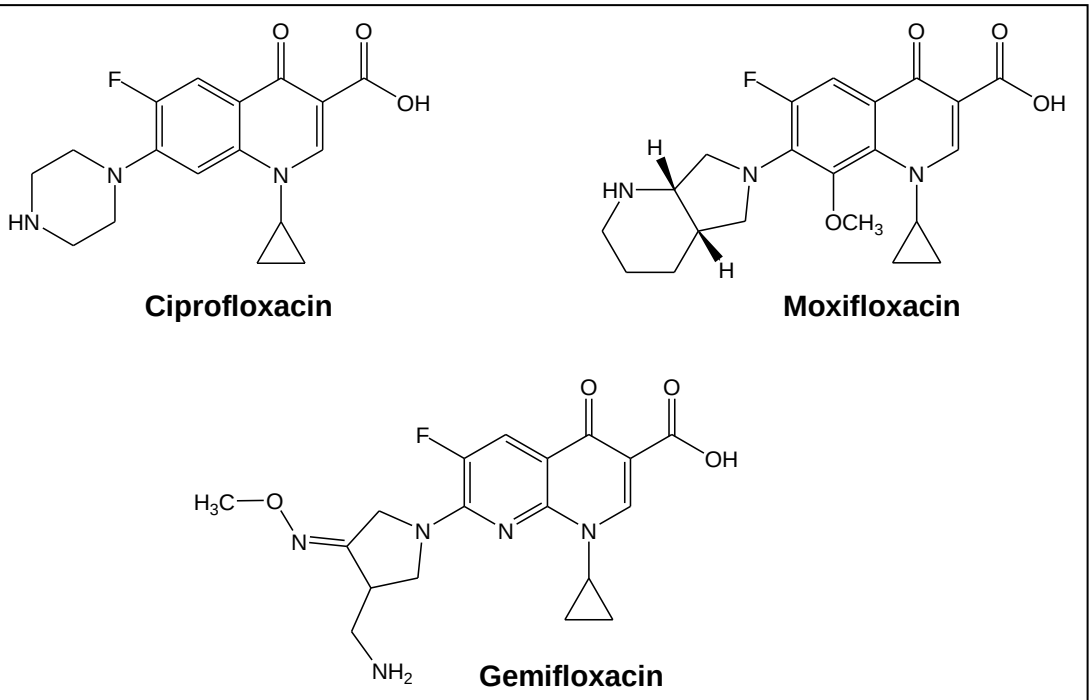

Figure 7. Structure representation of ciprofloxacin, moxifloxacin and gemifloxacin

Table 1. Pharmacokinetic parameters for ciprofloxacin, moxifloxacin and gemifloxacin.

quinolone	Dose (mg)	C_{max} (mg/L)	Vd (L/kg)	CL (L/h)	$t_{1/2}$ (h)	AUC (mg/L.h)	reference
Ciprofloxacin	500 PO	2.26	3.76	54.5	3.69	10	(LeBel et al. 1986)
	400 IV	4.5 ± 0.8	-	34.4	3.4 ± 0.5	12 ± 1.8	(Gonzalez et al. 1985)
Moxifloxacin	400 PO	2.5	3.1	11.6	15.6	29.8	(Stass and Kubitza 1999)
	400 IV	3.6	2.1	11.6	15.4	34.6	
Gemifloxacin	320 PO	1.5 ± 0.4	4.9	9.1	6.6 ± 1.3	9.8 ± 2.7	(Allen et al. 2000)

Cmax = peak plasma drug concentration; Vd = volume of distribution; CL = total body clearance; t1/2 = elimination half-life; AUC = arrea under the concentration-time curve; PO = oral; IV = intravenous

1.2.1. Ciprofloxacin

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid; MW 331.4) is a second-generation fluoroquinolone discovered in 1982, and still widely used nowadays. As we can see on the figure 7, it contains (i) a fluoro group at position 6 which has considerably increased the activity of this class of compounds since the discovery of nalidixic acid, (ii) a piperazinyl group at position 7 which markedly increases its activity against Gram negative bacteria, and (iii) a cyclopropyl group at position 1 which is also thought to increase the activity of the molecule.

The pharmacokinetic parameters of ciprofloxacin (see Table 1) are better than those of nalidixic acid and other first-generation fluoroquinolones, especially due to the cyclopropyl group at position 1 which increases the volume of distribution, and the piperazynil group at position 7 which augments the half-life of the molecule (Van Bambeke et al. 2005).

In addition to the general adverse effects produced by fluoroquinolones, ciprofloxacin is an inhibitor of human cytochrome P450 1A2 (CYP1A2)-mediated metabolism, thus coadministration with other drugs primarily metabolized by CYP1A2 (such as theophyllin) results in increased plasma concentrations of these drugs and could lead to clinically significant adverse effects ((Antoniou et al. 2011); (Peterson et al. 2004)).

Ciprofloxacin is marketed worldwide, and is used to treat infections caused by Gram negative organisms such as *Enterobacteriaceae* (see Table 2 for in vitro MICs of ciprofloxacin). It is indicated for the treatment of complicated urinary tract infections, gastroenteritis with severe diarrhea, prostatis infections, skin and soft tissue infections, osteomyelitis, intra-abdominal infections and pneumonia due to aerobic Gram negative bacteria (http://www.ema.europa.eu/docs/fr_FR/document_library/Referrals_document/Ciprofloxacin_Nycomed_29/WC500008740.pdf). Because ciprofloxacin was the first fluoroquinolone with a broad spectrum, a high activity, and a favorable pharmacokinetic profile, it has been widely used, and this overuse has led to the emergence of resistant strains (in Gram negative as well as in Gram positive microorganisms). It has been shown that mutations in ParC or ParE subunits of topoisomerase IV were responsible for the resistance to ciprofloxacin in *Streptococcus pneumoniae* and *Staphylococcus aureus*

((Pan and Fisher 1996); (Perichon et al. 1997)). *Pseudomonas aeruginosa* has also developed resistance to ciprofloxacin ((Dalhoff 1994); (Thomson 1999)), but mostly due to the overexpression of an efflux pump system localized at the membrane of this microorganism which expel the drug out of the cell ((Dupont et al. 2005); (Zhang et al. 2001)). Development of those resistance mechanisms is the principal reason for the restricted use of an antibiotic to few infectious diseases. Ciprofloxacin being limited to Gram negative bacterial infections, new molecules have been developed with improved potency against Gram positive organisms and may less easily select for resistance.

1.2.2. Moxifloxacin

Moxifloxacin ((1'S,6'S)-1-Cyclopropyl-7-(2,8-diazabicyclo[4.3.0]non-8-yl)-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; MW 400.4 g/mol) is a third-generation fluoroquinolone which has been patented in 1989 in the USA, but is used since 1999. As we can see on figure 7, moxifloxacin structure differs from that of ciprofloxacin by (i) an amino-pyrrolidine group at position 7 which is bulkier than piperazinyl group and tends to expand the spectrum of activity to Gram positive bacteria such as *Streptococcus pneumoniae*, and (ii) a methoxy group at position 8 which increases the potency of the drug (and which causes less risks of phototoxicity associated to a C-8 halogen present in some molecules developed earlier). The bulkier side chain at position 7 allows also a better half-life and increases CNS penetration.

The pharmacokinetic parameters are described in Table 1, where we can see that moxifloxacin has longer elimination half-life and higher AUC than ciprofloxacin. It has been reported that moxifloxacin treatment can lead to the general side effects registered for all fluoroquinolones, and in particular acute liver failure or serious liver injury and QTc prolongation/torsades de pointes. But recent reviews (Andrade and Tulkens 2011; (Van Bambeke and Tulkens 2009)) showed that hepatotoxicity caused by this fluoroquinolone is less frequent than this caused with other class of antibiotics, and although causing QTc prolongation, no severe cardiac toxicity was reported in pharmacovigilance systems. Moxifloxacin is not believed to have significant drug interactions.

Moxifloxacin indications are quite limited because it is considered as a drug of last resort when all other antibiotics have failed. Its indications are restricted to respiratory tract infections (as acute exacerbations of chronic bronchitis, acute bacterial

sinusitis and community acquired pneumonia caused by multidrug resistant *Streptococcus pneumoniae*), uncomplicated and complicated skin and skin structure infections, and complicated intra-abdominal infections. Other indications could be applied for moxifloxacin, for example to treat tuberculosis or meningitis. The restricted use of this fluoroquinolone, and its better activity may have helped to control the emergence of resistance to this antibiotic in *S. pneumoniae* (Patel et al. 2011). Resistance to moxifloxacin is rarely due to active efflux mechanisms, because of its poor affinity for these proteins (as it has been shown that fluoroquinolones with a bulkier substituent at position 7 are less susceptible to efflux ((Takenouchi et al. 1996); (Beyer et al. 2000)), but it is mostly due to target enzymes mutations. In *S. pneumoniae*, moxifloxacin acts primarily on DNA gyrase (Pestova et al. 2000) as it has been shown for fluoroquinolones bearing a methoxy group at position 8. In vitro experiments conducted by De Vecchi and colleagues in 2009 (De Vecchi et al. 2009) showed that moxifloxacin has a lower propensity to select for resistance in *S. pneumoniae*. In *Mycobacterium tuberculosis*, which counts only one type II topoisomerase (the DNA gyrase), it has been shown that the resistance to moxifloxacin (which has a strong bactericidal activity against *M. tuberculosis*) is due to a stepwise process of additive mutations in the *gyrA* gene of DNA gyrase, and rarely in the *gyrB* gene ((Pantel et al. 2011); (van den et al. 2011); (Ginsburg et al. 2003) ; (Piton et al. 2010)).

1.2.3. Gemifloxacin

This fluoroquinolone (7-[(4*Z*)-3-(aminomethyl)-4-methoxyimino-pyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1,8-naphthyridine-3-carboxylic acid; MW 389.4 g/mol) was patented in the USA in 2003. As we can see on Figure 7, gemifloxacin (i) is a naphthyridone with a nitrogen in place of the carbon at position 8, which is thought to enhance its activity against both DNA gyrase and topoisomerase IV (Yoo et al. 2004), (ii) and has a pyrrolidine ring at position 7 which is substituted by an alkyloxime and an aminomethyl groups at positions 4 and 3 respectively (it has been shown that the elimination of the alkyloxime substituent caused a decrease in the activity of the molecule (Hong 2001)).

As other fluoroquinolones, gemifloxacin is well absorbed after oral administration, and despite its low serum concentration (1.4 mg/L compared to 3.2 mg/L for moxifloxacin), gemifloxacin has a good tissue penetration with drug concentrations in

bronchial mucosa, epithelial lining fluid and alveolar macrophages higher than those in serum (Wise and Honeybourne 1999). The most common adverse events occurring in treatments with gemifloxacin are hypersensitivity reactions (rash and pruritus), phototoxicity (which has not been reported for moxifloxacin), and hepatotoxicity, while QT prolongation is less risky with gemifloxacin than with moxifloxacin (Jivcu and Gotfried 2009).

Gemifloxacin is named a “respiratory” fluoroquinolone as moxifloxacin because of its enhanced activity against Gram positive organisms (see Table 2 for in vitro MICs of gemifloxacin), and particularly *Streptococcus pneumoniae*, and is restricted to the treatment of lower respiratory tract infections (Blondeau and Tillotson 2008) as community acquired pneumonia, and acute bacterial exacerbation of chronic bronchitis. Gemifloxacin shows better activity against sensitive and resistant *S. pneumoniae* than moxifloxacin (Appelbaum et al. 2004), and this may be due to its dual activity to inhibit DNA gyrase and topoisomerase IV. A single mutation in *parC* or *gyrA* gene will result in only a small increase in the MIC (Heaton et al. 2000). Azoulay-Dupuis and collegues (Azoulay-Dupuis et al. 2005) showed that gemifloxacin has the lowest MICs against *S. pneumoniae* harboring either (i) a single mutation in *gyrA*, *parC* or *parE*, or (ii) double or triple mutations in these genes, or (iii) also in mutants with efflux mechanism. LaPlante KL and colleagues (LaPlante et al. 2007) showed that gemifloxacin (as moxifloxacin) engenders less mutations in *gyrA* or *parC* than levofloxacin in *S. pneumoniae* strains, and even if susceptible to efflux (Lismond et al. 2011), gemifloxacin MICs remain low.

Table 2. MIC_{90} in µg/mL (range in parenthesis) for ciprofloxacin, moxifloxacin and gemifloxacin against commonly infectious pathogens (from (Hooper 2005b)).

	organism	Ciprofloxacin	Moxifloxacin	Gemifloxacin
Gram positive bacteria	*Staphylococcus aureus*, methicillin susceptible	0.5 (0.03-2)	0.12 (0.06-0.25)	0.06 (0.03-0.06)
	Staphylococcus aureus, methicillin resistant	≥32 (25-128)	4 (2-16)	8 (1-8)
	Streptococcus pneumoniae	2 (1-8)	0.25 (0.06-0.5)	0.06 (0.03-0.06)
	Listeria monocytogenes	1 (0.5-4)	0.5	0.25 (0.12-0.25)
	Enterococcus faecalis	(1-128)	(0.5-16)	2 (2-4)
Anaerobic and mycobacteria	*Clostridium difficile*	6.25-12.5	2-16	2
	Mycobacterium tuberculosis	1	0.125	8
	Anaerobic Gram positive cocci	2-6.25	1-2	0.125-0.5
Gram negative and chlamydial pathogens	*Burkholderia cepacia*	(2->256)	(4-256)	64
	Enterobacter aerogenes	0.5 (0.03->16)	2 (0.25->16)	0.25 (0.008-2)
	Escherichia coli	0.25 (0.015->128)	0.25 (0.008-32)	0.016-0.03 (0.004-32)
	Haemophilus influenzae	(≤0.015-0.06)	0.03-0.06	(≤0.004-0.03)
	Klebsiella pneumoniae	0.5 (0.05-0.5)	1 (0.13-1)	0.25 (0.008-32)
	Legionella spp.	(0.016-0.06)	0.06	0.003-0.008
	Neisseria gonorrhoeae	(0.001-2)	(0.015-1)	-
	Pseudomonas aeruginosa	(0.5-128)	(8->128)	4-8 (0.03-256)
	Salmonella spp.	(0.01-0.25)	0.12-0.25	(0.015-0.12)
	Shigella spp.	(0.008-≤0.06)	0.03-0.06	(≤0.015-0.25)
	Yersinia enterocolitica	(0.016-0.06)	0.06-0.12	0.015-0.03

2. EFFLUX PUMPS IN PROKARYOTIC AND EUKARYOTIC CELLS

For long time, it has been considered that biological membranes were only physical barriers which aimed to separate intra- and extracellular media. Nowadays, we know that the cellular membrane plays a preponderant role in physiological processes because it contains essential components for the cellular life and exchanges with its environment. Prokaryotic or eukaryotic cellular membranes are dynamic assemblies of (i) phospholipids organized in bilayer which protects the cell from hydrophilic molecules and allows a selective permeability, and (ii) proteins which can play different roles as receptors in order to transmit information from the extracellular to the intracellular medium, or transporters to allow some molecules to go in or out of the cell. Amphiphilic molecules can diffuse passively throughout the phospholipid bilayer and accumulate within the cells, which could engender potential cytotoxicity. Therefore, cells have developed some defense mechanisms to protect themselves against invasion by toxic diffusible molecules. One of these mechanisms consists of the expression of efflux proteins at the plasma membrane which can expel toxic substances out of the cell.

Efflux proteins are present in prokaryotic cells as well as in eukaryotic cells. They can be constitutively expressed and are thought to play an important role in cell survival (protein and lipid homeostasis, cell communication…). Their overexpression is also inducible to fight against potentially toxic invasions (chemotherapeutic drugs, xenobiotics in general). Two categories of proteins insure the crossing of molecules throughout membranes: channels and transporters. We will focus on efflux transporters, mainly on those playing a role in drug resistance in either prokaryotic or eukaryotic cells.

2.1. Efflux pumps in prokaryotic cells

Antibiotic resistance in bacteria can occur by four main mechanisms: alteration or protection of the antimicrobial target (usually by a mutation in the drug target), alteration of drug access to the target (expression of efflux pumps, or impermeabilization of the bacterial outer membrane), inactivation of the antimicrobial agent (expression of β-lactamases to inactivate β-lactam antibiotics for example), and/or existence of an alternative target, or multiplication of the target. Here we will focus our attention on the active drug efflux mechanisms which generally lead to low level of resistance, but which are important when combined to other resistance mechanisms. Such a mechanism was

observed for the first time for tetracyclines by McMurry and colleagues in 1980 (McMurry et al. 1980), and since this date, a large number of efflux pumps has been discovered and classified following their substrate specificity and their mechanism of action, as presented in Figure 8. Five families/superfamilies are better known and studied in prokaryotic cells (ATP-binding-cassette (ABC) transporters, major facilitator superfamily (MFS), resistance-nodulation-cell division (RND) superfamily, small multidrug resistance (SMR) family and multidrug antimicrobial extrusion (MATE) family), and I will briefly describe the three first which are of particular interest for this thesis .

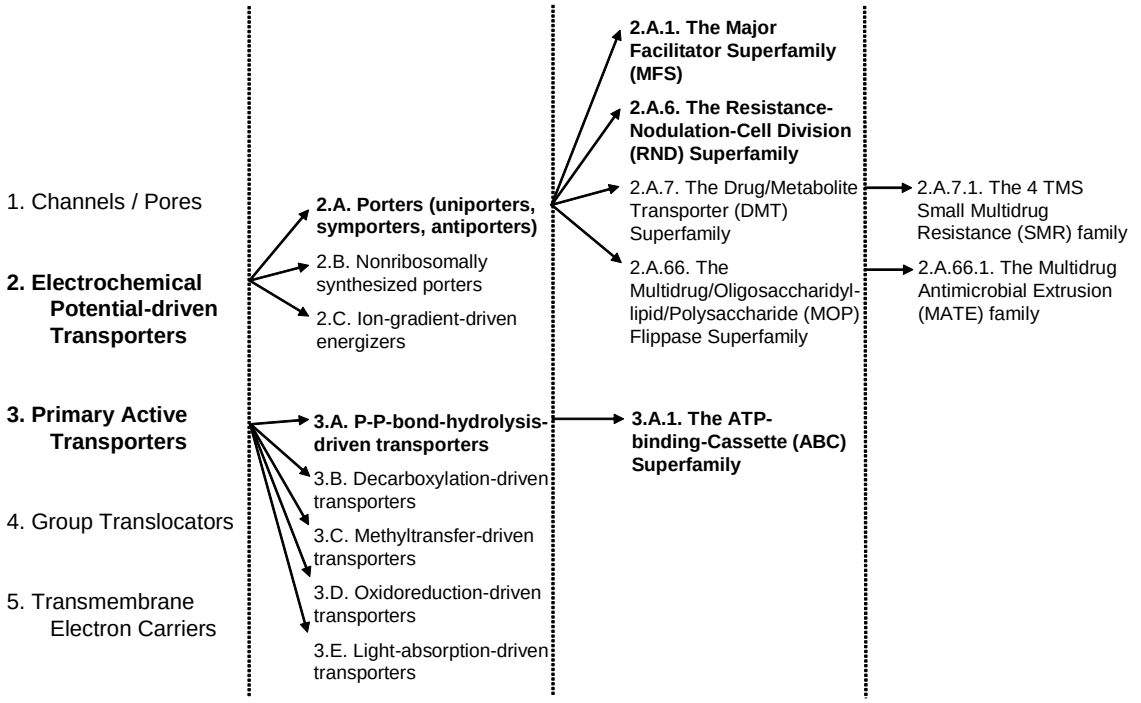

Figure 8. The transporters classification. In bold, the group, superfamilies and families of interest for this thesis. (From www.tcdb.org, Saier Lab Bioinformatics Group).

2.1.1. ABC, MFS and RND transporters : structure and mechanism of action

These three superfamilies of proteins are present in both eukaryotes and prokaryotes, and are able to transport physiological substrates as well as drugs, but we will focus here on the bacterial efflux proteins and transporters involved in the multidrug resistance (MDR). Figure 9 shows a schematic representation of these MDR transporters.

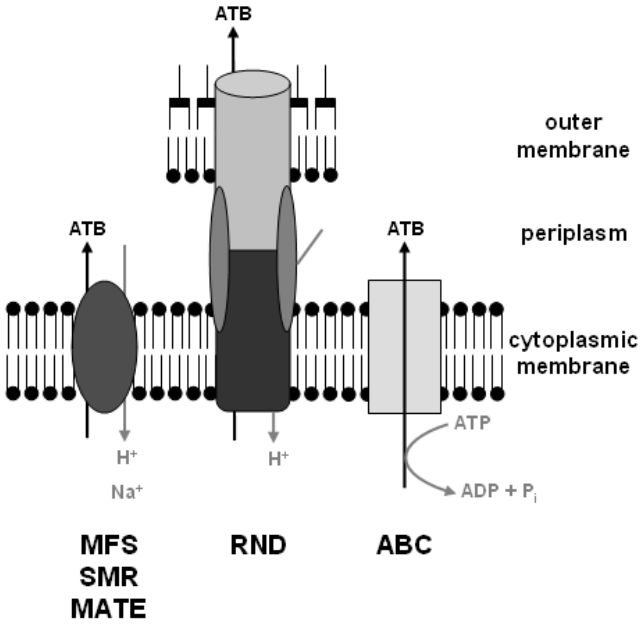

Figure 9. Schematic representation of the five families of bacterial transporters (ATB : antibiotic).

Porters from the ABC superfamily consist of two transmembrane domains (TMD) which form the core of the protein, and two cytoplasmic nucleotide-binding domains (NBD) where the ATP can bind. As we can see on Figure 8, ABC efflux proteins are included in the group of primary active transporters, and therefore use the energy of the ATP hydrolysis to transport drugs from the inside to the outside of cells. Their structure and mechanism of action will be described in more details in the next chapter (Efflux pumps in eukaryotic cells), because they are more commonly found in eukaryotic cells. Briefly, when the substrate enters in the protein, ATP is hydrolyzed in ADP and inorganic phosphate, inducing a modification of the protein conformation, leading to the release of the substrate in the extracellular medium.

MFS and RND transporters are superfamilies of the electrochemical potential-driven transporters and are part of the porters group (we will focus here in the antiporters). They are structurally different: MFS transporters consist of one protein and are found mostly in Gram positive bacteria, whereas RND transporters consist of a tripartite structure made of three proteins (a membrane-associated drug/proton antiporter, membrane fusion proteins, an outer membrane channel-forming protein (OMF)) and are found mostly in Gram negative organisms. As they are part of the same

group called porters, they share the same mechanism of action: they utilize a carrier-mediated process to catalyze the efflux reaction of the drug where two species are transported in opposite directions in a tightly coupled process not directly linked to a form of energy other than chemiosmotic energy. For the MFS antiporters, it seems that the protein works using a rocker-switch mechanism ((Tamura et al. 2003); (Law et al. 2008); (Gbaguidi et al. 2004)): while the substrate binds to its binding-site in the efflux protein (which is thought to be at the N-terminal extremity helices of the protein), the proton translocation starts at the C-terminal extremity of the protein, some salt bridges formation and breakages appear between the residues involved in the binding of the substrate, and a global conformational change of the protein induces the translocation of the substrate and a proton in the opposite sides. For RND pumps, which are tripartite proteins, drug is captured in the outer leaflet of the inner membrane and extruded in a coupled exchange with a proton (as for MFS). The drug goes then through the channel formed by the outer membrane protein to gain the outside of the bacteria (membrane fusion proteins mediate contacts between OMF and the membrane-associated drug/proton antiporter) ((Pos 2009); (Nikaido and Takatsuka 2009); (Misra and Bavro 2009)).

It is known that some of these efflux proteins can be drug-specific, and confer resistance to one class of antibiotics, or they can expel structurally different compounds, thus confering resistance to two or more classes of antibiotics (see (Nikaido 1994); (Hooper 2005a); (Cattoir 2004); (Lomovskaya and Watkins 2001)). Genes encoding drug-specific pumps are often found on mobile elements (plasmids or transposons) and most of them are inducibly expressed by given antibiotics, whereas those encoding MDR pumps are mostly chromosomal and seem to have a physiological role in the bacterial cell metabolism (Butaye et al. 2003). For the MFS transporters, it seems that there is probably an individual transporter for each class of antibiotic extruded, and that a few amino acid residues at the single substrate-binding site determines the specificity of each transporter for its cognate substrate (Law et al. 2008). Compared to MFS transporters, RND proteins expel a large variety of drugs, which is thought to be principally due to the multiple sites of interactions for various structurally compounds contained in large periplasmic loops of the membrane-associated drug/proton antiporter (Mao et al. 2002).

2.1.2. Mex-Opr efflux system in *Pseudomonas aeruginosa*

Since their discovery in the 1990s ((Li et al. 1994); (Li et al. 1995)), the RND transporters have been extensively investigated and it is now possible to determine their structure: one protein spanning the cytoplasmic membrane is associated with two other proteins to expel the drug from the cytoplasm of the bacterium to the extracellular medium. There are several transporters in *Pseudomonas aeruginosa* which constitute the Mex-Opr efflux system: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK-OprM and MexXY-OprM, where MexB/D/F/K/Y are the cytoplasmic membrane-associated drug-proton antiporter of the RND family, MexA/C/E/J/X are the periplasmic membrane fusion proteins (MFP) and OprM/J/N are the outer membrane channel-forming proteins (OMF) (Poole 2001). These RND transporters can be constitutively expressed, as MexAB-OprM ((Li and Nikaido 2009); (Morita et al. 2001)) or MexXY-OprM, conferring an intrinsic resistance to a variety of antibiotics. But they can be expressed only when mutations occur in their repressor or activator genes as MexCD-OprJ and MexEF-OprN (Schweizer 2003), consisting of an inducible resistance mechanism.

This Mex-Opr efflux system in *Pseudomonas aeruginosa* is able to expel a large variety of antibiotics as chloramphenicol, tetracyclines, fluoroquinolones, β-lactams, macrolides, aminoglycosides (Cattoir 2004; (Piddock 2006); (Nikaido 1996); (Nikaido 1998)). Focusing on fluoroquinolones (which are substrates of MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Schweizer 2003)), it has been shown that they act as inducers of the expression of these transporters (Kohler et al. 1997), with molecules of the second generation like ciprofloxacin, norfloxacin, ofloxacin and enoxacin inducing mainly *mexEF-oprN* (see also (Llanes et al. 2011) for the induction of *MexEF-OprN* by ciprofloxacin) and *mexCD-oprJ*, and fluoroquinolones of the first generation like nalidixic acid, oxolinic acid, flumequine and pipemidic inducing mainly *mexAB-oprM* and *mexEF-oprN*. Tohidpour A and colleagues (Tohidpour et al. 2009) showed that ciprofloxacin induces the overexpression of the Mex-Opr efflux system in 35% of resistant *Pseudomonas aeruginosa*. Usually, resistance to fluoroquinolones in *Pseudomonas aeruginosa* can result only from the overexpression of the Mex-Opr efflux system, but for some fluoroquinolones, it needs a target enzyme alteration to be clinically relevant ((Speciale et al. 2000); (Dunham et al. 2010)).

2.1.3. NorA in *Staphylococcus aureus*

Due to the increase in methicillin-resistant *Staphylococcus aureus* (MRSA), some quinolones were used to treat infections caused by this pathogen, but the emergence of quinolone resistance has been rapidly reported (Shalit et al. 1989). In 1989, Ubukata K and colleagues (Ubukata et al. 1989) cloned the *norA* gene which confers resistance to fluoroquinolones in *S. aureus*. NorA belongs to the MFS family of transporters, and is thus working as an antiport which needs the proton motive force to expel the drug outside of the bacteria. This protein is encoded by a chromosomal gene and is composed of 388 amino acids which formed 12 transmembrane-spanning regions (Yoshida et al. 1990). This efflux pump has been shown to have 44% amino acid identity and 67% similarity with Bmr, a multidrug efflux pump from *Bacillus subtilis* (Piddock 2006), and that NorA is structurally similar to the TetA, TetB and TetC proteins which efflux tetracyclines only (Neyfakh 1992).

It is now known that NorA is able to expel chloramphenicol and fluoroquinolones only. NorA expression has a complex regulation, but it has been shown that some proteins could bind to the *norA* promoter region and induce the overexpression of the NorA protein (Truong-Bolduc et al. 2003), and that mutations in the promoter or in the coding region of the *norA* gene can also lead to the resistance to fluoroquinolones ((Schmitz et al. 1998); (Ohshita et al. 1990)). Kaatz GW and Seo SM (Kaatz and Seo 1995) showed that the NorA-mediated multidrug resistance in *S. aureus* is inducible by exposure of the bacteria to a substrate of the NorA efflux pump as ciprofloxacin. An important point in the quinolone resistance conferred by NorA is that all fluoroquinolones have not the same susceptibility to the NorA-mediated efflux: indeed, it has been observed that NorA can transport hydrophilic fluoroquinolones as norfloxacin, ciprofloxacin or enoxacin, but not the lipophilic ones as moxifloxacin or sparfloxacin ((Neyfakh et al. 1993); (Truong-Bolduc et al. 2003); (Gootz et al. 1999) ; (Fukuda et al. 1998)). The reason why NorA transports hydrophilic fluoroquinolones more effectively than hydrophobic ones remains undetermined (Kaatz et al. 1993), but the development of new fluoroquinolones should take into account these properties to decrease the sensitivity to the efflux conferred by this efflux pump.

2.1.4. PatA/PatB in *Streptococcus pneumoniae*

Among the efflux pumps found in *Streptococcus pneumoniae*, PmrA (from the MFS family of transporters) was the first discovered which was thought to confer resistance to fluoroquinolones ((Gill et al. 1999); (Poole 2000)). But this efflux pump has been shown to confer fluoroquinolone resistance in *S. pneumoniae* only when overexpressed. The existence of another efflux system for fluoroquinolones in this microorganism has been proposed, which would be more effective than PmrA ((Piddock and Johnson 2002); (Piddock et al. 2002); (Varon and Gutmann 2000); (Martinez-Garriga et al. 2007)). Brenwald NP and colleagues (Brenwald et al. 2003), and Pestova E and colleagues (Pestova et al. 2002) in 2002 observed an ATP energized efflux of fluoroquinolones in *S. pneumoniae* which is caused by PatA and PatB efflux proteins ((Marrer et al. 2006a); (Marrer et al. 2006b)). This kind of efflux pumps are from the ABC superfamily and need the hydrolysis of ATP to efflux the drug in the extracellular media. Recent studies have shown that this PatA/PatB efflux pump is responsible for the fluoroquinolone resistance phenotype in *S. pneumoniae*, and that this efflux mechanism is inducible by fluoroquinolones ((Avrain et al. 2007) (Garvey and Piddock 2008) (Garvey et al. 2011) (El Garch et al. 2010)). Literature about this PatA and PatB efflux system is scarce because of its recent discovery, but this efflux pump is now to take into account for the development of new fluoroquinolones which would not be affected by efflux.

2.2. Efflux pumps in eukaryotic cells

As for prokaryotes, active efflux of drugs is a known mechanism of resistance in eukaryotes. The proteins involved in these efflux mechanisms are of the same families as in bacteria (Van Bambeke et al. 2000), but the primary active transporters are predominant in eukaryotes. This chapter will be devoted to the ATP-binding cassette (ABC) transporters, the best studied of the efflux pumps superfamilies. The existence of such efflux pumps in eukaryotic cells has been known since the discovery of P-glycoprotein (P-gp) (Juliano and Ling 1976), but it is only in 1986 that Chen (Chen et al. 1986) identified the multidrug transporter gene *MDR1* from a multidrug-resistant carcinoma cell line. To date, many ABC transporters have been found to play an important role in the physiology of cells by transporting hydrophobic compounds, and thus in various diseases when affected in their function.

2.2.1. Classification, structure and mechanism of action of ABC transporters

ABC transporters superfamily consists of 49 genes encoding 49 transmembrane proteins in humans. These genes are localized in different chromosomes, and following their organization and the homology between amino acids, they are classified into seven families from ABCA to ABCG (Dean et al. 2001).

Their general structure is described as followed: to be functional, an ABC transporter must have two transmembrane domains (TMD), and two nucleotide binding domains (NBD) ((Lage 2003); (Kos and Ford 2009)). TMDs are hydrophobic, membrane-embedded domains of 6 transmembrane α-helices, and are believed to determine the specificity for the substrate molecules transported by the ABC transporter. NBDs are localized in the cytoplasm of cells, and can bind ATP molecules to hydrolyze and thus provide the energy for the function of the transporter. NBDs are constituted by 3 characteristic sequences: (i) the Walker A (GxxGxGKS/T) and (ii) Walker B (ΦΦΦΦDE) motifs, where Φ is any aliphatic residue, which are involved in the binding of the ATP, and are commonly present in all proteins which have an ATPase activity (Walker et al. 1982), and (iii) the C signature consensus sequence (LSGGQ), which is localized between the two Walker motifs, and is determinant for the belonging to the ABC transporters superfamily (Hyde et al. 1990). As we can see on Figure 10 showing the organization of TMDs and NBDs for the different ABC transporters, (i) some families are considered as half transporters (for example ABCG family), containing only one TMD and one NBD, and which form dimers to be functional, (ii) some proteins are called long ABC transporters because of the presence of an additional TMD containing 5 α-helices at the N-terminus extremity of the protein.

The mode of action of ABC transporters is not yet fully elucidated, but some studies have tried to determine a mechanism which can be summarized as followed : when a substrate enter in the binding site of the ABC transporter (it is not clear if it enters by the cytoplasm or by the membrane, even if recent studies favor the second mechanism), two ATP molecules will bind and lead to the closing of the NBDs, which modifies the conformation of the TMDs and leads to the release of the substrate in the extracellular medium. ATP molecules are then hydrolyzed in ADP and inorganic phosphate, the NBDs are separated and the ABC transporter returns to its initial conformation (Figure 11) ((Seeger and van Veen 2009); (Eckford and Sharom 2009); (Linton 2007)).

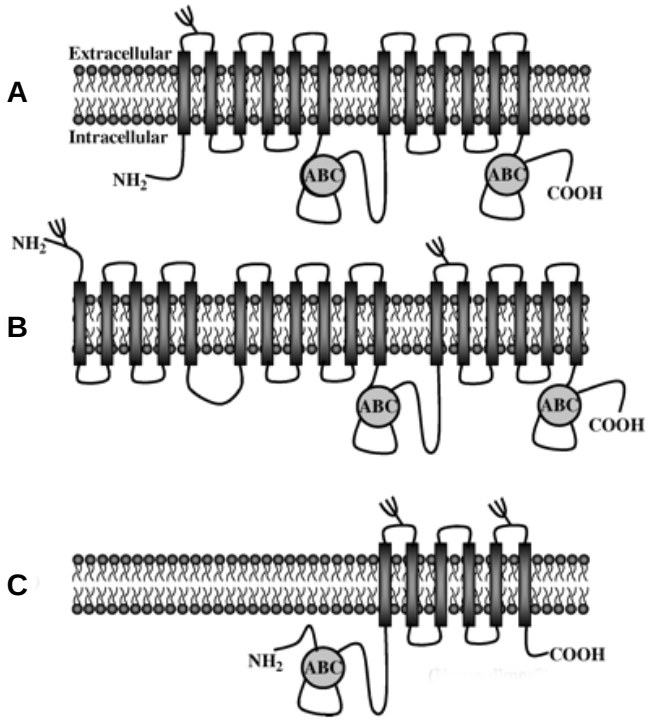

Figure 10. (Ford J et al., 2004). Membrane topology of ABC transporters. ABC = ATP Binding Cassette (= NBD). **A**. ABC transporter with 2 NBDs, 2 TMDs (= 2 x 6 helices α). **B**. Long ABC transporter with 2 NBDs, 3 TMDs (2 x 6 helices α + 5 helices α at the N terminal extremity). **C**. A half ABC transporter with 1 TMD, 1 NBD.

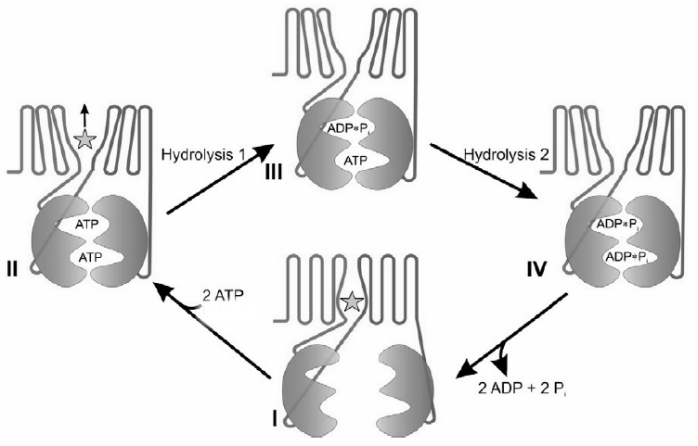

Figure 11. (Seeger MA and Van Veen HW, 2009). Schematic drawing of the mechanistic model for ABC transporters. **I**. NBDs are separate and one drug is bound at the inner membrane leaflet. **II**. Binding of ATP closes the NBD dimer and induces the high to low affinity transition. **III** and **IV**. Two processive hydrolysis steps destabilize the NBD dimer interface.

2.2.2. Localization, substrates and functions of ABC transporters

(Table 3 shows a summary of localization, physiological and pharmacological substrates of P-gp, MRP1, MRP2, MRP4 and BCRP).

