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Louvain Drug Research Institute (LDRI)
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**An integrated pharmacokinetic,
pharmacogenomic and pharmacodynamic study
of fluoroquinolones in the context of
intracellular infections**

Gwenaëlle MAHIEU
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Promotor: Prof. Françoise Van Bambeke
Co-promotor: Prof. Laure Elens

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*"The greatest glory in living lies not in never falling,
but in rising every time we fall"*

Nelson Mandela

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JURY

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PROMOTOR

Prof. Françoise VAN BAMBEKE

Cellular and Molecular Pharmacology (FACM), Louvain Drug Research Institute (LDRI), Université catholique de Louvain, Brussels, Belgium

CO-PROMOTOR

Prof. Laure ELENS

Integrated PharmacoMetrics, PharmacoGenomics, and Pharmacokinetics (PMGK), Louvain Drug Research Institute (LDRI), Université catholique de Louvain, Brussels, Belgium

COMMITTEE MEMBERS

Prof. Pierre SONVEAUX (President)

Pole of Pharmacology and Therapeutics, Institut de Recherches Expérimentales et Cliniques (IREC), Université catholique de Louvain, Brussels, Belgium

Prof. Vincent HAUFROID

Department of Clinical Chemistry, Cliniques Universitaire Saint-Luc, Louvain Center for Toxicology and Applied Pharmacology (LTAP), Institut de recherche expérimentale et clinique (IREC), Université catholique de Louvain, Brussels, Belgium

Prof. Ana BELOQUI GARCIA (Secretary)

Advanced Drug Delivery and Biomaterials (ADDB), Louvain Drug Research Institute (LDRI), Université catholique de Louvain, Brussels, Belgium

JURY

Prof. Alexia CHAUZY

UFR Médecine-Pharmacie, INSERM U1070, Université de Poitiers, Poitiers, France

Prof. Xavier DECLEVES (External member)

Department of Pharmacology and Toxicology, UMRS 1144, Université DE Paris Cité, Paris, France

Prof. Sylvain GOUTELLE (External member)

Département Pédagogique de Pharmacologie, Physiologie et Toxicologie, UMR CNRS 5558, Laboratoire de Biométrie et Biologie Évolutive, Université Lyon 1, Lyon, France

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SUMMARY

SUMMARY

Staphylococcus aureus, a Gram-positive bacterium, is a human commensal organism that colonizes 30-50% of individuals on the skin or mucous membranes, such as the nose. However, it can also act as an opportunistic pathogen, causing severe infections, including osteomyelitis, pneumonia, or endocarditis, associated with significant mortality. *S. aureus* infections represent a major public health challenge due to its ability to develop antimicrobial resistance (AMR), such as methicillin-resistant *S. aureus* (MRSA), and its capacity to persist intracellularly. This intracellular lifestyle allows bacteria to evade immune responses and reduces antibiotic efficacy, leading to persistent and recurrent infections. *S. aureus* can invade both phagocytic and non-phagocytic cells, exploiting intracellular niches such as phagolysosomes or escaping into the cytosol.

Consequently, effective treatments require antibiotics that can act extracellularly, penetrate host cells, and act intracellularly. Fluoroquinolones (FQs), such as moxifloxacin (MXF) and ciprofloxacin (CIP), are promising candidates for treating intracellular infections. However, their efficacy is influenced by cellular transporters, particularly ATP-binding cassette (ABC) transporters and their genetic polymorphisms, notably single nucleotide polymorphisms (SNPs), which can affect drug accumulation and contribute to inter-individual variability in drug responses. Additionally, *in vivo* fluctuations in drug levels further impact pharmacokinetics (PK), compromising FQ efficacy against *S. aureus* and contributing to the emergence of resistance.

This work aims to investigate the interplay between PK and PD of FQs in intracellular *S. aureus* infections, focusing on the role of ABC transporters and their SNPs, as well as to develop an *in vitro* PD intracellular model to simulate

SUMMARY

clinically relevant drug exposures. We first demonstrated that ABCB1 significantly impacts MXF transport, while ABCC4 is more crucial for CIP transport. Moreover, we showed that specific ABCB1 haplotypes reduce ABCB1 efflux activity towards these antibiotics. Finally, a PD model, based on the hollow fiber system, the gold standard for evaluating the PD of antibiotics *in vitro*, was adapted to study intracellular infections. This model enhanced MXF activity against intracellular *S. aureus*, while it decreased CIP efficacy compared to the traditional static model, which fails to reproduce serum concentration fluctuations.

This thesis highlights the importance of integrating PK, PD, and pharmacogenomics in assessing antibiotic efficacy against intracellular pathogens. It provides valuable perspectives and a novel, validated tool for optimizing treatment strategies against *S. aureus* infections. This model is now ready to be applied to other pathogens and/or drugs, offering a promising approach for optimizing dosing regimens and considering intracellular reservoirs.

LIST OF ABBREVIATIONS

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ABC	ATP-binding cassette
ABCB1/G2/C4	ATP-binding cassette sub-family B, G, C member 1, 2, 4
ABCP	Placental ABC transporter
ADME	Absorption, Distribution, Metabolism, Excretion
AMR	Antimicrobial resistance
AUC	Area under the curve
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming unit
CIP	Ciprofloxacin
CL	Clearance
C_{max}	Maximum serum concentration
EMA	European Medicines Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FnBPA/B	Fibronectin-binding proteins A/B
FQs	Fluoroquinolones
FTC	Fumitremorgin C
HFIM	Hollow-fiber infection model
ICS/ECS	Intracellular space / Extracellular space
IV	Intravenous
ke	Elimination constant
Ki	Inhibitory constant
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration

LIST OF ABBREVIATIONS

MOI	Multiplicity of infection
MPC	Mutant prevention concentration
MRP	Multidrug resistance-associated protein
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
MXF	Moxifloxacin
MXR	Mitoxantrone resistance protein
NBD	Nucleotide-binding domain
NK	Natural killer
NSAIDs	Nonsteroidal anti-inflammatory drugs
OCT/OAT	Organic cation transporter/Organic anion transporter
Omp	Outer membrane porins
P-gp	P-glycoprotein
PAE	Post-antibiotic effect
PBMCs	Peripheral blood mononuclear cells
PBP2a	Penicillin-binding protein 2a
PD	Pharmacodynamics
PK	Pharmacokinetics
PMA	Phorbol-12-myristate-13-acetate
PMNs	Polymorphonuclear leukocytes
PMQR	Plasmid-mediated quinolone resistance
PRRs	Pattern recognition receptors
PVL	Panton-Valentine leukocidin
QRDR	Quinolone resistance-determining region
ROS	Reactive oxygen species
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. pneumonia</i>	<i>Streptococcus pneumonia</i>
SCVs	Small-colony variants
SEM	Scanning electron microscopy

LIST OF ABBREVIATIONS

SNPs	Single nucleotide polymorphisms
Spa	Staphylococcal protein A
T_{1/2}	Half-life
T_{inf}	Time of infusion
TKC	Time-kill curve
TKI	Tyrosine kinase inhibitor
T_{max}	Time to maximum concentration
TMD	Transmembrane domain
TMH	Transmembrane helix
UTI	Urinary tract infection
Vd	Volume of distribution
VISA	Vancomycin intermediate <i>Staphylococcus aureus</i>
VRSA	Vancomycin resistant <i>Staphylococcus aureus</i>
WHO	World Health Organization
ZW	Zwitterionic

CHAPTER I – INTRODUCTION

I.1 Context of the thesis

Staphylococcus aureus (*S. aureus*) infections represent a major public health challenge, especially because of its capacity to develop antibiotic resistance, such as in the case of methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. Indeed, the emergence of resistance to multiple antibiotics and the limited availability of therapeutic alternatives have led both the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) to recognize MRSA as one of the high-priority pathogens [2,3]. Furthermore, *S. aureus* has the ability to invade and persist in various host cell types, including endothelial and epithelial cells, but also in polymorphonuclear leukocytes (PMNs) and macrophages [4,5]. This intracellular lifestyle is problematic because it allows the bacteria to escape immune detection and reduces their susceptibility to antibiotic treatments, leading to chronic and relapsing infections [6,7]. Therefore, to be effective against *S. aureus*, antibiotics must not only exhibit potent extracellular activity but also effectively accumulate within host cells and express their activity in the intracellular milieu to act on intracellular bacteria. This property is crucial to prevent the emergence of intracellular niches where *S. aureus* can survive and potentially develop resistance if exposed to subtherapeutic antibiotic concentrations [8].

Fluoroquinolones (FQs), such as moxifloxacin (MXF) and ciprofloxacin (CIP), are promising options for the treatment of intracellular infections. Indeed, these antibiotics are widely used in clinics due to their broad-spectrum activity, high bactericidal activity [9,10], and proven efficacy against intracellular pathogens [11–14]. The ability of FQs to penetrate and accumulate within infected host cells is essential for their therapeutic efficacy against intracellular bacteria, including intracellular *S. aureus* [15].

However, the degree of their intracellular accumulation, and thus their efficacy, can be influenced by the activity of efflux transporters [16–18]. Particularly, ABC efflux transporters can influence the general pharmacokinetics (PK) of FQs when expressed in epithelial barriers, as well as their cellular PK in non-polarized cells, through active extrusion of substrates from cells [19]. Furthermore, these transporters can be affected by genetic polymorphisms, such as single nucleotide polymorphisms (SNPs), which can lead to inter-individual variability in drug response, thereby influencing the efficacy of FQ therapy across patients [20]. Finally, fluctuations in drug levels within the body can also affect both cellular and general PK. Together, these factors may result in suboptimal serum drug concentrations, thereby compromising FQ efficacy (PD) against intracellular *S. aureus*, contributing to the emergence of resistance.

In conclusion, these elements highlight the complex interaction between cellular mechanisms (PK), genetic variability (pharmacogenomics), and antibiotic efficacy (PD) in the treatment of intracellular *S. aureus* infections. These three dimensions are the key factors upon which this thesis is articulated.