In general, ABC transporters are ubiquitously distributed, meaning that they are localized in the whole body, but mainly at the level of epithelial barriers of organs as brain, intestine, kidney, liver, placenta, lungs, and some of them are found in subcellular compartments such as the mitochondria or lysosomes (see Dean et al. 2001 for all 48 ABC transporters localization, (Marquez and Van Bambeke 2011) for the ABC transporters localization involved in the MDR, and (Oostendorp et al. 2009) for the ABC transporters present at the intestinal barrier).

Because of their ubiquitous presence, and their very wide variety of physiological substrates, ABC transporters have important physiological functions. Indeed, these efflux proteins are able to facilitate the transport of various endogenous as well as exogenous substances such as amino acids, peptides, proteins, lipids, heavy metallic ions, sugars or hydrophobic compounds. They therefore have essential physiological roles for example in the excretion of toxins by the liver or kidneys, or in the lipid homeostasis of cells, or in the limitation of penetration of toxic compounds in the brain or placenta. Some of the ABC efflux transporters are well known to transport drugs, thus making them key players in the pharmacokinetic properties (absorption, distribution, metabolism and excretion) of many drugs. Numerous pathologies are associated to an altered physiological function of ABC transporters ((Dean and Allikmets 2001); (Ueda 2011)), among the most known: Tangier disease (loss of circulating HDL and build-up of cholesterol in the peripheral tissues) is due to a mutation in the *ABCA1* gene which alters the ability of the protein to export phospholipids and cholesterol, Dubin-Johnson syndrome (increased bilirubin in the serum) is caused by the alteration of ABCC2 in the liver, and the Cystic Fibrosis or mucoviscidosis (impairment of various organs by the production of abnormal secretions leading to mucous build-up) is due to a mutation in the *ABCC7* gene (Cystic Fibrosis Transmembrane conductance Regulator = CFTR) which prevents this chloride channel to be functional.

In addition to their physiological functions, it is well known that some of these ABC transporters are involved in the multidrug resistance (MDR), particularly for the chemotherapy drugs used in the treatment of various cancers. Three subfamilies of the ABC transporters contain proteins involved in the MDR: the ABCB subfamily (which

includes ABCB1 also known as P-glycoprotein (P-gp)), the ABCC subfamily (which includes ABCC1 also known as MRP1 (Multidrug Resistance Related Protein 1)), and the ABCG subfamily (which includes ABCG2 also known as BCRP (Breast Cancer Resistance Protein)). These efflux pumps have been discovered as factors overexpressed in MDR cell lines in culture, and they have since been detected in MDR tumors in patients. When cells are in contact with toxic agents (such as anticancer drugs), the MDR phenotype will develop as a defense mechanism in order to eliminate the toxic substance (as for antibiotics in bacteria). Among several resistance mechanisms developed by eukaryotic cells, the overexpression of an efflux pump is the best characterized. We will discuss hereafter about three ABC transporters: BCRP and P-gp in general, and MRP4 in particular.

Table 3. Localization, physiological and some pharmacological substrates of 5 ABC transporters

ABC transporter	Localization	Tissue distribution	Physiological substrates	Pharmacological substrates (anti-infectives and anti-cancer agents)
P-gp (ABCB1)	Apical	Kidney, adrenal gland, liver, pancreas, intestine, lung, BBB, placenta	Phospholipids, cytokines, steroids	Tetracyclines, β-lactams, macrolides, FQ, methotrexate, docetaxel, paclitaxel, etoposide, doxorubicin, topotecan
MRP1 (ABCC1)	Basolateral (except in placenta and BBB)	Kidney, lung, testis, skeletal and cardiac muscles, placenta	Glutathiones, glutathiones conjugates (LTC_4, DNP-SG), bilirubin glucuronides, bile salts	Macrolides, FQ, methotrexate, vinblastine, doxorubicin, irinotecan
MRP2 (ABCC2)	Apical	Liver, kidney, small intestine, placenta	LTC_4, DNP-SG, bilirubin glucuronides, sulphated bile salts	β-lactams, macrolides, ketolides, FQ, methotrexate, docetaxel, paclitaxel, etoposide, doxorubicin, irinotecan
MRP4 (ABCC4)	Apical (kidney, BBB) or basolateral (prostate, choroid plexus)	Prostate (low expression in liver, kidney, brain, pancreas, ovary, testis, lung, intestine)	cAMP, cGMP, bile salts, folate, conjugated steroids, prostaglandins (PGE1, PGE2)	Tetracyclines, β-lactams, FQ, methotrexate, irinotecan, topotecan
BCRP (ABCG2)	apical	Placenta, breast, BBB, liver, intestine	Vitamins (riboflavin, biotin), porphyrins, estrogen sulfate conjugates	β-lactams, FQ, methotrexate, doxorubicin, irinotecan, topotecan

BBB : blood-brain barrier ; LTC_4 : leukotriene C_4 ; DNP-SG : 2,4-dinitrophenyl-S-glutathione ; FQ : fluoroquinolones.
To see all the pharmacological substrates, (Marquez and Van Bambeke 2011).

2.2.3. P-glycoprotein and BCRP in multidrug resistance

2.2.3.1. P-glycoprotein

P-gp, also referred as MDR1 or ABCB1, is a transmembrane protein of 1280 amino acids (170 kDa) which contains 12 transmembrane segments, and is found at the apical part of the plasma membrane of cells from several tissues (intestine, liver, kidney, blood-brain barrier, testis, placenta and lung). The three-dimensional structure of the murine P-gp has been recently resolved at a 3.8 Å resolution (Figure 12), and will be very helpful for the understanding of the substrate binding and the mechanism of transport of the ABC transporters in general.

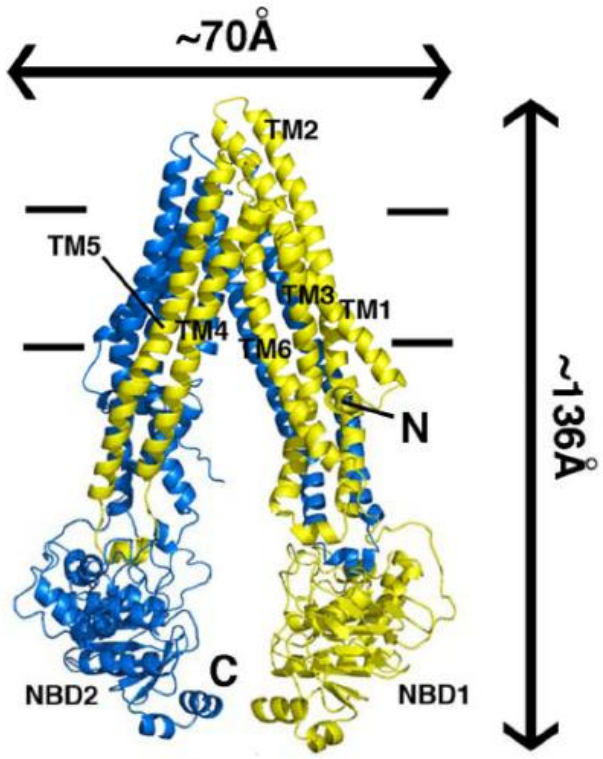

Figure 12. Structure of P-gp (inward facing) (Aller et al. 2009), front view. Transmembrane segments (TM) 1 to 6 (yellow) are labeled on the figure, and TM 7 to 12 are represented in blue (not labeled on this figure). The N- and C-terminal of the protein are labeled as N and C. The nucleotide binding domains are labeled as NBD1 and NBD2. Horizontal bars represent the approximate positioning of the lipid bilayer.

As we have mentioned before, P-gp has a large variety of pharmacological substrates ((Marquez and Van Bambeke 2011); (Fromm 2004)), and this transporter will therefore modulate the pharmacokinetics of numerous drugs among which drugs used in chemotherapy, hypertension, allergy, infection, immunosuppression, neurology and inflammation. The apical localization of P-gp in hepatocytes, enterocytes, and proximal tubular cells in the kidney and on the luminal side of the BBB suggests that this transporter plays a significant role in the bioavailability, distribution and excretion of its drug substrates. Its physiological protective role has been evidenced for example in mice where *Mdr1a* (the gene coding for P-gp in mouse) was inactivated (Lankas et al. 1997): ivermectin (an antiparasitic agent substrate of P-gp) accumulation in brains of mice where P-gp wasn't functional was 80-fold higher than in mice where P-gp was functional, and leads to neurotoxicity.

But the downside of P-gp's ability to pump xenobiotics out is that it interferes with the delivery of drugs to their target tissues or cells. This phenomenon is now well-known in chemotherapy, where the *mdr1* gene is overexpressed following a chemotherapy using a P-gp drug substrate. In 2003, Scotto KW (Scotto 2003) showed that the *mdr1* gene expression can be induced by a variety of chemotherapeutic agents, and a lot of studies show an overexpression of P-gp, in response to chemotherapy, which leads to therapeutic failure. P-gp regulation is complex and differs following the cell line used, and the drug used: indeed, it has been shown that a demethylation of the *mdr1* promoter induced the expression of the gene ((El Osta and Wolffe 2000); (El Osta et al. 2002)) ; a high *mdr1* mRNA stability can lead to the overexpression of P-gp (Lee et al. 2008), and atorvastatin treatment can induce a decrease in mdr1 mRNA stability therefore an underexpression of P-gp (Rodrigues et al. 2009); a mutation in the mdr1 promoter can induce the expression of P-gp (Rund et al. 1999); genetic rearrangement, or duplication of the gene were also evidenced in resistant cells where P-gp is overexpressed ((Mickley et al. 1997); (Knutsen et al. 1998); (Albertson 2006)).

P-gp inhibitors have been developed, such as verapamil, but this kind of molecules is used only *in vitro* as they are generally too toxic to be used in clinic (in addition, inhibitors target efflux pumps in all body compartments, which can lead to affect the physiological role of these proteins). With the recent discovery of the crystal structure of P-gp, new studies about the structure-activity relationship can be investigated and thus new chemotherapeutic drugs (not substrate of P-gp) can be developed.

2.2.3.2. BCRP

BCRP, also designated as ABCG2 or MXR (mitoxantrone resistance factor), is a member of the G subfamily of ABC transporters. It is a half transporter (75 kDa) which contains 6 transmembrane domains and one NBD (Ni and Mao 2011), and therefore is dimerized to be a functional efflux pump. It has been discovered in 1998 in a multidrug resistant human breast cancer cell line MCF-7 (Doyle et al. 1998).

This transporter is widely distributed, as all ABC transporters, and it is found in tissues including intestine, liver, testis, placenta and the BBB (Meyer zu Schwabedissen and Kroemer 2011). As P-gp, it has physiological roles such as pumping vitamins into milk or it can be useful as a xenobiotic clearance mechanism for the mother (Vlaming et al. 2009). But BCRP can also play a critical role in the MDR in that its substrates include a large amount of chemotherapeutic drugs, such as mitoxantrone, doxorubicin, topotecan, or antibiotics as fluoroquinolones. Selection of cells with antitumor drugs cited before results in significant induction of BRCP, leading to a lower accumulation and prevention of anticancer drugs to reach their intracellular targets ((Honjo et al. 2002); (Kawabata et al. 2001); (Robey et al. 2001); (Takara et al. 2009)). The overexpression of BCRP can be due to several factors, among which: a demethylation of CpG islets in the ABCG2 promoter resulting in increased gene transcription ((Bram et al. 2009); (Turner et al. 2006)), a gene amplification (Bram et al. 2007), or a truncation at the 3'UTR of the ABCG2 mRNA associated with a loss of the miRNA-159c binding site conferring higher mRNA stability ((Apati et al. 2008); (Sandberg et al. 2008); (To et al. 2008); (To et al. 2009)). Here again, BCRP inhibitors have been discovered and developed such as fumitremorgin C (FTC) and its analogue Ko143, anti-HIV protease inhibitors as ritonavir, or the tyrosine kinase inhibitors gefitinib and imatinib. Among these inhibitors, some are very promising because of their IC_{50} values in a nM range (Ko143 (Allen et al. 2002) (van Loevezijn et al. 2001)), GF120918 (de Bruin et al. 1999), gefitinib (Ozvegy-Laczka et al. 2004), imatinib (Houghton et al. 2004)), and their high selectivity for BCRP only. But data about the drug binding and the mechanism of transport of this efflux pump (as all ABC transporters) remain still poor, and these domains need to be investigated in order to develop new molecules that would not be substrate for BCRP.

2.2.4. Multidrug resistant associated protein 4 (Mrp4)

2.2.4.1. Structure, localization, substrates and function

MRP4, also referred as ABCC4, is a transmembrane protein of the C subfamily of ABC transporters discovered in 1997, and contains 12 transmembrane segments and 2 NBDs as P-gp (in contrast to some MRP have a third TMD which contains 5 α helices and where the N-terminal extremity of the protein is in the extracellular medium) (Choudhuri and Klaassen 2006). MRP4 is expressed at high level in prostate, and at low to moderate levels in ovary, testis, adrenals, lung and intestine (Deeley et al. 2006)), and as MRP1, it can be localized at the apical membrane of cells in the kidney proximal tubule and in brain capillaries, but at the basolateral membrane in prostate cells, choroid plexus and hepatocytes (Keppler 2011).

The first identified substrate of MRP4 was PMEA (9-(2-phosphonylmethoxyethyl)adenine), an antiretroviral drug (Schuetz et al. 1999). Its physiological substrates were discovered in the 2000 years and count about 20 molecules among which the best known are: cGMP, cAMP, leukotriene C_4, prostaglandin E_2, folate and bile acids with reduced glutathion. The physiological role of MRP4 as a transporter of cAMP could be involved in cell signalling and proliferation (Sassi et al. 2008), and Van de Ven and colleagues (van, V et al. 2008) shows that MRP4 was an important factor for the dendritic cell migration. The differential localization of MRP4 is ideal for the pharmacokinetic of multiple drugs in that it can transport a substrate from hepatocytes to blood in the liver (apical localization), or from renal cells to the lumen in the kidney (basolateral localization).

Inhibitors directed against MRP4 may be of therapeutic interest to interfere with the cellular release of proinflammatory mediators such as prostaglandins and leukotrienes. But inhibitors developed until now are not specific against one MRP in particular, but they inhibit all MRPs and are used in research to demonstrate a transport by these proteins. As an example, the leukotriene D_4 receptor antagonist MK571 is the best inhibitor of MRPs while probenecid, gemfibrozil, or cyclosporin A are less specific , inhibiting also other anion transporters or P-gp.

2.2.4.2. MRP4 in multidrug resistance and its regulation

As for the majority of the MRP transporters, MRP4 is known to be involved in the transport of drugs and therefore involved in multidrug resistance. As MRP4 is described to transport purine and nucleotide analogues, it can mediate resistance to the class of pharmaceutical therapeutics used in the treatment of hepatitis B, such as PMEA (adefovir). Indeed, the MRP4 gene was found to be overexpressed in a cell line resistant to the nucleotide analogue PMEA (Schuetz et al. 1999), and other studies with nucleotide analogues and anti-viral treatments showed that they are able to induce the expression of MRP4: PMEA was shown to induce MRP4 expression in lymphoblastoid cell line and that this MRP4 overexpression can also induce resistance to azidothymidine (AZT) (Sampath et al. 2002); NIH3T3 cells transfected with MRP4 showed resistance to methotrexate and PMEA treatment (Lee et al. 2000); AZT treatment of human macrophages confers resistance to this drug (Jorajuria et al. 2004), and AZT combined with lamivudine and abacavir lead also to an overexpression of MRP4 in T-lymphoblastoid cell line (Turriziani et al. 2006); MRP4 can be overexpressed by a stepwise selection of human T-lymphoblastoid leukemia cell line with 6-mercaptopurine (Peng et al. 2008), and can confer resistance to a range of base, nucleoside and nucleotide analogues such as 6-mercaptopurine, thioguanine, PMEG (9-(2-phosphonomethoxyethyl)guanine), PME diaminopurine, and ganciclovir (Borst et al. 2004). A few studies showed that MRP4 is able to transport and confer resistance to the anticancer agent cisplatin ((Aleksunes et al. 2008); (Savaraj et al. 2003)), or to the non-steroidal anti-inflammatory drug celecoxib (Gradilone et al. 2008), or to the anti-topoisomerase I agents topotecan (Leggas et al. 2004) and irinotecan (Norris et al. 2005).

The mechanism by which MRP4 is overexpressed after a drug treatment is often unknown, but recent studies have investigated the regulation of MRP4 and have determined some regulatory pathways. This kind of studies has been mostly assessed in hepatocytes, and therefore involved the nuclear receptors. The regulation of MRP4 has been thus shown to be regulated by : Nrf2 (Nuclear factor-E2-realted factor 2) ((Maher et al. 2007); (Maher et al. 2008) ; (Aleksunes et al. 2010) ; (Cheng et al. 2011)), CAR (Constitutive androstane receptor) (Maher et al. 2005), PPARα (Peroxisome proliferator-activated receptor) ((Moffit et al. 2006) ; (Maher et al. 2008)), and AhR (aryl hydrocarbon receptor) (Xu et al. 2010). MRP4 regulation has also been recently investigated in

prostate cancer cells (because of its high expression level in prostate), and MRP4 has been identified as an androgen receptor-regulated gene as dihydrotestosterone induced its expression in prostate cancer cells ((Cai et al. 2007); (Ho et al. 2008)).

2.3. Fluoroquinolone efflux and ABC transporters in eukaryotic cells

As explained before, nine ABC transporters are involved in the multidrug resistance, namely ABCB1 (P-gp), members of the C subfamily (ABCC1-6 and ABCC10, also referred as MRP1-7), and ABCG2 (BCRP). Their role in the MDR is well described for the resistance to anticancer drugs in eukaryotic cells, but as we have seen, they are able to transport a wide variety of substrates belonging to unrelated pharmacological classes, among which antibiotics, and in particular fluoroquinolones. With a bioavailability of nearly 90%, fluoroquinolones are rapidly absorbed and distributed in body tissues, but they have been reported to undergo efflux, which could explain the lower bioavailability of some of these antimicrobials (ciprofloxacin and norfloxacin bioavailability is 50-80% and 30-40% respectively). Indeed, Rabbaa L. and colleagues (Rabbaa et al. 1997) were the first to hypothesize that ciprofloxacin and ofloxacin could be P-gp substrates, and undergo intestinal elimination. More recent studies have reported that ciprofloxacin and enrofloxacin are efficiently transported by BCRP, therefore are actively secreted into milk and could reach toxic concentrations. These efflux transporters are clearly involved in the fluoroquinolone distribution in body tissues, as some experiments showed that the brain distribution of sparfloxacin was higher in mdr1a-/- mice compared to that in wild-type mice where Pgp is present (de Lange et al. 2000).

This fluoroquinolone active efflux is to be considered as a major problem, as it can affect their bioavailability thus decreasing their activity against microbial pathogens. Moreover, active efflux can lead to higher concentrations of fluoroquinolones in some body compartments which could increase the incidence of adverse side effects. Table 4 summarizes all studies that have been conducted to evidence fluoroquinolone efflux mediated by ABC transporters.

Table 4. Fluoroquinolone efflux is mediated by P-gp, MRP1, MRP2, MRP4 and BCRP. List of all the fluoroquinolones which have been reported to be effluxed in eukaryotic cells. Each number represents a publication which reports the efflux of a fluoroquinolone by one or several of the ABC transporters listed in the table.

	P-gp	MRP1	MRP2	MRP4	BCRP
ciprofloxacin	[4] [19] [32]			[10]	[1] [10] [13]
moxifloxacin	[3] [4]				
ofloxacin	[2] [18] [23]	[27] [33]			[1] [13]
levofloxacin	[2] [4] [11] [18] [24] [28]				
grepafloxacin	[4] [12] [15] [19] [21] [24] [25] [28] [29]	[17] [21] [25]	[12] [15]		[1]
norfloxacin	[4] [29]	(MRP1 or MRP2 or MRP4) [30] [31]			[13]
sparfloxacin	[5] [7] [19] [24] [25] [29]				
gatifloxacin	[2] [6]		[6]		
enrofloxacin					[16]
danofloxacin	[9] [22]		[22]		
tosufloxacin	[29]				
ulifloxacin					[1]
CNV 97100	[8] [20]				
HSR-903	[14] [26]		[26]		

References for this table are listed hereafter as their PMID number (PubMed) : [1] 17639028; [2] 21830912; [3] 19188390; [4] 19822706; [5] 9756763; [6] 20573570; [7] 11102735; [8] 17410527; [9] 17188652; [10] 21930826; [11] 9262363; [12] 11877335; [13] 16434544; [14] 10381759; [15] 11796340; [16] 16846465; [17] 15948026; [18] 8878593; [19] 12637104; [20] 12434394; [21] 15131241; [22] 17211460; [23] 19172473; [24] 15543082; [25] 10991972; [26] 11015683; [27] 10817732; [28] 10992002; [29] 11906478; [30] 1730899; [31] 1402686; [32] 8467353; [33] 9527807.

3. CONTEXT OF THE STUDY

3.1. Cellular pharmacokinetics of fluoroquinolones

Cellular pharmacokinetics are centered on evaluation of the penetration, distribution, metabolization and efflux of drugs in individual cells. These parameters are closely related to general pharmacokinetics because the cellular disposition of a drug governs its general fate in the body (Van Bambeke et al. 2006). Cellular pharmacokinetic studies are of particular interest for fluoroquinolones as these antibiotics are able to accumulate in cells, and therefore are useful to combat intracellular infections.

As amphiphilic molecules, fluoroquinolones generally enter cells by passive diffusion ((Easmon et al. 1986); (Cao et al. 1992); (Dorian et al. 1998); (Carlier et al. 1990)). But they could also be substrates for active uptake transporters in polarized epithelial cells. To support this hypothesis, recent studies, conducted by Takaai M and colleagues (Takaai et al. 2007) and Fukumori S and colleagues (Fukumori et al. 2008), demonstrated that levofloxacin apical uptake in Caco-2 cells was partly due to an active influx transporter not yet identified, but which was inhibited by molecules such as nicotine or L-carnitine. Moreover, Van Wert and colleagues (Vanwert et al. 2008) also showed that ciprofloxacin interacts with organic anion transporter 3 (OAT3), an uptake transporter found basolateraly in renal cells. And one publication has reported that active transport of quinolones could take place in human monocytes (non-polarized cells) (Bounds et al. 2000).

Fluoroquinolones have been found to accumulate in non phagocytic (as fibroblasts or epithelial cells) and in phagocytic cells (polymorphonuclear leukocytes and macrophages), but not in non-nucleated cells such as erythrocytes (Carlier et al. 1990). Considering accumulation, fluoroquinolone cellular concentrations are generally 4- to 10-fold higher than the extracellular ones for reasons which are not yet elucidated. When inside cells, these antibiotics are thought to be localized in the cytosol as during fractionation studies, all the quinolones were found in the soluble fraction (Seral et al. 2003a), but they are probably able to diffuse in the subcellular compartments.

Despite the rapid uptake and accumulation of fluoroquinolones inside cells, efflux is considered very fast when cells are transferred in drug-free medium. As described in Table 4, three of the widely used fluoroquinolones (ciprofloxacin, moxifloxacin and levofloxacin) are substrates of ABC efflux transporters. The presence of these multidrug

transporters contributes to reduce the absorption or to accelerate the elimination of these antibiotics when expressed at epithelial barriers, and to reduce the cellular accumulation of the drug in non-polarized cells. All together, this leads to suboptimal serum drug concentrations and to a decreased pharmacological activity of the quinolone toward intracellular targets.

A recent study has compared pharmacokinetic parameters of ciprofloxacin when administered orally or intrapulmonary (Chono et al. 2007), and is particularly interesting to understand the importance of pharmacokinetics. The intrapulmonary administration of ciprofloxacin avoids the general distribution of the drug in all body compartments by blood circulation, thus leading to higher intracellular ciprofloxacin concentrations in alveolar macrophages and epithelial lining fluid. Therefore, it leads to higher AUC ratio of epithelial lining fluid to plasma compared to oral administration, meaning that ciprofloxacin could have difficulty to cross the pulmonary endothelial cells (when orally administered), and/or that the distribution of ciprofloxacin to blood could be inhibited by a MDR transporter on the alveolar epithelium (when intrapulmonary administered).

3.2. Cellular pharmacodynamics/activity of fluoroquinolones

Fluoroquinolones are the only antibiotics which are bactericidal against intracellular bacteria. Many reports have shown that they are strongly active against intracellular *Legionella pneumophila* ((Edelstein et al. 1990); (Edelstein et al. 1992); (Edelstein et al. 1996) ; (Baltch et al. 1998) ; (Jonas et al. 2001)), *Staphylococcus aureus* ((Paillard et al. 2002); (Seral et al. 2003a)), *Mycobacterium tuberculosis* ((Sato et al. 2003); (Tomioka et al. 2000)), *Chlamydophila pneumonia* (Kutlin et al. 2002), and *Listeria monocytogenes* ((Facinelli et al. 1997); (Carryn et al. 2002)). Although fluoroquinolones accumulate in eukaryotic cells, their intracellular activity cannot be predicted from their accumulation level. Indeed, three parameters can modify the intracellular activity of a drug compared to its extracellular one: the cellular bioavailability of the drug, the intracellular expression of antibiotic activity, and the intracellular bacterial responsiveness to the drug.

It has been observed, for some *in vitro* experiments, that despite moxifloxacin accumulation level was higher in THP-1 monocytes than that of levofloxacin, moxifloxacin intracellular activity against *S. aureus* was not better compared to that of levofloxacin (Nguyen et al. 2007); and although ciprofloxacin and moxifloxacin

accumulation levels were almost the same in THP-1 monocytes, their intracellular activity was very different against *L. monocytogenes* (Carryn et al. 2002). Seral C and colleagues (Seral et al. 2005) also showed that, intracellularly, 4 fluoqoruinolones (ciprofloxacin, levofloxacin, garenoxacin and moxifloxacin) were 5 to 10 times less potent against *L. monocytogenes*, and at least 100 times less potent against *S. aureus* ; and in 2003 (Seral et al. 2003b), this team showed that ciprofloxacin and moxifloxacin activities against *S. aureus* are defeated in acidic pH medium. Taken together, these data confirmed that (i) cellular accumulation of a drug is not the only determinant of its intracellular activity (MICs must be taken into account), and (ii) local environmental conditions are critical for the activity of fluoroquinolones (probably due to different chemical characteristics of molecules). And although fluoroquinolones are bactericidal, it has been sometimes observed a lack of eradication of the infection even when concentrations used are beyond the MIC (for example with *L. monocytogenes* (Carryn et al. 2002), or with *S. aureus* (Paillard et al. 2002)). This could be explained by the inaccessibility or metabolically insensitive bacterial inoculums to fluoroquinolones.

However, the intracellular activity of fluoroquinolones is partially defeated by the acidic pH prevailing in the phagolysosomes where many bacteria are localized. In this context, two novel molecules in development (delafloxacin and finafloxacin) are promising because they show an increased activity in broth at acidic pH. Accordingly, they are also much more efficient against intracellular *S. aureus* and *L. pneumophila*, two phagolysosomal bacteria ((Lemaire et al. 2011b); (Lemaire et al. 2011a)). In addition, these molecules accumulate at higher levels the pH is acid (as in phagolysosomes).

3.3. Ciprofloxacin and moxifloxacin in eukaryotic cells: previous studies

Previous works of our laboratory showed different behavior for ciprofloxacin and moxifloxacin in terms of accumulation level and efflux in mouse J774 macrophages. Indeed, while ciprofloxacin accumulation is increased and efflux is lowered in J774 cells in the presence of MRP inhibitors (as probenecid, gemfibrozil and MK-571), moxifloxacin accumulation and efflux are fast and not affected by the presence of a MRP inhibitor ((Michot et al. 2004); Michot et al.2005). In addition, it has been demonstrated that the intracellular activity of ciprofloxacin was increased against *L. monocytogenes* when

MRPs were inhibited in J774 cells (Seral et al. 2003a). Therefore, it has been concluded that ciprofloxacin was a Mrp substrate while moxifloxacin was not.

It is known that prolonged exposure of eukaryotic cells to a toxic drug can select for resistance mechanisms, and particularly for the overexpression of a MDR transporter. Thus, J774 wild-type mouse macrophages chronically exposed to increasing concentrations (0.1 to 0.2 mM) of the Mrp substrate ciprofloxacin (Michot et al. 2006) showed a marked decrease in ciprofloxacin accumulation together with a faster efflux as compared to wild-type cells, while the accumulation and efflux of moxifloxacin remained unchanged. Moreover, ciprofloxacin accumulation level was higher in conditions of ATP depletion or in the presence of MRP inhibitors. Later, Marquez B and colleagues (Marquez et al. 2009) have shown that the ciprofloxacin decreased accumulation and faster efflux in ciprofloxacin-exposed cells was due to the overexpression of an Mrp transporter, identified as Mrp4 by using real-time PCR and western blot. To confirm this hypothesis, they also showed that ciprofloxacin accumulation was increased in cells exposed to this antibiotic when the *mrp4* gene expression was silenced using specific siRNA. All together, these data demonstrate that J774 wild-type cells exposed to ciprofloxacin have developed a resistance mechanism which consists of an increased ciprofloxacin efflux due to the overexpression of the Mrp4 efflux pump.

As previously described, antibiotic efflux from eukaryotic cells is a major problem as it will affect the pharmacokinetic of the drug and its intracellular activity. Accordingly, it has been demonstrated in the laboratory (Lismond et al. 2008) that (i) the intracellular activity of ciprofloxacin against *L. monocytogenes* was decreased when the bacterium was internalized in ciprofloxacin-exposed cells, but (ii) that of moxifloxacin was unchanged.

AIMS OF THE THESIS

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Antibiotics exert selective toxicity against bacterial cells. Yet, they are used to kill bacteria in order to treat animal and human infections. Therefore, they are in contact with eukaryotic cells, towards which they can cause toxicity. Anticancer drugs, which are highly toxic for eukaryotic cells, easily select for cells expressing resistance mechanisms, including overexpression of efflux systems or mutation in their pharmacological targets. In this thesis, we wondered whether fluoroquinolone antibiotics could lead to the development of resistance mechanisms in eukaryotic cells. To this effect, wild-type mouse macrophages (WT) were exposed to increasing concentrations of two fluoroquinolones, ciprofloxacin (a known substrate of the efflux transporter Mrp4), and moxifloxacin (non substrate for Mrp4). While ciprofloxacin-resistant cells (CIP-R) were previously described in our laboratory as displaying an overexpression of Mrp4 which is at the origin of a decreased accumulation and faster efflux of ciprofloxacin (Michot et al, 2006; Marquez et al, 2009), moxifloxacin-resistant cells (MXF-R) were not yet characterized.

The first aim of this thesis was therefore to better characterize both ciprofloxacin- and moxifloxacin-resistant cells with respect to their resistance phenotype. To this effect, we examined the kinetics of Mrp4 overexpression along the stepwise selection in CIP-R cells and determined the molecular mechanism for this overexpression. We also studied the phenotype of MXF-R cells with respect to the expression of ABC transporters. In parallel we examined the effect of ciprofloxacin and moxifloxacin on topoisomerases activity in WT, CIP-R and MXF-R cells, as these enzymes are homologous to their bacterial target.

The second aim of this thesis was to investigate the recognition and transport of fluoroquinolones by the Mrp4 transporter, to better characterize the structure-activity relationship defining substrate specificity and the consequences of this transport for fluoroquinolone pharmacological activity. To this effect, we compared the pharmacokinetic profiles of different fluoroquinolones in WT and CIP-R cells in relation with their activity against intracellular bacteria. We then compared the fluoroquinolone sensitivity to efflux by prokaryotic (NorA in *S. aureus,* PatA/PatB in *S. pneumoniae,* Mex/Opr in *P. aeruginosa*) and eukaryotic (Mrp4) efflux pumps in order to delineate common parameters important for recognition by these transporters by performing a combined biological and structural study.

RESULTS

CHAPTER I: RESISTANCE MECHANISMS INDUCED BY CIPROFLOXACIN AND MOXIFLOXACIN IN MOUSE MACROPHAGES

Ciprofloxacin and moxifloxacin, two fluoroquinolone antibiotics, have similar structures, but a different behavior with respect to efflux. Indeed, Michot JM and colleagues have shown in 2005 that ciprofloxacin is substrate for a Mrp efflux transporter while moxifloxacin is not. In 2006, Michot JM and colleagues showed that it was possible to select cells resistant to ciprofloxacin when J774 mouse macrophages were exposed to chronic and increasing concentrations of this antibiotic. Marquez B and colleagues, in 2009, characterized these ciprofloxacin-resistant cells by demonstrating an overexpression of the Mrp4 transporter, which expel the drug out of the cells.

The goals of this first chapter are:

- to determine the mechanism by which Mrp4 is overexpressed in ciprofloxacin-resistant cells

- to characterize phenotypically and genotypically cells chronically exposed to increasing concentrations of moxifloxacin

- to investigate the toxicity caused by these two fluoroquinolones in wild-type, ciprofloxacin- and moxifloxacin-resistant cells, in comparison to two anticancer agents (camptothecin and etoposide).

I.1. Characterization of Abcc4 gene amplification in stepwise-selected mouse J774 macrophages resistant to the topoisomerase II inhibitor ciprofloxacin

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PLoS ONE, December 2011, vol. 6, issue 12, e28368

OPEN ACCESS Freely available online

PLoS one

Characterization of *Abcc4* Gene Amplification in Stepwise-Selected Mouse J774 Macrophages Resistant to the Topoisomerase II Inhibitor Ciprofloxacin

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Abstract

Exposure of J774 mouse macrophages to stepwise increasing concentrations of ciprofloxacin, an antibiotic inhibiting bacterial topoisomerases, selects for resistant cells that overexpress the efflux transporter Abcc4 (Marquez et al. [2009] *Antimicrob. Agents Chemother.* 53: 2410–2416), encoded by the *Abcc4* gene located on Chromosome 14qE4. In this study, we report the genomic alterations occurring along the selection process. Abcc4 expression progressively increased upon selection rounds, with exponential changes observed between cells exposed to 150 and 200 μM of ciprofloxacin, accompanied by a commensurate decrease in ciprofloxacin accumulation. Molecular cytogenetics experiments showed that this overexpression is linked to *Abcc4* gene overrepresentation, grading from a partial trisomy of Chr 14 at the first step of selection (cells exposed to 100 μM ciprofloxacin), to low-level amplifications (around three copies) of *Abcc4* locus on 1 or 2 Chr 14 (cells exposed to 150 μM ciprofloxacin), followed by high-level amplification *of Abcc4* as homogeneous staining region (hsr), inserted on 3 different derivative Chromosomes (cells exposed to 200 μM ciprofloxacin). In revertant cells obtained after more than 60 passages of culture without drug, the *Abcc4* hsr amplification was lost in approx. 70% of the population. These data suggest that exposing cells to sufficient concentrations of an antibiotic with low affinity for eukaryotic topoisomerases can cause major genomic alterations that may lead to the overexpression of the transporter responsible for its efflux. Gene amplification appears therefore as a mechanism of resistance that can be triggered by non-anticancer agents but contribute to cross-resistance, and is partially and slowly reversible.

Citation: Marquez B, Ameye G, Vallet CM, Tulkens PM, Poirel HA, et al. (2011) Characterization of *Abcc4* Gene Amplification in Stepwise-Selected Mouse J774 Macrophages Resistant to the Topoisomerase II Inhibitor Ciprofloxacin. PLoS ONE 6(12): e28368. doi:10.1371/journal.pone.0028368

Editor: Hendrik W. van Veen, University of Cambridge, United Kingdom

Received June 16, 2011; **Accepted** November 7, 2011; **Published** December 5, 2011

Funding: This work was supported by the Fonds National belge de la Recherche Médicale (grants no. 3.4.597.06 and 3.4.583.08), the Belgian Fonds de la Recherche Scientifique (grants no. 1.5.195.07 and 1.5.137.10), and the Belgian Federal Science Policy Office (Research project P6/19 [research action P6]). C.M.V. is Boursier of the Belgian Fonds pour la Recherche dans l'Industrie et l'Agriculture (F.R.I.A.), B.M. was successively post-doctoral fellow of the First post-doc program of the Belgian Region wallonne and of the Fonds spéciaux de recherche de l'Université catholique de Louvain, F.V.B. is Maître de Recherches of the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Overexpression of multidrug transporters (MDR) from the ATP-binding cassette (ABC) family is now widely recognized as a mechanism of resistance to cytotoxic drugs and is associated with therapeutic failures in patients receiving anticancer chemotherapy [1]. Several mechanisms have been described as leading to the overexpression of multidrug transporters, like induction of gene transcription (possibly caused by the drug itself ([2] for review)), increase in mRNA stability [3], epigenetic changes [4,5], or gene amplification [6]. These mechanisms have been explored so far mainly in hepatocytes exposed to different xenobiotics, where activation of gene transcription by nuclear receptors has been well documented [7]. In cells exposed to anticancer agents, chromosomal alterations have also been reported after selection *in vivo* [8] or *in vitro* upon chronic exposure to drugs [9], but the underlying mechanisms can be much more diverse (see for a few examples [10–12]).

By its efflux properties, the multidrug transporter ABCC4 (MRP4) protects cells against toxicity induced by antimetabolites, such as methotrexate or analogues of purines and nucleosides, or by type I topoisomerase inhibitors, such as camptothecins [13–15]. ABCC4 overexpression has been reported in cancer cells, such as in prostate tumors [16] or human leukemic cells (with *in vitro* acquired resistance to 6-mercaptopurine [17]), and is associated with a poor clinical outcome in neuroblastoma [18]. Moreover, single nucleotide polymorphisms in *ABCC4* gene have been shown to modulate the therapeutic response to methotrexate in children suffering from acute lymphoblastic leukemia [19].

Because of their broad substrate specificity, multidrug transporters can also reduce the cellular accumulation of other drugs and impair their activity if their pharmacological target is

intracellular [2]. Conversely, these drugs can also trigger the overexpression of their transporters, as demonstrated for ABCC4 with the antiviral agent adefovir [20] and the fluoroquinolone antibiotic ciprofloxacin [21]. Fluoroquinolones are potent and widely used antibacterial agents that show a marked accumulation in eukaryotic cells, which explains their activity against a large array of intracellular bacteria (see [22] for review). Fluoroquinolones act by inhibiting the prokaryotic type II topoisomerase enzymes (DNA gyrase and topoisomerase IV). Although 100 to 1000-fold more active against bacterial enzymes than against their mammalian homologue topoisomerase II [23], fluoroquinolones can also cause genotoxic and clastogenic effects in eukaryotic cells at high concentrations [24], which has raised concerns about potential toxicities if used at supratherapeutic concentrations [25].

Applying to J774 macrophages a method widely used *in vitro* to select tumor cell lines resistant to anticancer drugs [26] and which consists in exposing cells to progressively increasing concentrations of the drug of interest, we were able to select, after about 50 passages in the presence of ciprofloxacin, cell lines in which the accumulation of this fluoroquinolone was markedly reduced [27]. This phenotype is associated with an accelerated efflux of ciprofloxacin that has been ascribed to an increased expression of *Abcc4* (*Mrp4*) mRNA [21]. We also showed that Abcc4 protein overexpression was only slowly reversible, as more than 60 passages in the absence of ciprofloxacin were needed to obtain cells displaying a phenotype similar to that of the wild-type cell line (similar level of ciprofloxacin accumulation [27] despite a residual slight increase in Abcc4 protein content [21]). All together these data suggested that overexpression of *Abcc4* could be driven through gene amplification.

The present study therefore focuses on the characterization of the progressive acquisition of multidrug resistance in J774 macrophages collected along the selection process with ciprofloxacin and examines possible genomic amplification of *Abcc4* in these cells by fluorescence in situ hybridization (FISH) and multicolor FISH (mFISH). Our data show that *Abcc4* gene amplification indeed occurs in the resistant cells and that it is slowly reversible. We also evidence other clonal chromosomal alterations developing along the selection process, which may reflect the genomic instability induced in eukaryotic cells when exposed to high concentrations of ciprofloxacin.

Results

Characterization of ciprofloxacin accumulation and Abcc4 expression in cell lines resistant to different concentrations of ciprofloxacin

We showed previously that mouse J774 macrophages resistant to 200 μM ciprofloxacin were characterized by a markedly reduced accumulation of this drug [27] that was attributed to the overexpression of the ABC transporter Abcc4 [21].

To further understand the mechanisms leading to this overexpression, we have now compared the accumulation of ciprofloxacin and the expression of Abcc4 (protein and mRNA levels) in wild-type cells *vs.* cells resistant to ciprofloxacin concentrations of 100, 150 and 200 μM. The data illustrated in Figure 1 show that the accumulation of ciprofloxacin was decreased in parallel with the increased level of resistance. However, the process was not linearly related to the drug concentration used for selection, most of the effect being obtained only in cells resistant to the highest concentration. This reduction of ciprofloxacin accumulation was associated with a commensurate increase in the expression of Abcc4, both at the mRNA and protein levels. The lowest panel of Figure 1 shows the correlation between ciprofloxacin accumulation and protein levels of Abcc4.

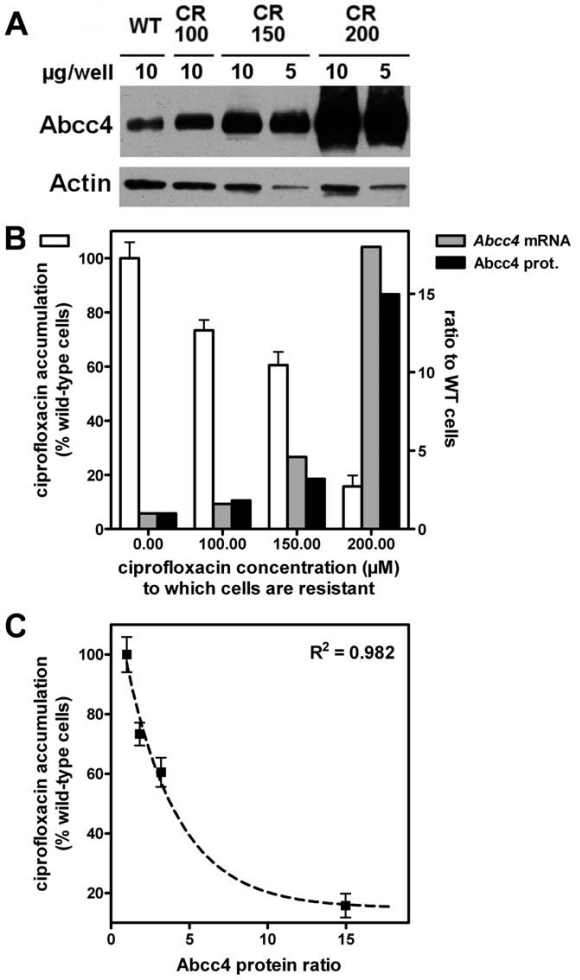

Figure 1. Relationship between cellular accumulation of ciprofloxacin and Abcc4 expression. A. Upper panel: western blot of Abcc4 (and actin as loading control) in cell lysates from wild-type (WT) macrophages and from cells resistant to different concentrations of ciprofloxacin (100 μM [CR100], 150 μM [CR150] and 200 μM [CR200]). B. Ciprofloxacin accumulation and Abcc4 mRNA and protein relative expression in cells made resistant to increasing concentrations of ciprofloxacin; (i) left axis (open bars): accumulation of ciprofloxacin in % (mean ± SD [n = 3]) of the value measured in wild-type cells incubated during 2 h with 50 μM ciprofloxacin [absolute value: 162 ng/mg prot.]); (ii) right axis: Abcc4 mRNA (grey bars) and protein (black bars) levels as a ratio to the value observed in wild-type cells (set to 1). C. Correlation between ciprofloxacin residual accumulation and Abcc4 relative expression in these cells. The curve corresponds to a best fit based on an inverse logarithmic function ($y = 11.5 \times e^{-0.3037\ X} + 14.92$).
doi:10.1371/journal.pone.0028368.g001

Characterization of the *Abcc4* locus amplification in ciprofloxacin-resistant and revertant cell lines compared to wild-type macrophages

Conventional karyotype coupled to Multicolor FISH (mFISH) showed that the wild-type J774 cell line was characterized by a near-triploid karyotype (Figure S1) and exhibited particularly a

derivative of Chr 14 characterized by the replacement of its telomeric end by a part of Chr 3 (Figure 2, Chr B) in addition to the two apparently normal Chromosomes 14. The *Abcc4* locus localized at Chr 14qE4 was analyzed by FISH using BAC (Bacterial Artificial Chromosome) probes, on metaphases in comparison to a more centromeric control locus (see Figure S2 for typical images and Figure 3 for a schematic representation of chromosomes with *Abcc4* loci observed in the different cell lines and a quantification of the different clones).

Wild-type macrophages remained disomic for *Abcc4* locus, as we observed 2 different copies of *Abcc4* on two apparently normal Chromosomes 14 (Figure 2, Chr A), in comparison to the control probe (normally located at Chr 14qA3), which exhibited a third signal on the derivative Chromosome 14 (Figure 2, Chr B). In the intermediate resistant cell line CR100, we predominantly observed the wild-type pattern (almost 67% of the metaphases). A third copy of the *Abcc4* locus was identified in about 1/3 of the cells, either on an additional apparently normal Chromosome 14 with both *Abcc4* and control probes (27% of the metaphases, Figure 3), or on a marker Chromosome (7% of the metaphases). At the next step of selection with ciprofloxacin (CR150 cells), only 21% of the analyzed metaphases displayed the wild-type pattern while all the others showed different clones characterized by a low level chromosomal amplification of *Abcc4* (approximately 3 copies) located either on one or on two Chromosomes 14, or associated to a marker Chromosome which did not exhibit the Chromosome 14 centromeric control probe.

In the fully ciprofloxacin-resistant cell line CR200, we observed *Abcc4* high-level amplification as a homogenous staining region (hsr) in all metaphases (Figure S2) on different marker Chromosomes that did not exhibit the Chromosome 14 centromeric control probe. Of note, all metaphases observed for the CR200 cell line displayed 2 apparently normal Chr 14 and no *Abcc4* copy on the derivative Chr 14, as in wild-type cells. To identify the marker Chromosomes with *Abcc4* hsr, we performed a mFISH analysis followed by *Abcc4* hybridization (Figure 2 and Figure S2). Upon examination of 73 metaphases, the hsr was found with a localization that was either centromeric to a derivative Chr 5 (72.5% of the metaphases, clone I) (Figure 2, Chr C) or telomeric to a derivative Chr 16 (10%, clone III) (Figure 2, Chr D). Interestingly, in 13.5% and 4% of the observed metaphases, the *Abcc4* amplification was found on either two (clone II) or one (clone IV) smaller marker Chromosome(s) (Figure 2, Chr M). These contained three heterochromatic regions aside of the centromere and were suspected to correspond to a derivative of Chr 13 by mFISH, although the chromosomal origin of the centromeric region remained uncertain (Figure 2, Chr M). Other additional genomic alterations were acquired in the resistant cells, namely −der(9)T(9;19),+13,−idic(13) in all clones, +9, in clones I and II, and +der(1)T(1;2)(H?;?) in clone III, as identified by mFISH.

In the revertant cell line Rev200 for which 31 metaphases were examined, we observed the wild-type phenotype (2 apparently normal Chr 14, and one without the *Abcc4* locus) in around half of them (16), but 9 metaphases displayed a remaining high-level amplification of *Abcc4* on the small marker Chr M (Figure 3), as already observed in the CR200 cell line. Moreover, in the remaining 6 metaphases, we observed an additional copy of *Abcc4*, located on a very small unidentified marker Chromosome (Chr N; Figures 2 and S2). All these observations were in agreement with the analysis of interphasic cells, made on a larger cell population (200 nuclei), for each cell line (data not shown).

Characterization of the *DnajC3* locus amplification in resistant cell lines

As *DnajC3* is located close to *Abcc4* at Chromosome 14qE4, and because we already detected DnajC3 protein overexpression in the

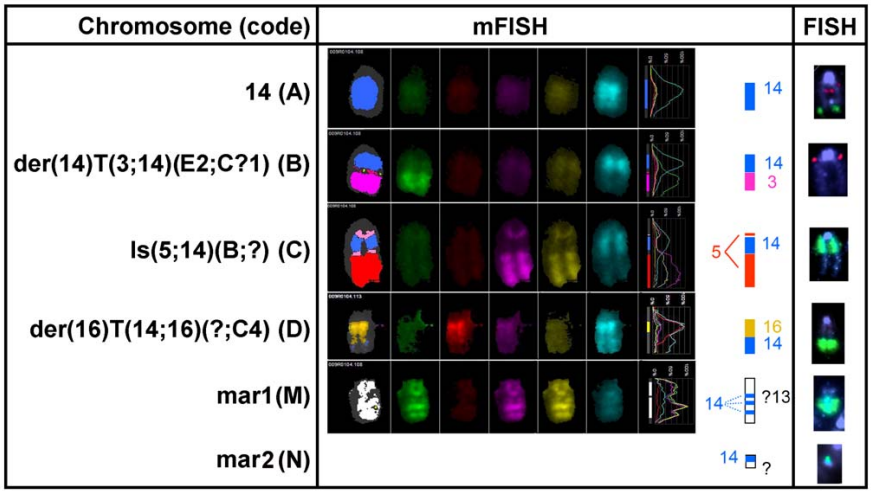

Figure 2. Molecular cytogenetics of relevant chromosomes in wild-type, fully ciprofloxacin-resistant, and revertant macrophages. mFISH and FISH experiments (red: control probe; green: *Abcc4* probe) for relevant chromosomes in wild-type macrophages, in the clones of the fully ciprofloxacin-resistant macrophages CR200, and in the revertant macrophages (Rev200). Karyotypes of the cell lines (abnormalities illustrated in the figure are highlighted in bold): a) wild-type cells: 72<3n>,X,der(X)T(X;11)(E or F1;?B5),der(1)T(1;6)(C?;B?3), +der(1)(1A1→1C?::6B?3→6D~F::X?→X?),−3,+der(5;17)(5A1→5C2::17A1→17?), der(6)T(1;6)(?D;B3),+der(6),+der(9)T(9;19)(?B;C2),+?Del(12)(?B),−13,idic(13), **der(14)T(3;14)(E2;C?1)**,idic(15),−18,−19,idic(19),ace(3)x2,ace(18)x2; b) CR200 cells ("idem" refers to the chromosomal abnormalities stated in the wild-type cells karyotype): – clone I: 72,idem,**Is(5;14)(B;?)**,+9,−der(9)T(9;19),+13,−idic(13),ace(3)x2~3, ace(?14)x0~2,ace(18)x1~2[cp17]; – clone II: 72,idem,?+9,−der(9)T(9;19),+13,−idic(13),**mar1x2~3**, ace(?14)x1~2,ace(18)x1~2[cp3]; – clone III: 71,idem,+der(1)T(1;2)(H?;?),−der(9)T(9;19),+13, −idic(13), **der(16)T(14;16)(?;C4)**,ace(3)x3,ace(?14)x0~2[cp2].
doi:10.1371/journal.pone.0028368.g002

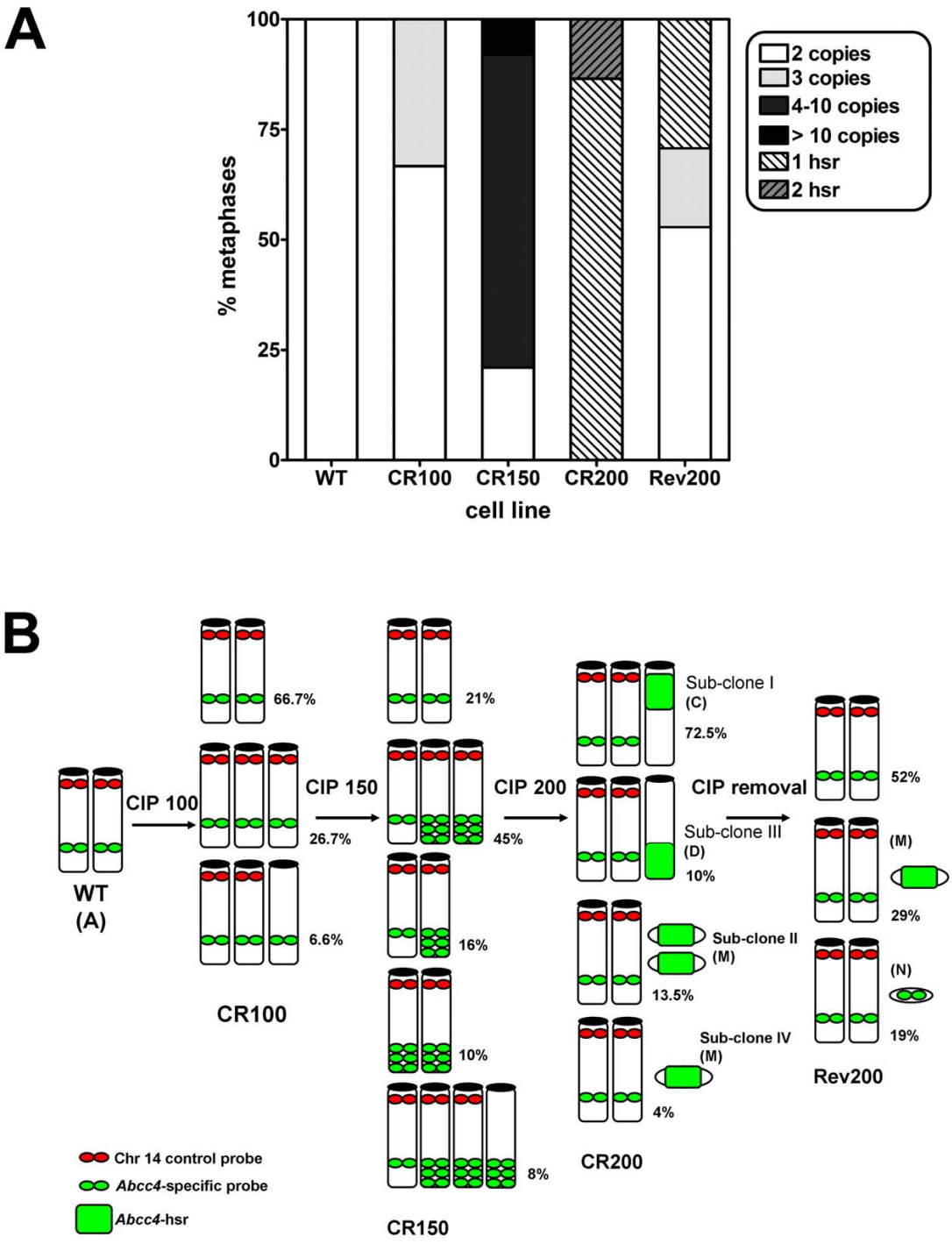

Figure 3. *Abcc4* copies detected by FISH on metaphases of cultured macrophage cell lines. A. Relative abundance of the clones according to number of *Abcc4* copies detected by FISH in the different cell lines (WT: wild-type cells; CR100, 150, and 200: cells resistant to 100, 150, and 200 μM of ciprofloxacin; Rev200: revertant cells). Hsr, homogeneous staining region. B. Schematic representation of chromosomes with *Abcc4* copies in the same cell lines (only chromosomes with *Abcc4* copies are shown; Chr der(14)T(3;14)(E2;C?1) (Chr B), present in almost all clones, but which lacks *Abcc4* locus, is not represented). Percentages refer to the relative abundance of each clone, and letters between brackets to Chromosomes as identified in Figure 2.

doi:10.1371/journal.pone.0028368.g003

fully ciprofloxacin-resistant cells in an ongoing proteomic study [Caceres *et al.*, in preparation], we looked for a possible co-amplification of *DnajC3* with *Abcc4*. FISH analysis with the *DnajC3* specific probe combined with the control probe on Chromosome 14 indeed revealed similar patterns of gene amplification in all the resistant cell lines, like those observed with *Abcc4* probe (data not shown). Moreover, when we hybridized together *Abcc4* and *DnajC3* probes, we observed the co-localization of both probes in amplified regions, either at Chromosome 14 in intermediate resistant cells (CR150 cell line, Figure 4, left panel), or in the hsr in the fully ciprofloxacin-resistant CR200 cell line (Figure 4, right panel).

Influence of ABCC4 on ciprofloxacin accumulation in murine and human cells

As all the work presented so far had been performed with a murine cell line, we examined whether ciprofloxacin is also a substrate of the human homologue transporter. To this effect, we compared the accumulation of ciprofloxacin (a) in wild-type J774 macrophages (basal expression of Abcc4) and in J774 macrophages resistant to 200 μM of ciprofloxacin and (b) in HEK293 human cells and HEK293/4.63 transduced with the cDNA coding for human ABCC4 and expressing it to high levels [28]. The accumulation of ciprofloxacin was drastically reduced in HEK293/4.63 cells compared to HEK293 cells, and was increased by gemfibrozil, a broad-spectrum inhibitor of MRP transporters (see Figure S3), demonstrating that ciprofloxacin is also a substrate of the human homologue of Abcc4.

Discussion

The present study shows that an antibiotic is capable of inducing at large concentrations a series of clonal chromosomal alterations in an eukaryotic cell, leading, among other changes, to the overexpression of a multidrug transporter responsible for its efflux. This effect is slowly and only partially reversible at the genomic level. The demonstration remains so far limited to ciprofloxacin and the murine multidrug transporter Abcc4 (Mrp4).

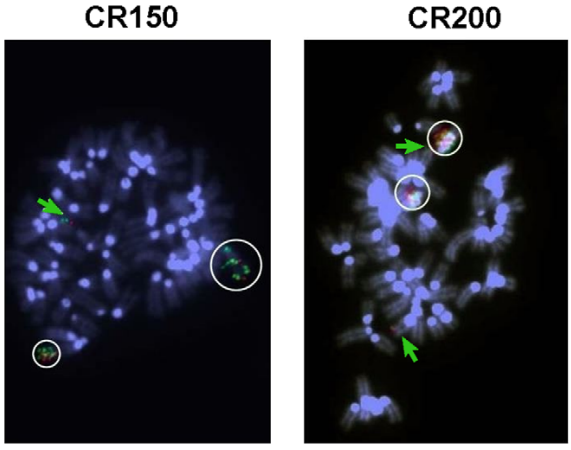

Figure 4. *DnajC3* and *Abcc4* FISH analysis in selected resistant cell lines. Metaphase spreads of CR150 (left) and CR200 (clone II; right) cells were subjected to FISH analysis with an *Abcc4* BAC probe (green) and a *DnajC3* BAC probe (red). Chromosomes were counterstained with DAPI. Colocalization of both probes in amplified regions is highlighted with white circles; green arrow indicates Chr 14 with *Abcc4* and *DnajC3* copies.
doi:10.1371/journal.pone.0028368.g004

However, we show here that ciprofloxacin is also substrate for ABCC4, the human homologue of this transporter (as also found by others [29]), suggesting that similar mechanisms could possibly take place in humans. This opens interesting perspectives in terms of interactions of antibacterial agents with the host in relation to potential cell toxicity, and, in a broader context, in the manner eukaryotic cells deal with exogenous compounds.

ABCC4 gene amplification as an hsr has already been described in a human T lymphoid cell line made resistant to the antiviral drug adefovir [20], another well known ABCC4 substrate. In this case, however, amplification was located at the distal end of chromosome arm 13q, which is compatible to the normal location of human *ABCC4* (13q32). Hsr and double minute chromosomes are the two common patterns of gene amplification observed upon selection by anticancer agents [6]. Interestingly, we do not observe double minute chromosomes at any step of the selection, in contrast with what has been shown for *Abcb1* (P-glycoprotein) in colchicine-resistant J774.2 cells [30], or for *ABCG2* in mitoxantrone-resistant glioblastoma cells [31], which display double minutes at low drug concentrations, but hsr at high concentrations. In other instances, both patterns are present at the same time, as described for *ABCC1* in a doxorubicin-resistant human tumor cell line [32].

We showed that the karyotypes of the fully resistant clones mainly differ from the one of the wild-type cell line by 3 different marker Chromosomes with *Abcc4* high level amplification. These observations suggest a random chromosomal insertion of the 14qE4 genomic amplification as an hsr, which provides a proliferative advantage by enabling cells to resist to the strong pressure of selection and leading to clonal expansions. Of interest, gene amplification was not restricted to *Abcc4*, since *DnajC3*, another gene located at 14qE4, was co-amplified, and the corresponding protein overexpressed [Caceres *et al.*, in preparation]. Since both *Abcc4* and *DnajC3* genes are constitutively expressed in the wild-type macrophages, they do not need another genetic event in addition to genomic amplification for overexpression, such as a "switch on" induced by a promoter capture or by insertion of retroviral sequences [33,34]. We did not observe extrachromosomal elements, such as double minutes (dmin) or episomes that are classically reported [6]. However, we cannot exclude their occurrence at the first steps of selection before integration of the amplicons within chromosomes detected in CR200 cells. In cancer cells, genomic amplification has been shown to occur at common chromosomal fragile sites, or to result from defects in DNA replication or telomere dysfunction. It is noteworthy to mention that the chromosomal band 14qE4, with Abcc4 and DnajC3 loci, has been shown to be a common fragile site that may favor genomic instability [35].

The phenotypic reversion is associated with a drastic regression of the *Abcc4* amplification, with loss of the hsr-like amplification of *Abcc4* to Chr 5 and to Chr 16. The persistence of a copy of a marker Chromosome (Chr M, with *Abcc4* hsr) in nearly 30% of the revertant cells, and the time required to obtain phenotypic reversion, underline the stability of the gene amplification. As the revertant cells display a similar phenotype as wild-type cells regarding ciprofloxacin accumulation [27] and almost similar levels of *Abcc4* mRNA [21], it is likely that the *Abcc4* amplification observed on this marker in single copy is not associated with efficient transcription.

In vitro, gene amplification is likely to be initiated by a DNA double-strand break in cells that lack appropriate cell-cycle checkpoints [6]. Chemotherapeutic drugs targeting topoisomerase II promote DNA double-strand break, favoring thereby the development of therapy-related leukaemias. In bacteria, fluoro-

quinolones form a reversible ternary complex linking together DNA and prokaryotic type II topoisomerase enzymes to impair the progression of the replication fork. This leads to several lethal damages, including SOS response induction and, possibly chromosome fragmentation, which explains their rapid bactericidal activity [36]. While no genotoxicity has been reported so far for ciprofloxacin *in vivo* or in cultured cells [37–39], chromosomal aberrations have been described in cultured human lymphocytes exposed to supratherapeutic concentrations similar to those used in this study [40,41], as well as an increase of sister-chromatid exchange [42] and in DNA single strand breaks frequencies [43] in mouse bone marrow cells. Although the concentrations needed to observe these alterations (100–200 μM, i.e. 40–80 mg/L) are well above the serum levels observed in patients receiving conventional therapies (1–4 mg/L), one needs to take into consideration that ciprofloxacin accumulates in tissues (with tissular levels reaching values 2–7 fold higher than serum levels) as well as in body fluids, with the highest concentrations (200–900 mg/L) being found in urine. This suggests that *in vivo* exposure may be more important than anticipated based on serum levels only.

Although pending for further investigations aimed at elucidating the mechanism leading to gene amplification, our data thus indicate that ciprofloxacin may induce in eukaryotic cells chromosomal aberrations leading to overexpression of the transporter responsible for its efflux. Whether such alterations may occur *in vivo* will clearly depend on the concentration of the drug, and probably also on a combination of its recognition by efflux transporters and its capacity to interact with DNA-topoisomerase complexes. Other fluoroquinolones may, indeed, be much more toxic than ciprofloxacin in this context [41,44], with many of them having been withdrawn or not accepted for registration in many countries for unsatisfactory benefit to risk ratio involving, among other untoward reactions, clastogenic effects (see [45] for an example with gemifloxacin). It is interesting to note that N-substituted piperazinyl quinolones derived from ciprofloxacin or other clinically-used fluoroquinolones show *in vitro* cytotoxicities that are as high as those seen with etoposide, a well known inhibitor of topoisomerase II [46,47]. These compounds are now evaluated as potential anticancer agents.

Materials and Methods

Chemicals

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Ciprofloxacin HCl (potency 85%) was received from Bayer HealthCare (Wuppertal, Germany) as microbiological standard. Other chemical products were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and culture conditions

J774 mouse macrophage-like cells [48] (referred to as wild-type cells) were cultured and maintained as already described [49]. Ciprofloxacin-resistant macrophages and their revertant were fully described in a previous publication of our group [27]. Resistant cells were obtained by chronic exposure to progressively increasing concentrations of ciprofloxacin (100 μM, 150 μM, and 200), yielding to cell lines referred to here as CR100 (used at the 5th passage), CR150 (used at the 3rd passage) and CR200 (used at the 76th passage) respectively. Revertant cells (referred to here as Rev200) were obtained by returning CR200 cells to ciprofloxacin-free and used here at their 84th passage in the absence of selective pressure. Human Embryonic Kidney HEK293 cells and HEK293/4.63 cells transduced with human *ABCC4* cDNA were obtained from P. Borst (*Het Netherlands Kanker Instituut*, Amsterdam, The Netherlands). They were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum [28].

Accumulation and efflux of fluoroquinolones

These experiments were performed exactly as previously described [21,27,50]. Cell-associated ciprofloxacin was assayed by fluorimetry ($\lambda_{exc} = 275$ nm and $\lambda_{em} = 450$ nm) and its cellular concentration expressed by reference to the total cell protein content as measured by the Lowry's method [51].

Quantification of *Abcc4* by real-time PCR and Western blot Analysis

Abcc4 expression was assessed at mRNA and proteins levels, as described previously [21]. Real-time PCR experiments were performed starting from 1 μg of total purified RNA transcribed into cDNA, and using SYBR Green detection. Two housekeeping genes, *Ywhaz* and *Rpl13a* (mouse geNorm normalization kit, PrimerDesign Ltd., Southampton, UK) were used for normalization. The relative quantification of *Abcc4* gene in cell lines of interest was done using levels measured for wild-type J774 macrophages as baseline, based on Pfaffl's equation [52]. Western-blots were performed on cell crude extracts. After electrophoresis on acrylamide gel and transfer to nitrocellulose membrane, proteins of interest were detected using anti-ABCC4 monoclonal antibody (M_4I-10; Alexis Biochemicals, Lausen, Switzerland) or anti-actin polyclonal antibodies (Sigma-Aldrich) (dilution 1/1000), followed by appropriate horseradish peroxidase-coupled secondary antibodies (dilutions 1/600). Blots were then revealed by chemiluminescence assay (SuperSignal West Pico, Pierce, Thermo Fisher Scientific Inc., Rockford, IL).

Cytogenetic analysis

Metaphase chromosomes were obtained according to standard methods for mouse cell lines [53]. Cells were grown for 48 h into 6-well plates (initial density of 5×10^4 cells per cm^2) and fed with fresh medium 4 h before addition of a mitotic spindle inhibitor (KaryoMAX® Colcemid™ Solution, Invitrogen) at a final concentration of 0.02 μg/ml. After 45 min of incubation at 37°C, cells were washed with PBS, detached by trypsinization and collected by centrifugation (900 rpm, 10 min). They were then submitted to hypotonic choc by resuspension in KCl 75 mM and incubation for 10 min at room temperature. After centrifugation (900 rpm, 5 min), cell pellets were resuspended in Carnoy's fixative (methanol/glacial acetic acid, 3/1) and incubated 15 min at room temperature. This fixation step was repeated three times. Metaphase chromosomes were obtained after spreading and air-drying of fixed cells onto microscope glass slides. Metaphase cells were banded with trypsin denaturation followed by a Wright staining. Metaphases were analysed with Ikaros Imaging System (MetaSystems, Altlussheim, Germany). Karyotypes were described according to The Rules for Nomenclature of Chromosome Aberrations (Revised: January 2005) from the International Committee on Standardized Genetic Nomenclature for Mice (http://www.informatics.jax.org/mgihome/nomen/anomalies.shtml). A total of 8 and 21 metaphasic cells were analyzed, for wild-type and resistant macrophage (CR200) cell lines, respectively.

Fluorescence in situ hybridization analysis

Fluorescence in situ hybridization (FISH) analysis was performed using Bacterial Artificial Chromosome (BAC) probes (from the Roswell Park Cancer Institute, Buffalo, NY) specific for *Abcc4* (clone RP23-390O15) and *DnajC3* loci (clone RP23-378H12) (both

located at Chr 14qE4), and for a more centromeric locus of Chromosome 14 which served as control (clone RP23-364J13, located at Chr 14qA3). BAC localizations have been checked by end sequencing analysis on a Beckman CEQ2000 sequencer (Beckman Coulter, Fullerton, CA). After extraction, BAC probes were labeled by random priming with fluorescent dUTP (Cy3-dUTP [red] or fluorescein-12-dUTP [green]), using the BioPrime® DNA Labeling System (Invitrogen). Hybridization and fluorescence detection were carried out according to standard procedures [54]. Briefly, fluorescent probes (*Abcc4* or *DnajC3* probes: 20 ng/µl; control probe: 24 ng/µl), denatured at 70°C for 5 min and preannealed with a 50-fold excess of mouse Cot-1 DNA (Invitrogen), were hybridized overnight at 37°C to cytogenetic slides pretreated with pepsin 0.01%/HCl 0.01 M at 37°C for 13 min, and then denatured at 75°C for 2 min 20 sec in 70% formamide/2xSSCP (saline sodium citrate phosphate buffer). After washing (2 min in 0.4xSSC [saline sodium citrate buffer] at 30°C), cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and observed with a Zeiss Axioplan2 microscope equipped with a HBO103W lamp (Zeiss, Oberkochen, Germany). Images were captured with the Isis Imaging System (MetaSystems). For each hybridization, a minimum of 30 metaphases and of 200 interphasic cells were analyzed.

Multicolour FISH (mFISH)

Fresh metaphase spreads were pretreated with RNase A (100 µg/ml)/2xSSC for 45 min at 37°C, then denatured as previously described. The multicolour probe kit (21XMouse, MetaSystems) was denatured according to manufacturer's instructions, and then hybridized to metaphases at 37°C for 3 to 4 days. Post-hybridization washes and counterstaining were performed according to manufacturer's instructions, using DAPI/antifade (MetaSystems). Ten metaphases were analyzed for wild-type macrophage cells, and more than 20 for resistant cells CR200. When mFISH was combined with *Abcc4* probe hybridization, we compared metaphases hybridized first with mFISH probes, then washed and hybridized again with the *Abcc4* probe.

Supporting Information

Figure S1 mFISH karyotype of wild-type J774 macrophages. Chromosomes are displayed with false colors, as indicated by rounds; squares indicate the combination of true colors given by the probes.
(TIF)

Figure S2 *Abcc4* FISH and mFISH analysis in wild-type J774 macrophages, in cells resistant to increasing concentrations of ciprofloxacin, and in revertant cells. A: Metaphase spreads of J774 wild-type (upper left panel), CR100 (upper right panel), CR150 (middle panel) and Rev200 (lower panel) cells were subjected to FISH analysis with an *Abcc4* BAC probe (green) and a control BAC probe located on Chr 14 (red). Chromosomes were counterstained with DAPI. Representative metaphases of the different clones observed are shown. Green arrow indicates Chr 14 with *Abcc4* copy, red arrow points to the control BAC probe (red) located on Chr 14, green circle indicates *Abcc4* amplification, and green square *Abcc4* additional copy. B: Metaphase spreads of the main three clones (I, II, III) observed in CR200 cells hybridized first with mFISH probes and subsequently with the *Abcc4* BAC probe (green) and the control BAC probe (red) located on Chr 14 (chromosomes counterstained with DAPI).
(TIF)

Figure S3 Comparison of ciprofloxacin accumulation in murine and human cells with basal or overexpression of Abcc4/ABCC4. Cellular accumulation of ciprofloxacin in J774 mouse macrophages (WT or CR200) and in human embryonic kidney cells (HEK293, parental cells; HEK293/4.63 transduced with the human cDNA coding for ABCC4 and overexpessing the transporter to high levels [28,29]). Cells were incubated during 2 h with an extracellular concentration of 20 mg/L (50 µM) of ciprofloxacin in the absence of in the presence of 500 µM gemfibrozil. Data are expressed in percentage of the value measured in control condition in the parental cell line and are the mean ± SD of 3 independent determinations.
(TIF)

Acknowledgments

We thank M. C. Cambier, V. Mohymont, and S. Nonckreman for skilful technical assistance. We acknowledge the kind help and advice of J. Britton-Davidian and J. Catalan (*Institut des Sciences de l'Evolution*, *Laboratoire Génétique et Environnement*, *Université Montpellier II*, Montpellier, France) in mouse karyotyping. We are grateful to the NKI (*Netherlands Kanker Instituut*, Amsterdam, The Netherlands) for providing us with HEK293 and HEK293/4.63 cell lines.

Author Contributions

Conceived and designed the experiments: BM HAP FVB. Performed the experiments: BM GA CMV. Analyzed the data: BM HAP FVB. Contributed reagents/materials/analysis tools: PMT HAP FVB. Wrote the paper: BM PMT HAP FVB.

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Characterization of *Abcc4* Gene Amplification in Stepwise-Selected Mouse J774 Macrophages Resistant to the Topoisomerase II Inhibitor Ciprofloxacin

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Figure S1: mFISH karyotype of wild-type J774 macrophages.

Chromosomes are displayed with false colors, as indicated by rounds; squares indicate the combination of true colors given by the probes.