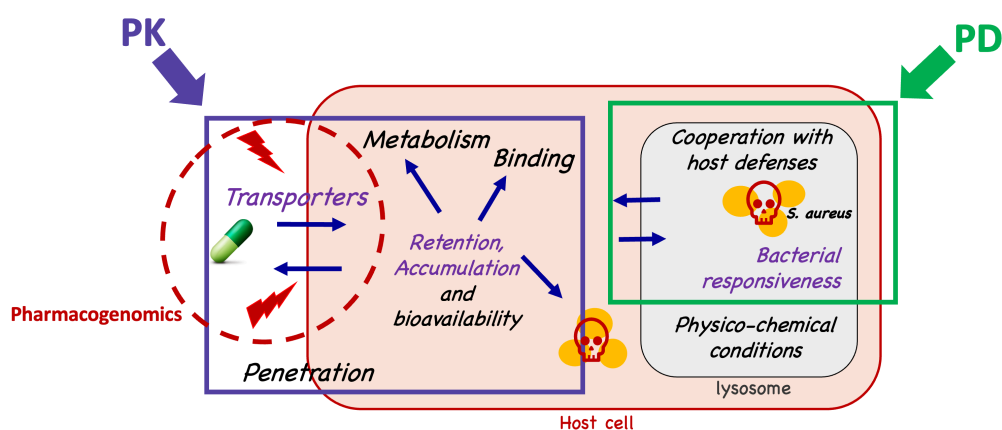


Figure I.1. Graphical summary of the three dimensions studied in my thesis (created in PowerPoint, adapted from [5]).

1.2 *Staphylococcus aureus* as a pathogen

1.2.1 Global overview

a) Etymology and historical discovery

In 1880, Sir Alexander Ogston, a Scottish surgeon, was the first to describe the bacteria in pus extracted from a surgical abscess in a knee joint: “The masses looked like bunches of grapes”. This observation inspired the name “**Staphylococcus**”, a combination of two Greek words: *staphyle* (meaning bunch of grapes) and *kokkos* (meaning berry), the latter referring to the round shape of the bacteria as observed under the microscope [21]. A few years later, in 1884, the German surgeon Anton J. Rosenbach named the pigmented appearance of the bacteria “**aureus**”, derived from the Latin word *aurum* (meaning gold), referencing the golden color of the bacterial colonies, attributed to their production of carotenoid pigments [21,22].



Figure 1.2. Sir Alexander Ogston (image from [23]).

b) General characteristics, pathogenicity, and infection mechanisms

S. aureus is a Gram-positive, cocci-shaped bacterium with a diameter ranging from 0.5 to 1.0 μm [24]. It is an aerobic and anaerobic facultative organism that grows between 18 and 40°C [25]. In the general population, this commensal bacterium is often present asymptotically in 30-50% of healthy adults [26]. The bacteria typically colonize the skin and mucous membranes, such as the nasal passages, without causing infection [27]. Notably, around 20-30% of the population is persistently colonized by *S. aureus* in the anterior nares [28].

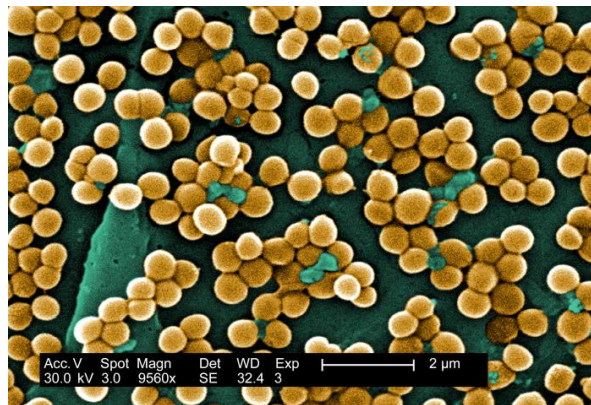


Figure I.3. Digitally colorized scanning electron microscopical (SEM) image depicted numerous clumps of MRSA bacteria (image from [29]).

However, if *S. aureus* enters the tissues or spreads in the bloodstream through a wound or a breach in the skin or mucosal barriers, it can become a significant human pathogen [26,30]. In fact, most nosocomial infections caused by *S. aureus* are acquired through contact with healthcare worker hands, after having been colonized from their own reservoir, or from contact with colonized patients [26]. Once colonization happens, the infection process is often triggered by some predisposing factors, including prolonged hospitalization, immunosuppression, surgery, use of invasive medical devices (e.g., catheters), or chronic metabolic

diseases (e.g., diabetes) [30]. *S. aureus* infections can be both nosocomial and community-acquired, with clinical manifestations varying from localized skin and soft tissue infections (e.g., impetigo, furuncle, cellulitis, abscesses) to severe, widespread infections (e.g., endocarditis, osteomyelitis, toxic shock syndrome, pneumonia, and sepsis) [24,26,30]. Infections can be acute, recurrent, or chronic and persistent [31].

The pathogenic success of *S. aureus* is attributed to a wide combination of virulence factors that facilitate host colonization and immune system evasion. These include: (1) the production of **surface proteins** known as adhesins, promoting colonization of host tissues; (2) the synthesis of a **protective polysaccharidic capsule** and **staphylococcal protein A** (Spa), both inhibiting phagocytosis; (3) the secretion of **various enzymes** such as lipases, proteases, and hyaluronidases causing tissue destruction; and (4) the release of **toxins**, notably Panton-Valentine leukocidins (PVL) and superantigens, causing damage in host tissues and inducing disease symptoms [24,30,32]. Furthermore, this pathogen can also form communities of bacteria encased in a self-produced extracellular matrix called “biofilm” [6,33] and persist intracellularly within host cells, such as macrophages, allowing bacteria to be protected from both the immune system and antimicrobial therapies [31]. Collectively, these adaptations make *S. aureus* infections particularly challenging to treat and eradicate.

c) Treatment strategies and antimicrobial resistance

The management of *S. aureus* infections relies on the type of infection and the antibiotic susceptibility of the strain. Indeed, for methicillin-sensitive isolates (MSSA), penicillin remains the first-choice treatment. Therapy for MSSA infections typically includes the administration of β -lactams antibiotics such as nafcillin, oxacillin, or cefazolin [34]. However, the emergence of MRSA strains has significantly complicated treatment approaches and constitutes a significant public health concern [2,3]. This resistance is primarily due to the acquisition of the *mecA* gene, encoding for an altered penicillin-binding protein 2a (PBP2a) with a reduced affinity for β -lactam antibiotics. This modification renders most β -lactam antibiotics, including methicillin, ineffective against MRSA [1]. Furthermore, MRSA strains have additional resistance genes, conferring resistance to a broad range of antibiotics [30]. For severe MRSA infections, vancomycin is considered the first-line treatment. However, in the case of vancomycin-intermediate and resistant strains (VISA/VRSA), or when vancomycin is not tolerated or contraindicated, last-line alternatives such as linezolid, daptomycin, or ceftaroline may be considered [30,34].

The duration of antibiotic therapy ranges from 2 to 4-6 weeks, depending on infection severity and type of infection as well as patient-specific risk factors. Finally, in addition to pharmacological interventions, non-pharmacological interventions, such as drainage of abscesses and the removal of infected foreign devices, are often performed to ensure infection control and prevent recurrence [34].

d) Intracellular lifestyle of *S. aureus* and its role in antibiotic resistance

AMR is not the only factor responsible for therapeutic failure in *S. aureus* infections. This pathogen employs different strategies to protect itself from antibiotics. A significant strategy is the adoption of an **intracellular lifestyle**, where bacteria evade both antibiotics and host immune response, making it even more difficult and challenging to eliminate the infection [26]. Indeed, while traditionally viewed as an extracellular pathogen, *S. aureus* has shown the ability to survive within a range of cell types, including **phagocytic cells**, such as neutrophils and macrophages, as well as **non-phagocytic** cells like epithelial cells, endothelial cells, and osteoblasts [4,7].

- **Invasion of non-phagocytic cells**

To infiltrate non-phagocytic cells, *S. aureus* employs a *zipper-like mechanism* mediated by fibronectin (Fn)-binding proteins A and B (FnBPA, FnBPB), which are surface adhesins. These adhesins attract and bind to fibronectin on the bacterial surface, forming a complex that interacts with $\alpha 5 \beta 1$ integrins located on the surface of host cells. This interaction forms a bridge between bacterial adhesins and host cell integrins, activating intracellular signaling pathways in the host cell, causing cytoskeletal rearrangements. These changes result in membrane invagination and the endocytosis of the bacteria into a vacuole (endosome). Once inside the vacuole, *S. aureus* inhibits the fusion of the vacuole with lysosomes, preventing bacterial destruction [35]. The bacteria can also escape from the vacuole to the host cell cytoplasm, where they persist and establish an intracellular reservoir. In the cytoplasm, *S. aureus* can replicate, eventually cause cell lysis, and escape back into the extracellular environment [36].

- **Survival in phagocytic cells**

The process of entry is different for phagocytic cells. Indeed, these immune cells automatically recognize and ingest pathogens through the process of phagocytosis, a process initiated by pattern recognition receptors (PRRs) such as Toll-like receptors. These receptors recognize bacterial components, which initiate the formation of pseudopodia, which engulf pathogens. Additional receptors on the macrophage surface, including scavenger receptors, complement receptors, and Fc receptors, facilitated the internalization of the pathogen into a phagosome. Then, the phagosome fuses with lysosomes to form a phagolysosome, where the pathogen is exposed to substances such as reactive oxygen species (ROS) and degradative enzymes, leading to its destruction [37,38]. However, once again, *S. aureus* has developed several mechanisms to survive within phagocytic cells. These include the production of enzymes such as catalase and superoxide dismutase, which neutralize ROS [36]. Additionally, *S. aureus* can also escape into the cytoplasm and adopt specific phenotypes, such as the formation of small colony variants (SCVs), characterized by reduced metabolism and a slow growth rate, enhancing its capacity to persist within host cells [35,36]. Alternatively, the bacteria may enter a transitory dormant state, metabolically inactive with no replication, called “persistence” [39].