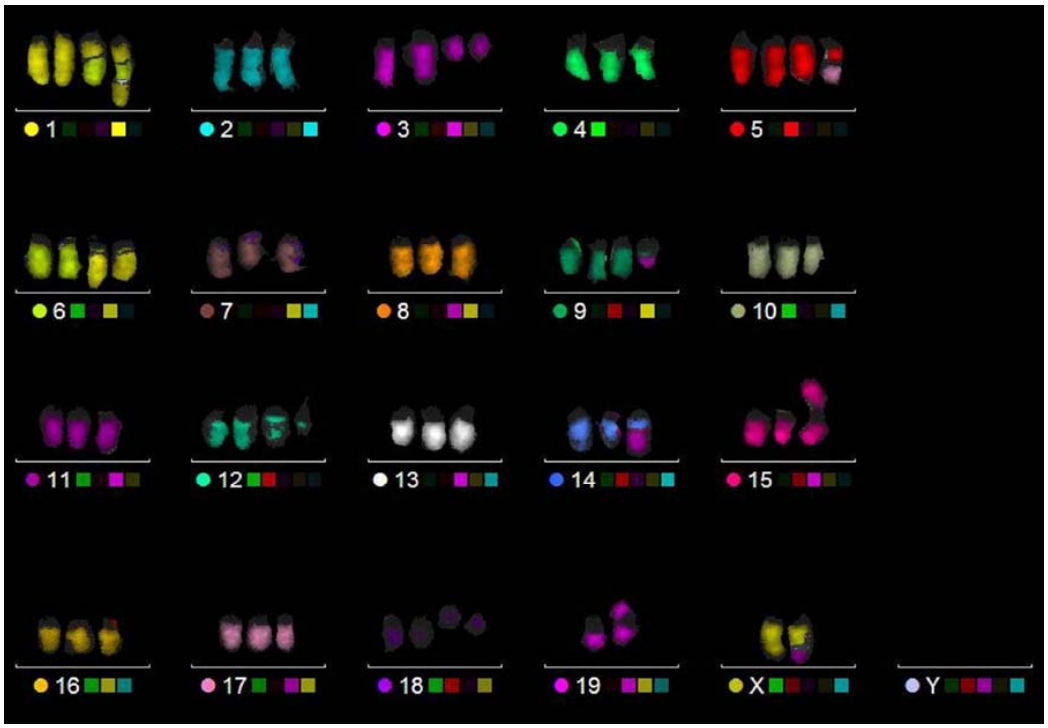


Figure S2. *Abcc4* FISH and mFISH analysis in wild-type J774 macrophages, in cells resistant to increasing concentrations of ciprofloxacin, and in revertant cells.

A: Metaphase spreads of J774 wild-type (upper left panel), CR100 (upper right panel), CR150 (middle panel) and Rev200 (lower panel) cells were subjected to FISH analysis with an *Abcc4* BAC probe (green) and a control BAC probe located on Chr 14 (red). Chromosomes were counterstained with DAPI. Representative metaphases of the different clones observed are shown. Green arrow indicates Chr 14 with *Abcc4* copy, red arrow points to the control BAC probe (red) located on Chr 14, green circle indicates *Abcc4* amplification, and green square *Abcc4* additional copy.

B: Metaphase spreads of the main three clones (I, II, III) observed in CR200 cells hybridized first with mFISH probes and subsequently with the *Abcc4* BAC probe (green) and the control BAC probe (red) located on Chr 14 (chromosomes counterstained with DAPI).

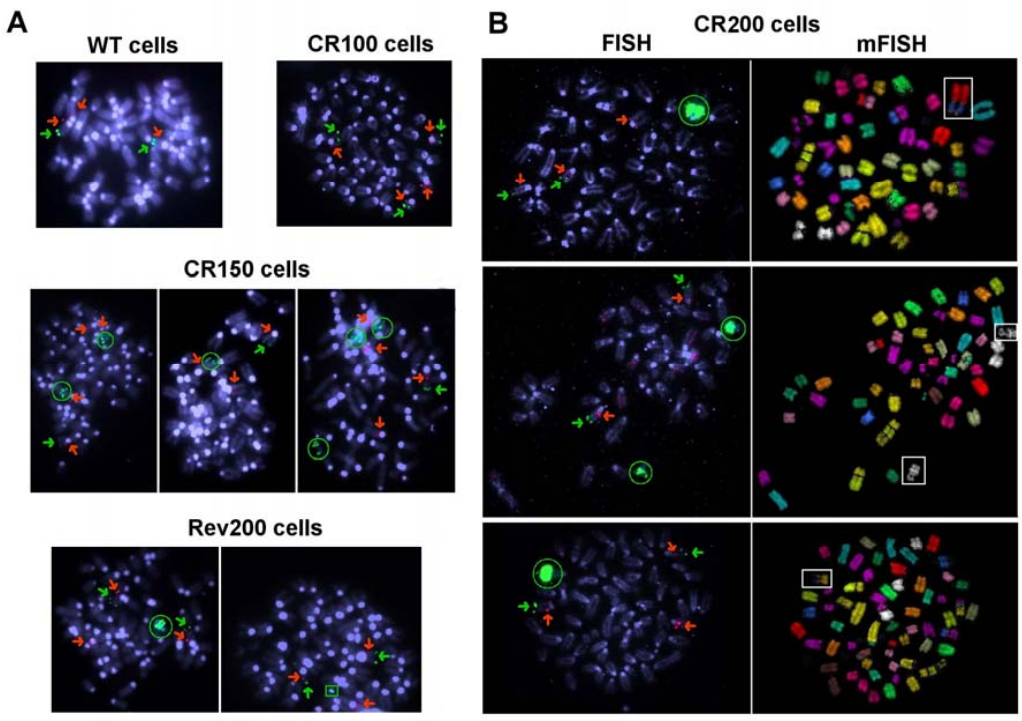
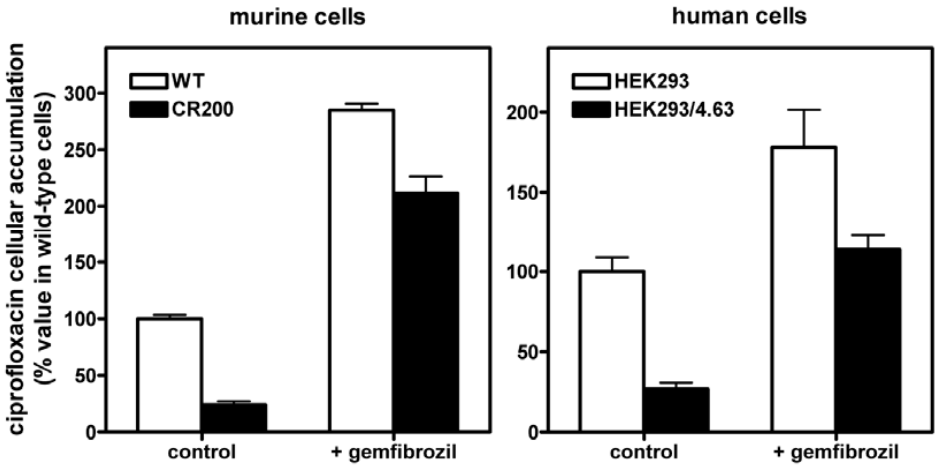


Figure S3. Comparison of ciprofloxacin accumulation in murine and human cells with basal or overexpression of Abcc4/ABCC4.

Cellular accumulation of ciprofloxacin in J774 mouse macrophages (WT or CR200) and in human embryonic kidney cells (HEK293, parental cells; HEK293/4.63 transduced with the human cDNA coding for ABCC4 and overexpessing the transporter to high levels [28,29]). Cells were incubated during 2 h with an extracellular concentration of 20 mg/L (50 µM) of ciprofloxacin in the absence of in the presence of 500 µM gemfibrozil. Data are expressed in percentage of the value measured in control condition in the parental cell line and are the mean ± SD of 3 independent determinations.



I.2. Modulation of the expression of ABC transporters in murine (J774) macrophages exposed to large concentrations of the fluoroquinolone antibiotic moxifloxacin

Coralie M. Vallet, Béatrice Marquez, Naïma Nhiri, Ahalieyah Anantharajah, Marie-Paule Mingeot-Leclercq, Paul M. Tulkens, Jean-Yves Lallemand, Eric Jacquet, Françoise Van Bambeke

Toxicology, September 2011, vol. 290, p. 179-187

Toxicology 290 (2011) 178–186

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

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

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ARTICLE INFO

Article history:
Received 12 August 2011
Received in revised form
10 September 2011
Accepted 12 September 2011
Available online 17 September 2011

Keywords:
Moxifloxacin
Ciprofloxacin
Efflux
Mrp4
P-gp
Bcrp

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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doi:10.1016/j.tox.2011.09.003

(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

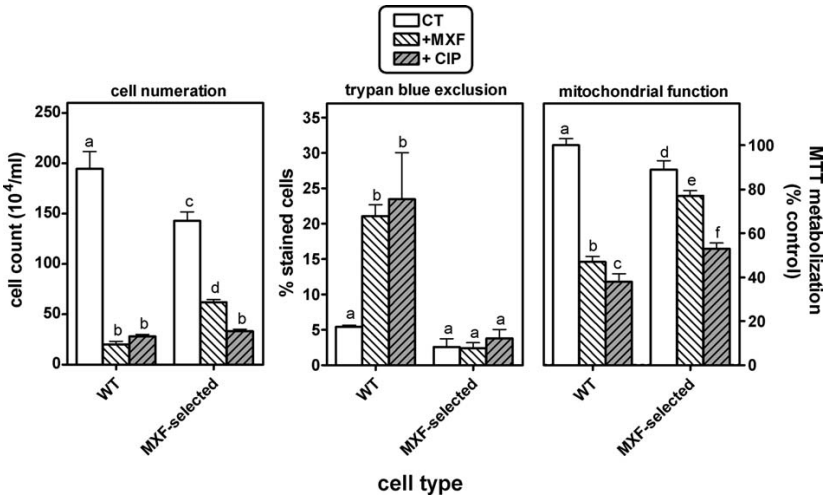

Fig. 1. Effect of fluoroquinolones on cell proliferation, viability and mitochondrial metabolism in wild-type (WT) or moxifloxacin-selected (MXF-selected) cells. Cells were plated for 48 h in drug-free medium (open bars) or in medium containing 0.2 mM moxifloxacin (left-diagonally hatched bars with white 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

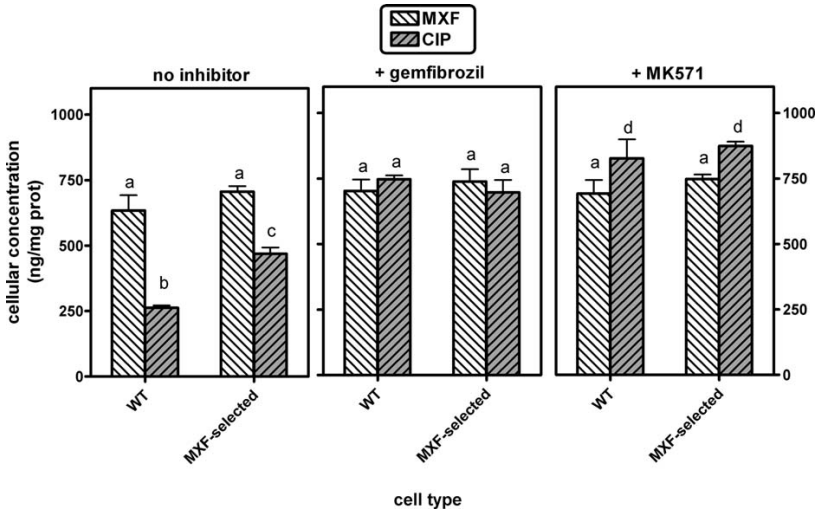

Fig. 2. Cellular concentration of moxifloxacin (MXF; left-diagonally hatched bars with white background) compared to ciprofloxacin (CIP; right diagonally hatched bars with grey background) in wild-type (WT) or moxifloxacin-selected (MXF-selected) cells after 2 h incubation at an extracellular concentration 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 $p < 0.05$]).

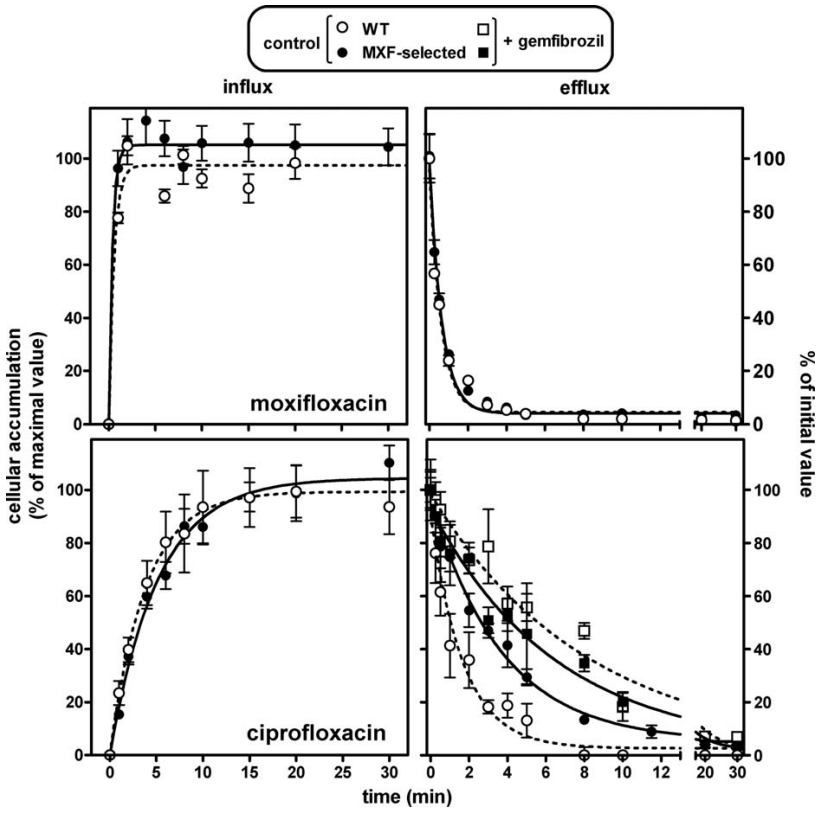

Fig. 3. Kinetics of accumulation and efflux of moxifloxacin (top) and ciprofloxacin (bottom) in wild-type (WT) and moxifloxacin-selected (MXF-selected) macrophages. Cells were incubated in the presence 50 μM fluoroquinolone for 120 min (graph cut at 30 min), washed and re-incubated in antibiotic free 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

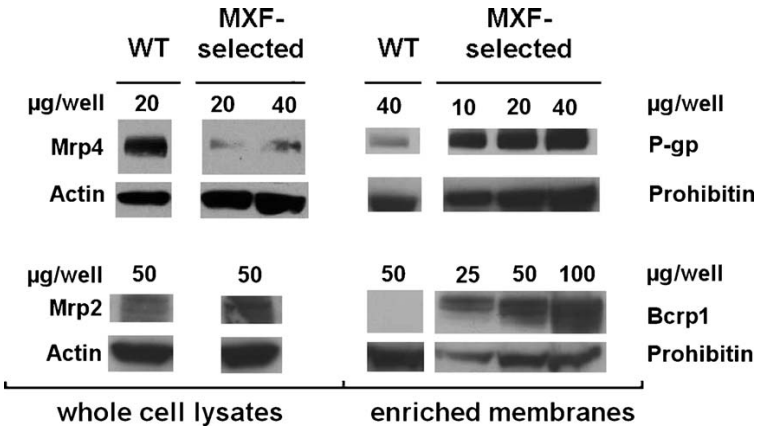

Fig. 4. Western blots of proteins prepared from wild-type (WT) or moxifloxacin-selected (MXF-selected) cells. Gels were loaded with the indicated amounts of proteins. Left: whole cell lysates, revelation with rat anti-Mrp4 (1:2000) or with mouse anti-Mrp2 (1:300), or with rabbit anti-actin (1:1000) antibodies, 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

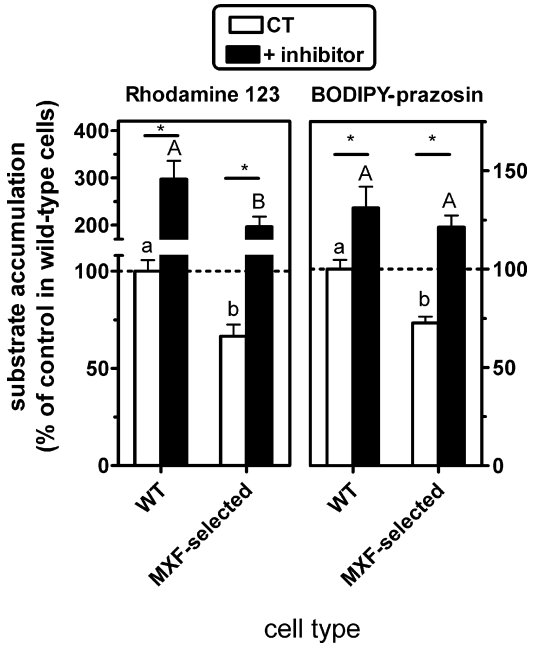

Fig. 5. Cellular accumulation of preferential substrates of P-glycoprotein or Bcrp1 and influence of inhibitors in wild-type (WT) or moxifloxacin-selected (MXF-selected) cells. Left: cells were incubated during 2 h with 10 μM rhodamine 123 in the absence (CT; control) or in the presence of 100 μM verapamil (+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.

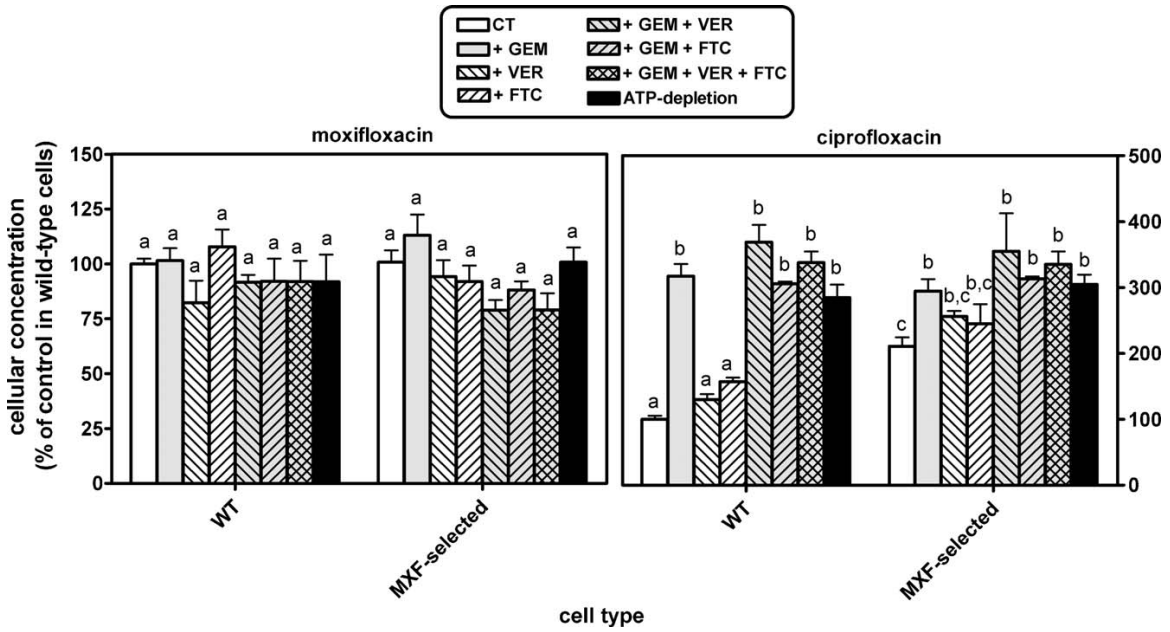

Fig. 6. Influence of preferential inhibitors of efflux transporters on ciprofloxacin and moxifloxacin accumulation. Wild-type (WT) or moxifloxacin-selected (MXF-selected) cells were incubated during 2 h with an extracellular concentration of 50 μM antibiotic alone (control, CT) or in the presence of 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

This study was supported by the Belgian *Fonds de la Recherche Scientifique Médicale* [grants 3.4.597.06 and 3.4.583.08], the Belgian *Fonds de la Recherche Scientifique* [grant 1.5.195.07], the Belgian Federal Science Policy Office [Research projects P5/33, P6/19], and a grant-in-aid from the French Association *Mucoviscidose: ABCF2*. C.M.V. is Boursier of the Belgian *Fonds pour la Recherche dans l'Industrie et l'Agriculture* (F.R.I.A.), B.M. was post-doctoral fellow of the First post-doc program of the Belgian *Région wallonne*, N.N. was supported by a Post-Doctoral Grant from ICSN, and F.V.B. is *Maître de Recherches* of the Belgian *Fonds de la Recherche Scientifique* (*F.R.S.-FNRS*).

Acknowledgments

We thank R. Robey (National Institutes of Health, Bethesda, MA) for the kind gift of fumitremorgin C and M.C. Cambier for skillful technical assistance.

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

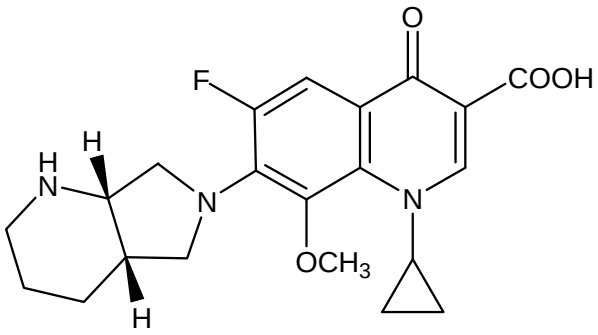
Table SP1: Sequence of the primers used for sequencing of the *Mrp4* ORF, temperature for PCR amplification and size of the corresponding amplicons

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

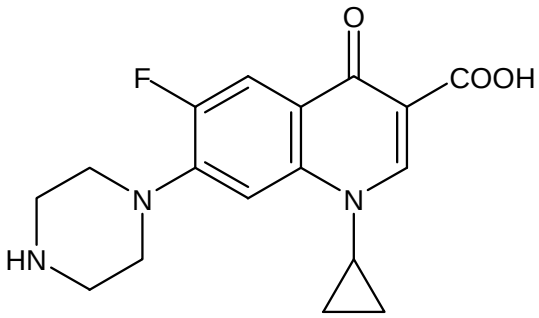
Gene	Assay ID	Gene	Assay ID
Abca1	Mm00442646_m1	Abcc10	Mm00467403_m1
Abca2	Mm00431553_m1	Abcc12	Mm00556685_m1
Abca3	Mm00550501_m1	Abcd1	Mm00431749_m1
Abca4	Mm00492004_m1	Abcd2	Mm00496455_m1
Abca5	Mm00461656_m1	Abcd3	Mm00436150_m1
Abca6	Mm00461636_m1	Abcd4	Mm00436180_m1
Abca7	Mm00497010_m1	Abce1	Mm00649858_m1
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

I.3. Cytotoxicity of ciprofloxacin and moxifloxacin, and topoisomerases activity in wild-type, ciprofloxacin- and moxifloxacin-resistant cells

(This study was performed in collaboration with Ahalieyah Anantharajah, a researcher student in pharmacy)

3. Cytotoxicity of ciprofloxacin and moxifloxacin, and topoisomerases activity in wild-type, ciprofloxacin- and moxifloxacin-resistant cells

3.1. Background

We have shown that ciprofloxacin (CIP) and moxifloxacin (MXF), two fluoroquinolone antibiotics, are able to induce resistance mechanisms in eukaryotic cells. Indeed, ciprofloxacin is substrate of an efflux pump (Mrp4) in J774 macrophages, and chronic exposure of wild-type macrophages (WT cells) to large concentrations of ciprofloxacin leads to the selection of ciprofloxacin-resistant cells (CIP-R cells) ((Michot et al. 2004) (Michot et al. 2005) (Michot et al. 2006)). It has been shown that the Mrp4 transporter was overexpressed in those cells, inducing the markedly decreased accumulation of ciprofloxacin (Marquez et al. 2009). In comparison, we have shown that moxifloxacin accumulation in wild-type macrophages was not affected by the Mrp4 transporter, and chronic exposure of those cells to large concentrations of moxifloxacin leads to moxifloxacin-resistant cells (MXF-R cells). These cells showed a lower expression of the Mrp4 transporter, and an overexpression of other MDR transporters (but which do not affect moxifloxacin accumulation) (Vallet et al. 2011).

As the resistance to moxifloxacin is not related to an increased efflux of the antibiotic, moxifloxacin-resistant cells should have developed another resistance mechanism in order to survive in the presence of large concentrations of this antibiotic. We know that type II topoisomerases are the target of fluoroquinolones in bacteria, and some evidence shows that some fluoroquinolones, at large concentrations, can interact with these enzymes in eukaryotic cells.

The aim of this study is:

- to evaluate and compare the cytotoxicity induced by antibiotics (CIP and MXF) and anticancer agents (camptothecin (CPT) and etoposide (ETO)) known to inhibit topoisomerases in WT, CIP- and MXF-R cells.
- to evaluate and compare the effects of antibiotics (CIP and MXF) and anticancer agents (CPT and ETO) on topoisomerases of WT, CIP- and MXF-R cells.
- to find a link between the cytotoxicity observed and the resistance mechanisms induced by the antibiotics.

3.2. Material and methods

Cell lines and culture conditions. J774 mouse macrophages were used in this study as WT cells and were cultured and maintained as already described (Michot et al. 2005). CIP-R and MXF-R cells, derived from wild-type cells, were obtained by long-term exposure to chronically and progressively increasing concentrations of the corresponding antibiotic, and are maintained in culture with 0.2 mM of CIP or MXF. Details of the method used to obtain those cells have been described previously in (Michot et al. 2006) for CIP-R cells and in (Vallet et al. 2011) for MXF-R cells.

Evaluation of the metabolic activity: MTT test. This test was used to evaluate the metabolic activity of cells (thus, indirectly the viability of cells) exposed to potential toxic agents. WT, CIP-R and MXF-R cells were incubated in 96-well plates for 48 h with increasing concentrations of CIP or MXF (50 to 2000 µM), or 24 h with increasing concentrations of CPT (0 to 10000 nM) or ETO (0 to 1000 µM), or 24 h with combination of CPT+CIP, CPT+MXF, ETO+CIP, or ETO+MXF (the same ranges of concentrations were used for CPT and ETO, but the concentration of fluoroquinolones were fixed at 200 µM). Then cells were rinsed with Phosphate Buffer Saline (PBS), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was added at a concentration of 0.5 mg/mL to each well. After 1h of incubation at 37°C, 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm.

Evaluation of cell viability

Detection of apoptosis by DAPI. WT, CIP-R and MXF-R cells were seeded in 6-well plates at day 0, and allowed to grow for 24 h. At day 1 cells were incubated for 24 h with 200 µM CIP, or 200 µM MXF, or 500 nM CPT, or 10 µM ETO, or in combination (CPT+CIP, CPT+MXF, ETO+CIP, ETO+MXF). Induction of apoptosis by the drugs was determined with the DAPI method as already described in (Servais et al. 2005). In brief, cells were collected by trypsinization and pelleted at 290g for 7min, then washed three times with ice cold PBS, and finally resuspended in formol 3.7% to fix them. Formol was eliminated by centrifugation, pellets were resuspended in PBS and spread on polylysine-coated slides which were dried before staining with a solution of 4',6'-diamidine-2'-phenylindole (DAPI). Three slides per condition were assayed and 500 cells per slide were counted using a Zeiss light microscope.

Detection of necrosis by Lactate Dehydrogenase (LDH) release measurement. Cells were plated and incubated as previously described for the DAPI method. Necrosis induced by the drugs was assayed by measuring the activity of the cytosolic enzyme

lactate dehydrogenase (LDH) released from cells in the culture medium as previously described in (Montenez et al. 1999). Results were expressed as the percentage of LDH activity found in the medium over the sum of the activities found in the medium and in cells.

Topoisomerases activity

Preparation of nuclear extracts. Cells were plated in 58 cm² Petri dishes, and incubated for 20 h either in culture medium (control), or in the presence of 0.2 mM MXF, 0.2 mM CIP, 1 µM CPT, or 10 µM ETO, washed and scraped in cold PBS, pelleted at 800 x g for 3 min, and resuspended in TEMP buffer (Tris-HCl 10 mM pH7.5, EDTA 1 mM, $MgCl_2$ 4 mM, PMSF 0.5 mM). All subsequent operations were carried out at 4°C. After left for swelling during 10 min, cells were disrupted in a Dounce homogenizer (tight-fitting pestle B). The preparations were then subjected to centrifugation at 1,500 x g for 10min, the supernatants were removed and pellets (containing the nuclei) resuspended in 1 mL TEMP buffer, and centrifuged again at 1,500 x g for 5min. Nuclei collected in the final pellet were then lysed in TEP buffer (Tris-HCl 10mM pH7.5, EDTA 1mM, PMSF 0.5mM) with addition of NaCl at a final concentration of 1M. After 45 min incubation on ice, nuclear lysates were centrifuged at 15,000 x g for 15min, and the supernatants (nuclear extracts) were collected. Protein content was determined by the Bradford assay and samples stored at -80°C after addition of 20 % glycerol until needed for assay.

Topoisomerase assay. Topoisomerase activity was assay using a method adapted from that of (Fabian et al. 2006). For topoisomerase I assay, 12.5 ng of nuclear proteins were added to a reaction mixture containing, in a final volume of 20 µL, 20 mM Tris-HCl pH7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 30 µg/mL bovine serum albumin, and 250 ng of pBR322 supercoiled DNA plasmid (Fermentas, ThermoFisher Scientific, Waltham, MA). For topoisomerase II assay, the same incubation mixture was used except that the buffer also contained 2 mM ATP. After incubation at 37°C for 30 min, the reactions were terminated by adding 5 µL of stopping buffer (0.5% bromophenol blue, 50 mM EDTA pH8, 0.5% SDS, 20% glycerol). The reaction products were analysed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Evaluation of the cellular and nuclear accumulation of ciprofloxacin, camptothecin and etoposide. WT, CIP-R and MXF-R cells were incubated for 20 h with 200 µM CIP, or 500 nM CPT, or 10 µM ETO, or in combination (CPT+CIP or ETO+CIP). Cells were washed with PBS, collected in 1/4M sucrose, and homogenized with a dounce as described in (Seral et al. 2003a). One fraction (homogenate) of each sample was

conserved to assay the total cellular concentration of the drugs, and one fraction was centrifuged at low speed to separate nucleus and cytosol. The concentration of drugs in each fraction was assayed (i) by microbiologic assay for CIP (using *E. coli* ATCC25922 and antibiotic medium 11), and by liquid scintillation for CPT and ETO (cells were incubated in the presence of 0.75 µCi/mL [^{3}H]camptothecin (specific activity = 4.5 Ci/mmol) and 1 µCi/mL [^{3}H]etoposide (specific activity = 300 mCi/mmol)). The activity of lactate deshydrogenase, N-acetyl-betahexosaminidase and cytochrome-c-oxydase was assayed in all fractions as markers of the soluble proteins (cytosol), of lysosomes and of mitochondria respectively. The drug content was expressed with reference to the protein content of each fraction.

3.3. Results

Mitochondrial activity of cells exposed to ciprofloxacin, moxifloxacin, camptothecin, etoposide or combination. MTT test was assayed to evaluate the mitochondrial activity in WT, CIP-R and MXF-R cells exposed to large ranges of fluoroquinolones (50 to 2000 µM) or anti-topoisomerases agents (0 to 10.000 nM for camptothecin; 0 to 1000 µM for etoposide) or combination of one fluoroquinolone and one anti-topoisomerase agent. Dose-effect curves (not shown here) were used to determine the IC_{50} values (concentrations of drugs decreasing the mitochondrial function of 50%) presented in figure 1. We observed that 200 µM of both fluoroquinolones can reduce the mitochondrial activity of 50% in WT cells (Fig. 1.A.). In comparison, IC_{50} were higher in CIP-R and MXF-R cells for both fluoroquinolones (6 fold and 2.5 fold for CIP and MXF respectively in CIP-R cells, and about 2 fold for both fluoroquinolones in MXF-R cells). Only about 200 nM camptothecin and 7 µM etoposide were needed to reduce the mitochondrial activity of 50% in all three cell types (Fig. 1.B. and 1.C.). But when camptothecin or etoposide were co-incubated with a fluoroquinolone, IC_{50} were markedly increased in all three cell types, except in CIP-R cells when co-incubated with ciprofloxacin.

These data suggest that: (i) CIP-R cells are highly resistant to ciprofloxacin but only partly to moxifloxacin whereas MXF-R cells are resistant to both fluoroquinolones, and (ii) ciprofloxacin seems to protect cells from the toxicity of camptothecin and etoposide because of the higher IC_{50} values in all cell types except in CIP-R cells where ciprofloxacin is largely effluxed.

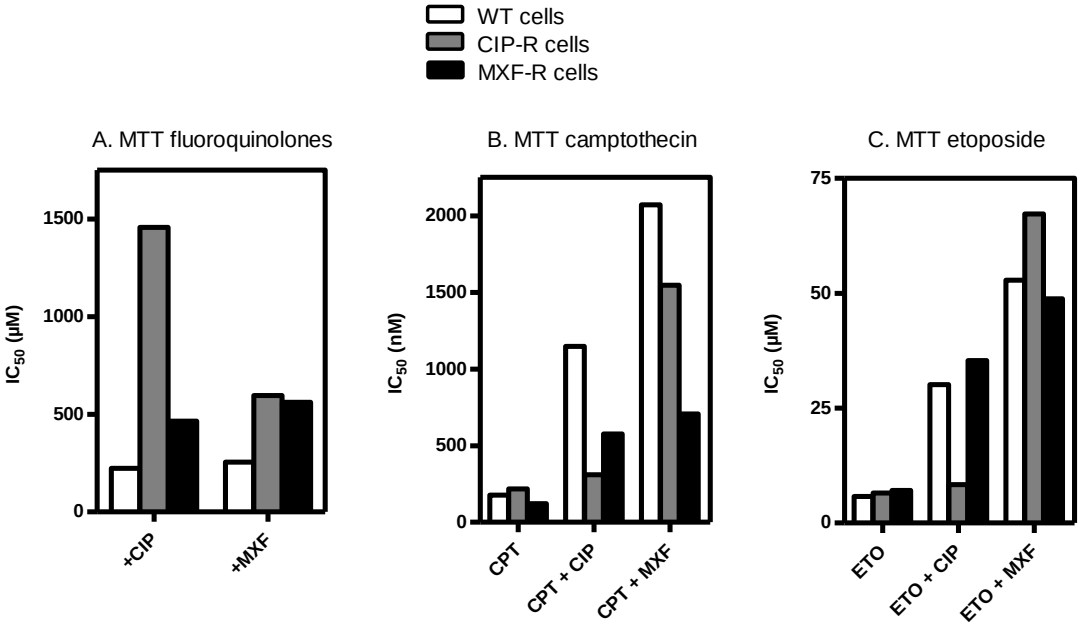


Figure 1. Effects of fluoroquinolones or anti-topoisomerases agents or combinations of both on the mitochondrial activity in wild-type (WT in white), ciprofloxacin-resistant (CIP-R in grey) and moxifloxacin-resistant (MXF-R in black) cells. **A**. Cells were incubated for 48 h with ciprofloxacin (CIP) or moxifloxacin (MXF). **B**. Cells were incubated for 24 h with camptothecin (CPT) alone, or in combination with CIP or MXF. **C**. Cells were incubated for 24 h with etoposide (ETO) alone or in combination with CIP or MXF.

Cytotoxicity of fluoroquinolones, anti-topoisomerases agents and their combinations on WT, CIP-R and MXF-R cells. Apoptosis (Fig 2.A.) and necrosis (Fig 2.B.) caused by CIP, MXF, CPT, ETO or combination of one fluoroquinolone and one anti-topoisomerase agent were evaluated in WT, CIP-R and MXF-R cells. First, we showed that both fluoroquinolones used alone (Fig 2.A. and 2.B. left panels) do not induce apoptosis nor necrosis in the three cell types. Second, camptothecin alone (Fig 2.A. and 2.B. middle panels) induced about 30% apoptosis and more than 70% necrosis in all three cell types. When camptothecin was co-incubated with ciprofloxacin or moxifloxacin, percentages of apoptotic cells and LDH release were markedly decreased, except in CIP-R cells when co-incubated with ciprofloxacin. Finally, etoposide (Fig 2.A. and 2.B. right panels) induced about 40% apoptosis and LDH release in WT and CIP-R cells, while this percentage is decreased in MXF-R cells (only 15% of apoptotic cells, and 30% of LDH release). When etoposide was co-incubated with ciprofloxacin or moxifloxacin, we evidenced decreased apoptosis and necrosis compared to those induced by etoposide alone, except again in CIP-R cells when co-incubated with ciprofloxacin.

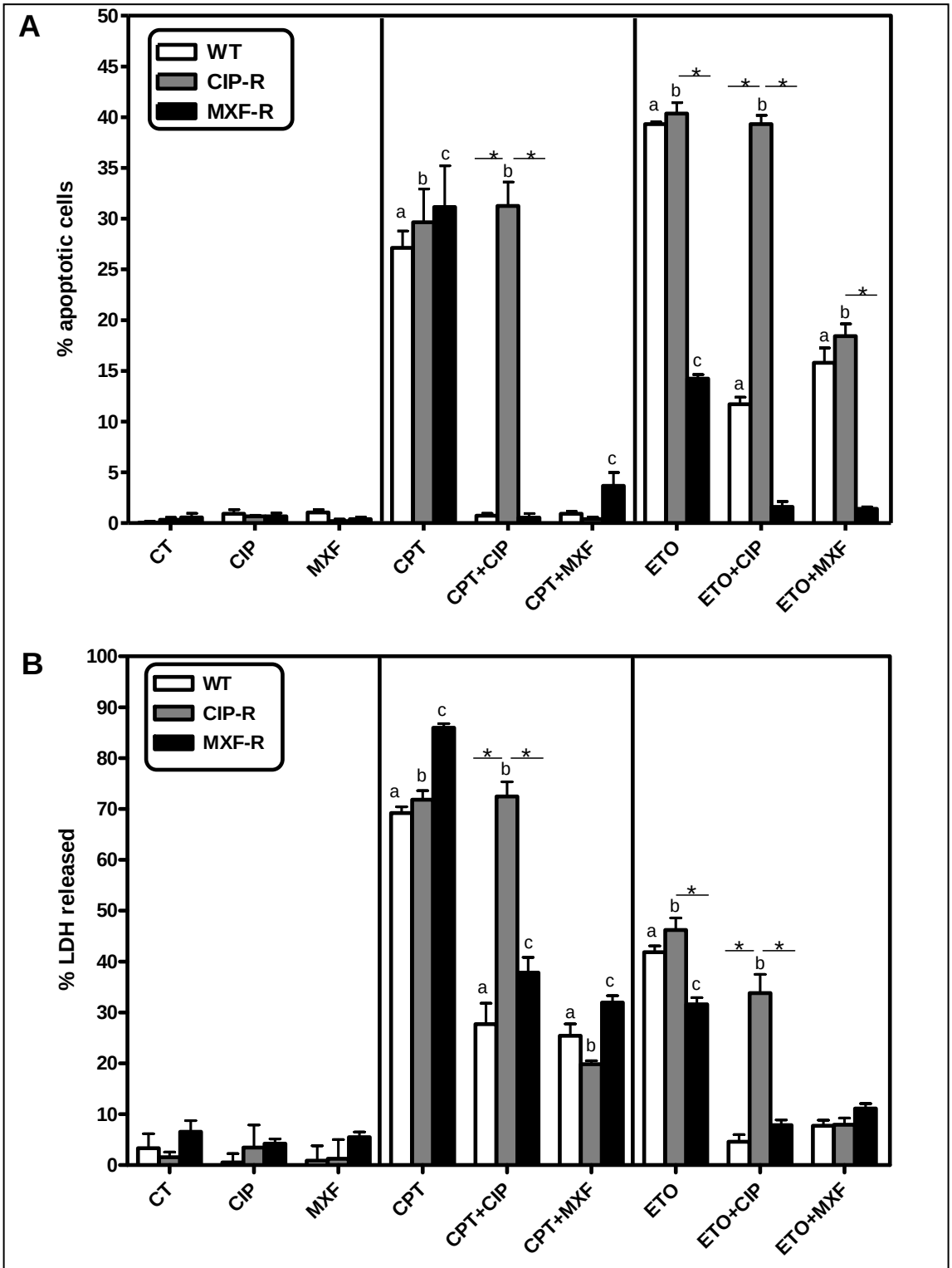


Figure 2. Percentage of apoptotic cells determined by the DAPI method (A), and percentage of LDH released (B) in WT, CIP-R and MXF-R cells incubated for 24 h without any drugs (CT), or with ciprofloxacin 200 µM (CIP), or with moxifloxacin 200 µM (MXF), or with camptothecin 500 nM (CPT), or with etoposide 10 µM (ETO), or with combination as indicated in the figure. Values are means of three independent experiences. Statistical analysis (ANOVA Tukey post-hoc test, $p<0.05$) : letters indicate a significant difference within a cell type with the control without drug (WT(a), CIP-R(b), MXF-R(c)), and asterisks mean that there is a significant difference within cell types in the same incubation conditions.

Evaluation of the topoisomerases activity. Fluoroquinolones target topoisomerases in bacteria, and are able to inhibit topoisomerases in eukaryotic cells. Ciprofloxacin and moxifloxacin were used to evaluate their possible inhibitory effect on topoisomerases from WT, CIP-R and MXF-R cells. In addition, we compared the effects of those two antibiotics with the effects of the two known anticancer agents camptothecin and etoposide (type I and type II topoisomerase inhibitor respectively), and the combination of one fluoroquinolone and one anticancer agent.

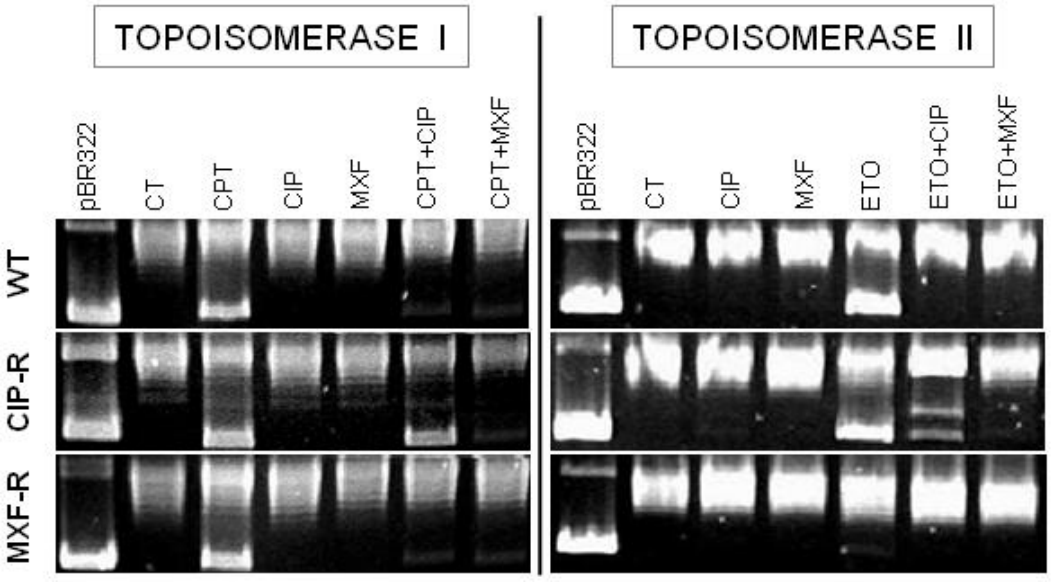


Figure 3. Activity of type I topoisomerase (left panel) and type II topoisomerase (right panel) in WT, CIP-R and MXF-R cells. Nuclear extracts were prepared from cells incubated without drugs (CT), or with 1 µM camptothecin (CPT), 200 µM ciprofloxacin (CIP), 200 µM moxifloxacin (MXF), 10 µM etoposide (ETO), or combinations of one fluoroquinolone and one anti-topoisomerase agent (CPT+CIP; CPT+MXF; ETO+CIP; ETO+MXF). Topoisomerase activity was determined using pBR322 supercoiled plasmid as a substrate (lower band on gel).

We observed on figure 3 that neither ciprofloxacin nor moxifloxacin inhibit type I or type II topoisomerases in all three cell types. Camptothecin was used as a control for type I topoisomerase inhibition, and this anticancer agent was effective in all three cell types (Fig. 3 left panel). When camptothecin was co-incubated with one fluoroquinolone, its inhibitory effect was annihilated, except in CIP-R cells when co-incubated with ciprofloxacin. Etoposide was used as a control for type II topoisomerase inhibition, and this anticancer agent was effective only in WT and CIP-R cells (Fig. 3 right panel), but not in MXF-R cells, as we observed a total activity of type II topoisomerase even in the

presence of etoposide. When etoposide was co-incubated with one fluoroquinolone, its inhibitory effect was annihilated as for camptothecin, except in CIP-R cells when co-incubated with ciprofloxacin.

Evaluation of the cellular and nuclear accumulation of ciprofloxacin, camptothecin, and etoposide. The total cellular and nuclear concentrations of ciprofloxacin, moxifloxacin, camptothecin and etoposide alone or in combination were evaluated in WT, CIP-R and MXF-R cells.

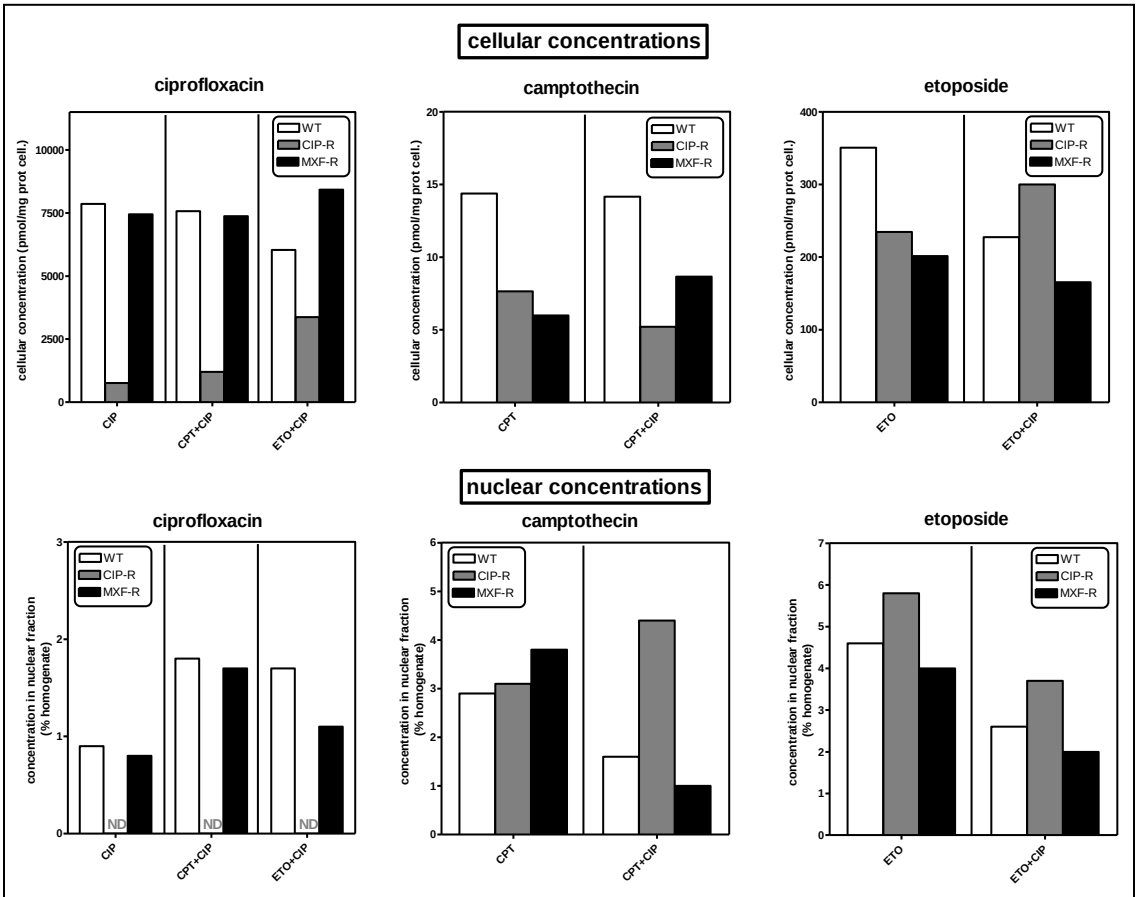


Figure 4. Concentrations of ciprofloxacin (left panel), camptothecin (middle panel), and etoposide (right panel) in the homogenate (upper panels) or in the nuclear fraction (lower panels) in WT, CIP-R and MXF-R cells incubated during 20 h with 200 µM ciprofloxacin (CIP), 500 nM camptothecin (CPT), 10 µM etoposide (ETO) or combinations (CPT+CIP, ETO+CIP). Nuclear concentrations are expressed in percentage of the homogenate quantity. Values are the results of unique samples.

Ciprofloxacin cellular accumulation (Fig. 4 upper left panel) is lower in CIP-R cells compared to that in WT and MXF-R cells. No marked difference was noted when cells

where co-incubated with camptothecin, while ciprofloxacin accumulation was increased in CIP-R cells co-incubated with etoposide. Considering now the nuclear fraction (Fig. 4 lower left panel), we observed that the concentration of ciprofloxacin was increased in WT and MXF-R cells upon co-incubation with an anticancer agent, but ciprofloxacin remained undetectable in the nuclear fraction collected from CIP-R cells. Camptothecin and etoposide cellular concentrations (Fig. 4 upper middle and right panels) were lower in CIP-R and MXF-R cells than in WT cells (possibly due to an efflux of these agents by transporters present in those cells [camptothecin is known to be substrate for MRP4, BCRP and P-gp, and etoposide for P-gp (Marquez B and Van Bambeke F, 2011)]). No major modifications of their cellular contents were observed when co-incubated with ciprofloxacin (except for etoposide which showed a decreased cellular concentration in WT cells when co-incubated with ciprofloxacin). The nuclear content in camptothecin and etoposide (Fig. 4 lower middle and right panels), was grossly similar in the three cell types. Interestingly, however, it was decreased in wild-type and MXF-R cells co-incubated with ciprofloxacin

3.4. Conclusion and discussion

In this study, we wanted to know more about the toxicity of ciprofloxacin and moxifloxacin in WT (J774) mouse macrophages, ciprofloxacin- and moxifloxacin-resistant cell lines. The cytotoxicity of both fluoroquinolones was compared to that of two anticancer agents known to inhibit eukaryotic topoisomerases: camptothecin as type I topoisomerase inhibitor, and etoposide as type II topoisomerase inhibitor. We have shown that (i) both fluoroquinolones do not induce the same toxicity in the three cell types, (ii) the mechanism of resistance in MXF-R cells may be due to an alteration of the activity of type II topoisomerases (compared to the efflux-mediated resistance mechanism in CIP-R cells), and (iii) fluoroquinolones can “protect” cells against the toxicity of anticancer agents, a phenomenon strictly dependent on the ability of fluoroquinolones to accumulate in cells.

Although fluoroquinolones are antibiotics, meaning that they are toxic for bacteria, our results show that they can be toxic for eukaryotic cells when large concentrations are used. Data presented in this study showed that both fluoroquinolones are toxic for WT cells, and partially toxic for MXF-R cells, whereas only moxifloxacin is toxic for CIP-R cells. Other studies have examined the cytotoxicity of fluoroquinolones in eukaryotic cells, and they show that this phenomenon is time- and concentration-

dependent: ciprofloxacin is able to induce apoptosis in cancer cells with 2 to 6 fold the concentrations used in this study, and with incubation times higher than 48 h ((Pessina et al. 2007); Aranha et al. 2000; (Herold et al. 2002)). In addition, it has been shown that the cell type can influence the toxicity caused by fluoroquinolones (Herold et al. 2002).

Cytotoxicity of fluoroquinolones in eukaryotic cells could be related to their ability to inhibit eukaryotic topoisomerases ((Anderson and Osheroff 2001); (Elsea et al. 1992); (Elsea et al. 1993)). Some studies have shown that ciprofloxacin can inhibit type II topoisomerases which stimulate the DNA cleavage ((Robinson et al. 1991); (Bromberg et al. 2003); (Pessina et al. 2001)). But these studies were made in conditions where the antibiotic was added to the nuclear extract (which maximizes the contact between topoisomerase and the molecule), while in our study, topoisomerase activity was tested on nuclear extracts from cells incubated only 20 h with the antibiotic. This could explain our data which showed no cytotoxic effects of ciprofloxacin nor moxifloxacin in all three cell types (Fig. 2.A. and 2.B.) after 24 h of incubation and no inhibition of type I and II topoisomerases by both fluoroquinolones (Fig. 3).

The resistance mechanism in CIP-R cells has been demonstrated to be due to the overexpression of the Mrp4 transporter ((Marquez et al. 2009); (Marquez and Van Bambeke 2011)), but this in MXF-R cells remains still unknown. Results presented in figures 2 and 3 suggest that exposure of cells to high concentrations of moxifloxacin can induce an alteration of type II topoisomerase activity, as those cells are less sensitive to the effect of etoposide than WT and CIP-R cells. This alteration could be due to a mutation in the enzyme gene, which could decrease the binding of the pharmacologic agent to the enzyme or increase the DNA strands religation activity despite the presence of the inhibitor (Robert and Larsen 1998).

In order to better understand the effects of fluoroquinolones on topoisomerases, we investigated the possible synergistic effects of fluoroquinolones with known topoisomerase inhibitors. Surprisingly, we have shown a protective role of fluoroquinolones towards the toxicity induced by camptothecin or etoposide (while opposite observations were made in several publications which showed a synergistic effect of combination of fluoroquinolones and anticancer agents: (Reuveni et al. 2008); (Fabian et al. 2006); (El Rayes et al. 2002). This protection is strictly linked to their ability to accumulate in cells. Indeed, ciprofloxacin didn’t protect against camptothecin or etoposide toxicity when co-incubated in CIP-R cells from which ciprofloxacin is largely effluxed (except in the presence of the MRP inhibitor gemfibrozil, which prevents

ciprofloxacin efflux from cells (data not shown)). Data in our study showed that this protection is due to the decreased ability of camptothecin and etoposide to inhibit type I and type II topoisomerases respectively in the presence of fluoroquinolones. The mechanism leading to this protection has not been elucidated yet, but we have shown in preliminary experiments that nuclear concentrations of camptothecin and etoposide were lower when co-incubated with fluoroquinolones, which could explain the decreased inhibition activity of these anticancer agents on their targets.

CHAPTER II: INFLUENCE OF STRUCTURE ON CELLULAR PHARMACOKINETIC PROFILE

The accumulation level of a drug is thought to govern its intracellular activity. Thus a drug which reaches high intracellular concentration should be more active than a drug which accumulates at a lower level. Yet, several pharmacokinetic and pharmacodynamic studies have shown that it is not true for every drug, and that each drug has its own behavior when inside cells. Indeed, a drug can bind to intracellular components, or be sequestered in intracellular compartments, or be effluxed out of cells by active transporters. All these parameters will contribute to decrease the contact between a drug and its target, therefore leading the drug to be less effective.

In this chapter, the two goals are:

- to describe the efflux profile, and the intracellular activity of three fluoroquinolones which accumulate to very different levels in J774 macrophages.

- to ascertain the molecular determinants of fluoroquinolones which manage their efflux by transporters in prokaryotic and eukaryotic cells.

II.1. Cellular accumulation of fluoroquinolones is not predictive of their intracellular activity: studies with gemifloxacin, moxifloxacin, and ciprofloxacin in a pharmacokinetic/pharmacodynamic model of uninfected and infected macrophages.

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International Journal of Antimicrobial Agents, May 2011, vol. 38, p. 249-256

International Journal of Antimicrobial Agents 38 (2011) 249–256

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Cellular accumulation of fluoroquinolones is not predictive of their intracellular activity: studies with gemifloxacin, moxifloxacin and ciprofloxacin in a pharmacokinetic/pharmacodynamic model of uninfected and infected macrophages

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ARTICLE INFO

Article history:
Received 22 March 2011
Accepted 17 May 2011

Keywords:
Ciprofloxacin
Moxifloxacin
Gemifloxacin
Mrp4
Staphylococcus aureus
Listeria monocytogenes
Macrophages

ABSTRACT

Fluoroquinolones enter eukaryotic cells but the correlation between cellular accumulation and activity remains poorly established. Gemifloxacin is known to accumulate to a larger extent than most other fluoroquinolones in tissues. Using murine J774 macrophages and human THP-1 monocytes, we show that gemifloxacin accumulates more than ciprofloxacin and even moxifloxacin. Whilst showing indistinguishable kinetics of accumulation in J774 macrophages, gemifloxacin was released at an approximately two-fold slower rate than ciprofloxacin and its release was only partial. Gemifloxacin was also a weaker substrate than ciprofloxacin for the efflux transporter Mrp4 active in J774 macrophages. In cells infected with *Listeria monocytogenes* or *Staphylococcus aureus* (typical cytoplasmic and phagolysosomal organisms, respectively), gemifloxacin was equipotent to moxifloxacin and ciprofloxacin in concentration-dependent experiments if data are normalised based on the minimum inhibitory concentration (MIC) in broth. Thus, larger cellular concentrations of gemifloxacin than of moxifloxacin or ciprofloxacin were needed to obtain a similar target effect. Fractionation studies showed a similar subcellular distribution for all three fluoroquinolones, with approximately two-thirds of the cell-associated drug recovered in the soluble fraction (cytosol). These data suggest that cellular accumulation of fluoroquinolones is largely a self-defeating process as far as activity is concerned, with the intracellular drug made inactive in proportion to its accumulation level. Whilst these observations do not decrease the intrinsic value of fluoroquinolones for the treatment of intracellular infections, they indicate that ranking fluoroquinolones based on cell accumulation data without measuring the corresponding intracellular activity may lead to incorrect conclusions regarding their real potential.

1. Introduction

Fluoroquinolone antibiotics are important in the current therapeutic arsenal because of their broad spectrum, highly bactericidal activity and favourable pharmacokinetic properties [1]. Their wide tissue distribution allows them to reach therapeutic concentrations in deep body compartments as well as in the intracellular milieu, which may be an advantage in the treatment of intracellular infections. Accumulation and activity in cells are usually linked when considering a given fluoroquinolone in a specific cell type, as demonstrated for ciprofloxacin in relation to the intracellular forms of *Listeria monocytogenes* in J774 macrophages in experiments where the drug's cellular concentration was modulated by inhibition or overexpression of the constitutive ciprofloxacin efflux transporter Mrp4 [2,3]. There is, however, a lack of quantitative data comparing distinct fluoroquinolones in this context.

Gemifloxacin [4] accumulates to high levels in human polymorphonuclear leukocytes and is active against intracellular bacteria [5,6]. This prompted us to compare it with other fluoroquinolones for cellular pharmacokinetics and activity in an established model of murine J774 macrophages [7]. Ciprofloxacin and moxifloxacin, when needed, were used as comparators as these antibiotics show low and high accumulation, respectively, in relation to differential susceptibility to efflux [8–11]. We also examined THP-1 cells, where no active fluoroquinolone efflux has been evidenced so far.

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doi:10.1016/j.ijantimicag.2011.05.011

We found that gemifloxacin accumulates to higher levels than ciprofloxacin and moxifloxacin in both cell types and that all three drugs have a similar subcellular distribution. Yet gemifloxacin showed no improved activity against two types of intracellular bacteria, *L. monocytogenes* and *Staphylococcus aureus*, localised in the cytosol and in phagolysosomes, respectively.

2. Materials and methods

2.1. Antibiotics and main reagents

Gemifloxacin mesylate (LG Life Sciences, Seoul, South Korea) and ciprofloxacin HCl and moxifloxacin HCl (Bayer HealthCare AG, Leverkusen, Germany) were obtained as microbiological standards (potencies 79%, 85% and 91%, respectively). Gemfibrozil was from Sigma–Aldrich (St Louis, MO), human serum was from Lonza Ltd. (Basel, Switzerland) and cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA).

2.2. Cell lines

Murine J774 macrophages (wild-type cells [9]) and their ciprofloxacin-resistant derivatives overexpressing the Mrp4 efflux transporter [8,11] were used for most experiments. Human THP-1 cells (ATCC TIB-202; American Tissue Culture Collection, Manassas, VA) [12,13] were used for comparison purposes. ATP depletion was achieved as previously described [9].

2.3. Determination of cellular accumulation of fluoroquinolones

A previously described protocol was used [9,14]. Cell-associated fluoroquinolones were assayed by fluorimetry (see [10] for ciprofloxacin and moxifloxacin; for gemifloxacin, the conditions were $\lambda_{ex.} = 270$ nm, $\lambda_{em} = 402$ nm; lowest limit of detection 50 μg/L; linearity 0–1.5 mg/L). The cell drug content was expressed by reference to the total cell protein content [15]. The apparent total cellular concentration was then calculated using a conversion factor of 3.08 μL of cell volume per mg of cell protein [9].

2.4. Cell fractionation studies in J774 cells

The main subcellular organelles were separated by differential centrifugation as previously described [2]. The protein and antibiotic content of each fraction was determined in parallel with the activity of marker enzymes of the main organelles (cytochrome *c* oxidase for mitochondria, *N*-acetyl-β-hexosaminidase for lysosomes, and lactate dehydrogenase for cytosol [7]).

2.5. Bacterial strains and susceptibility testing

Listeria monocytogenes strain EGD and *S. aureus* strain ATCC 25923 were used. Minimum inhibitory concentration (MIC) determinations were made according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [16] using tryptic soy broth for *L. monocytogenes* [13] and Mueller–Hinton broth for *S. aureus* [14].

2.6. Cell infection and assessment of antibiotic intracellular activities

Cell infection was performed as described previously [2], with pharmacological comparison between drugs and bacteria based on concentration-dependent effects analyses [14] to determine (i) the relative minimal and maximal efficacies (E_{min} and E_{max}, respectively, in $\log_{10}$ units) and (ii) the relative potencies (EC_{50}) and static concentrations (C_s). This type of analysis and its usefulness for comparing antibiotics and the response of different bacteria have been described in detail in previous publications [14,17–19]. As discussed previously [20], the large dilution of samples before spreading on agar plates for colony-forming unit (CFU) counting ensures the absence of a carry-over effect.

2.7. Curve fitting and statistical analyses

Curve fitting analyses were done using GraphPad Prism® 4.03 (GraphPad Software Inc., San Diego, CA). Statistical analyses were made with the same software for comparing concentration–response functions, and with GraphPad InStat® v3.06 (GraphPad Software Inc.) for other studies.

3. Results

3.1. Cellular pharmacokinetics

We first compared the cellular accumulation of gemifloxacin with that of ciprofloxacin and moxifloxacin and examined the influence of gemfibrozil, a broad-spectrum inhibitor of anion transporters including the Mrp transporters, on this accumulation. Fig. 1A shows that (i) gemifloxacin accumulated to a larger extent than the other two fluoroquinolones both in J774 and THP-1 cells; (ii) the accumulation of gemifloxacin and moxifloxacin was not influenced by gemfibrozil; (iii) in contrast, ciprofloxacin, which accumulated to the lowest extent in J774 macrophages, reached a cellular concentration similar to that of moxifloxacin in these cells in the presence of gemfibrozil, as already observed in the same model [10]; and (iv) the level of accumulation of ciprofloxacin was similar to that of moxifloxacin in THP-1 cells and was not influenced by the addition of gemfibrozil.

We then compared the kinetics of accumulation and efflux of gemifloxacin with that of ciprofloxacin using J774 macrophages only, as this is where the largest difference of accumulation was observed. Fig. 1B shows that the two fluoroquinolones could not be distinguished with respect to accumulation kinetics but displayed marked differences for efflux. Thus, gemifloxacin release (i) occurred at the same rate as its uptake (compare k_{in} and k_{out} parameters); (ii) was approximately two-fold slower than that of ciprofloxacin, including in the very initial period (see inset); (iii) was only partial, with ca. 25% of the accumulated drug remaining cell-associated in an apparent stable fashion after 30 min of incubation in drug-free medium compared with negligible amounts for ciprofloxacin.

We next measured the level of accumulation of gemifloxacin compared with that of ciprofloxacin in J774 macrophages overexpressing the ciprofloxacin efflux transporter Mrp4 (ciprofloxacin-resistant cells), using normal conditions and conditions of ATP depletion (which inhibits all ATP-dependent active transporters, including Mrp4). Fig. 2A shows that (i) gemifloxacin accumulation was reduced (but in a non-statistically significant manner) in ciprofloxacin-resistant cells compared with wild-type cells; (ii) ATP depletion increased its accumulation both in wild-type and ciprofloxacin-resistant cells, but with a significant difference in the latter cells only; (iii) ciprofloxacin accumulation was significantly reduced in ciprofloxacin-resistant cells, but was markedly increased by ATP depletion, reaching a value similar to that observed in wild-type cells after ATP depletion; and (iv) in line with our previous observations [11], ATP depletion markedly increased the accumulation of ciprofloxacin in wild-type cells.

Because the ciprofloxacin efflux transporter is saturable in a 10–200 mg/L range [9], we measured the accumulation of gemifloxacin both in wild-type J774 macrophages and in ciprofloxacin-resistant cells over increasing concentrations of

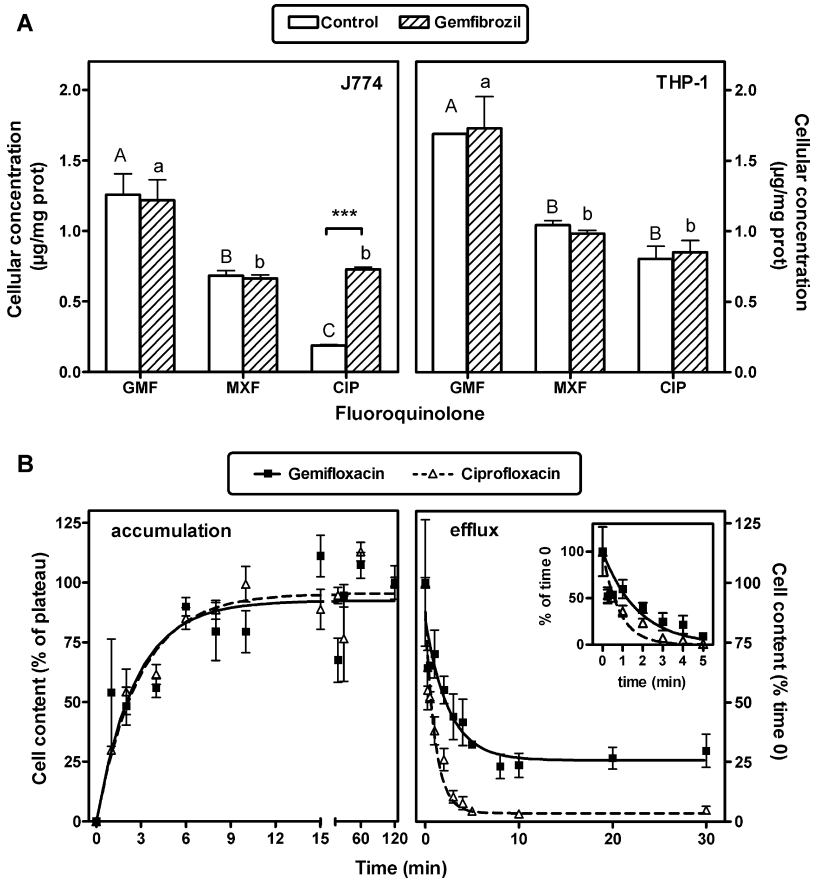

Fig. 1. Accumulation and efflux of fluoroquinolones. (A) Accumulation of gemifloxacin (GMF), moxifloxacin (MXF) and ciprofloxacin (CIP) in wild-type J774 mouse macrophages (left) and human THP-1 monocytes (right) incubated for 2 h with 20 mg/L in control conditions or in the presence of the Mrp inhibitor gemfibrozil (500 μM). All values are the mean of three independent determinations ± standard deviation. Statistical analysis (ANOVA): control vs. gemfibrozil, $^{***}P < 0.001$; comparison between fluoroquinolones, bars with different letters are different from one another ($P < 0.001$; upper case letters, control conditions; lower case letters, + gemfibrozil). (B) Kinetics of accumulation (left) and efflux (right) of gemifloxacin compared with ciprofloxacin in J774 macrophages (see [10] for efflux of moxifloxacin). For accumulation studies, cells were transferred to medium containing a fixed amount of drug (20 mg/L) and were collected at the times indicated on the abscissa. For efflux, cells were first exposed to the drug for 2 h at a concentration of 20 mg/L, gently washed, transferred to drug-free medium and collected at the times indicated on the abscissa. Data were used to fit a one-phase exponential association function for influx [$y = y_{max}x(1 - e^{-k_{in}xt})$] and a one-phase exponential decay function for efflux [$y = y_{max}xe^{-k_{out}xt}$ + plateau)] by non-linear regression. Regression parameters for influx: (a) gemifloxacin, $R^2 = 0.780$, $k_{in} = 0.386 \pm 0.123\ min^{-1}$; (b) ciprofloxacin, $R^2 = 0.922$, $k_{in} = 0.348 \pm 0.066\ min^{-1}$. Regression parameters for efflux: (i) main graph, (a) gemifloxacin, $R^2 = 0.897$, $k_{out} = 0.403 \pm 0.122\ min^{-1}$, plateau = 25.71 ± 4.63; (b) ciprofloxacin, $R^2 = 0.949$, $k_{out} = 0.949 \pm 0.204\ min^{-1}$, plateau = 3.56 ± 3.24; (ii) inset: data for the initial stage of efflux (0–5 min) and corrected for differences in plateau reached after 10 min, (a) gemifloxacin, $R^2 = 0.658$, $k_{out} = 0.571 \pm 0.138\ min^{-1}$; (b) ciprofloxacin, $R^2 = 0.909$, $k_{out} = 1.216 \pm 0.209\ min^{-1}$. Statistical analysis (paired *t*-test two-tailed): influx, no significant difference in rate constants; absolute values of plateaus of accumulation were different and in line with data of Fig. 1. Efflux: main graph, comparison of all values, $P < 0.001$; plateaus values only, $P < 0.001$; k values only, $P < 0.001$; inset, comparison for all values, $P = 0.016$; k_{out} values only, $P < 0.001$.

gemifloxacin in that range. Fig. 2B shows that whilst gemifloxacin accumulation was not significantly influenced by its extracellular concentration in wild-type cells, there was a significant increase over the range of concentrations investigated for ciprofloxacin-resistant cells. In contrast, and as described previously [9], ciprofloxacin showed a marked increase in its accumulation over the same concentration range in wild-type cells. For ciprofloxacin-resistant cells, the increase in cell accumulation of ciprofloxacin was much less marked in the range of drug concentrations investigated owing to overexpression of the Mrp4 transporter (see [11]).

These results suggest that gemifloxacin could be a poor, albeit still recognised, substrate for efflux transport in J774 macrophages if Mrp4 is overexpressed. We therefore compared the kinetics of gemifloxacin efflux in ciprofloxacin-resistant vs. wild-type cells. Whilst the plateau values observed at 30 min remained close to each other, denoting an incomplete release of gemifloxacin in both cases, its rate of efflux was significantly accelerated in ciprofloxacin-resistant cells compared with wild-type cells ($k_{out} = 2.393 \pm 0.907\ min^{-1}$ vs. $0.403 \pm 0.122\ min^{-1}$; $P < 0.001$) (see graphical representation in Supplementary Fig. 1).

3.2. Intracellular activity

To examine the correlation between cellular accumulation and intracellular activity, we compared all three fluoroquinolones in a pharmacological model of intracellular infection [14,17] using J774 macrophages as host cells since this is where the largest differences in accumulation levels had been observed. *Listeria monocytogenes* and *S. aureus* were selected as bacterial targets as they represent a typical cytoplasmic and phagolysosomal organism, respectively. Data presented in Fig. 3A (with analysis of the key pharmacological descriptors in Table 1) show that all three antibiotics induced essentially a similar response when expressed as a function of equipotent

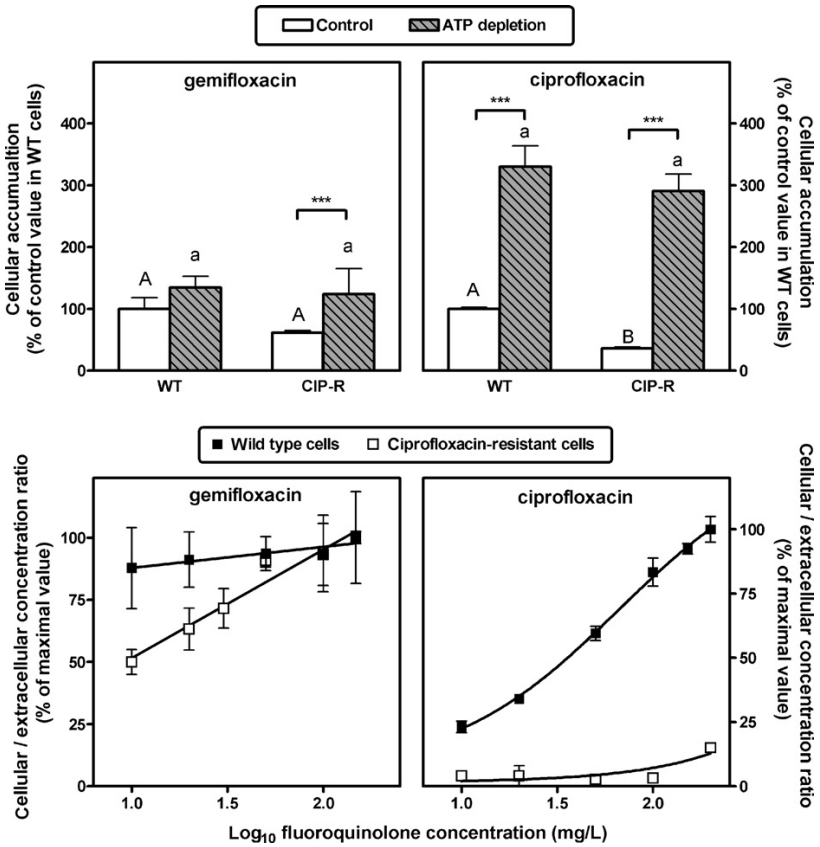

Fig. 2. Cellular accumulation of gemifloxacin compared with ciprofloxacin in wild-type (WT) or ciprofloxacin-resistant (CIP-R) J774 mouse macrophages (see [11] for the corresponding data for moxifloxacin). (A) Cells were incubated for 2 h in control conditions or ATP-depleted with a fixed concentration (20 mg/L) of gemifloxacin (left) or ciprofloxacin (right) with WT or CIP-R cells. Data are expressed as percentage of the value measured in WT cells in control conditions for each fluoroquinolone. All values are the means of three independent determinations ± standard deviation (S.D.). Statistical analysis (ANOVA): control vs. ATP depletion, $^{***}P < 0.001$; WT vs. CIP-R cells: bars with different letters are different from one another ($P < 0.05$; upper case letters, control conditions; lower case letters, ATP depletion). (B) Influence of the extracellular concentration of gemifloxacin (left) and ciprofloxacin (right) on their cellular-to-extracellular concentration ratio in WT or CIP-R J774 mouse macrophages measured after 2 h of incubation. The cellular concentration was expressed as μg per mg protein. Data are expressed as percentage of the highest value observed in WT cells for each fluoroquinolone. All values are the means of three independent determinations ± S.D.

extracellular concentrations (multiples of the MIC). Thus, in all cases, a single sigmoid function could be fitted to the individual responses of each antibiotic (see Supplementary Fig. 2 and the pertinent regression parameters and pharmacological descriptors in Table 1). As no statistically significant difference was observed between the three sets of experiments with respect to relative minimal efficacies (E_{min}; growth in the absence of antibiotic), maximal relative efficacies (E_{max}; maximal antibiotic-related killing), relative potencies (EC_{50}) and static concentrations (C_s; in multiples of the MIC), all data were pooled to fit a single function shown in Fig. 3A. We then calculated for each fluoroquinolone which cellular drug concentration would be needed to reach two predefined pharmacodynamic targets (static effect and a 1 or 2 $\log_{10}$ CFU decrease). The results (with the mode of calculation) are presented in Fig. 3B and show that the potencies of the drugs with respect to their intracellular targets were in inverse proportion to their respective cellular accumulations.