- **A “Trojan Horse” strategy**

Through these adaptations, *S. aureus* employs a “Trojan horse” strategy, hiding within immune or non-immune cells to evade both immune detection and antibiotic treatments. This led to the creation of intracellular niches where the bacteria can survive, persist, replicate, and disseminate throughout the body.

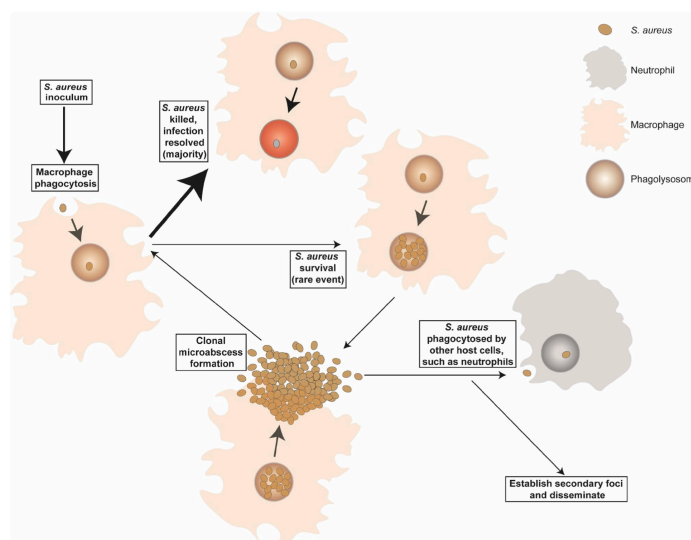


Figure I.4. Dynamics of *S. aureus* clonal populations (image from [40]).

1.2.2 Challenge in treating intracellular infections

For most antibiotics, penetrating intracellular spaces and achieving sufficient concentrations to exert their activity remain a significant challenge. For instance, while β -lactams can passively diffuse through cell membranes, they fail to accumulate effectively inside phagocytes, thereby limiting their intracellular efficacy [11]. This limitation can be explained by their **weak acidic character**, which makes it difficult to accumulate in acidic intracellular compartments such as lysosomes or vacuoles [15,41]. To counteract this phenomenon, high-dose administration over extended periods can enhance the effectiveness of β -lactams against intracellular infections [11]. In contrast, **weak bases** such as macrolides accumulate inside cells and become trapped in lysosomes and vacuoles due to protonation in acidic compartments [5]. Linezolid, another weak base, is highly diffusible in body tissues but accumulates only modestly in cells (approximately 1-2x), which limits its effectiveness against phagocytosed *S. aureus* [42]. Meanwhile, aminoglycosides, vancomycin, or daptomycin, due to their **high polarity or**

molecular size, are unable to cross the cell membrane passively [41], thus decreasing their efficacy against intracellular pathogens such as *S. aureus* when compared to their proven efficacy towards the extracellular forms [11]. Similarly to β -lactams, prolonged exposure to aminoglycosides has been shown to enhance their intracellular activity by promoting endocytosis-mediated uptake into the host cells. Nonetheless, this approach exposes to the risk of increased side effects [5].

Moreover, even if antibiotics successfully penetrate cell membranes and accumulate within host cells, they need to be active in the acidic pH of the intraphagolysosomal compartment. Many antibiotics, including gentamicin and macrolides, show reduced intracellular concentrations and diminished activity under acidic conditions due to **protonation**, affecting their ability to combat intracellular pathogens [41]. Additionally, inside the cell, bacteria such as *S. aureus* can adopt specific metabolic adaptations to survive in hostile environments (SVCs, persisters), making antibiotic classes, targeting active cellular processes, less active.

Given these challenges, FQs present a promising alternative for combating intracellular infections. Indeed, they possess favorable pharmacokinetic (PK) and pharmacodynamic (PD) properties, including efficient diffusion across cell membranes and accumulation within host cells, allowing them to achieve therapeutic concentrations inside phagocytes [15]. Moreover, they remain active at acidic pH levels, such as those found within phagolysosomes, preserving their bactericidal activity against bacteria residing in these intracellular compartments [43]. The possibility to administer them orally enhances patient adherence. This makes them particularly suitable for long-term therapy [44]. Additionally, FQs exhibit favorable PD properties, such as a broad spectrum of activity (Gram-positive and Gram-negative bacteria), and have already demonstrated efficacy towards intracellular pathogens [9,11,12].

Key insights

- *S. aureus* is a major pathogen with clinical manifestations ranging from **localized skin infections** to severe **disseminated diseases**.
- *S. aureus* **virulence factors** facilitate **host colonization** and **immune response evasion**.
- The rise of **MRSA** infections constitutes a **major public health concern**.
- The **intracellular lifestyle of *S. aureus*** allows it to persist within **host cells**.
- Treatment of intracellular infection is **challenging** due to **limited intracellular antibiotic efficacy**, although **FQs** are **promising alternatives**.

I.3 Fluoroquinolone generalities: moxifloxacin and ciprofloxacin

I.3.1 Chemical structure and structure-activity relationships

The first quinolone, nalidixic acid, was discovered by George Lesher in 1962 as an impurity during the synthesis of chloroquine [45,46]. In 1967, nalidixic acid was licensed for the treatment of uncomplicated urinary tract infections (UTIs) [47]. However, its initial use was limited due to its narrow spectrum of activity (primarily targeting Gram-negative bacteria), low serum concentrations, high minimum inhibitory concentration (MIC) required for efficacy, and toxicity [48].

The emergence of resistant bacterial strains in the 1980s created a need for new treatments, leading to structural optimization of nalidixic acid [49]. Consequently, this effort gives rise to second-, third-, and fourth generation of FQs, classified depending on their spectrum of activity and their therapeutic indications. **Figure I.5** summarizes the structure-activity relationship of FQs, illustrating how core substitutions affect their activity.

The major feature was the formation of the flumequine by the substitution of a fluorine atom at position 6 (C6) of nalidixic acid. This modification markedly enhanced bactericidal activity against Gram-negative bacteria and gave the class its name, “fluoroquinolones”[49]. Then, from this compound, emerged the second generation of FQs, including norfloxacin and **CIP**, characterized by the introduction of a piperazine group at position 7 (C7) and a cyclopropyl group at position 1 (N1) for CIP. These changes improved bioavailability, potency, and activity against Gram-negative pathogens.

Subsequent alkylation of the piperazine group (C7) and the substitution of a methoxy (-OCH₃) group at position 8 (C8) led to molecules active on some Gram-positive bacteria (ofloxacin and levofloxacin). [48] The third generation, represented by **MXF**, conserved the key modifications of its predecessors, including the methoxy group at C8 and the cyclopropyl ring at position N1. However, it introduced a bicyclic group (azabicyclo group) at position 7 (C7), which enhanced activity against anaerobic organisms and Gram-positive bacteria, but also improved tissue penetration by increasing lipophilicity and prolonged the drug's half-life [48].

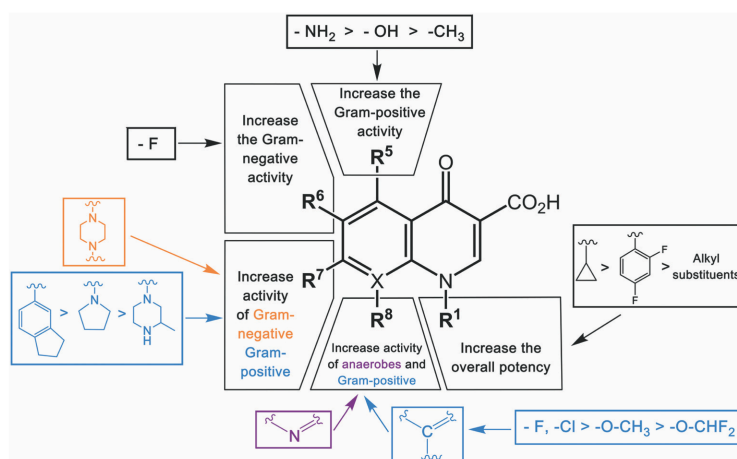


Figure I.5. Structure-activity relationship of quinolones, depicting core substitutions and their effects (image from [48]).

Finally, these successive modifications have resulted in an optimized pharmacophore with enhanced efficacy, reduced resistance, and improved safety profiles. Key structural features include a cyclopropyl ring at N1, a methoxy group at C8, either a pyrrolidine or piperazine group at C7, and a fluorine atom at C6. The fourth generation, characterized by the removal of the fluorine at C6, is represented by garenoxacin [49], this generation is active towards respiratory agents and atypical organisms [50]. In this thesis, particular attention is given to CIP and MXF, which differ in their lipophilicity, $T_{1/2}$, and spectrum of activity. Their respective structures are presented in **Figure I.11** and **Figure I.12**.

1.3.2 Mechanism of action and resistance mechanisms

FQs exhibit bactericidal activity by penetrating bacterial cells and forming a ternary complex with DNA and their target enzyme, DNA gyrase and topoisomerase IV [51]. Their amphiphilic character at physiological pH facilitates their diffusion across bacterial membranes via porin channels in Gram-negative bacteria and passively in Gram-positive bacteria. Inside the cell, FQs act on DNA gyrase (topoisomerase II) in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria [10,52]. DNA gyrase introduces negative supercoiling to DNA, crucial for DNA replication and transcription [47]. However, topoisomerase IV also facilitates the relaxation of supercoiled DNA and decatenation of interlinked daughter chromosomes after replication, permitting cell division [45].

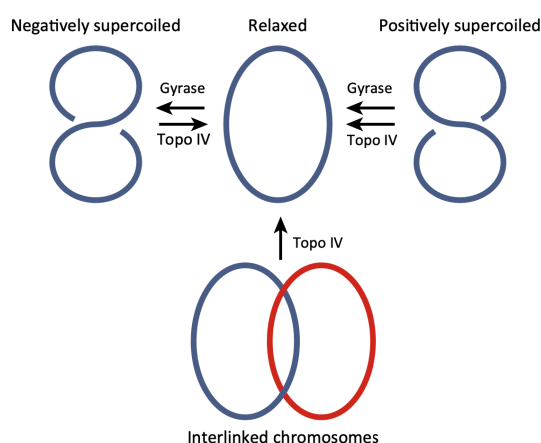


Figure I.6. Action of DNA gyrase and topoisomerase IV on DNA (image from [53]).

Both enzymes are composed of two types of subunits; DNA gyrase is composed of two GyrA and two GyrB, while topoisomerase IV is composed of two ParC and two ParE subunits (known as GrlA and GrlB in *S. aureus*), respectively. The catalytic cycle of these enzymes depends on their subunits: GyrA and ParC mediate DNA cutting and re-ligation, while GyrB and ParE bind ATP, required for the conformational change during enzyme activity [48,51].

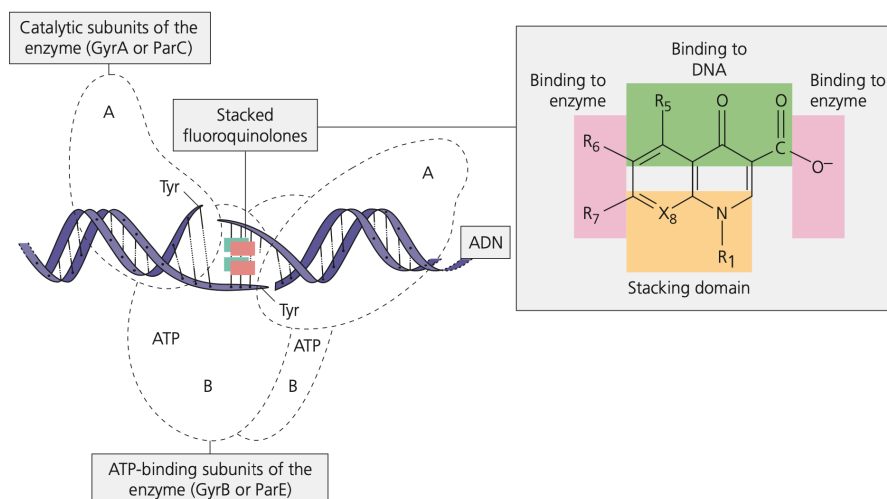


Figure I.7. Ternary complex between DNA, enzyme, and stacked fluoroquinolones (image from [51]).

FQs establish interaction with both subunits of the targeted enzyme. During the “gate opening step” of the double-stranded DNA, four FQ molecules are stacked on the single-stranded DNA, stabilizing the cleavable complex, preventing the re-ligation of the cleaved DNA strands, thus blocking replication and transcription, leading to slow bacterial cell death [51]. However, if the enzyme dissociates from the complex, DNA breaks can occur, leading to the accumulation of ROS and rapid bacterial cell death [45]. **Figure I.8** illustrates how FQs interact with bacterial DNA, inhibiting DNA gyrase and topoisomerase IV leading to DNA damage and bacterial cell death.

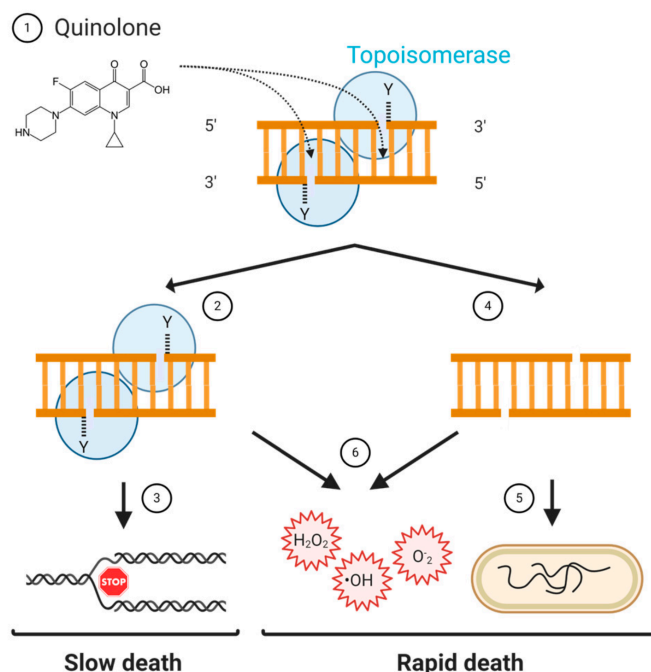


Figure I.8. Mechanism of action of fluoroquinolones: (1) Stabilization of bacterial DNA gyrase or topoisomerase IV complex, leading to either slow (3) or rapid (6,5) bacterial cell death depending on enzyme removal (2, 4) (Image from [45]).

Despite the potent antibacterial properties of FQs, bacteria have developed resistance through several mechanisms. **Figure I.9** provides an overview of the key factors contributing to bacterial resistance to FQs. These include the **up-regulation of efflux pumps** (e.g., NorA in *S. aureus*), which actively expel the drug from the bacterial cells, and **mutations or down-regulations of outer membrane porins** (Omp), such as OmpF (*E. coli*), which alter drug transport inside Gram-negative bacteria. Resistance can also arise through the acquisition of **plasmid-mediated resistance genes** (PMQR) (e.g., *qnr*) protecting enzymes from FQ binding. Additionally, **chromosomal mutations in genes encoding DNA gyrase** (*gyrA*) or **topoisomerase IV** (*parC*), known as quinolone resistance determining region (QRDR), can reduce their affinity for FQ by structurally modifying the targeted protein [49,54].

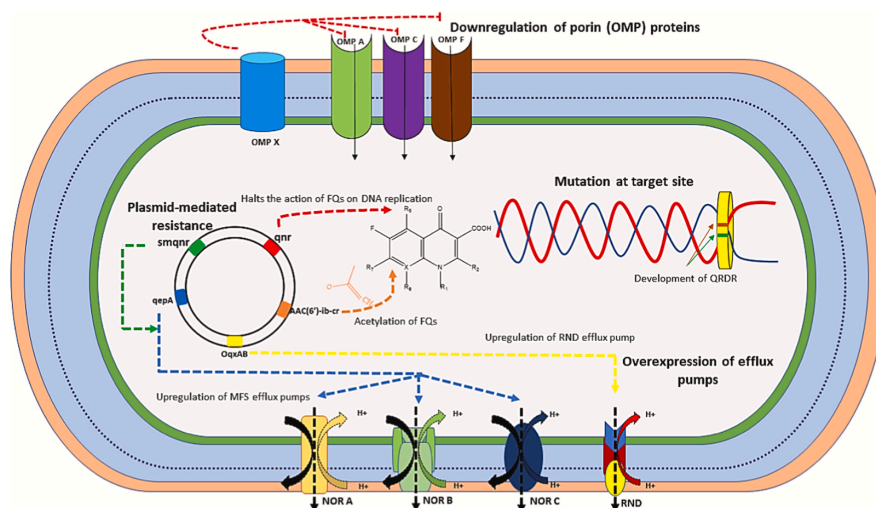


Figure I.9. Summary of FQ resistance mechanisms including QRDR, PMQR, efflux pumps overexpression and reduced membrane permeability through down-regulation of OMP (image from [54]).

1.3.3 Pharmacokinetic and pharmacodynamic parameters

The pharmacokinetics (PK) describes how the body influences drug concentration through absorption, distribution, metabolism, and elimination mechanisms. Most FQs exhibit excellent bioavailability, ranging from 50% to 98% of the administered dose [55]. This high bioavailability allows rapid switching between oral and intravenous (IV) routes, making these antibiotics suitable for both ambulatory and hospitalized patients [49], thus improving compliance and reducing treatment costs [52]. Indeed, FQs are absorbed passively in the duodenum and jejunum after rapid dissolution in the gastrointestinal tract [56]. While food intake does not significantly impact absorption, the formation of chelates with cations (e.g., aluminum, magnesium, calcium, zinc, and iron), frequently found in some medications as well as some dairy products, can reduce absorption and tissue penetration [10,55]. In fact, FQs demonstrate good tissue and body fluid penetration [49], achieving therapeutic concentrations in various compartments

such as lungs, prostate, skin, and bones, making them active for treating infections in these areas [52,57]. Maximum peak serum concentration ($C_{\max}$) is achieved within 1 to 2 hours of administration [57], with $C_{\max}$ values ranging from 1 to 7 mg/L [50]. Serum levels are comparable between the oral and IV routes; however, they are generally low, requiring fractionated dosing [49]. The $T_{1/2}$ of FQs varies significantly among molecules, ranging from 1.5 to 18 hours, with most drugs requiring dosing every 12 to 24 hours [50]. Protein binding also varies widely, from less than 30% to more than 90%, depending on the FQ [50]. Another important characteristic of FQs is their ability to accumulate in PMNs and macrophages, resulting in higher intracellular concentrations, which enhances their efficacy against intracellular pathogens [12,58]. Lastly, FQs are metabolized and eliminated through both renal (20-85%) and hepatic (15-50%) pathways, necessitating dose adjustments for patients with renal or hepatic impairments [59]. This is further detailed later in the manuscript for ciprofloxacin (CIP) and moxifloxacin (MXF), see section I.3.3 a) and b) where their renal and hepatic elimination and the key enzymes involved in their metabolism are discussed in detail.