3.3. Subcellular distribution

Lastly, we compared the subcellular distributions of ciprofloxacin, moxifloxacin and gemifloxacin. Fig. 4 shows that all three fluoroquinolones shared essentially the same distribution, with ca. 70% recovered in the soluble fraction, ca. 10% of ciprofloxacin and gemifloxacin and 18% of moxifloxacin in the nuclei/unbroken cells fraction, and the remainder in the organelles/membranes fraction. As previously described [7], lactate dehydrogenase was mostly recovered in the soluble fraction, and cytochrome oxidase and *N*-acetyl-β-hexosaminidase in the granules/membranes fraction, indicating that the fractionation method effectively separated the corresponding subcellular entities with only a very low proportion of unbroken cells left after homogenisation.

4. Discussion

Gemifloxacin, approved for clinical use in over 27 countries [21], is characterised by very low MICs against Gram-positive bacteria [22,23], related to the presence of an oximinomethyl group [4] in its C7 side chain, and by a high tissue accumulation [24]. Human pharmacokinetic/pharmacodynamic studies show that gemifloxacin achieves higher area under the concentration–time curve (AUC)/MIC ratios in epithelial lining fluid and alveolar macrophages than other currently used fluoroquinolones, suggesting an advantage in terms of availability and efficacy at the site of infection [25,26]. However, the present study shows that the

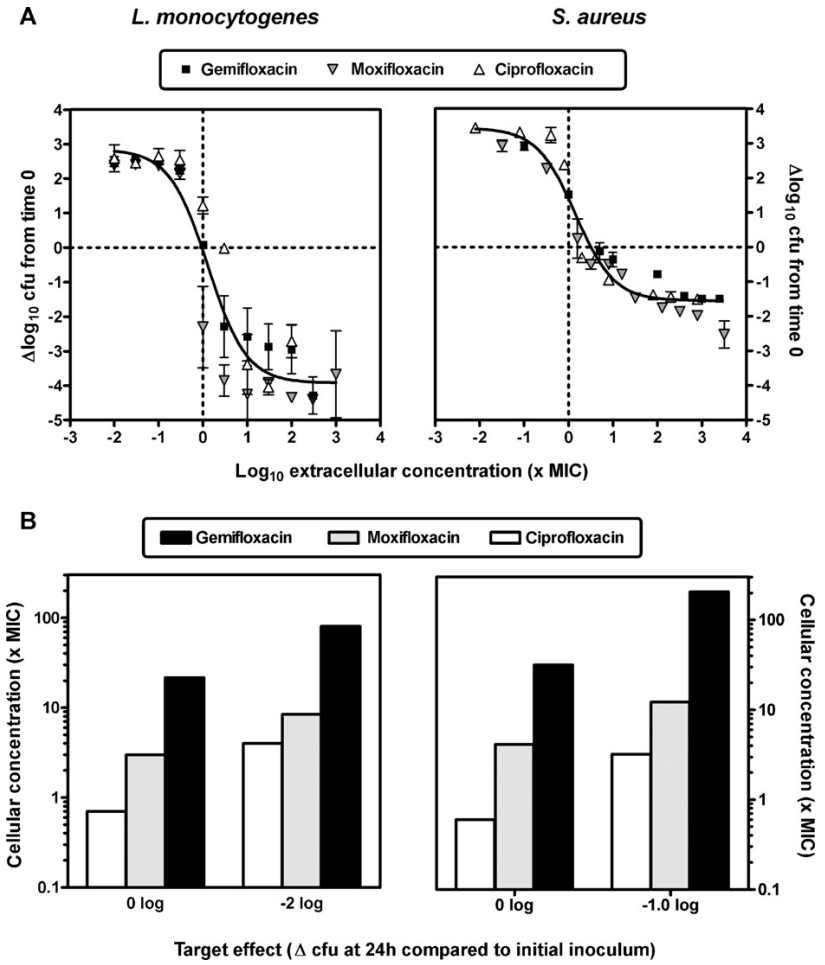

Fig. 3. Concentration–response of the activities of gemifloxacin, moxifloxacin and ciprofloxacin against phagocytosed *Listeria monocytogenes* EGD (left) and *Staphylococcus aureus* ATCC 25923 (right) in wild-type J774 macrophages. (A) After phagocytosis and elimination of extracellular bacteria, cells were incubated for 24 h with increasing concentrations of antibiotic (total drug) covering a minimum inhibitory concentration (MIC) range of ca. 0.01× to ca. 1000× MIC [MICs were 1 mg/L and 0.125 mg/L (ciprofloxacin), 0.5 mg/L and 0.03 mg/L (moxifloxacin) and 0.5 mg/L and 0.008 mg/L (gemifloxacin) for *L. monocytogenes* and *S. aureus*, respectively]. The graphs show the change in the number of colony-forming units (CFU) (log scale) per mg of cell protein compared with the initial post-phagocytosis inoculum (ordinate) as a function of the extracellular concentration of each drug expressed in multiple of its MIC (abscissa). In each graph, the horizontal dotted line corresponds to an apparent static effect and the vertical line to the MIC of the drug. A single sigmoidal regression has been fit to all data sets (see Supplementary Fig. 2 for individual regression curves). The pertinent regression parameters and numerical values of the four key pharmacological descriptors (E_{min}, E_{max}, EC_{50} and C_s) are shown in Table 1 for each drug–bacteria combination. (B) The ordinates show the calculated cellular concentrations (total drug, in multiples of MIC) needed to achieve two predefined activity levels (targets) shown on the abscissa [static effect (no apparent change in CFU) and 2 (*L. monocytogenes*) or 1 (*S. aureus*) $\log_{10}$ CFU decrease compared with the initial post-phagocytosis inoculum]. The cellular concentrations were calculated by (i) using the concentration–response curves shown in (A) to determine the extracellular concentrations needed to achieve the target effects (graphical interpolation) and (ii) using the data of Fig. 2 (lower panel; wild-type cells) to calculate the corresponding apparent total cellular concentrations of gemifloxacin and ciprofloxacin (for moxifloxacin, the accumulation data published in [10] was used) based on a conversion factor of 3.08 μL of total cell volume per mg protein as determined experimentally for wild-type J774 macrophages in previous studies [9].

higher accumulation of gemifloxacin in J774 macrophages (i) is not associated with differences in influx rates compared with a fluoroquinolone with lower accumulation (ciprofloxacin); (ii) does not preclude and cannot be explained by differences in active efflux transport (in comparison with moxifloxacin); and (iii) does not lead to higher intracellular activity. This goes against commonly accepted pharmacokinetic and pharmacodynamic concepts that tend to link accumulation and lack of efflux on the one hand, and accumulation and activity on the other hand. Our model may be questioned, but it is important to note that it reproduces (i) with respect to pharmacokinetics, what is observed in human alveolar macrophages where the concentrations of ciprofloxacin, moxifloxacin and gemifloxacin are, respectively, 2–5×, 20–40× and 90× higher than serum levels [26–28] and (ii) with respect to intracellular activity, what has been observed in human polymorphonuclear leukocytes infected by *S. aureus* [5].

Mechanistically, differences in accumulation of drugs in cells and tissues usually result from commensurate differences in influx or efflux rates, or from differential trapping by intracellular organelles or constituents.

Considering influx first, a faster drug accumulation is usually related to a higher lipophilicity (which is supposed to facilitate transmembrane diffusion) or due to the activity of transporters. This does not seem to apply to gemifloxacin, as this fluoroquinolone (i) is not globally more lipophilic than ciprofloxacin (see Supplementary Table 1 for experimental and calculated log *P* and log *D* values) and (ii) is probably not the substrate of a specific influx transporter when compared with ciprofloxacin (same rate accu-

Table 1
Pertinent regression parameters[a] [with confidence intervals (CI)] and statistical analyses of data from experiments examining the concentration–response activities of ciprofloxacin, moxifloxacin and gemifloxacin (shown in Fig. 3A). The first three rows show the analysis for each individual antibiotic and the last row shows the analysis made for all pooled data.

Antibiotic	*Listeria monocytogenes*					*Staphylococcus aureus*				
	R^2	E_{min}[b] (CI)	E_{max}[c] (CI)	EC_{50}[d] (CI)	C_S[e]	R^2	E_{min}[b] (CI)	E_{max}[c] (CI)	EC_{50}[d] (CI)	C_S[e]
Ciprofloxacin	0.95	2.82 (1.83 to 3.81)	−3.96 (−5.44 to −2.48)	2.94 (1.00 to 8.66)	0.49	0.96	3.80 (2.92 to 4.68)	−1.60 (−2.26 to −0.94)	1.36 (0.64 to 2.89)	3.1
Moxifloxacin	0.95	2.84 (2.05 to 3.63)	−4.48 (−5.21 to −3.56)	1.30 (0.62 to 2.74)	0.47	0.95	2.98 (2.11 to 3.86)	−1.85 (−2.28 to −1.43)	1.62 (0.78 to 3.38)	2.6
Gemifloxacin	0.97	3.03 (1.79 to 4.27)	−3.55 (−4.28 to −2.82)	0.65 (0.27 to 1.59)	0.74	0.98	3.07 (2.32 to 3.83)	−1.30 (−1.64 to −0.97)	2.02 (1.01 to 4.03)	4.8
All three fluoroquinolones	0.91	3.44 (2.95 to 3.94)	−3.92 (−4.53 to −3.32)	1.44 (0.95 to 2.19)	0.93	0.94	3.44 (2.95 to 3.94)	−1.55 (−1.81 to −1.29)	1.44 (0.95 to 2.19)	3.2

MIC, minimum inhibitory concentration; CFU, colony-forming units.
Statistical analysis: the raw data obtained for each individual antibiotic, and the corresponding Hill functions, compared using one-way analysis of variance (ANOVA) (parametric) and Kruskal–Wallis (non-parametric) tests were found to be not significantly different (*L. monocytogenes* $P = 0.420$ and 0.152, respectively; *S. aureus* $P = 0.351$ and 0.249, respectively). The analysis was then repeated for comparison of antibiotic pairs (ciprofloxacin vs. gemifloxacin, ciprofloxacin vs. moxifloxacin and moxifloxacin vs. gemifloxacin) using unpaired *t*-test and showed no significant difference for any comparison ($P > 0.18$).
[a] By use of all data for antibiotic concentrations ranging from ca. 0.01× to ca. 1000× MIC [ciprofloxacin 0.01–100 mg/L (*L. monocytogenes*) and 0.001–100 mg/L (*S. aureus*), moxifloxacin 0.005–30 mg/L (*L. monocytogenes*) and 0.001–100 mg/L (*S. aureus*) and gemifloxacin 0.005–150 mg/L (*L. monocytogenes*) and 0.0008–20 mg/L (*S. aureus*)].
[b] Relative minimal efficacy: change in CFU (in $\log_{10}$ units) at 24 h from the initial post-phagocytosis inoculum, as extrapolated for an infinitely low antibiotic concentration.
[c] Relative maximal efficacy: change in CFU (in $\log_{10}$ units) at 24 h from the initial post-phagocytosis inoculum, as extrapolated for an infinitely large antibiotic concentration.
[d] Relative potency: extracellular concentration (in multiple of MIC) yielding a change in CFU at 24 h half way between E_{min} and E_{max}.
[e] Static concentration: concentration (in multiples of MIC) resulting in no apparent bacterial growth (no change in CFU from the initial post-phagocytosis inoculum) as determined by graphical interpolation [MIC values are 1 mg/L and 0.125 mg/L (ciprofloxacin), 0.5 mg/L and 0.03 mg/L (moxifloxacin) and 0.5 mg/L and 0.008 mg/L (gemifloxacin) for *L. monocytogenes* and *S. aureus*, respectively].

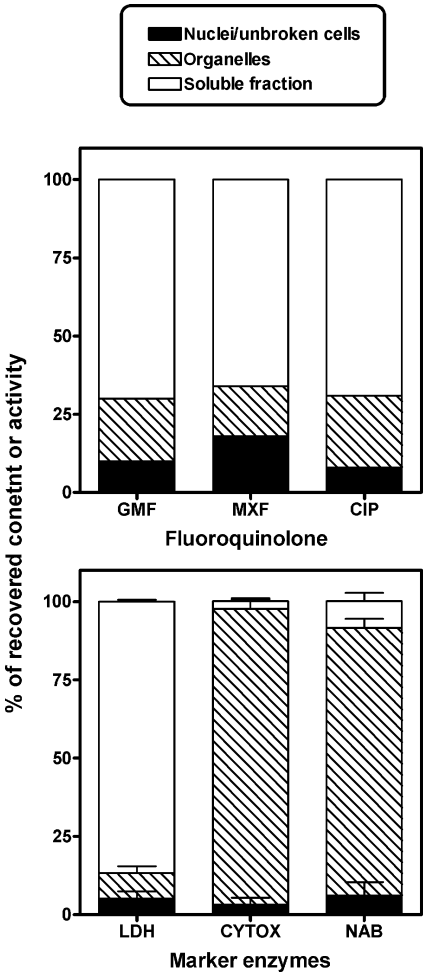

Fig. 4. Subcellular distribution of gemifloxacin (GMF), moxifloxacin (MXF) and ciprofloxacin (CIP) in J774 mouse macrophages incubated for 2 h with 50 mg/L of each drug. The upper panel shows the antibiotic content in the nuclei/unbroken cells, organelles and soluble fraction expressed as a percentage of the total recovered amount (each bar corresponds to a separate experiment). The lower panel shows the distribution of lactate dehydrogenase (LDH; marker of the cytosol), cytochrome *c* oxidase (CYTOX; marker of mitochondria) and *N*-acetyl-β-hexosaminidase (NAB; marker of lysosomes) as the mean values (±standard deviation) of the three experiments (corresponding to each of the individual experiment shown in the upper panel).

mulation constants). Non-specific influx transporter(s) observed in polymorphonuclear leukocytes and human monocytes [29–31] can probably be dismissed here as those belong to the solute carrier organic anion (SLCO) family [32] that is inhibited by gemfibrozil, which was not the case here.

Considering efflux, Mrp4 has been proposed as the main transporter responsible for the lower accumulation of ciprofloxacin in J774 macrophages compared with levofloxacin, garenoxacin and moxifloxacin. Indeed, these fluoroquinolones reach a similar level of accumulation when Mrp4 is made inactive by ATP depletion or inhibited by the addition of gemfibrozil [10]. Moreover, ciprofloxacin accumulation is significantly increased by silencing the gene coding for Mrp4 [8]. The present data show that this conclusion cannot be generalised to all fluoroquinolones and all situations. Thus, gemifloxacin not only accumulates more than moxifloxacin in J774 macrophages under conditions of ATP depletion or in the presence of gemfibrozil, but also in THP-

1 macrophages in which no gemfibrozil-inhibited efflux can be demonstrated. Another compelling reason to disregard efflux as being the main cause for the differential accumulation of gemifloxacin vs. ciprofloxacin and moxifloxacin is that gemifloxacin actually appears to be a weak but nevertheless effective substrate of Mrp4 in J774 macrophages, whereas we know that moxifloxacin is not. Thus, globally and in contrast to what we proposed for moxifloxacin, the higher cellular concentration of gemifloxacin compared with other fluoroquinolones must find an explanation beyond considerations of influx and efflux rates only.

Considering intracellular trapping, a model has been presented [33] that relates fluoroquinolone accumulation in eukaryotic cells to their trapping under a protonated form in lysosomes owing to the acid pH (~5.4) prevailing therein. This, however, is unlikely because fluoroquinolones are not weak bases but zwitterionic compounds. Moreover, differences in accumulation of drugs in acidic membrane-bounded compartments should result from commensurate differences in the number and/or the pK_a of their basic functions (see [34]), which is not the case for the three fluoroquinolones studied here (see individual basic pK_a values in Supplementary Table 1). More factually, cell fractionation studies show a predominant association of the cell-associated fluoroquinolones with the cytosol rather than with lysosomes, in line with the results of previous studies with ciprofloxacin [2,35] (studies using the same technique have shown that macrolides, which are weak bases, are predominantly associated with lysosomes in J774 macrophages [2,36,37]). Lastly, experimental studies have shown a lack of effect of monensin (an H^+ ionophore that collapses the cytosolic–lysosomal ΔpH) on ciprofloxacin accumulation under conditions in which it drastically reduces the accumulation of azithromycin in J774 macrophages [9].

Actually, a more likely explanation for the larger cellular accumulation of gemifloxacin compared with moxifloxacin and ciprofloxacin could be its tighter binding to still undefined cellular constituents such as soluble proteins. This hypothesis would account for the pharmacokinetic and subcellular distribution data presented here, including (i) the lower efflux rate of gemifloxacin compared with ciprofloxacin (which, however, may also result from the less efficient recognition of gemifloxacin by the Mrp4 efflux transporter, both mechanisms being not mutually exclusive) and (ii) its incomplete release upon transfer of the cells to drug-free medium. It is also consistent with the larger serum protein binding of gemifloxacin (55–73%) compared with moxifloxacin (39–52%) and ciprofloxacin (30% only) [38,39].

Determining the molecular nature of the intracellular binding sites for fluoroquinolones still requires further investigation, but the mechanism proposed provides a rational explanation for the main critical observation made here, namely that all three fluoroquinolones are equipotent against intracellular bacteria despite their differences in cellular accumulation. Indeed, we show that it is the MIC of each drug that drives its intracellular potency (as defined by the C_s and EC_{50} pharmacological descriptors) since all three fluoroquinolones show superimposable concentration–effect relationships once the data are normalised on the basis of multiples of the MIC. MICs are measured in broth where little protein binding takes place, which means that their values must essentially be interpreted as corresponding to free drug levels [40]. Intracellularly, a static effect (C_s) for gemifloxacin was obtained for an extracellular concentration corresponding to its MIC, although its intracellular concentration is much higher. It is therefore tempting to speculate that only a fraction of the total intracellular gemifloxacin is available for activity, corresponding essentially to its free form. Moxifloxacin should show an intermediate behaviour with intracellular activity also driven by its MIC (as measured in broth), which is what we observe. Thus, the larger cellular accumulation of some fluoroquinolones, taking gemifloxacin as an example, would essentially be a self-defeating process as far as activity is concerned (assuming all comparisons are made on basis of the MIC), leading to a larger concentration of bound drug with, however, no or little difference in their free form. This confirms and extends previous work showing that the intracellular activity of fluoroquinolones was weaker and not in proportion to what could be anticipated from the level of their cellular accumulation [13,41–43].

In conclusion, the present work documents that (i) recording the cellular accumulation of fluoroquinolones does not allow prediction of their intracellular activity and (ii) higher cellular accumulation may depend on other parameters than influx and efflux rates and/or the activity of specific transporters. This calls for both more mechanistic studies and more comprehensive structure–activity analyses where these two important elements of the pharmacological properties of fluoroquinolones will be examined in a systematic fashion.

Acknowledgments

The authors are grateful to M.C. Cambier, C. Misson and M. Vergauwen for dedicated technical assistance. They also thank Oscient Pharmaceuticals Corp. and Bayer HealthCare for the kind gift of gemifloxacin and of moxifloxacin and ciprofloxacin, respectively.

Funding: CMV is Boursier of the Belgian Fonds pour la Recherche dans l'Industrie et l'Agriculture (FRIA); BM was postdoctoral fellow of the First post-doctoral programme of the Belgian Région wallonne; SL and FVB are Chargé de Recherches and Maître de Recherches, respectively, of the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS). This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale (grants no. 3.4.597.06 and 3.4.583.08) and the Belgian Fonds de la Recherche Scientifique (grant no. 1.5.195.07).

Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2011.05.011.

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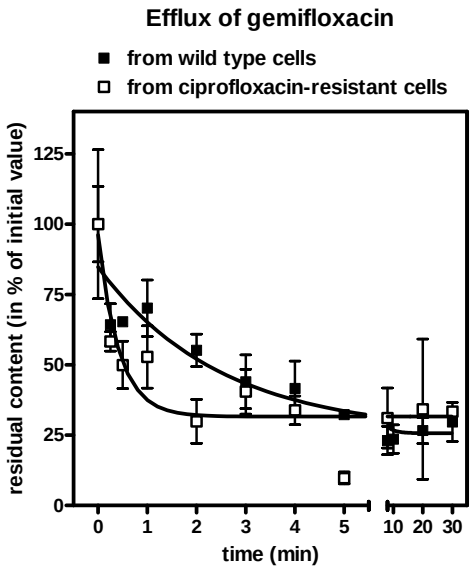
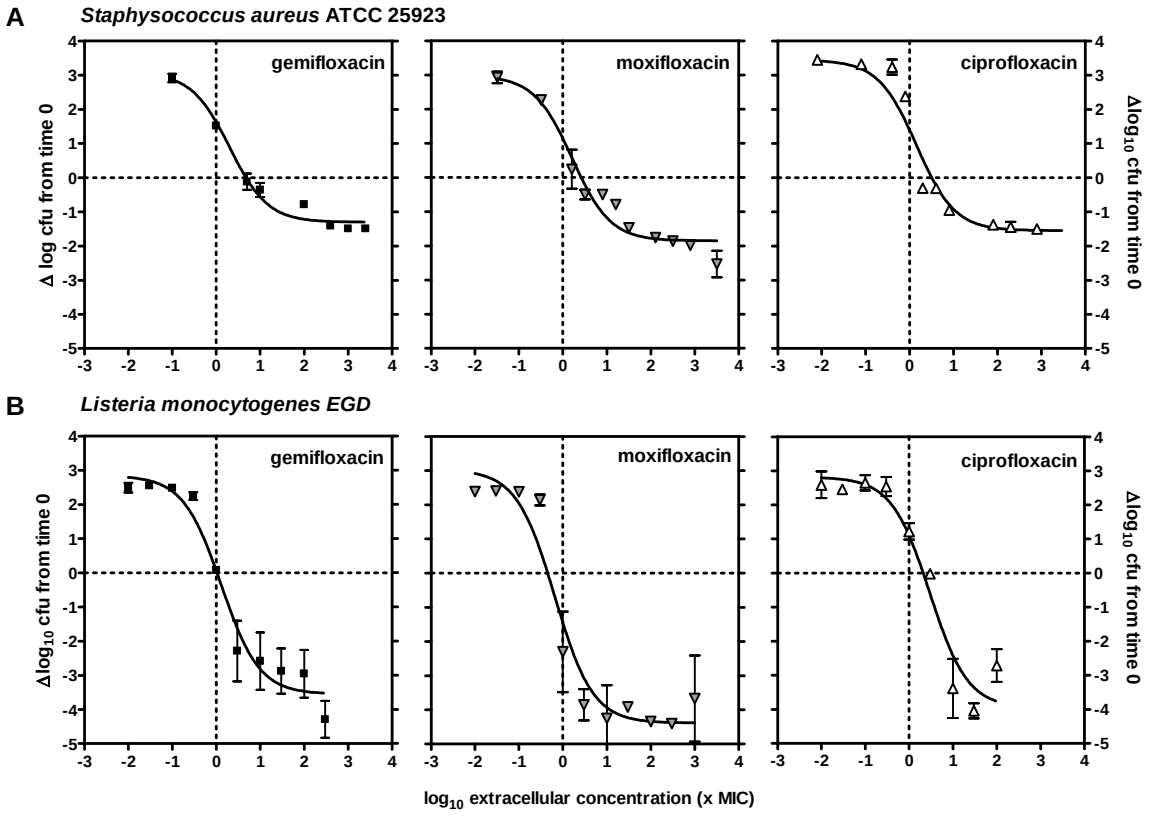
Vallet *et al.* Intracellular activity of fluoroquinolones - Supplementary Material

Figure SP1



Caption to Figure SP1: Kinetics of efflux of gemifloxacin from wild-type and ciprofloxacin-resistant J774 cells. Cells were exposed to gemifloxacin (20 mg/L) for 2 h and then transferred to antibiotic-free medium for up to 30 min. Regression parameters: (a) wild-type cells (same data as in Figure 2), $R^2 = 0.896$, $k_{out} = 0.403 \pm 0.122$ min^{-1}, *plateau* $= 25.71 \pm 4.63$; (b) ciprofloxacin-resistant cells, $R^2 = 0.830$, $k_{out} = 2.39 \pm 0.907$ min^{-1}, *plateau* $= 31.6 \pm 4.0$).

Figure SP2



Caption to Figure SP2: Concentration-response of the activities of gemifloxacin, moxifloxacin, and ciprofloxacin (CIP) against *S. aureus* ATCC25923 (top) and *L. monocytogenes* EGD (bottom) in wild-type J774 macrophages. Cells were incubated with increasing concentrations of antibiotic (total drug) for 24 h. Each graph shows the change in the number of cfu (log scale) per mg of cell protein compared to the initial post-phagocytosis inoculum (ordinate) as a function of the extracellular concentration of each drug expressed in multiples of its MIC (abscissa). In each graph, the horizontal dotted line corresponds to an apparent static effect and the vertical line to the MIC of the drug. A sigmoidal regression has been fitted to each set of data (see Table 1 for the pertinent regression parameters and numerical values of the four key pharmacological descriptors (E_{min}, E_{max}; EC_{50}, C_s).

Table SP1: Physico-chemical properties of fluoroquinolones and azithromycin at physiologically-relevant pHs

The data indicate that (i) ciprofloxacin, moxifloxacin, and gemifloxacin display quite similar biophysical properties although showing distinct cellular accumulation levels (gemifloxacin > moxifloxacin > ciprofloxacin; see Results) that are not correlated to the minor differences seen; (ii) these properties are very different from those of azithromycin, a drug known to accumulate extensively in lysosomes by proton-trapping (see Discussion). The pHs considered are those of the extracellular (7-7.4) and of the lysosomal (5-5.4) milieus, respectively.

Drug	pKa_1 [a] (acidic)	pKa_2 [a] (basic)	species in solution (calculated %) [a]						logP [b]		calculated logD [a,c]	
			pH 7.4			pH 5.4			calculated [a,d]	experimental [e]	pH 7	pH 5
			cationic	zwitterionic	anionic	cationic	zwitterionic	anionic				
ciprofloxacin	5.8	8.7	2	**93**	5	**69**	30	0	1.63'	2.30	-1.38	-1.62
moxifloxacin	5.6	9.4	2	**97**	1	**66**	34	0	1.90	2.90	-1.72	-1.33
gemifloxacin	5.5	9.5	1	**98**	1	**47**	**52**	0	1.04	2.30	-2.54	-1.78
azithromycin	-	8.9 [f] 9.6	**96.98** [g]	0.02	0	**99.97** [g]	0	0	2.44	4.02	-1.99	-4.41

[a] calculated using Reaxys (http://www.reaxys.com) with the ChemAxon's Marvin plug-in calculators (http://www.chemaxon.com/marvin). The actual values of the pK_a of the acidic function may be about 0.5 units higher due to the influence of the vicinal carbonyl function [1].

[b] logP: partition coefficient (log of the ratio of the concentrations of the unionized compound between a non polar [octanol] and a polar [water] phases);

[c] logD: distribution coefficient (log of the ratio of the sum of the concentrations of all forms of the compound [ionized plus un-ionized] in each of thee two phases at a given pH)

[d] These values are the arithmetic average of three methods of calculations (Viswanadhan's fragmentation; Klopman's fragmentation; and PHYSPROP© database [see https://www.reaxys.com/static/marvin/marvin_5_3_7/help/calculations/partitioning.html for details]).

[e] value as reported in Drugbank (see http://www.drugbank.ca and [2]

[f] azithromycin is a dicationic drug

[g] dicationic form (monocationic form: 3 % at pH 7.4 and 0.03 % at pH 5.4; a zwitterionic form is virtually inexistent (< 0.001 %) at these pH values).

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II.2. Substrate recognition by efflux pumps in prokaryotes (NorA in *Staphylococcus aureus*, PatA/PatB in *Streptococcus pneumoniae*, Mex/Opr in *Pseudomonas aeruginosa*) and in eukaryotes (Mrp4 in murine J774 macrophages) : a combined biological and structural study with 25 fluoroquinolones.

(This study was performed in collaboration with Martine Prévost and Julien Dupont (Université Libre de Bruxelles) for the structural and modelling experimentations).

2. Substrate recognition by efflux pumps in prokaryotes (NorA in *Staphylococcus aureus*, PatA/PatB in *Streptococcus pneumoniae*, Mex/Opr in *Pseudomonas aeruginosa*) and in eukaryotes (Mrp4 in murine J774 macrophages): a combined biological and structural study with 25 fluoroquinolones.

2.1. Background

Fluoroquinolones are widely used antibiotics, showing favorable properties such as broad spectrum, intense bactericidal activity and excellent bioavailability. Fluoroquinolones accumulate in eukaryotic cells, an interesting property which makes them very helpful to treat intracellular infections. Although active efflux of antimicrobial agents is a well-known mechanism of resistance in prokaryotic cells, antibiotic transporters are also found in eukaryotic cells, where they modulate the distribution of these drugs. But little is known about the mechanism of recognition of the drug by the transporter in prokaryotic cells as well as in eukaryotic cells.

The aim of this study was (i) to compare the transport of fluoroquinolones by four efflux proteins (three in prokaryotic cells: NorA in *S. aureus*, PatA/PatB in *S. pneumoniae*, Mex/Opr efflux system in *P. aeruginosa*; and one in eukaryotic cells: Mrp4 in murine macrophages), and (ii) to compare the *S. aureus* NorA and the mouse macrophages Mrp4 transporters with respect to recognition of fluoroquinolones.

2.2. Materials and methods

Chemicals and quinolone antibiotics. Gemfibrozil and reserpine were purchased from Sigma-Aldrich. Structures of quinolones and their solubility are shown in Table 1. Norfloxacin, enoxacin and pefloxacin were purchased from Sigma-Aldrich. The other molecules were received from their manufacturers: ciprofloxacin and moxifloxacin from Bayer HealthCare AG (Leverkusen, Germany), difloxacin and sarafloxacin from Abbott Laboratories (Abbott Park, Illinois), lomefloxacin from G.D. Searle and Co., delafloxacin from Rib-X Pharmaceuticals Inc. (New Haven, CT), garenoxacin from Bristol-Myers Squibb (New Brunswick, CT), sparfloxacin from Rhône-Poulenc Rorer (Antony, France), gemifloxacin from LG Life Sciences Ltd (Seoul, Korea), trovafloxacin from Pfizer (Groton, CT), levofloxacin from Aventis Pharma (Antony, France), and marbofloxacin from Vetoquinol (Lure, France). Pradofloxacin and its derivatives (FQ2, FQ4, FQ5, FQ6, FQ7, FQ8), and derivatives of ciprofloxacin (enrofloxacin, FQ10, FQ13) were a kind gift from Dr. Wetzstein, Bayer Healthcare, Leverkusen, Germany.

Cell culture and cell lines. J774 wild-type mouse macrophages and its derived ciprofloxacin-resistant cell line (obtained by a chronical treatment of wild-type cells with increasing concentrations of ciprofloxacin from 0.1 to 0.2 mM in the culture medium, as it is described in Michot et al. 2006) were used in this study. Their culture conditions were the same as described previously in Michot et al. 2006.

Cellular accumulation of quinolones. These experiments were performed as described by Michot et al. 2005. Briefly, wild-type and ciprofloxacin-resistant cells were incubated for 2 h with 20 mg/L of each quinolone in the presence or in the absence of 500 µM gemfibrozil. Cells were then washed with PBS and collected by gentle scrapping in a medium appropriate for the detection of each quinolone as described in the table. The cellular content in quinolones was measured using a method adapted for each quinolone (see Table 2; fluorimetric assay (as described in Michot et al. 2004 and 2005), disc-plate microbiological assay, or HPLC (using a column Xterra RP18, 5µm, 4.6x150mm cartridge from Waters, a PDA detector, and a ACN/Na_2HPO_4 25mM pH3 buffer as mobile phase). The fluorimetric assay was used in most cases (Michot et al. 2004). Yet, 10 fluoroquinolones among the 25 tested in this work were not detectable by this method. When sensitive enough (see Table 2), the microbiological assay (disc-plate assay), was used as an alternative and preferred to HPLC. Despite the fact HPLC is more sensitive than the other methods, it presents specific limitations for measuring cell-associated fluoroquinolones. These antibiotics are known to bind to DNA or other intracellular components (Edlund et al. 1988), leading to a co-precipitation of fluoroquinolones during the extraction process, which may lead to an underestimation of their concentration. Scholl and colleagues (Scholl et al. 1987) have shown that a preliminary liquid extraction using acetonitrile is necessary to release the tissue-bound ciprofloxacin for HPLC detection, which is what we did here. All three methods were validated by comparing the cellular concentration of ciprofloxacin; we showed that ciprofloxacin concentration detected by the three methods yielded identical results (see Table A for LODs, linearity, R^2 and the intracellular concentration of ciprofloxacin measured for the three methods of ciprofloxacin detection). This confirmed a previous study showing that both bioassay and HPLC methods yielded identical results (Zeiler et al. 1987).

Table A. Validation of methods used for the detection of ciprofloxacin.

Detection method	LOD	Linearity	R^2	[CIP] (ng/mg prot)
fluorimetry	10 ng/mL	20 to 100 ng/mL	0.9914	345.50 ±16.67
Disc-plate assay	125 ng/mL	0.25 to 16 µg/mL	0.9952	303.10 ± 42.57
HPLC	1.5 ng/mL	25 to 500 ng/mL	0.9992	377.89 ±20.53

LOD = Limit Of Detection.

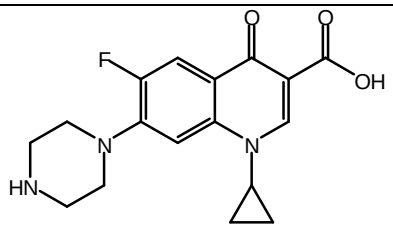
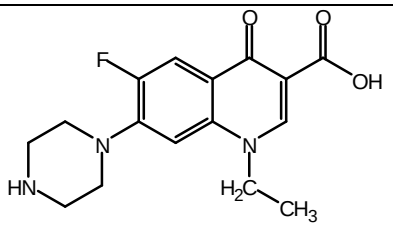
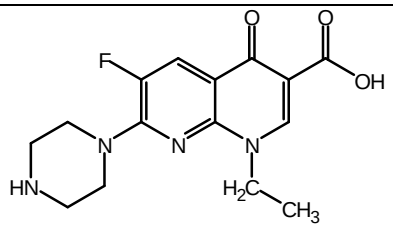
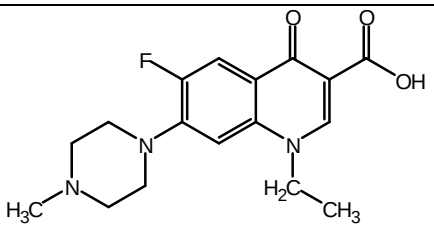
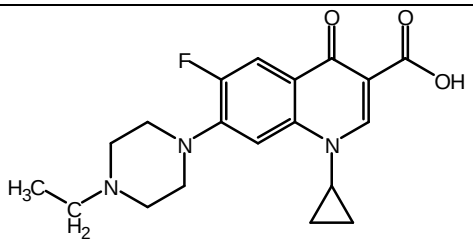
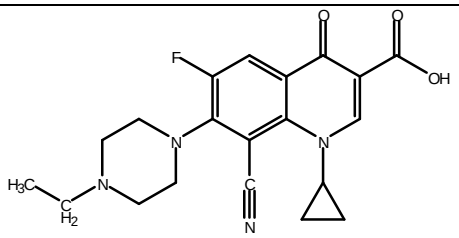
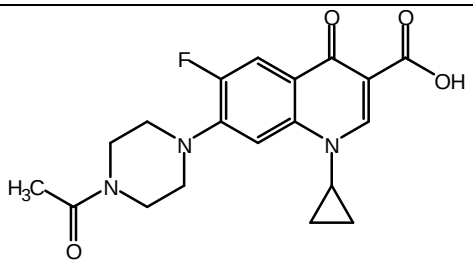
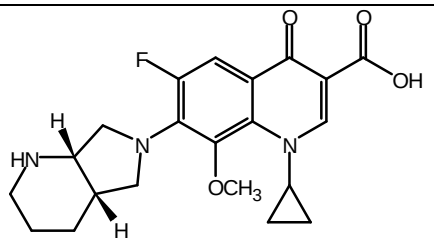
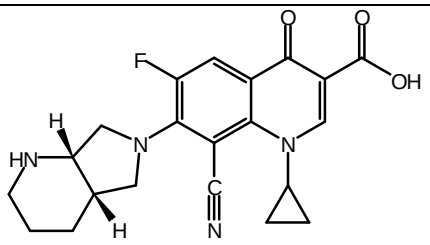
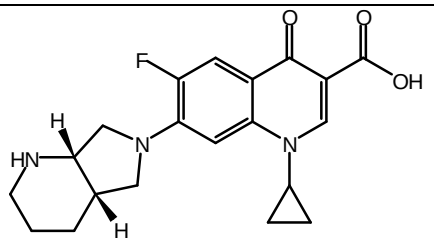
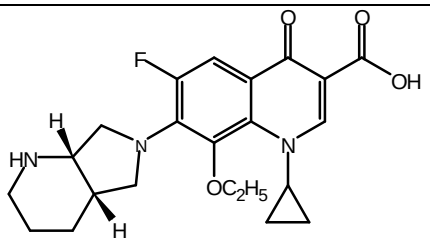
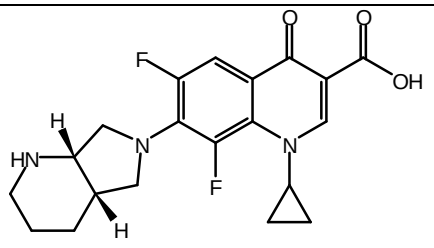
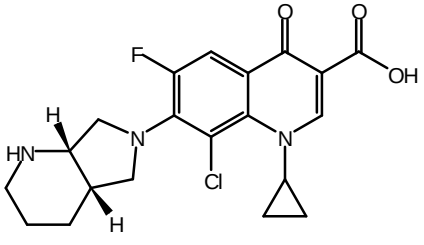
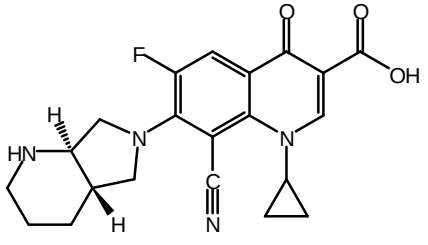
[CIP] = intracellular concentration of ciprofloxacin measured by the three methods when cells were incubated with 20 mg/L of ciprofloxacin.