Pharmacodynamics (PD), which commonly refers to the effect of the drug on the body (or on bacteria), illustrates, in this case, the antibacterial activity of FQs. These antibiotics are bactericidal, and their activity is concentration-dependent, meaning that higher drug concentrations correlate with greater activity [52]. Two key PK/PD parameters are used to describe their efficacy: the ratio of the peak serum concentration ($C_{\max}$) to the MIC ($C_{\max}/\text{MIC}$) and the ratio of the area under the curve (AUC) to the MIC (AUC/MIC). To minimize the selection of resistant mutants and to optimize treatment efficacy, FQs require a $C_{\max}/\text{MIC}$ ratio > 10 and an AUC/MIC ratio of at least 30 for Gram-positive bacteria and of 125 or higher for Gram-negative bacteria [55,60]. Furthermore, achieving a ratio > 250 enhances bacterial eradication for both Gram-positive and Gram-negative bacteria [61].

These PK/PD thresholds have been validated not only in in vitro and animal models but also in clinical studies [61,62]. Additionally, FQs exhibit a post-antibiotic effect (PAE), during which bacterial growth remains inhibited for 1 to 2 hours after drug concentrations fall below the MIC [52].

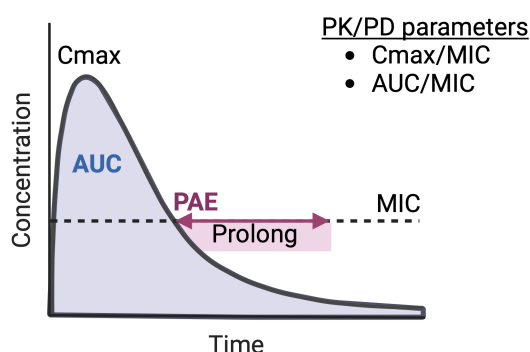


Figure I.10. Important PD parameters for the efficacy of fluoroquinolones, post-antibiotic effect (PAE) (image created in Biorender.com, adapted from [63]).

This section will explore CIP and MXF respective physiochemical properties as well as PK/PD parameters to further understand their clinical applications.

a) Ciprofloxacin

CIP, a second generation of quinolone introduced in the 1980s, is considered one of the most active agents against Gram-negative bacteria [49], including *Enterobacteriaceae*, *Neisseria* spp., *Haemophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas* spp., and *Acinetobacter* spp [47,64,65]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has set the susceptibility breakpoint¹

¹ Breakpoints are part of a system for categorizing microorganisms as susceptible (S and I) and resistant (R) to agents approved for use in the treatment of infectious diseases.

A microorganism is categorized as "**Susceptible, standard dosing regimen**", when there is a high likelihood of therapeutic success using a standard dosing regimen of the agent. A microorganism is categorized as "**Susceptible, Increased exposure**" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection. A microorganism is categorized as "**Resistant**" when there is a high likelihood of therapeutic failure even when there is increased exposure.

for CIP at ≤ 1 mg/L [66]. Clinically, CIP is effective in treating a wide range of infections, such as UTIs, gastrointestinal infections (e.g., infectious diarrhea), osteomyelitis, gonorrhea, respiratory tract infections (e.g., pneumonia, sinusitis, otitis), and bone and joint infections [47,52].

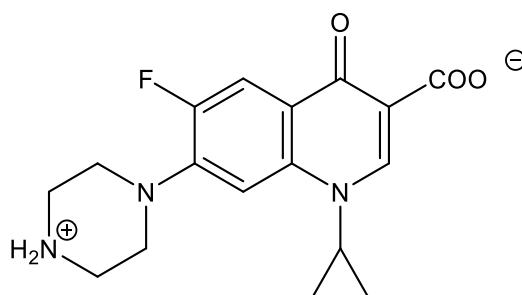


Figure I.11. Representation of the chemical structure of CIP at physiological pH.

CIP PK profile is important for its clinical use. Administered orally or intravenously, CIP has an oral bioavailability of approximately 70%. Following administration, the drug is rapidly absorbed, reaching a C_{max} within 1 to 2 hours, with values ranging from 0.56 to 3.7 mg/L depending on the administered dose (250-750 mg). CIP is well-distributed in the body, with a volume of distribution (V_d) of 2.1-5 L/kg, reflecting broad tissue penetration [64,65]. This characteristic is attributed to the addition of a cyclopropyl group at N-1, which enhances lipophilicity, while the piperazine ring at position C-7 maintains a degree of hydrophobicity ($\text{Log } D$ at pH 7 = -1.20 [67]), facilitating its penetration through Gram-negative bacteria and increasing its activity against these bacteria [48]. CIP is normally administered twice daily, with oral regimens ranging from 500 to 1500 mg/day and IV regimens from 400 to 800 mg/day, infused over 1 hour to avoid venous irritation. The therapy duration varies between 5 to 14 days, with shorter therapy for uncomplicated infections (e.g., cystitis, diarrhea) and longer therapy for complex infections (e.g., bones and joint infections).

Its $T_{1/2}$ ranges from 3 to 5 hours, and the drug is mainly eliminated unchanged in the urine (40-50%), with 25-35% excreted in the feces, and only a small part (15-25%) undergoes hepatic metabolism via CYP1A2 [64,65].

Regarding PD parameters, CIP acts essentially by inhibiting DNA gyrase in Gram-negative bacteria [64], disturbing DNA replication and transcription, and causing bacterial cell death. However, its hydrophilic character ($\log D$ at pH 7 = -1.20) [67], necessitates entry through porin channels. Consequently, resistance mechanisms, such as reduced porin expression (e.g., OmpF) or increased efflux transporter expression (e.g., NorA), can reduce its efficacy [10,68]. The overuse and misuse of CIP, favored by its advantageous PK and PD parameters, have led to significant resistance, particularly in the case of community-acquired respiratory tract infections and Gram-positive bacteria such as *S. aureus* and *Streptococcus pneumoniae* (*S. pneumoniae*) [49]. Consequently, to address resistance challenges, newer FQs, such as MXF, have been developed, offering broader activity against Gram-positive bacteria and reduced susceptibility resistance mechanisms.

b) Moxifloxacin

MXF, a third generation of FQs (also classified as a fourth generation of FQs) introduced in the early 2000s [50], is characterized by a significant improvement in its spectrum of activity compared to its predecessors. While conserving efficacy towards Gram-negative bacteria, MXF shows enhanced activity towards Gram-positive bacteria [47]. Its broad-spectrum activity covers Gram-positive bacteria such as *S. pneumoniae*, *Listeria monocytogenes* (*L. monocytogenes*), and *S. aureus*, as well as Gram-negative bacteria like *Hemophilus influenzae*, *Moraxella catarrhalis*, and *Legionella* spp. Additionally, MXF is effective against atypical bacteria, including *Chlamydia* spp., *Mycoplasma* spp., and *Ureaplasma*, and

anaerobes like *Bacteroides fragilis* [47,50,69]. EUCAST defines the susceptibility breakpoint for MXF at MIC $\leq$ 0.5 mg/L [66]. Clinically, it is prescribed to treat respiratory tract infections (e.g., community-acquired pneumonia, bronchitis, and sinusitis) and skin and soft tissue infections [69].

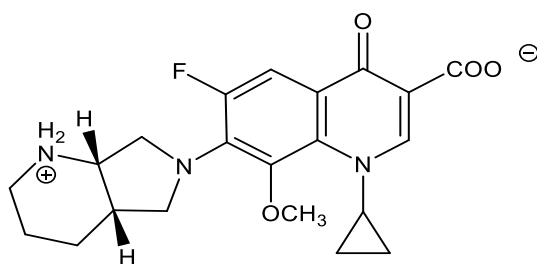


Figure I.12. Representation of the chemical structure of MXF at physiological pH.

MXF has favorable PK parameters. Administered orally or intravenously, it has an oral bioavailability of approximately 90%. Following a single 400 mg dose, the drug reaches $C_{\max}$ within 1 to 3 hours, with levels ranging from 3.1 to 4.5 mg/L. MXF demonstrates excellent tissue penetration, with a V_d of approximately 2 L/kg [69,70]. The drug is typically given once daily, with a standard oral or IV dose of 400 mg and a treatment duration of 5-21 days, depending on the type and severity of the infection [70]. Its $T_{1/2}$ is approximately 12 hours and is eliminated via both renal and hepatic pathways, with about 20% excreted unchanged in urine and 25% excreted unchanged in feces. The remainder is metabolized into inactive metabolites, primarily M1 (a sulfo-conjugate formed via sulfotransferase (SULT) 2A1) and M2 (an acyl-glucuronide conjugate formed via UDP-glycuronosyltransferase (UGT) 2A1) [69,71,72].

In terms of PD, MXF targets both DNA gyrase and topoisomerase IV [45], displaying concentration-dependent bactericidal killing [48]. Notably, the addition of a methoxy group at C8 and a cyclopropyl ring in N1 increases its potency against Gram-positive bacteria by stabilizing the ternary complex formed between

bacterial DNA, the enzyme, and the drug. Furthermore, the cyclopropyl group enhances the lipophilicity of the drug ($\log D$ at pH 7 = -0.53 [67]), facilitating bacterial cell penetration and extending its $T_{1/2}$. Additionally, a dibenzyl group at C7 (azabicyclo groups) limits efflux via pumps like NorA in *S. aureus*, counteracting resistance mechanisms associated with CIP [48]. Moreover, the methoxy group at C8 reduces the selection of resistant mutants [49]. While MXF is highly effective against respiratory pathogens, it is less potent than CIP against Gram-negative bacteria like *Pseudomonas aeruginosa* [50,52]. The concept of the mutant prevention concentration (MPC) has been introduced with MXF to mitigate resistance development. MPC is defined as the lowest drug concentration required to inhibit the growth of the least susceptible bacteria subpopulations, also called single-step mutants. Compared to older FQs, MXF shows a lower MPC towards Gram-positive bacteria, such as *S. pneumoniae* ($MPC_{50}/MIC_{50} = 2 \mu\text{g/mL}$) [49,73]. This lower MPC reduces the susceptibility of resistance development during therapy, as effective bacterial killing can be achieved at lower serum concentrations [48].

1.3.4 Adverse effects

Although FQs are widely used antibiotics effective against a broad spectrum of bacterial infections, their potential for serious adverse effects necessitates prudent use. FQ therapy should be reserved primarily for cases where alternative antibiotics are ineffective. For mild and uncomplicated infections, where alternative antibiotics are commonly recommended, recurrent lower UTIs, traveler's diarrhea prevention or treatment, non-bacterial infections, or infections that may resolve on their own or are not severe, the risks associated with FQs may outweigh the benefits; thus, they should not be used. Additionally, FQ should be avoided in patients with a history of serious side effects related to their use [74].

Adverse effects can range from mild but frequent events to rare but severe complications. These effects may be class-specific, affecting all FQs, or depending on substituents within the class. The structure-toxicity relationship of FQs, highlighting how specific functional groups contribute to their adverse effects, is illustrated in **Figure I.13** [49].

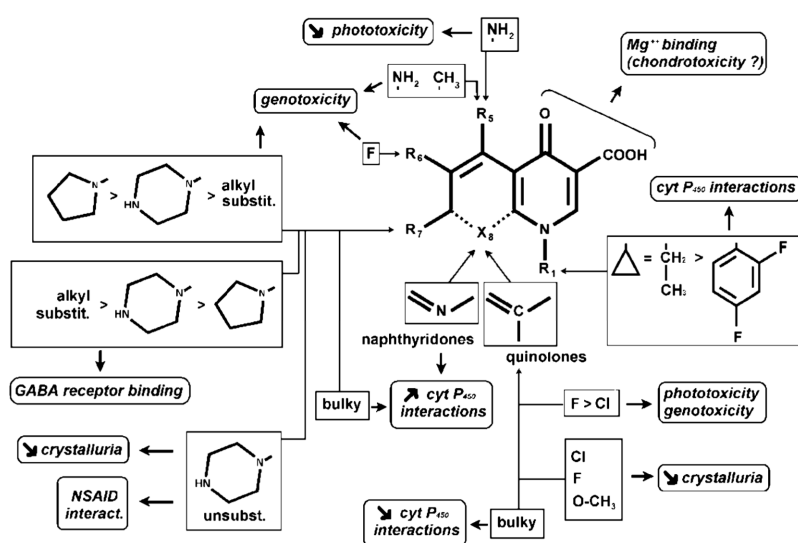


Figure I.13. Structure-toxicity relationship (image from [49]).

One of the common adverse effects involves **gastrointestinal disturbances**, affecting approximately 10% of patients, a side effect frequently associated with antibiotics. These include nausea, diarrhea, gastric pain, and vomiting. The use of FQs can also lead to FQ-resistant *Clostridium difficile* (<0.1%). In addition to gastrointestinal effects, more severe and potentially life-threatening complications have also been reported. Among these are **tendinopathy and tendon rupture**, especially affecting the Achilles tendon. Although rare (<0.4%), the risk increases up to 2% in older individuals (>60 years), patients with kidney disease or solid organ transplantation, athletes in training, and in those receiving concurrent systemic corticosteroids.

Other significant adverse effects include alteration in glucose metabolism, leading to both **hyperglycemia** and **hypoglycemia** (<0.1%), cardiovascular risks including **QTc prolongations and cardiac arrhythmias**, especially when FQs are combined with other drugs that induce hypokalemia, prolong QTc interval, or inhibit CYP450 (<10%). **Peripheral neuropathy** (numbness and pain), though rare (<0.1%), is another serious complication that can occur after the first dose and may lead to permanent damages. Furthermore, the risk of **aortic aneurysm and dissection**, while also rare, remains a dangerous concern due to the potential for life-threatening bleeding. **Central nervous system** (<10%) and **psychiatric effects** (<1%) including headaches, agitations, hallucinations, psychosis, and convulsions, have also been reported, necessitating monitoring of vulnerable patients, particularly those taking NSAIDs or CYP450 inhibitors. **Hepatotoxicity**, often indicated by elevated transaminase levels (<10%), is another risk associated with FQs. **Retinal detachment**, which can lead to permanent vision loss, **hypersensitivity**, and **photosensitivity reactions**, have also been observed (<2.5%). Finally, **genotoxicity** could also occur but remains insufficiently studied due to quinolone restrictions on pregnant women [49,75].

Considering these risks, several black box warnings were issued to restrict their use, and several FQs have been withdrawn from the market a few years after their approval due to serious adverse effects. For example, sparfloxacin was removed for QTc prolongation and phototoxicity, and gemifloxacin was removed due to reports of erythematous rashes [76]. In Belgium, the availability of systemic FQs has been significantly reduced, with only three agents currently available on the market: CIP, MXF, and levofloxacin [77].

1.3.5 Intracellular activity

FQs, including CIP and MXF, exhibit potent activity against intracellular pathogens such as *L. monocytogenes* (cytosol) [13,78,79], *Legionella pneumophila* (phagosomes) [12,80], *S. aureus* (phagolysosome) [11,81–83] and *Mycobacterium tuberculosis* (phagosomes) [14]. However, their intracellular activity cannot be only predicted based on their intracellular accumulation within host cells [84]. Indeed, despite demonstrating concentration-dependent activity, their intracellular efficacy may be significantly lower than expected, even when intracellular concentrations represent several multiples of the MIC [5,17]. For example, while gemifloxacin, another FQ, accumulates more than CIP and MXF, it shows similar activity in the cytosol (against *L. monocytogenes*) and in phagolysosomes (against *S. aureus*) [84]. This variability in activity arises from several critical factors influencing the intracellular activity of the drug. These factors include bioavailability, subcellular localization, pH of the compartment, efflux mechanisms, and the metabolic state of the pathogens [11,13].

a) Drug bioavailability and subcellular localization

FQs show significant accumulation within host cells, with CIP reaching levels 4 times higher and MXF up to 13 times higher compared to extracellular fluid, as observed in murine J774 macrophages [85]. This ability to concentrate within cells extends to both phagocytic cells (PMNs and macrophages) and non-phagocytic cells (epithelial cells) [17]. Accumulation of FQs occurs primarily through rapid passive diffusion, typically within 5 to 60 minutes; however, transporters involved in purine transport in eukaryotic cells may also contribute, particularly for CIP [41]. Once inside the cell, FQs predominantly localize in the cytosol, as observed in the soluble fraction during fractionable studies [12,84,86].

However, they also distribute to other subcellular compartments, such as lysosomes and phagolysosomes [5]. Subcellular localization is a critical determinant of their efficacy. For instance, MXF, which is more lipophilic than CIP, tends to accumulate more efficiently in the cytosol and acidic compartments such as phagolysosomes. This characteristic allows MXF to achieve superior potency against pathogens residing in acidic environments such as *S. aureus* [83].

b) Efficacy in specific intracellular compartments

The intracellular environment, particularly the pH of subcellular compartments, is another key determinant of FQ activity. As zwitterionic compounds, FQs can interact with both anion and cation transporters, influencing their distribution within cells [17]. In acidic compartments, such as the phagolysosome (pH~5), FQs can become protonated, reducing their membrane permeability and thus antimicrobial efficacy [43]. CIP is more affected by pH changes than MXF, leading to reduced activity in acidic conditions [83]. Consequently, even with high intracellular concentrations, FQs efficacy can be diminished if they are sequestered in compartments where their activity is neutralized. Studies have shown that both CIP and MXF exhibit decreased activity in acidic conditions against *S. aureus* [83]. Specifically, FQs are reported to be 5-20 times less potent against *L. monocytogenes* and up to 100 times less potent against *S. aureus* in acidic environments compared to extracellular pathogens [85].

c) Efflux mechanisms

Efflux transporters, such as ABCB1 (P-glycoprotein (P-gp) or multidrug resistance protein 1 (MDR1)) and ABCC (multidrug resistance-associated proteins (MRPs)), also significantly influence the intracellular activity of FQs by actively exporting the drug out of host cells [49].

In macrophages J774, CIP efflux has been identified as being ABCC4 (MRP4) dependent [67,87], resulting in lower intracellular concentrations and reduced activity towards *L. monocytogenes* [88]. Prolonged antibiotic exposure can exacerbate this problem by inducing the overexpression of transporters such as ABCB1, ABCG2 (BCRP), and/or ABCC4, as observed in stepwise drug exposure studies mimicking clinical conditions [88]. Efflux inhibitors, however, can ameliorate this limitation [17,87]. Indeed, probenecid, gemfibrozil, and MK571 [17] inhibit ABCC4, potentially enhancing CIP intracellular activity towards *L. monocytogenes* [86]. In contrast, MXF, which is not a substrate of ABCC4, remains unaffected by ABCC inhibitors [88]. This characteristic, combined with its higher lipophilicity, may contribute to its superior efficacy in treating intracellular infections compared to CIP [67]. The role of efflux transporter in FQ PK is discussed in detail in Chapter I – section 1.4.

d) Bacterial responsiveness

Finally, the metabolic state of intracellular pathogens significantly influences their susceptibility to antibiotics. Pathogens like *L. monocytogenes*, which actively replicate in the cytosol, are more susceptible to antibiotics due to their high metabolic activity [85,86]. In contrast, other pathogens can enter in a metabolically dormant state or be protected by the intracellular environment, making them less susceptible to treatment. For example, *S. aureus* residing in the phagolysosome is shielded by its acidic environment and limited nutrient availability, inducing a dormant or slow-growing state, reducing its responsiveness to antibiotics [11,85].

In this thesis, the role of efflux transporters in eukaryotic cells was specifically investigated as key modulators of CIP and MXF intracellular activity.

Key insights

- **FQs, CIP and MXF, evolved from nalidixic acid through key modifications at C6, N1, C7, and C8, enhancing efficacy and spectrum of activity.**
- **CIP and MXF target DNA gyrase and topoisomerase IV, with resistance developing from efflux pumps, porin expression changes, target protection, or target mutations.**
- **FQs have high bioavailability, tissue penetration, and concentration-dependent killing.**
- **CIP targets Gram-negative bacteria, while MXF is effective against both Gram-negative and Gram-positive bacteria.**
- **FQs can induce serious side effects, warranting cautious use and restriction to cases where alternatives are effective.**
- **Intracellular efficacy can be affected by bioavailability and subcellular localization of the drug, local pH, and efflux mechanisms.**

I.4 Fluoroquinolone transport in eukaryotic cells

The transport of FQs in eukaryotic cells relies on a dynamic balance between efflux and influx transporters, which collectively regulate their systemic and cellular PK and PD.