Bacterial strains. 3 bacteria and their isogenic derivative strains were used in this study: *Staphylococcus aureus* ATCC25923 as wild-type strain, and its derivative SA-1 overexpressing the efflux pump NorA (Ba et al. 2006); *Streptococcus pneumoniae* SP335, which overexpresses PatA/PatB, and its 2 derivatives where the genes *patA* and *patB* were inactivated (El Garch et al. 2010); *Pseudomonas aeruginosa* PAO1 with basal expression of efflux systems and its multidisruptant derivative PAO509 (PAO1 Δ(*mexAB-oprM*)Δ(*mexCD-oprJ*)Δ(*mexEF-oprN*)Δ(*mexJK*)Δ (*mexXY*)) (Mima et al. 2007).

Minimal inhibitory concentration (MIC). Microdilution method was used according to CLSI recommendations, and using Mueller-Hinton broth for *Staphylococcus aureus*, Mueller-Hinton broth cation adjusted (MHBCa) supplemented with 5% lysis horse blood for *Streptococcus pneumoniae,* and MHBCa for *Pseudomonas aeruginosa.*

Modelling and docking. Three-dimensional models were constructed by homology modelling using the cristal structures of the prokaryotic lactose permease (LacY) for NorA, and of the eukaryotic P-glycoprotein for Mrp4. Docking was performed using an induced fit procedure in the 3D models. The putative binding site of NorA was defined as that of the lactose analog in LacY complex structure, and that of Mrp4 identified using mutagenesis data (El Sheikh et al. 2008).

Table 1. Structure of the 25 fluoroquinolones (and their solubility (S) in water at pH7 and 25°C)

quinolone	structure	quinolone	structure
Ciprofloxacin (S = 0.46 g/L)		Norfloxacin (S = 0.35 g/L)	
Enoxacin (S = 0.16 g/L)		Pefloxacin (S = 0.4 g/L)	
Enrofloxacin (S = 0.14 g/L)		FQ10 (8-cyano-N-ethyl-ciprofloxacin)	
FQ13 (N-acetyl-ciprofloxacin)		Moxifloxacin (S = 0.033g/L)	
Pradofloxacin (S = 0.039 g/L)		FQ2 (8-desmethoxy-moxifloxacin)	
FQ4 (8-ethoxy-moxifloxacin)		FQ5 (8-desmethoxy-8-fluoro-moxifloxacin)	
FQ6 (8-desmethoxy-8-chloro-moxifloxacin)		FQ7 (deoxy-finafloxacin)	

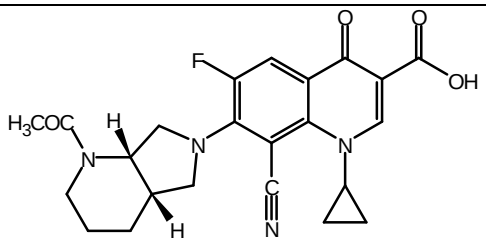
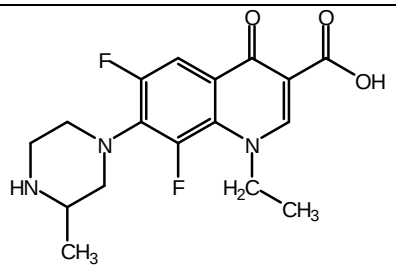
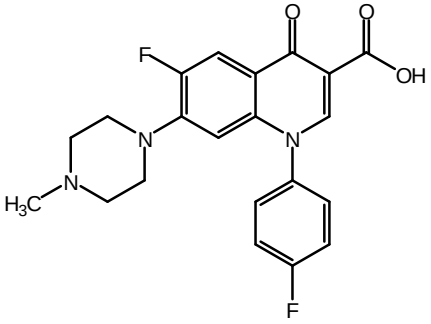
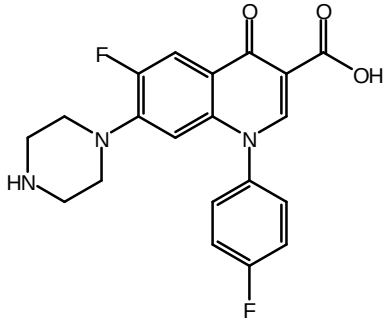
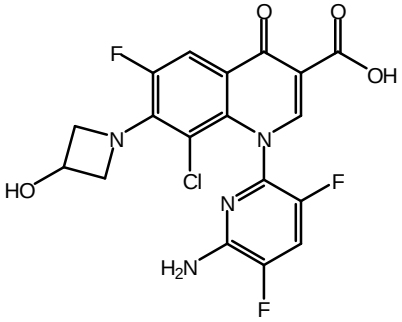
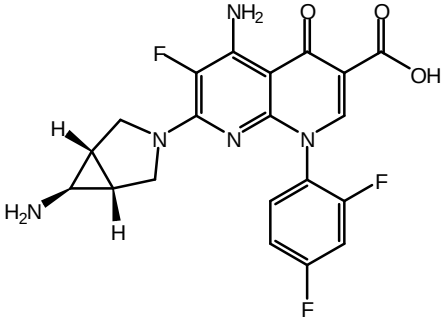
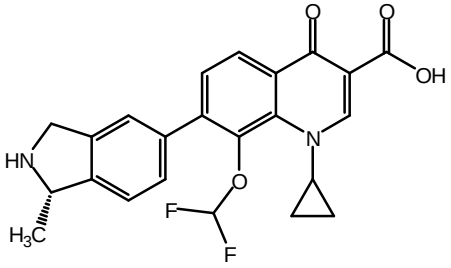
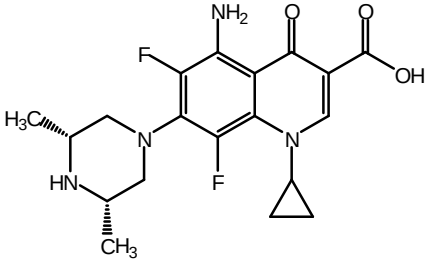
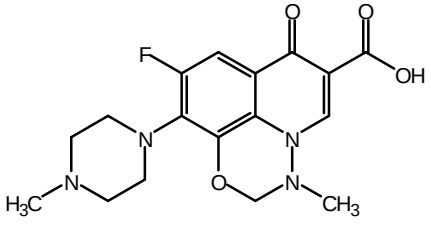
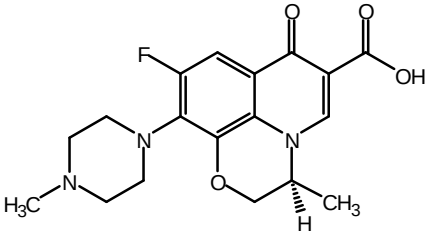
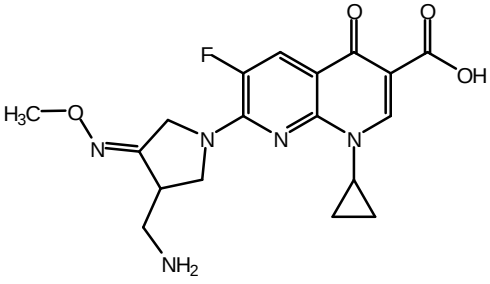
FQ8 (N-acetyl-8-desmethoxy-8-cyano-moxifloxacin)		Lomefloxacin (S = 0.12 g/L)	
Difloxacin (S = 1.6 g/L)		Sarafloxacin (S = 2.2 g/L)	
Delafloxacin (S = 5.3 g/L)		Trovafloxacin (S = 0.62 g/L)	
Garenoxacin (S = 0.2 g/L)		Sparfloxacin (S = 0.043 g/L)	
Marbofloxacin (S = 3.6 g/L)		Levofloxacin (S = 0.18 g/L)	
Gemifloxacin (S = 0.019 g/L)			

Table 2. Detection of fluoroquinolones by different assays

Fluorimetric assay

quinolones	λ excitation (nm)	λ emission (nm)	LOQ[(c)] (ng/mL)
ciprofloxacin	275	450	20
enrofloxacin	276	448	10
norfloxacin	281	447	20
pefloxacin	276	440	5
enoxacin	340	395	250
FQ2	282	456	10
moxifloxacin	298	504	50
FQ4	295	508	50
FQ5	290	480	20
difloxacin	280	458	15
sarafloxacin	277	463	25
lomefloxacin	284	458	25
garenoxacin	292	414	200
levofloxacin	298	500	20
marbofloxacin	297	510	100

Microbiologic assay

quinolones	Bacterial strain	Medium [(a)]	LOQ[(c)] (ng/mL)
ciprofloxacin	*E. coli* ATCC25922	Antibiotic medium 11	125
delafloxacin	*S. aureus* ATCC25923	Antibiotic medium 11	62.5
trovafloxacin	*S. aureus* ATCC25923	Antibiotic medium 11	500
gemifloxacin	*S. aureus* ATCC25923	Antibiotic medium 11	250
sparfloxacin	*E. coli* ATCC25922	Antibiotic medium 11	250

HPLC assay

quinolones	% ACN / Na_2HPO_4 [(b)]	λ detection (nm)	LOQ[(c)] (ng/mL)
ciprofloxacin	20 / 80	275	50
FQ10	20 / 80	285	50
FQ13	30 / 70	285	50
pradofloxacin	20 / 80	285	50
FQ6	30 / 70	293	50
FQ7	20 / 80	285	50
FQ8	30 / 70	285	50

[(a)] Difco antibiotic medium 11 (Becton Dickinson & co., Franklin Lakes, NJ, USA)
[(b)] Proportion of ACN and Na_2HPO_4 buffer used for the elution of the molecules. ACN = acetonitril
[(c)] LOQ: Lower Limit of Quantification

2.3. Results

Comparison of the transport of 25 quinolones by NorA in *Staphylococcus aureus*, PatA/PatB in *Streptococcus pneumoniae* and Mex/Opr in *Pseudomonas aeruginosa.* MICs were determined for the 25 fluoroquinolones on the different strains of *S. aureus*, *S. pneumoniae* and *P. aeruginosa*. For each bacterium, we selected a condition where efflux is considered as minimal (-) (no expression of efflux pumps [in *S. pneumoniae* SP335 ΔpatA or ΔpatB or in *P. aeruginosa* PAO509] or presence of efflux pump inhibitor [in *S. aureus* ATCC25923 in the presence of reserpine]), and a condition where the efflux is considered as maximal (+) (basal expression when constitutively expressed [in *P. aeruginosa* PAO1], or overexpression of efflux systems [in *S. aureus* SA-1 or in *S. pneumoniae* SP335]). MICs difference in fold dilution between conditions where efflux is maximal and minimal were calculated for all quinolones in the three bacteria (Table 3), and these values were used to establish possible correlations between the efflux of quinolones in the 3 bacteria (Fig. 1).

As shown in Table 3, in conditions where efflux is minimal, all fluoroquinolones displayed high activity against *S. aureus* ATCC25923 and *P. aeruginosa* PAO509, (MIC≤0.5 mg/L in general), but they were generally less active against *S. pneumoniae* SP335 with MIC being in general 2 to 5 dilutions higher than against *S. aureus*. When comparing for each molecule the MIC in conditions where efflux is minimal or maximal, we observed a high variability in Gram positive bacteria, with some molecules showing almost no change in MIC upon expression of efflux systems (see e.g. difloxacin or moxifloxacin vs. *S. aureus* [difference in MIC of 0 and 1 dilution respectively], and others showing a major impact (see e.g. ciprofloxacin and norfloxacin vs. *S. aureus* for which MICs increased of 6 dilutions due to active efflux. The same type of observations was made for *S. pneumoniae, with, interestingly enough,* the same molecules appearing as being substrates or not. On the contrary, all fluoroquinolones were markedly effluxed by the Mex/Opr efflux system in *P. aeruginosa* (with changes of MICs ranging from 4 to 8 dilutions).

Table 3. MICs of 25 quinolones in 3 bacteria and MICs difference between conditions where efflux is minimal and maximal.

Quinolones	*Staphylococcus aureus*			*Streptococcus pneumoniae*				*Pseudomonas aeruginosa*		
	MICs (mg/L)		MICs ≠ (fold dilutions) *	MICs (mg/L)			MICs ≠ PatA / PatB (fold dilutions) *	MICs (mg/L)		MICs ≠ (fold dilutions) *
	SA-1 (+)	ATCC 25923 (-)		SP335 (+)	Δ PatA (-)	Δ PatB (-)		PAO1 (+)	PAO509 (-)	
Norfloxacin	16	0.25	6 (1.00)	64	4	4	4 (1.00) / 4	0.5	0.03-0.06	4 (0.8)
Enoxacin	8	0.5	4 (0.67)	64	16	16	2 (0.50) / 2	1	0.03	5 (1.00)
Ciprofloxacin	4	0.06	6 (1.00)	16	1	1	4 (1.00) / 4	0.125-0.25	0.008	5 (1.00)
FQ13	4	0.25	4 (0.67)	32	4	4	3 (0.75) / 3	64	0.25-0.5	7 (1.40)
FQ7	1	0.06-0.125	4 (0.67)	2-4	0.5	0.25-0.5	3 (0.75) / 3	8	0.125	6 (1.20)
Levofloxacin	0.5	0.125	2 (0.33)	4	1	1	2 (0.50) / 2	0.5	0.016	5 (1.00)
Lomefloxacin	4	0.25	4 (0.67)	32	8	8	2 (0.50) / 2	1	0.03	5 (1.00)
Pradofloxacin	0.25	0.03	3 (0.50)	0.5	0.125	0.06	2 (0.50) / 3	2	0.008	8 (1.60)
Delafloxacin	0.016	0.002-0.004	3 (0.50)	0.125	0.03	0.016	2 (0.50) / 3	1	0.016	6 (1.20)
FQ8	8	1	3 (0.50)	32	8	8	2 (0.50) / 2	64	0.5-1	6 (1.20)
Marbofloxacin	1	0.25	2 (0.33)	4	2	2	1 (0.25) / 1	0.5	0.016	5 (1.00)
Sarafloxacin	1	0.125	3 (0.50)	8	1	1	3 (0.75) / 3	0.25	0.016	4 (0.80)
FQ2	0.25	0.03	3 (0.50)	0.5	0.125	0.125	2 (0.50) / 2	1	0.008	7 (1.40)
Pefloxacin	0.5	0.25	1 (0.17)	16	8	8	1 (0.25) / 1	2	0.03	6 (1.20)
FQ5	0.03	0.008	2 (0.33)	0.125	0.06	0.06	1 (0.25) / 1	0.5	0.008	6 (1.20)
Garenoxacin	0.03	0.016	1 (0.17)	0.06	0.03	0.03	1 (0.25) / 1	2	0.03	6 (1.20)
FQ10	1	0.5	1 (0.17)	16	8	8	1 (0.25) / 1	16	0.125-0.25	6 (1.20)
Trovafloxacin	0.03	0.008-0.016	1 (0.17)	0.25-0.5	0.25	0.25	1 (0.25) / 1	2	0.016	7 (1.40)
FQ6	0.016	0.008	1 (0.17)	0.06	0.03	0.03	1 (0.25) / 1	1	0.008	7 (1.40)
Sparfloxacin	0.125	0.06	1 (0.17)	0.5	0.25	0.25	1 (0.25) / 1	1	0.008-0.016	6 (1.20)
Difloxacin	0.25-0.5	0.25	0 (0.00)	4	4	2	0 (0.00) / 1	2	0.03	6 (1.20)
Gemifloxacin	0.125	0.008	4 (0.67)	0.125	0.03	0.016	2 (0.50) / 3	0.25	0.016	4 (0.80)
FQ4	0.125-0.25	0.03	2 (0.33)	0.25-0.5	0.25	0.25	1 (0.25) / 1	8	0.125	6 (1.20)
Enrofloxacin	0.06-0.125	0.06	1 (0.17)	2	1	0.5-1	1 (0.25) / 1	4	0.03	7 (1.40)
Moxifloxacin	0.06	0.03	1 (0.17)	0.25	0.25	0.25	0 (0.00) / 0	2	0.03	6 (1.20)

- ATCC25923 = wild-type *S. aureus*; value measured in the presence of 10 mg/L reserpine; SA-1 = ATCC25923 *S. aureus* strain overexpressing the NorA efflux pump
- SP335 = *S. pneumoniae* which overexpresses PatA and PatB ; Δ PatA or Δ PatB = SP335 where *patA* or *patB* are disrupted
- PAO1 = wild-type strain of *P. aeruginosa* ; PAO509 = PAO1 where the genes coding for MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK-OprM, and MexXY-OprM are disrupted.
- MICs ≠ is the difference of MIC (in $\log_2$) dilutions between strains with minimal efflux (-) and maximal efflux (+) (for ATCC25923 *S. aureus*, MICs were performed in the presence of reserpine). A difference of 2 $\log_2$ dilutions is considered as significant (Ericsson and Sherris 1971).
* Values in parenthesis give these ratios relative to the value calculated for ciprofloxacin (set to a reference value of 1.00).

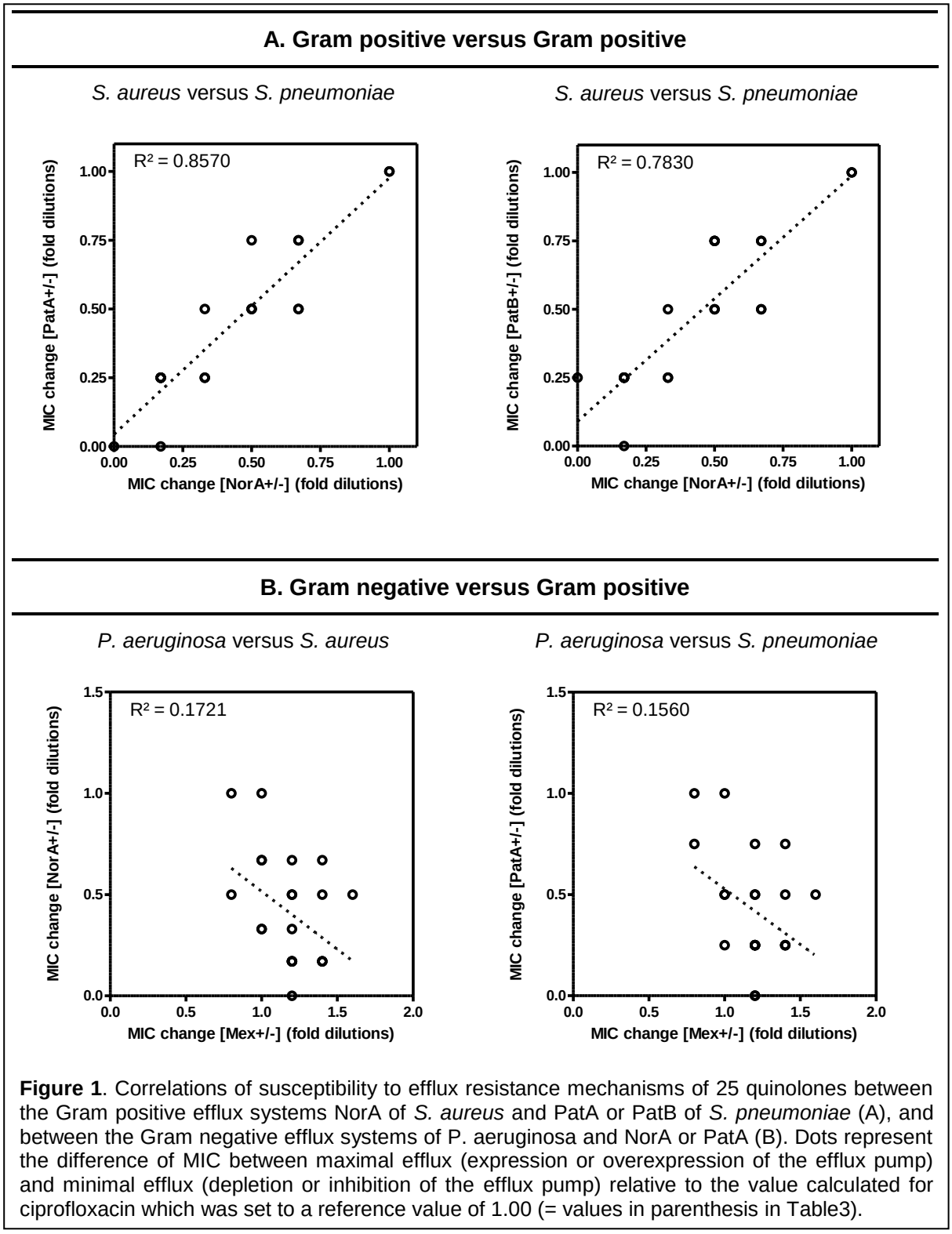


Figure 1. Correlations of susceptibility to efflux resistance mechanisms of 25 quinolones between the Gram positive efflux systems NorA of *S. aureus* and PatA or PatB of *S. pneumoniae* (A), and between the Gram negative efflux systems of P. aeruginosa and NorA or PatA (B). Dots represent the difference of MIC between maximal efflux (expression or overexpression of the efflux pump) and minimal efflux (depletion or inhibition of the efflux pump) relative to the value calculated for ciprofloxacin which was set to a reference value of 1.00 (= values in parenthesis in Table3).

Figure 1 shows the correlation between the susceptibility to efflux of fluoroquinolones when tested against both Gram positive bacteria, or against Gram positive and Gram negative bacteria. A high degree of correlation is observed between efflux mediated by NorA in *S. aureus* and PatA (R^2 = 0.85) or PatB (R^2 = 0.78) in *S. pneumoniae* (Figure 1.A.). On the contrary, all quinolones are good substrates for the Mex/Opr efflux systems in *P. aeruginosa* (as seen in Table 3), and this makes correlations between NorA or PatA/PatB and Mex/Opr largely irrelevant (R^2 = 0.17 and 0.15 for *S.aureus* vs *P. aeruginosa* and *S. pneumoniae* vs *P. aeruginosa* respectively, see Figure 1.B.).

Comparison of the transport of 25 quinolones by efflux pumps of prokaryotic cells (NorA, PatA/PatB and Mex/Opr) and eukaryotic cells (Mrp4). The accumulation of the 25 quinolones was determined in wild-type J774 mouse macrophages and in their derived ciprofloxacin-resistant cell line where Mrp4 is overexpressed. Gemfibrozil was added to inhibit the transport of fluoroquinolones by Mrp4 in wild-type cells. The susceptibility of fluoroquinolones to efflux by Mrp4 was estimated by the ratio of accumulation observed in WT macrophages in the presence of gemfibrozil (minimal efflux) to that observed in macrophages overexpressing Mrp4 (maximal efflux) (see Table 4). This ratio was compared to the difference of MICs observed for the three efflux pumps in the three corresponding bacteria (see Figure 2).

We first observed in Table 4 that all fluoroquinolones tested accumulate at a higher extent than ciprofloxacin in wild-type macrophages, except norfloxacin, FQ13 and FQ8. A few molecules (FQ5, trovafloxacin, sparfloxacin, gemifloxacin and FQ2) showed a 6- to 11-fold higher accumulation compared to that of ciprofloxacin. We then evidenced that fluoroquinolones are differently recognized by the Mrp4 transporter by showing that Mrp4 affected differently the accumulation levels of fluoroquinolones. Indeed, norfloxacin, enoxacin and FQ13 were well effluxed by Mrp4 as ciprofloxacin (ratio values between accumulation levels in WT cells in the presence of gemfibrozil and ciprofloxacin-resistant cells were higher than 10). On the contrary, difloxacin, gemifloxacin, FQ4 and enrofloxacin were not or poorly effluxed by Mrp4 as moxifloxacin (values of the ratio for these quinolones are very low and near from 1.00). Other quinolones have an intermediate efflux (in Table 4, quinolones are classified from top to bottom in decreasing ratio, meaning in a decreasing efflux by Mrp4).

Table 4. Intracellular accumulation of 25 quinolones in wild-type cells in the absence of gemfibrozil (WT), or in the presence of 500 µM gemfibrozil (WT + Gem), and in ciprofloxacin-resistant cells in the absence of gemfibrozil (CIP-R), or in the presence of 500 µM gemfibrozil (CIP-R + Gem)

quinolones	Intracellular accumulation (in percentage of ciprofloxacin accumulation in WT cells)				Ratio WT+Gem/CIP-R
	WT	WT + Gem (-)	CIP-R (+)	CIP-R + Gem	
Norfloxacin	30	244	16	186	15.12 (1.27)
Enoxacin	329	563	47	436	11.96 (1.01)
Ciprofloxacin	100	340	28	251	11.90 (1.00)
FQ13	28	113	11	46	10.13 (0.85)
FQ7	215	395	42	328	9.41 (0.79)
Levofloxacin	121	195	25	215	7.68 (0.65)
Lomefloxacin	261	543	73	383	7.41 (0.62)
Pradofloxacin	154	187	26	186	7.15 (0.60)
Delafloxacin	94	144	28	144	5.16 (0.43)
FQ8	63	84	16	71	5.05 (0.42)
Marbofloxacin	179	202	42	174	4.72 (0.40)
Sarafloxacin	191	305	68	325	4.45 (0.37)
FQ2	1102	1230	468	1124	2.62 (0.22)
Pefloxacin	327	391	217	390	1.80 (0.15)
FQ5	687	656	380	597	1.72 (0.14)
Garenoxacin	280	299	179	163	1.66 (0.14)
FQ10	176	192	123	172	1.56 (0.13)
Trovafloxacin	670	753	531	604	1.42 (0.12)
FQ6	341	241	173	203	1.39 (0.12)
Sparfloxacin	643	702	539	811	1.30 (0.11)
Difloxacin	288	314	248	313	1.26 (0.11)
Gemifloxacin	651	704	562	826	1.25 (0.11)
FQ4	450	502	436	583	1.15 (0.10)
Enrofloxacin	391	354	310	471	1.14 (0.10)
Moxifloxacin	232	273	254	312	1.07 (0.09)

Values are expressed in percentage of the accumulation of ciprofloxacin (considering 100%) in WT cells. The last column represents for each quinolone the ratio of the values observed in WT cells with gemfibrozil (minimal efflux (-)) and in ciprofloxacin-resistant cells (maximal efflux (+)), and values in parenthesis are the ratio reported to that of ciprofloxacin (considering 1.00 for ciprofloxacin).

Figure 2 shows a high degree of correlation between efflux of fluoroquinolones mediated by the eukaryotic Mrp4 efflux pump and the prokaryotic NorA and PatA efflux pumps (present in *S. aureus* and *S. pneumoniae* respectively, both Gram positive bacteria), as all the points are clustered in the blue "cone" (except one which correspond to gemifloxacin). On the contrary, there is no correlation between the efflux caused by Mrp4 in eukaryotic cells and that caused by Mex/Opr efflux system in prokaryotic *P. aeruginosa*, a Gram negative bacterium (data not shown).

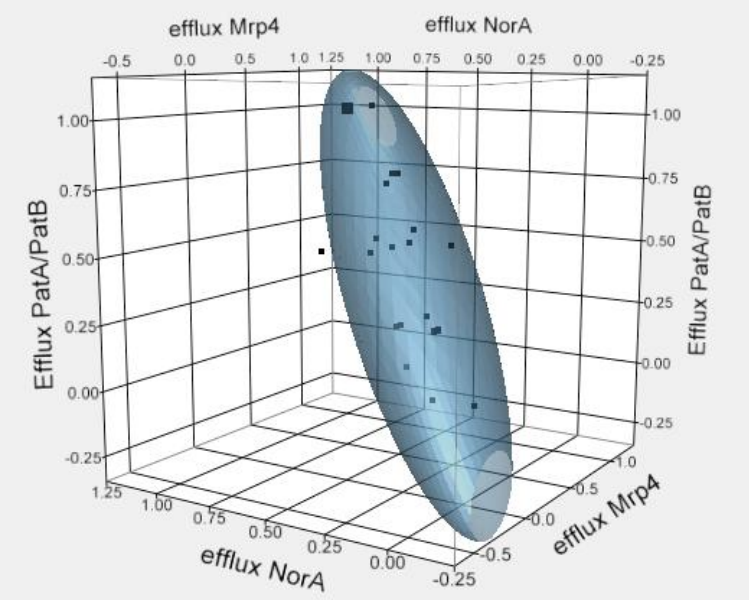


Figure 2. Multivariate analysis of the correlation between sensitivity to efflux mediated by Mrp4, NorA and PatA/PatB. Statistical analysis: correlation by pairs (R^2 = 0.75 for PatA/PatB vs Mrp4; 0.84 for NorA vs Mrp4; 0.85 for PatA/PatB vs NorA). Data analysis and graph generation with JMP Software version 9.0.3.

Correlation between the physicochemical properties of the 25 quinolones and their efflux by NorA and Mrp4. Physicochemical parameters (as logP, logD, molecular weight …) were calculated with the Reaxys web-based software (http://www.reaxys.com) for each fluoroquinolone (Table 5). They were analyzed by principal component analysis to check if one of these parameters could govern fluoroquinolone efflux by NorA or PatA/PatB or Mrp4, and results are presented in Figure 3. Each parameter is represented by an arrow, and is placed on the circle in function of the correlation found

with the other parameters, meaning that more arrows are close to each other, better is the correlation between parameters.

We observed a high degree of correlation between efflux mediated by NorA and PatA/PatB as both arrows representing these parameters are close to each other. In addition, because arrows for efflux mediated by Mrp4 and the prokaryotic efflux pumps are close from one another, we confirm the high degree of correlation between fluorquinolone recognition by these three pumps. Surprisingly, no correlation was evidenced between transport by the different efflux pumps (Mrp4, NorA or PatA) and the global lipophilicity of the molecules or any of the other physicochemical parameters examined (no arrows representing a physicochemical parameter is close to arrows representing efflux).

Figure 3. Principal component analysis of the correlations between physicochemical properties of fluoroquinolones and efflux mediated by Mrp4, NorA and PatA/PatB.The diagram shows a projection of multidimensional set of axes onto a 2-dimensional plane (arrows of the same length are on the same plane; arrows of different length have different orientations). The closeness of the arrows denotes the degree of correlation. Data analysis and graph generation with JMP software version 9.0.3.

Table 5. Physicochemical parameters calculated for the 25 fluoroquinolones

	Accumul.	Accumul. Max.	Efflux Mrp4	Efflux NorA	Efflux PatA/PatB	Molecular weight (g/mol)	pK COOH	pK NH_2	pI	logP	logD at pI	LogD at pH7	logD at pH5	Polar surface at pH7	% Uncharged species at pH7	% Negative species at pH7	% Positive species at pH7	% Zwitterionic species at pH7	Charge at pH7
Norfloxacin	0.30	0.72	1.27	1.00	1.00	319.33	5.77	8.68	7.37	1.44	-1.48	-1.48	-1.73	80.29	0.12	1.94	5.44	92.50	0.08
Enoxacin	3.30	1.65	1.01	0.67	0.5	320.32	5.50	8.59	7.20	1.42	-1.68	-1.68	-1.78	93.18	0.00	2.42	3.00	94.50	0.03
Ciprofloxacin	1.00	1.00	1.00	1.00	1.00	331.34	5.76	8.68	7.35	1.55	-1.38	-1.38	-1.62	80.29	0.11	1.95	5.28	92.66	0.08
FQ13	0.28	0.33	0.85	0.67	0.75	373.38	6.06	-0.81	3.03	1.16	1.16	0.18	1.13	83.99	10.29	89.71	0.00	0.00	-0.90
FQ7	2.15	1.16	0.79	0.67	0.75	396.41	5.39	9.40	7.55	1.98	-1.97	-1.97	-1.38	104.08	0.00	0.38	2.40	97.21	0.04
Levofloxacin	1.21	0.57	0.65	0.33	0.50	361.37	5.46	6.20	5.97	1.56	0.65	0.07	0.35	76.15	3.23	85.95	0.38	10.44	-0.79
Lomefloxacin	2.61	1.59	0.62	0.67	0.50	331.35	5.64	8.70	7.32	2.00	-1.06	-1.06	-1.20	80.29	0.00	1.89	4.13	93.89	0.06
Pradofloxacin	1.54	0.55	0.60	0.50	0.50	396.41	5.39	9.40	7.55	1.98	-1.97	-1.97	-1.38	104.08	0.00	0.38	2.40	97.20	0.04
Delafloxacin	0.94	0.43	0.43	0.50	0.50	440.76	5.62	1.06	4.00	2.56	2.55	1.16	2.46	122.82	3.98	96.02	0.00	0.00	-0.96
FQ8	0.64	0.25	0.42	0.50	0.50	447.89	5.94	0.13	3.04	2.34	2.34	1.25	2.29	83.99	8.08	91.92	0.00	0.00	-0.92
Marbofloxacin	1.80	0.59	0.40	0.33	0.25	362.36	5.38	6.16	5.92	0.69	-0.25	-0.86	-0.51	79.39	2.76	87.06	0.31	9.88	-0.81
Sarafloxacin	1.91	0.90	0.37	0.50	0.75	385.36	5.74	8.68	7.36	2.94	-0.01	-0.01	-0.24	80.29	0.11	1.96	5.41	92.83	0.08
FQ2	11.02	3.61	0.22	0.50	0.50	371.41	5.76	9.46	7.76	2.12	-1.56	-1.53	-1.16	80.29	0.00	0.33	5.39	94.27	0.10
Pefloxacin	3.27	1.15	0.15	0.17	0.25	333.36	5.66	6.47	6.22	1.83	0.87	0.49	0.38	66.92	4.52	76.29	1.05	18.14	-0.67
FQ5	6.88	1.93	0.14	0.33	0.25	389.40	5.65	9.39	7.67	2.27	-1.46	-1.43	-1.03	80.29	0.00	0.39	4.25	95.35	0.08
Garenoxacin	2.81	0.88	0.14	0.17	0.25	426.41	6.05	9.46	7.91	3.98	0.57	0.59	0.73	86.28	0.00	0.31	10.07	89.59	0.18
FQ10	1.76	0.57	0.13	0.17	0.25	384.40	5.32	6.27	5.94	2.15	1.03	0.47	0.78	90.71	2.09	84.07	0.33	13.51	-0.79
Trovafloxacin	6.71	2.21	0.12	0.17	0.25	416.35	5.41	9.44	7.58	2.40	-1.53	-1.53	-0.76	104.21	0.00	0.35	2.51	97.12	0.05
FQ6	3.41	0.71	0.12	0.17	0.25	405.85	5.64	9.42	7.68	2.73	-1.02	-1.00	-0.58	80.29	0.00	0.37	4.17	95.45	0.08
Sparfloxacin	6.43	2.06	0.11	0.17	0.25	392.40	5.75	8.79	7.42	2.35	-0.70	-0.70	-0.85	106.31	0.00	1.50	5.28	93.13	0.09
Difloxacin	2.88	0.92	0.11	0.00	0.00	399.39	5.64	6.45	6.20	3.32	2.36	1.95	1.88	66.92	4.29	77.15	0.95	17.63	-0.68
Gemifloxacin	6.51	2.07	0.11	0.67	0.50	389.38	5.53	9.53	7.68	1.35	-2.62	-2.54	-1.78	125.80	0.00	0.28	3.29	96.42	0.06
FQ4	4.50	1.47	0.10	0.33	0.25	445.46	5.76	9.42	7.74	2.32	-1.32	-1.29	-0.96	89.52	0.00	0.36	5.45	94.17	0.10
Enrofloxacin	3.92	1.04	0.10	0.17	0.25	359.39	5.69	6.58	6.33	2.29	1.14	0.88	0.61	66.92	3.83	66.67	1.56	27.93	-0.57
Moxifloxacin	2.32	0.80	0.09	0.17	0.00	401.43	5.69	9.42	7.71	1.97	-1.75	-1.72	-1.33	89.52	0.00	0.36	4.61	95.01	0.08

Accumul.: accumulation level in WT cells (considering 1 for that of ciprofloxacin); Accumul. Max.: accumulation level in WT cells in the presence of gemfibrozil (considering 1 for that of ciprofloxacin); Efflux Mrp4, efflux NorA, Efflux PatA/PatB: correspond to values in parenthesis in Tables 3 and 4; pK COOH, pK NH_2: correspond to pK values for the COOH and the NH_2 moieties of each fluoroquinolone; pI: isoelectric point.