Efflux transporters act as a defense mechanism, protecting cells from amphiphilic compounds that can diffuse passively across the phospholipid bilayer, accumulate intracellularly, and cause cytotoxicity. By actively exporting these compounds, efflux transporters maintain cellular homeostasis and limit toxic effects. Additionally, they play a key physiological role in facilitating the transmembrane transport of endogenous molecules, such as phospholipids, cytokines, nutrients, and metabolites [16].

While influx transporters primarily exist for polar compounds, most ABs, including FQs, are not preferentially recognized as substrates [16]. For example, CIP and MXF predominantly exist as zwitterions (ZW) at physiological pH, with CIP being 82% ZW and MXF 93% ZW. At acidic pH, such as urinary pH (6.3), these compounds partially convert to cationic (C) forms, CIP being 60% C and 39% ZW, while MXF becomes 49% C and 51% ZW [89]. These ionized states allow interactions with ABC but also SLC transporters such as organic cation and anion transporters (OCTs and OATs) [89]. Given this, we have chosen to focus exclusively this thesis on efflux transporters.

Efflux transporters influence both systemic and cellular PK of drugs, with their effects depending on their cellular orientation (**Figure I.14**). When localized at the apical membrane of polarized cells, efflux transporters accelerate drug clearance (CL) by actively transporting substrates out of the cells into luminal spaces (e.g., the intestinal lumen, bile, or urine).

Conversely, basolateral efflux transporters may promote drug retention by redirecting compounds back into circulation. While some efflux transporters, such as ABCB1, are ubiquitously expressed across various tissues, others exhibit specific distribution with specialized functions [16].

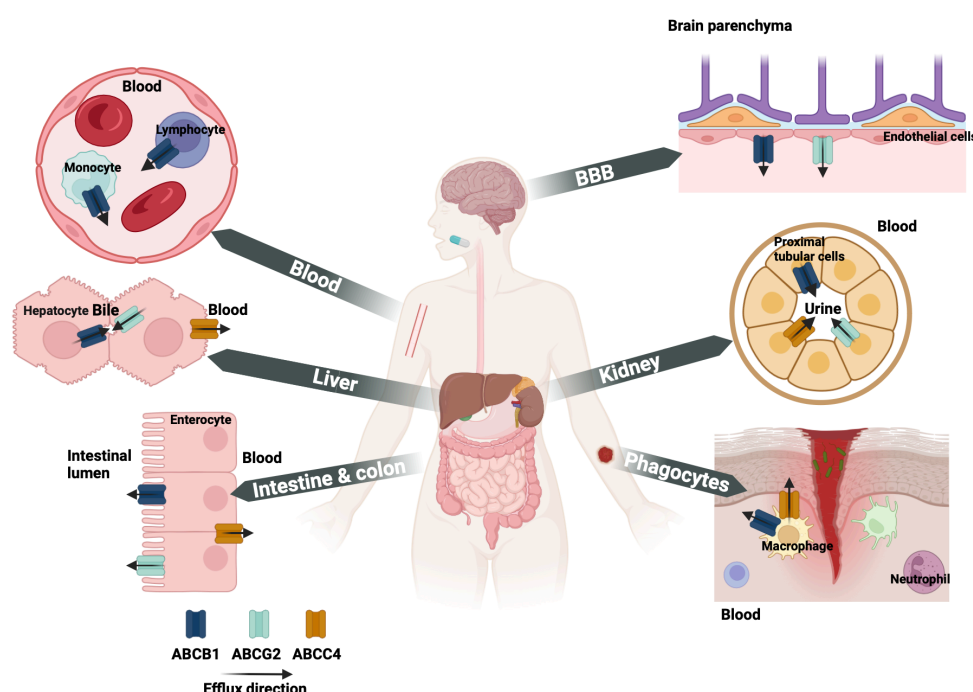


Figure I.14. Schematic representation of the localization of ABC transporters assessed in this thesis (image created in Biorender.com).

1.4.1 Efflux transporters and fluoroquinolone systemic pharmacokinetics

Efflux transporters modulate the systemic PK of FQs by influencing their absorption, distribution, metabolism, and excretion (ADME) [90]. For instance, CIP demonstrates an **oral bioavailability** of approximately 70%, which is partially influenced by transporter-mediated transintestinal elimination [91]. Indeed, in acidic environments where FQs are protonated, ABCG2 efflux transporter actively secrete CIP in the intestine, limiting its systemic absorption [90]. Similarly, **renal**

excretion of FQs is influenced by a combination of efflux and influx transporters. Levofloxacin is excreted via ABCB1 [92], while ofloxacin excretion relies on OCTs and OATs transporters [90]. ABCG2 facilitates the urinary secretion of CIP and grepafloxacin [93]. Efflux transporters also contribute to the **biliary CL** of FQs. For example, the biliary excretion of grepafloxacin involves partially ABCB1 [94] as well as ABCG2 for grepafloxacin and CIP [93]. Beyond systemic bioavailability, transporters can also **redirect FQs to specific compartments**, potentially leading to adverse or non-desired effects. For instance, ABCG2 facilitates the secretion of FQs into milk [95], while at the blood-brain barrier (BBB), ABCB1, ABCC1 (MRP1), and ABCG2 limit FQ penetration, limiting their efficacy against central nervous system infections (e.g., meningitis) but also protecting from seizures [16].

1.4.2 Efflux transporters and fluoroquinolone cellular pharmacokinetics

Beyond their systemic effects, efflux transporters regulate the cellular disposition of FQs, a critical determinant of their activity towards intracellular pathogens. Indeed, as explained earlier in this thesis, by actively exporting FQs from cells, efflux transporters limit their cellular concentration and reduce their therapeutic efficacy. However, studies have shown that inhibiting specific efflux transporters enhances cellular accumulation, leading to potentially improved antimicrobial activity. These findings highlight the potential role of efflux transporters in overcoming drug resistance mechanisms and optimizing therapeutic outcomes, particularly in infections caused by intracellular pathogens. This thesis will thoroughly evaluate the role of efflux transporters in cellular MXF and CIP disposition. **Table I.1** summarizes studies that have been conducted to provide evidence of CIP/MXF transport by efflux-mediated transporters.

CHAPTER I – INTRODUCTION

Superfamily	Transporters	Substrates	Cell lines	References
ATP-binding cassette (ABC)	ABCB1	MXF	Calu-3 ¹	[96,97]
	(P-gp, MDR1)	CIP	Calu-3, Caco-2 ²	[97,98]
	ABCG2 (BCRP)	CIP	Caco-2, MDCKII ³	[93,95,99]
	ABCC4 (MRP4)	CIP	Caco-2; HEK293 ⁴ J774 macrophages ⁵	[99] [17,67,87]
Multidrug And Toxin Extrusion (MATE)*	MATE1	MXF/CIP	HEK293, MDCKII ³	[100,101]

Table I.1. CIP and MXF efflux are mediated by ABCB1, ABCG2, ABCC4, and MATE1. ¹ Human lung cancer cell line; ² Human colorectal adenocarcinoma cell line; ³ Madin-Darby canine kidney cell line; ⁴ Human embryonic kidney cell line; ⁵ Murine macrophages. *Transporters that use the proton gradient (H⁺ antiport mechanism).

I.5 ATP-binding cassette (ABC) transporters

Membrane transport proteins are essential for maintaining cellular function by facilitating the movement of molecules across biological membranes [102]. Representing approximately 2% of the total genes in the human genome [19], these proteins are classified into four main categories: ion channels, transporters, aquaporins, and **ATP-powered pumps** [102,103]. Based on their mechanisms of action, they can transport substrates either passively or actively. Passive transporters move substrates down their concentration gradients, while active transporters couple the movement of one molecule or ion against its concentration gradient with the movement of another molecule or ion towards its gradient or use direct energy sources such as ATP [103].

Among active transporters, ABC transporters use the energy released from ATP hydrolysis to translocate a wide range of substrates across cellular membranes, often against their concentration gradients [19]. These pumps play a pivotal role in maintaining cellular homeostasis, supporting defense mechanisms, and influencing drug PK [104].

1.5.1 Classification and structure of ABC transporters

The concept of ABC transporter was introduced in 1992 by Christopher Higgins. To date, 49 human genes encoding efflux transporters have been identified, including one pseudogene [19,102]. Initially, these transporters were classified based on their function, such as multidrug resistance proteins (MDRs), but as functional roles were found to vary across members, a unified classification system based on sequence similarity and domain structure was adopted.

They are now categorized into eight subfamilies (ABCA to ABCI) [105] using the following criteria: (1) Proteins with more than 40% sequence identity are grouped into the same family (e.g., ABC); (2) within each family, proteins with more than 55% sequence identity are further classified into the same subfamily (e.g., ABCB); and (3) proteins sharing over 70% sequence identity are named as specific members, typically numbered in the order of their discovery (e.g., ABCB1) [103].

ABC transporters are defined by their highly conserved ABC domain, essential for ATP hydrolysis. While the transmembrane domains (TMDs) exhibit sequence variability, the nucleotide-binding domains (NBDs) display significant conservation, with three hallmark motifs [105] (see **Figure I.15**). The Walker A and Walker B motifs play critical roles, with Walker A interacting with the phosphate group of the ATP due to its glycine-rich structure, and Walker B, containing glutamate, facilitates binding to the γ -phosphate through nucleophilic attack. The ABC signature motif (C-signature) interacts with the Walker A motif of the opposite NBD, forming a “sandwich” configuration around ATP [19,102]. Additional motifs, such as the A-loop, Q-loop, D-loop, and H-loop, further coordinate ATP binding and hydrolysis. The Q-loop plays a specific role in binding the second ATP molecule [102,104].