In order to check the interactions of fluoroquinolones with the efflux protein, murine Mrp4 was modeled (by homology with the human P-gp crystal structure), and the two fluoroquinolones ciprofloxacin (a substrate of Mrp4), and moxifloxacin (described here as a non substrate of Mrp4) were docked in the binding site of the efflux transporter (see Figure 4).

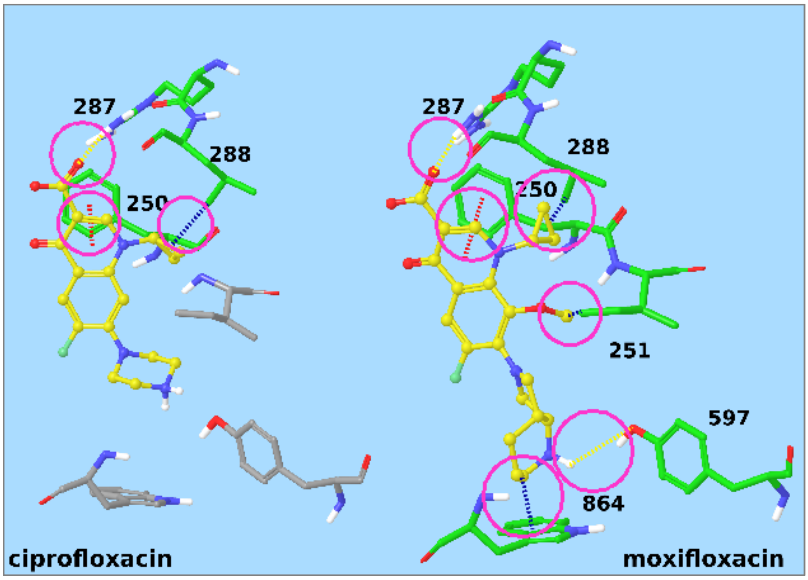


Figure 4. Docking of ciprofloxacin and moxifloxacin (in yellow) in the Mrp4 binding site. Amino acids of the binding site are represented in green when they interact with the fluoroquinolone, and in grey when there is no interaction. Interactions between fluoroquinolone and an amino acid are evidenced by pink circles.

Fig. 4 shows that ciprofloxacin establishes less interaction with the binding site of Mrp4 than moxifloxacin. Indeed, 3 interactions are evidenced for ciprofloxacin (clustered around the carboxylic and the cyclopropyl moieties of the fluoroquinolone), while 6 interactions can be observed for moxifloxacin (3 identical as those for ciprofloxacin, and 3 additional at the level of the 8-methoxy and the 7-hydropyrrolopyridine moieties). The same observations were made for ciprofloxacin and moxifloxacin in the binding site of the NorA efflux pump of *Staphylococcus aureus*: only 2 interactions were evidenced between ciprofloxacin and the NorA binding site, while 4 interactions were shown for moxifloxacin.

Other fluoroquinolones were docked in the murine Mrp4 model, and in the *S. aureus* NorA model (data not shown), and this structural analysis shows that molecules

poorly transported (as moxifloxacin) by the efflux pumps established more interactions with the binding sites of both NorA and Mrp4 than molecules highly transported (as ciprofloxacin).

2.4. Discussion

Only few studies were performed on the structure-activity relationship between substrates and efflux pumps, certainly due to the lack of crystal structures of these transport proteins. Thus we have almost no data which could help us in the understanding of the substrate recognition by an efflux pump, and which could explain the capacity of an efflux pump to transport a large variety of substrates. In this study, we have not been able to ascertain one physicochemical parameter for fluoroquinolones which could govern their recognition by Mrp4 nor by NorA. Brillault J and colleagues (Brillault et al. 2010) have shown a correlation between the lipophilicity of fluoroquinolones (only 6 have been studied in this paper) and their passive diffusion across Calu-3 cell membrane, but this lipophilicity was not correlated with the active transport of these drugs. Another study (Akanuma et al. 2011) observed that (i) anionic β-lactam antibiotics showed higher transport activity via MRP4 than did zwitterionic ones, and (ii) anionic character and high molecular weight are favorable for MRP4-dependent transport of these antibiotics. In addition, a study conducted by Takenouchi T and colleagues (Takenouchi et al. 1996) showed that the decreased activity of fluoroquinolones in NorA efflux-mediated resistant *S. aureus* was not attributed to their global hydrophilicity. But they demonstrated that the bulkiness of the C-7 substituent and the bulkiness and hydrophobicity of the C-8 substituent of fluoroquinolones were significantly correlated with their efflux by NorA. Thus not only one, but maybe two or more physicochemical parameters could be involved in the substrate recognition by an efflux pump.

Despite the fact that no physicochemical parameter could be assigned to the substrate recognition by Mrp4 in the present study, we can still show the importance of C-8 and C-7 substituents of fluoroquinolones. Indeed, a homogenous series of moxifloxacin displaying only a modification of the C-8 substituent, showed very different susceptibility to efflux, and can be ranked in order of increasing efflux as followed: moxifloxacin < FQ4 < FQ6 < FQ5 < FQ2 < pradofloxacin. Thus, we observed that fluoroquinolones having an ethoxy (FQ4) or a methoxy group (moxifloxacin) at C-8 position are not effluxed by Mrp4, while the presence of a halogen atom as fluorine or

chlorine (FQ5 and FQ6) can slightly increase the efflux of the molecules, as the absence of a C-8 substituent (FQ2). The presence of a cyano moiety (pradofloxacin) is the only substituent observed here that can significantly increase the efflux in this series of molecules. A second observation was made on a set of three fluoroquinolones where only the C-7 substituent was modified: we demonstrated that both ciprofloxacin and FQ13 (the latter having a $COCH_3$ moiety) are well effluxed by Mrp4, whereas the presence of an N-ethyl group (enrofloxacin) at the C-7 position prevents the efflux of the molecule. Therefore, more homogenous series should be investigated to better understand the substrate recognition by MRPs and the substrate susceptibility to efflux.

A surprising discovery in this study was that NorA, a MFS efflux pump in *S. aureus*, and PatA/PatB and Mrp4, ABC efflux pumps in *S. pneumoniae* and mouse macrophages, showed similar transport of fluoroquinolones. This suggests that the same molecular determinants could govern the fluoroquinolone recognition by efflux systems whatever their phylogenetic classification and whatever their eukaryotic or prokaryotic origin. An original paper (Venter et al. 2003) has observed that a primary-active transporter (ABC transporter) can act as a secondary-active transporter when nucleotide binding domains were lacking. Indeed, when the ABC transporter LmrA from *Lactococcus lactis* was truncated by the removal of its NBDs, it was able to transport the substrates by using a proton-motive force. This suggests that the substrate specificity does not depend on the energy source of the transporter, but rather of certain residues contained in the TMDs. Recent studies (Loo and Clarke 2008) confirm that NBDs are required for the ABC transporter activity, but not required for binding of drug substrates.

We also observed that the RND efflux system Mex/Opr in *P. aeruginosa* can expel all fluoroquinolones tested in this study to the same extent, thus allowing no correlation with efflux by the other transporters (NorA or PatA/PatB). This difference of efflux could be due to the broad spectrum efflux system in *P. aeruginosa* (RND transporter) compared to the narrow spectrum efflux system in *S. aureus* for example (MFS transporter). Two studies ((Mao et al. 2002); (Middlemiss and Poole 2004)) showed that the substrate recognition by the RND pumps in *P. aeruginosa* could be made by two large periplasmic loops (LPL) which are not present in other efflux pumps. These two LPLs contain multiple sites of interaction for various structurally diverse compounds, and do not form a “pocket binding-site” as in other efflux pumps. This could explain the wide variety of substrates for RND efflux pumps.

The last observation made in the present study was that fluoroquinolones considered as non substrate of Mrp4, meaning that they are not expelled by the efflux pump, established more interactions in the binding site than molecules considered as substrates. Michot and colleagues (Michot et al. 2005) showed that although moxifloxacin accumulation was not affected by the presence of the Mrp4 transporter, this fluoroquinolone was able to inhibit the ciprofloxacin efflux, thus demonstrating a possible interaction of moxifloxacin with Mrp4. This suggests that the lack of transport is not due to poor recognition but rather to the inability of strongly bound drugs to progress for efflux within the transporter. Although the mechanism of action of ABC exporters is not yet fully elucidated, it is now well established that a modification of the protein conformation due to the binding and hydrolysis of ATP to the NBDs allows the release of the substrate ((van Veen et al. 2000); (Gutmann et al. 2010); (Linton 2007) ; (Zou and McHaourab 2009)). Indeed, an ABC transporter can bind a drug to its high affinity drug binding-site, leading to a binding and hydrolysis of ATP to the NBDs. The closing of the NBDs induces conformational changes in the TMDs together with alterations in the substrate-binding affinity. Thus, the substrate binding site moves from a high affinity state (protein in inward-facing/nucleotide-free conformation) to a low affinity state (protein in outward-facing/nucleotide-bound conformation). This means that a communication between TMDs and NBDs should take place as it has been suggested by Becker JP and colleagues (Becker et al. 2009). Results obtained in our study may raise the possibility of an altered TMD-NBD communication when molecules as moxifloxacin (which binds to Mrp4, but is not effluxed) are present in the binding-site. Another hypothesis would be that the path from high affinity to low affinity binding site is more difficult in the case of moxifloxacin as it establishes more interactions with the binding site compared to an effluxed molecule as ciprofloxacin.

Despite recent advances and studies as the discovery of the mouse P-gp X-ray structure (Aller et al. 2009), it would be of particular interest to visualize drug binding to the drug binding pocket of ABC efflux proteins, and to have structure models for the “transient” state (high affinity to low affinity drug binding state) to better understand the poly-specificity of drug binding and the mechanistic details of the transport cycle. Findings of this study raise many unanswered questions (as what distinguishes the inhibitor from the transported ligand? Do inhibitors and ligands bind different sets of residues?), but are of prime importance to orient further investigations.

GENERAL DISCUSSION

One of the research interests of our laboratory consists in exploring the interactions between antibiotics and eukaryotic cells within a context of molecular and cellular pharmacology and toxicology. The studies performed by Michot in 2004, 2005 and 2006 have shown that fluoroquinolones are substrates of eukaryotic efflux pumps to various extents, and that exposure of eukaryotic cells to ciprofloxacin in particular allows for the selection of ciprofloxacin-resistant cells. A further study of Marquez in 2009 showed that ciprofloxacin-resistant cells overexpress the ABC efflux transporter Mrp4 which is at the origin of the massive efflux of ciprofloxacin. In the present work, we have particularly focused our interest on moxifloxacin, another fluoroquinolone not considered as an Mrp4 substrate, and on the comparison of its behavior with that of ciprofloxacin and other fluoroquinolones. Cellular pharmacokinetic and pharmacodynamic parameters of fluoroquinolones in bacteria are well studied, but little is known about their effects on eukaryotic cells. Studies have shown that antibiotics are able to penetrate in eukaryotic cells but they are substrates of eukaryotic efflux pumps, which reduce their cellular accumulation, and may impair their activity against intracellular pathogens. Moreover, literature is scarce about fluoroquinolone substrate recognition by MDR transporters. Yet, understanding their broad substrate specificity is of prime interest to try designing antibiotics that are poorly transported by efflux pumps and therefore may show more potent intracellular activity.

The present study was therefore designed to get further knowledge of the efflux-mediated resistance of fluoroquinolones in eukaryotic cells, to explore the role of Mrp4 expression in this resistance as well as the toxicological effects of ciprofloxacin and moxifloxacin in cells exposed to these fluoroquinolones, and to investigate the structure-activity relationship of fluoroquinolone recognition by the Mrp4 efflux pump.

1. MAIN FINDINGS OF THIS WORK

In this work, we showed first that the Mrp4 transporter was overexpressed at different levels in cells exposed to increasing concentrations of ciprofloxacin. Indeed, we demonstrated that the *mrp4* gene and the Mrp4 protein levels were increased step by step in cells selected with 0.1, 0.15 and 0.2 mM of ciprofloxacin. This progressive increase of Mrp4 has been explained by a progressive duplication of the *mrp4* gene locus during the selection steps of resistant cells with increasing concentrations of the

antibiotic. Chromosomal alterations and gene amplification have been reported in some studies where cells were chronically exposed to anticancer agents ((Knutsen et al. 1998); (Yasui et al. 2004); (Fojo 2007)), but these mechanisms are less known for antibiotic in eukaryotic cells as they are usually less toxic than anticancer drugs. While ciprofloxacin has not been reported to be genotoxic, some studies reported chromosomal aberrations in cells exposed to supratherapeutic concentrations of the fluoroquinolone. In our study, we also used high concentrations of ciprofloxacin (40 to 80 mg/L), but these can be relevant as it is known that fluoroquinolones accumulate in tissues where they can reach concentrations that are 2 to 7-fold higher than serum levels.

The study with moxifloxacin led to unanticipated results. We found indeed that this molecule, which is not affected by the efflux mediated by Mrp4, was able to down-regulate the Mrp4 expression in cells selected by increasing concentrations of this molecule. Taken as a whole, these results and those obtained previously with ciprofloxacin demonstrate that two closely related fluoroquinolones (ciprofloxacin and moxifloxacin) revealed opposite patterns in terms of sensitivity to the Mrp4 efflux, as well as in terms of modulation of the expression of Mrp4. This is, to our knowledge, the first *in vitro* study to show an under-expression of a MDR transporter upon drug exposure. These observations raise two important questions. First, what is the resistance mechanism developed by moxifloxacin-resistant cells? We noticed that moxifloxacin-resistant cells were partly resistant to moxifloxacin, but this resistance was not due to a massive efflux of the molecule outside the cells (as compared to ciprofloxacin in ciprofloxacin-resistant cells). We thus focused our attention on topoisomerase activity as fluoroquinolones target these enzymes in bacteria, and a few studies showed that high concentrations of fluoroquinolones are able to inhibit the eukaryotic topoisomerase activity. Our preliminary experiments have shown that type II topoisomerases could be less sensitive to the action of moxifloxacin in moxifloxacin-resistant cells compared to wild-type cells. Further studies are needed to determine whether alteration in topoisomerase is at the origin of the resistance to moxifloxacin. Second, why is Mrp4 under-expressed in moxifloxacin-resistant cells? Only one study, conducted in rats with obstructive cholestatis, showed an increase in Mrp4 expression in the liver, but a decrease in the kidney (Denk et al. 2004), which was attributed to a global adaptive response to the increase in plasma (and urine) concentrations of cAMP. This mechanism can probably not apply in our case, because we used an *in vitro* model consisting in a

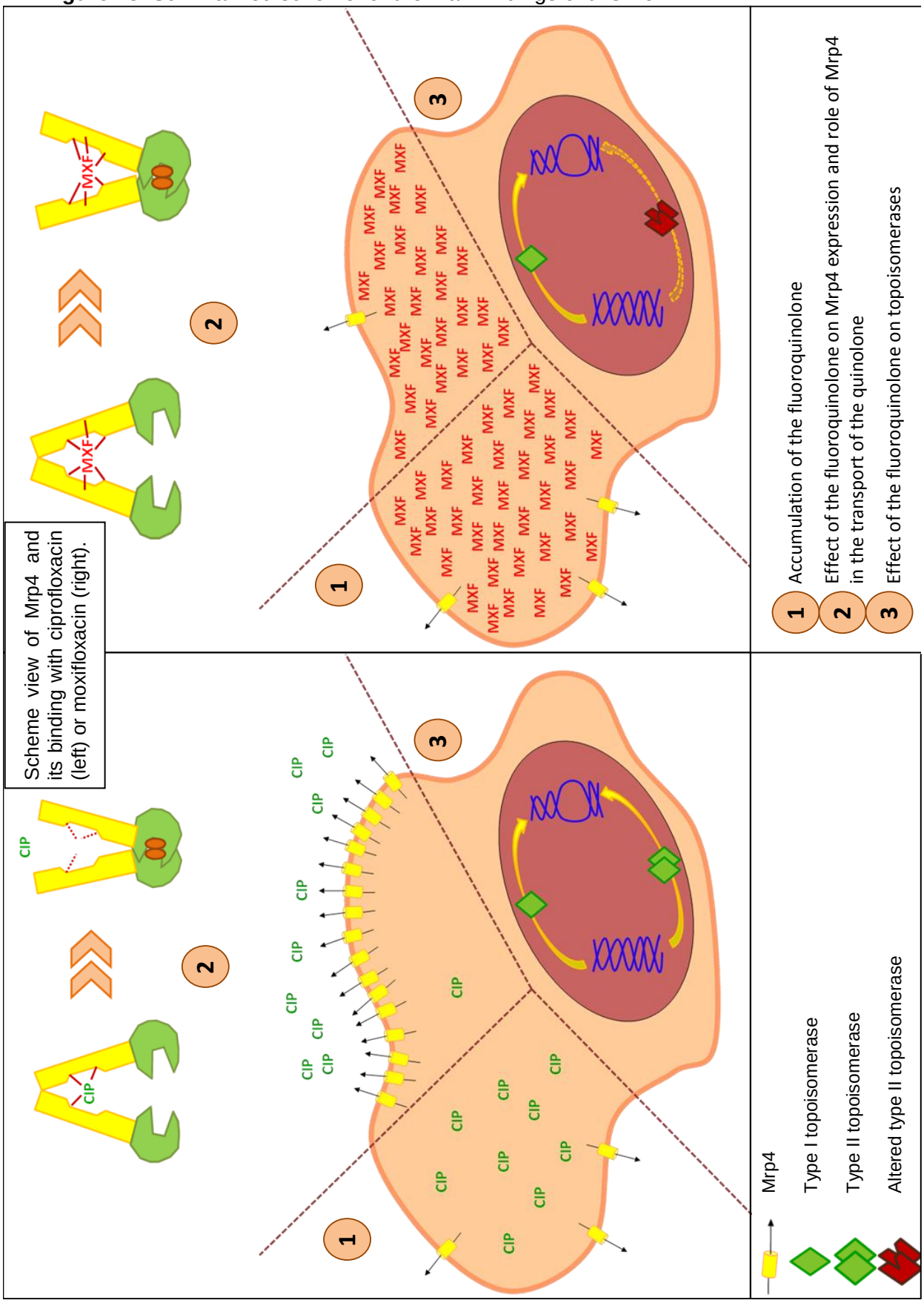
single cell line, incubated in a defined medium, making unlikely the influence of an exogenous signal molecule. An alternative explanation to this reduced Mrp4 expression (and changes in some other ABC transporters expression) in moxifloxacin-resistant cells could be a global adaptation of the cells to a major toxic stress. Recent studies and techniques ((Annereau et al. 2004); (Gillet et al. 2007); (Gillet et al. 2007)) evaluating the global expression profile of genes involved in toxic stress (as ABC transporters, enzymes involved in detoxification, cytochromes…) allow to show a modification of the expression of several genes in cell lines exposed to xenobiotics. In our study, we showed that not only Mrp4 expression was modified in moxifloxacin-resistant cells, but also non MDR transporters were overexpressed, confirming the global adaptation of these cells to the moxifloxacin pressure. This raises the question of the biological cost of the expression of ABC transporters for eukaryotic cells: reducing Mrp4 expression, even if not conferring resistance by itself, may help cells to survive by preventing them from using their energy sources for fueling a useless transporter. Moreover, additional preliminary studies performed by TLDA (as used for ABC transporters expression in Vallet et al. 2011) but not illustrated in the thesis have shown that genes as glutathione S-transferase M and P (which code for detoxifying enzymes) were up-regulated in moxifloxacin-resistant cells. These enzymes could contribute to prevent the toxic effect of moxifloxacin in the absence of active efflux.

A second part of this work consisted, in a first time, in comparing the cellular pharmacokinetics and pharmacodynamics of three fluoroquinolones in macrophages uninfected and infected with *L. monocytogenes* or *S. aureus*. We showed that gemifloxacin was not more active against the two intracellular pathogens used in this study compared to ciprofloxacin and moxifloxacin, despite higher accumulation and less sensitivity to efflux mediated by Mrp4 in J774 macrophages. This observation goes against the commonly accepted concept that accumulation is predictive of intracellular activity. It suggests that a more important actor could be the intracellular bioavailability of the drug, which could depend on its capacity to bind to some cellular constituents. We have no clue so far as whether this is the case for gemifloxacin but we may hypothesize it could bind to cellular proteins stronger than the other fluoroquinolones because (a) its release from cells is only partial and (b) it is known to bind to serum proteins to higher levels than ciprofloxacin or moxifloxacin.

Two additional important findings have been made when comparing the fluoroquinolone efflux in eukaryotic and prokaryotic cells and when analyzing the structure-efflux relationships for transport by Mrp4 and prokaryotic efflux transporters. First, we described a high correlation between the transport capacity for fluoroquinolones of two phylogenically-unrelated Gram positive bacterial efflux pumps (NorA for *S. aureus*, and PatA/PatB for *S. pneumoniae*) and an eukaryotic efflux pump (Mrp4 for J774 mouse macrophages). Mrp4 and PatA/PatB both belong to the ABC transporters superfamily but their structure is largely different, one being of prokaryotic origin and functioning probably as a heterodimer of two proteins, and the other one being of eukaryotic origin and encoded by a single gene. The similarity in substrate specificity between these transporters and NorA is quite surprising, because the latter belongs to the MFS superfamily, having thus a completely different transport mechanism (indeed, ABC exporters use the ATP hydrolysis as energy source, while MFS transporters use the proton motive force to expel molecules outside cells). Therefore, we can conclude that although phylogenetically different, NorA, PatA/PatB and Mrp4 must share common molecular determinants in their binding site for the substrate recognition. Second, we examined in more details the substrate recognition by Mrp4 and NorA using molecular modeling simulations. An astonishing observation was that all fluoroquinolones, whether affected by these transporters or not, can fit in the binding site of efflux pumps, suggesting they are all potential substrates. Unexpectedly also, a fluoroquinolone showing experimentally an effective transport (like ciprofloxacin) makes less interactions with the binding site than a fluoroquinolone that is not effectively transported (like moxifloxacin). We thus hypothesized that all molecules are substrates of efflux pumps, but that those which show a greater number of interactions may have a longer residence time within the protein, and behave rather as inhibitors than as substrates. We know indeed that moxifloxacin is capable of inhibiting ciprofloxacin transport by Mrp4 (Michot et al. 2005).

Figure 13 (see next page) shows a scheme wich summarizes the main findings of this work. It represents for ciprofloxacin and moxifloxacin: (i) their accumulation levels in J774 macrophages, (ii) their effect on Mrp4 expression and their transport by Mrp4, and (iii) their effect on topoisomerases.

Figure 13. Summarized scheme for the main findings of this work.



2. INTERESTS AND LIMITS OF THE CELLULAR MODEL USED IN THIS WORK

2.1. Interests of the J774 macrophages model

This work aimed at examining the fluoroquinolone efflux mediated by ABC transporters in eukaryotic cells, and mouse J774 macrophages represent a model with two main advantages.

First, if only few studies explored the multidrug resistance in J774 macrophages in the early 1990s ((Kirschner et al. 1992); (Lothstein et al. 1992)), those cells were well characterized and largely used in our laboratory since the 2000s to investigate the pharmacokinetic and the intracellular activity of many classes of antibiotics (as demonstrated by the large number of publications related to J774 macrophages in the FACM website www.facm.ucl.ac.be). In addition, Mrp proteins are well represented in these cells: after showing that fluoroquinolone accumulation was affected by a constitutive and basal expression of Mrp transporters at their plasma membrane ((Michot et al. 2004); Michot et al. 2005), Mrp4 was identified as the main ciprofloxacin transporter (Marquez et al. 2009). Moreover, among the 7 *mrp* genes (which code for Mrp proteins known to be involved in multidrug resistance), *mrp4* is from far the most abundantly expressed in wild type J774 macrophages (Marquez et al. 2009). It may be one of the reasons why this transporter is overexpressed when selecting cells with ciprofloxacin. And we demonstrated that P-gp and Bcrp, two other ABC transporters involved multidrug resistance, were also present at the plasma membrane of these cells but in very low amounts (Vallet et al. 2011). Nevertheless, their expression was inducible by moxifloxacin.

Second, macrophages play an important role in the defense of the organism against bacterial infection. As fluoroquinolones accumulate at high levels in cells, they are considered as antibiotics of choice for the treatment of intracellular infections. Indeed, fluoroquinolones are highly active against *Mycobacterium spp*. in macrophages ((Vacher et al. 1999); (Sato et al. 2000); (Sano et al. 2011)). Moreover, a recent study (Wendte et al. 2011) described that ciprofloxacin was more effective against intracellular *Yersinia pestis* than usually recommended antibiotics for treatment of plague such as gentamicin or doxycyclin. In addition, Carryn S and colleagues in our laboratory (Carryn et al. 2003) showed in 2003 that moxifloxacin remains the most active antibiotic against intracellular *L. monocytogenes* compared to β-lactams, gentamicin or azithromycin.

Thus, studying the influence of efflux transporters on fluoroquinolone accumulation within these cells is therefore of high importance. Intracellular infection models used in the laboratory to explore antibiotic intracellular activity was very useful as Seral C and colleagues (Seral et al. 2003a) demonstrated in 2003 that ciprofloxacin intracellular activity was enhanced in the presence of a Mrp inhibitor against *L. monocytogenes*, but not against *S. aureus* (which is contained in lysosomes). Moreover, it has been shown that fluoroquinolone activity against intracellular pathogens as *L. monocytogenes* was decreased when ABC transporters were present at the plasma membrane of J774 macrophages (Michot et al. 2006; (Lismond et al. 2008)). This cell model is therefore interesting to further explore pharmacokinetics and pharmacodynamics of fluoroquinolones in vitro.

2.2. Limits of the J774 macrophages model

The model we used also presents a series of limitations.

First, J774 macrophages are from mouse origin, thus expressing mouse proteins. Human and mouse *mrp4* genes display sequence homologies (67 to 92% following the exon, (Lamba et al. 2003)), but these differences may be sufficient to alter substrate specificity. Recent preliminary studies conducted in our laboratory in which human embryonic kidney cells (HEK293) overexpressing human MRP4 were used, have demonstrated that ciprofloxacin was substrate of human MRP4 while moxifloxacin was not (Marquez and Van Bambeke 2011). Although further studies are needed to compare more molecules, these observations suggest that the murine and human MRP4 transporters may share similar substrate specificity as far as fluoroquinolone recognition is concerned. In addition, the macrophage model is not often used to explore the multidrug resistance (resistance is mainly investigated for anticancer agents, using tumor cells). With respect to antibiotics, their active efflux transport has been mainly investigated in epithelial cells, and related to their pharmacokinetic profile ((Van Bambeke et al. 2003) for review).

Second, ABC transporters are indeed also expressed in epithelial cells where they contribute to barrier effects. The regulation of the expression of genes coding for these transporters may differ from one cell type to the other. For example, ABC transporter overexpression in hepatic or renal cells frequently involves a nuclear receptor regulation pathway, which allows a rapid adaptation of detoxifying organs to xenobiotic

exposure. Indeed, nuclear receptors as CAR (Constitutive androstane receptor), or PXR (pregnan X receptor) are involved in the overexpression of Mrps in hepatic cells (Mottino and Catania 2008); several therapeutic drugs activate directly these nuclear receptors and will lead to a direct transcriptional effect. Thus, the Mrp4 overexpression by gene amplification observed here in J774 mouse macrophages exposed to ciprofloxacin could be a resistance mechanism developed only in our particular conditions and cell type.

Third, we have selected resistant cells to ciprofloxacin and moxifloxacin by exposing wild-type mouse macrophages to increasing concentrations of these antibiotics, therefore leading to non-clonal resistant cells. The fact that we evidenced modulations in the expression of several transporters in moxifloxacin resistant cells together with alterations in topoisomerases activity may suggest the existence of subpoplulations with different resistance mechanism; but may also reflect the selection of a pleiotropic stress response. Yet the interest of this mode of selection is that it better reflects the way cells are exposed to xenobiotics *in vivo*, and therefore the way resistance can be selected (Gottesman et al. 1998).

Fourth, we used an *in vitro* model of J774 mouse macrophages, which may not reflect an *in vivo* situation. Indeed, in vivo, macrophages are stimulated by a variety of immunoagents as cytokines, interleukins and others. One study reported that the bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* was significantly increased in an *ex vivo* model of macrophages (meaning that macrophages are immunocompetent) compared to that in an *in vitro* model of J774 macrophages (Dalhoff 2005). It is also known that depending on their tissue localization, macrophages express different receptors at their surface, which may affect their response to inflammatory cytokines, for example. Intestinal macrophages illustrate this variability. Indeed, they are totally different from other tissue macrophages as they display no CD14 (LPS receptor), and they do not produce proinflammatory cytokines (as IL-1, IL-6, IL-10, …) ((Smythies et al. 2005) (Smith et al. 2011) (Bain and Mowat 2011)). Taken together, differences between *in vitro* and *in vivo* situation, and differences between macrophages themselves lead us to be cautious about the observations we made in our particular mouse macrophages *in vitro* model.

3. RELEVANCE OF THE FLUOROQUINOLONE CONCENTRATIONS USED IN THIS STUDY

The concentrations of fluoroquinolones used to obtain resistant cells (40 to 80 mg/L) may appear as supratherapeutic, as they are well above the serum levels observed in patients receiving conventional therapies (1 to 4 mg/L). But as already discussed, it is well known that fluoroquinolones accumulate not only in body fluids (like in urine, where concentrations can be as high as 900 mg/L) but also in tissues (where they can reach concentrations up to 7-fold higher than those in the serum). It has been shown for example that levofloxacin administered intravenously (1000 mg; (Conte, Jr. et al. 2007)) or orally (750 mg; (Nicolau et al. 2012)) to patients with chronic lung disease can reach concentrations as high as 76 or 38 mg/L in alveolar macrophages, whereas concentrations in plasma are only of 9 or 8 mg/L respectively. Moreover, over the last 10 years, new formulations have been developed to treat respiratory tract infections. These include inhaled formulations (aerosolized antibiotics (Geller 2009)), which allow for the deposition of high concentrations in the respiratory tract. Indeed, Chono and colleagues demonstrated that while ciprofloxacin concentration in alveolar macrophages is about 5 mg/L after oral administration, it is up to 40 mg/L after intrapulmonary administration (Chono et al. 2007). Aerosol delivery of antibiotics directly to the airways yields high concentrations at the site of infection while minimizing systemic exposure, hence leading to more potent antibiotics against *Pseudomonas aeruginosa* in cystic fibrosis patients (King et al. 2010).

4. PERSPECTIVES

4.1. What is the mechanism of Mrp4 down-regulation in moxifloxacin-resistant cells?

We showed that the Mrp4 protein level was decreased in moxifloxacin-resistant cells, but the *mrp4* gene level was unchanged compared to that in wild-type cells (Vallet et al. 2011). This observation tends to suggest a post-transcriptional regulation of Mrp4 in these cells, and microRNAs (miRNA) may be a possible mechanism to explore. A miRNA is a short RNA molecule which contains a few nucleotides (about 22) and which acts as a post-transcriptional regulator that binds to complementary sequences on target

messenger RNA transcripts (mRNAs) resulting in target degradation. Since their discovery in the early 1990s, and their recognized regulation activity in the 2000s, more and more studies demonstrate that miRNAs are involved in biological processes and negative regulation. Recent publications have shown that the expression of miRNAs is inversely correlated with the expression of ABC MDR transporters. Indeed, in 2011, Haenisch S and colleagues (Haenisch et al. 2011) demonstrated that after rifampicin treatment, HepG2 cells displayed a down-regulation of MRP2 related to a high level of miRNA-379, and Feng DD and colleagues (Feng et al. 2011) showed the same correlation between miRNAs and P-gp expression in leukaemia cell lines. It would thus be interesting to investigate miRNAs as possible Mrp4 down-regulators. To this effect, we could screen miRNAs present in mouse J774 macrophages and check their expression level in both wild-type and moxifloxacin-resistant cells.

4.2. Do topoisomerases play a role in the resistant phenotype of cells exposed to moxifloxacin?

4.2.1. Type II topoisomerase alteration as a resistance mechanism in moxifloxacin-resistant cells

While neither ciprofloxacin nor moxifloxacin seemed able to cause cell death by apoptosis, we have shown that moxifloxacin-resistant cells become to some extent resistant to apoptosis induced by the topoisomerase II poison etoposide. This may suggest that moxifloxacin exposure has selected for some kind of alteration in topoisomerase II activity making it resistant to etoposide action. It would be therefore of prime interest to sequence topoisomerase II gene in moxifloxacin-resistant cells vs. wild-type or ciprofloxacin-resistant cells to evidence a possible amino-acid modification that could account for these effects. In addition, because fluoroquinolones are obviously less toxic for macrophages than etoposide, it would be necessary to explore their potential capacity to also cause apoptosis after more prolonged exposure.

4.2.2. Protection against topoisomerase poison activity by fluoroquinolones

We have shown that co-incubation of ciprofloxacin or moxifloxacin with camptothecin or etoposide prevented the toxic effect of these anticancer agents alone (except in ciprofloxacin-resistant cells in conditions where ciprofloxacin does not accumulate). This is in opposition with other publications ((Fabian et al. 2006) or

(Reuveni et al. 2008)), which rather indicate that ciprofloxacin and moxifloxacin were able to enhance camptothecin- and etoposide-induced toxic and anti-topoisomerase effects. We do not have any explanation so far for these discrepancies, but can only point at this stage differences in the protocol used: Reuveni and colleagues incubated cells with a fluoroquinolone for 2 hours and then add camptothecin for an additional one hour, whereas we incubated at the same time fluoroquinolone and anticancer agent. We could therefore repeat our experiments comparing both protocols. In our experiments, however, the protection brought by fluoroquinolones is coherent with the demonstration of a reduced accumulation of topoisomerase inhibitors in the nuclei of cells that had been incubated with fluoroquinolones. Additional studies are needed to explain these changes in accumulation. We may think for example to the formation of a complex between fluoroquinolones and topoisomerase inhibitors preventing their access to their target. It would therefore be interesting to evidence a physical or a chemical incompatibility between fluoroquinolones and topoisomerase inhibitors; to this respect, we could assay the residual quantity of both fluoroquinolone and topoisomerase inhibitor by HPLC after their accumulation on cells (free fractions of drugs could be collected after dialysis).

4.3. Do fluoroquinolones bind to cellular proteins?

We have shown that the accumulation level of fluoroquinolones in eukaryotic cells was not predictive of their intracellular efficacy, as gemifloxacin displayed very high accumulation in J774 macrophages, but was not more active against intracellular pathogens than ciprofloxacin and moxifloxacin, suggesting a poor intracellular bioavailability. Yet, fluoroquinolone binding to intracellular components has never been explored. Mass spectrometry could help detecting changes in the mass of gemifloxacin when bound to a protein, but this supposes a covalent link which is unlikely here. Another possibility would be to measure the binding of gemifloxacin to cellular components by equilibrium dialysis against a cellular extract.

4.4. A crystal structure for Mrp4?

As we have shown in the chapter 2 of our results, the crystal structure of Mrp4 is not available yet. In our study, we have therefore used a three-dimensional model of the

mouse Mrp4 efflux pump made by homology with the crystal structure of the mouse P-gp. The putative binding site we have defined was identified based on mutagenesis analysis from a publication of El Sheikh and colleagues (El Sheikh et al. 2008) which reported the residues in transmembrane helices 6 and 12 that can affect the substrate binding in the human MRP4. A crystal structure would therefore be very useful in the understanding of the substrate binding and progression in the efflux pump, as well as for the design of new inhibitors for this transporter. A few bacterial efflux pumps have been crystallized (as Sav1866 from *S. aureus* (Dawson and Locher 2006), or MsbA from *E. coli* (Chang and Roth 2001)), and since 2009, a crystal structure has been resolved for the mouse P-gp (Aller et al. 2009). These works have allowed defining the binding site of a substrate, and are also used to go deeper inside the mechanism of action of these transporters.

4.5. Can we obtain a "pure" Mrp4 transport model?

If working with a cellular system as clear advantages in terms of physiological relevance, it also presents some limitations when trying to define molecular determinants involved in recognition by a specific transporter. We have seen indeed that J774 macrophages do express a large array of transporters, the expression of which is differently modified upon exposure to ciprofloxacin or moxifloxacin. The effects observed is thus the resultant of the potential effects of these transporters on the molecule investigated. To better compare the impact of Mrp4 on fluoroquinolones transporters, a simplified model of proteoliposomes containing Mrp4 as sole transporter would be worthwhile to use. This type of model has been developed by the team of Borst in 2003 (Reid et al. 2003). They prepared inside-out membrane vesicles starting from human or insect cells that contained a single ABC transporter (MRP1, 2, 3 or 4) in their membrane and used them to study the uptake of specific substrates. This kind of model vesicles could be helpful to better characterize the difference we evidenced in fluoroquinolone transport by Mrp4.

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