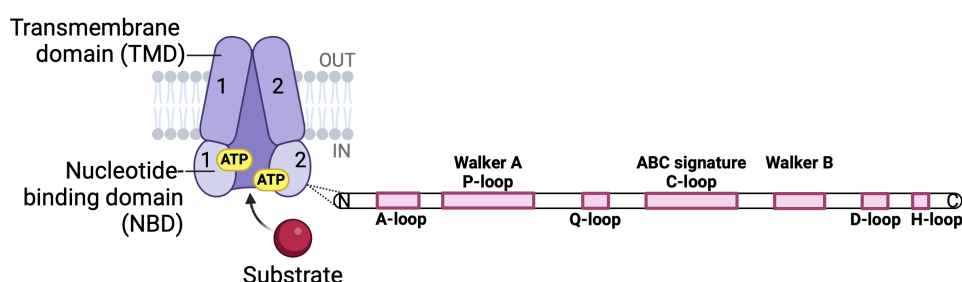


Figure I.15. Representation of ABC transporters with sequence alignment of hallmark motifs within the NBDs (image created in Biorender.com, adapted from [102]).

The core structural unit of a functional ABC transporter consists of four domains: two hydrophobic TMDs and two hydrophilic NBDs. The TMDs, typically formed by six hydrophobic α -helices (TMHs), create the substrate-binding site and determine substrate specificity. The NBDs, located on the cytoplasmic side, bind and hydrolyze ATPs to provide the energy required for substrate transport. Each TMD is paired with one NBD. Full ABC transporters have all four domains within a single polypeptide chain, while half transporters (e.g., ABCG2), contain only one TMD and one NBD. These half transporters require dimerization (either homodimerization or heterodimerization) to form a functional unit [102,103].

1.5.2 Mechanism of action

ABC transporters operate through an “alternating-access and release mechanism” to facilitate substrate translocation across cellular membranes [106] (**Figure I.16**). The process begins with the transporter in an inward-facing conformation, where the substrate-binding pocket within the TMDs is exposed to the intracellular side of the membrane. In this configuration, the substrate binds to the TMDs with high affinity, initiating the transport cycle. The substrate can enter either directly from the intracellular environment or, if hydrophobic, by accumulating within the membrane and laterally accessing the substrate pocket. Substrate binding triggers the binding of the two ATP molecules to the NBDs. ATP binding induces a conformational change, acting as a “molecular glue” that dimerizes the NBDs to form a closed nucleotide sandwich structure, with the ATP molecule “sandwiched” between the NBDs. The energy from ATP binding is transmitted to the TMDs, causing transporter shift from an inward-facing to an outward-facing conformation. This conformational change reduces the affinity of the binding pocket for the substrate, leading to its release on the extracellular side. Following substrate release, ATP hydrolysis occurs, generating a negative charge that disrupts the

nucleotide sandwich structure. This disruption separates the NBDs and releases the hydrolysis products (ADP and inorganic phosphates). Consequently, the TMDs return to their original inward-facing conformation, restoring the transport to its initial state and allowing the cycle to repeat [102,105].

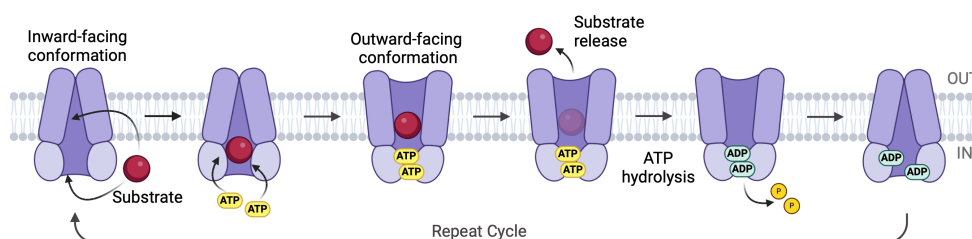


Figure I.16. Transport mechanism of ABC transporters (image created in Biorender.com, adapted from [105]).

1.5.3 Pharmacological significance

As previously discussed in this thesis, ABC transporters play crucial roles in drug PK. Modern PK integrates the concepts of elimination and transport as phase 0 (influx) and phase III or even IV (intracellular trafficking and efflux), both of which are directly influenced by transporters [106]. ABC transporters are highly versatile, capable of translocating a wide range of clinically toxicologically significant compounds [104]. These include anticancer drugs, HIV protease inhibitors, **antibiotics**, antidepressants, antiepileptics, and analgesics [106]. This broad substrate specificity highlights their essential role not only in drug PK but also in determining drug efficacy (PD). Moreover, they contribute significantly to the emergence of drug resistance [19,106]. Among the transporters involved in multidrug resistance, ABCB1 (also known as P-gp or MDR1), ABCC (MRPs) subfamily, and ABCG2 (BCRP) have been extensively studied [19,104]. In this work, we have chosen to focus on subfamilies B, C, and G, as they have been linked in the literature to the transport of CIP and MXF (**see Table I.1**). Specifically, we investigated the roles of three transporters: ABCB1, ABCC4, and ABCG2.

Protein name (synonyms)	Localization	Tissue distribution and Function(s)	Physiological substrates	Pharmacological substrates
ABCB1 (P-gp, MDR1)	Apical [106]	Adrenal gland > liver, gallbladder > brain (BBB), intestine, colon, kidney, ovary, fallopian tube, placenta, eye, immune cells (monocytes, T cells, B cells) [102,107] Involved in multidrug resistance [103,108,109]	Peptides, lipids, steroid hormones, phospholipids, cholesterol, cytokines [19,110,111]	Hydrophobic and amphipathic compounds [102,112] Anticancer drugs, HIV protease inhibitors, immunosuppressants, antiarrhythmics, calcium channel blockers, analgesics, antihistamines, antibiotics (FQs) , pesticides [102,109]
ABCC4 (MRP4)	Apical /Basolateral [106]	Prostate, lung, skeletal muscle, pancreas, spleen, thymus, testis, ovary, small intestine > kidney, gallbladder, tonsil [102,112], colon, fallopian tube > brain [113] Involved in nucleoside transport [103,108]	Cyclic nucleotides (AMPc, GMPc), ADP, eicosanoids (prostaglandins, leukotrienes), urate, conjugated steroids, hormones, folates, bile acids, glutathione [102]	Anticancer drugs, antiviral drugs, cardiovascular drugs, anti-inflammatory drugs, antibiotics (FQs) [102]
ABCG2 (BCRP, ABCP, MXR)	Apical [106]	Duodenum, small intestine, colon, rectum, seminal vesicle, endometrium, appendix > kidney, testis, placenta, smooth muscle >	Sulfate and glucuronide conjugates (hormones), nucleotides, urate [19,102]	Anticancer drugs, cardiovascular drugs, antibiotics (FQs) , vitamins, statins, gastrointestinal drugs, antidiabetic drugs [19,102]

	thyroid gland, adrenal gland, lung, heart muscle, brain Involved in detoxification and drug resistance [103,108,114]
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Table I.2. Localization, tissue distribution and function(s), physiological and pharmacological substrates of the three studied ABC transporters.

a) ATP-Binding Cassette Sub-Family B

The ABCB subfamily of transporters consists of 11 genes encoding four full transporters and seven half transporters [103,108]. Among them, ABCB1 is the most extensively studied member and was the first in this subfamily to be cloned and characterized due to its overexpression in certain drug-resistant tumor cell lines [102,108].

- **ABCB1: Gene location, structure, function, inhibition and polymorphisms**

Gene location, structure, and function

ABCB1 is encoded by the *ABCB1* gene, located on chromosome 7q21.1 [103]. This gene spans 29 exons and produces a protein of 1280 amino acids, with an approximate molecular weight of 170 kDa [19,115]. Structurally, the protein is composed of two half-transporters, each containing a TMD and an intracellular NBD [115]. ABCB1 undergoes post-translational modifications, phosphorylation, which may regulate transporter function, and N-glycosylation, which ensures routing to the plasma membrane and stability [115]. As summarized in **Table I.2**, ABCB1 is expressed in various tissues, particularly those involved in barrier and protective functions, where it regulates the absorption, distribution, and elimination of endogenous and exogenous compounds, particularly pharmacological agents.

ABCB1 substrates are generally amphipathic, hydrophobic molecules, with molecular weights ranging from 300 to 2000 Da [19]. These substrates can be anionic, cationic, or noncharged, and their affinity for ABCB1 is closely correlated with their lipophilicity and hydrogen bonding properties [19].

ABCB1 inhibitors

ABCB1 inhibitors block substrate transport through different mechanisms, including the disruption of ATP hydrolysis, the alteration of ABCB1 expression, or direct competition for substrate-binding sites [102]. These inhibitors are classified into three generations based on their specificity, potency, and toxicity [102].

1. First-generation inhibitors

These include verapamil, quinidine, and cyclosporine A, which are **also ABCB1 substrates**. These compounds inhibit ABCB1 by competing with substrates for binding sites, thereby blocking their efflux [102,112]. However, despite their effectiveness, these inhibitors exhibit low binding affinity to ABCB1, necessitating the use of high concentrations to achieve therapeutic effects, leading to significant toxicity [116]. Moreover, these inhibitors have **intrinsic pharmacological activities** of their own, which can lead to additional unwanted side effects in patients, thus limiting their clinical application [19,102,112].

2. Second-generation inhibitors

Second-generation inhibitors, such as valspodar (PSC-833, a cyclosporine A analogue) and elacridar (GF120918), are non-competitive inhibitors with greater potency and higher affinity for ABCB1 compared to first-generation agents [19]. They are less toxic and **lack pharmacological effects** [102]. However, they can still interact unpredictably with other enzymes, particularly CYP3A4, leading to potential PK interactions by altering CL of drugs metabolized by CYP3A4 [102]. Elacridar also inhibits ABCG2, which may cause off-target effects [112,116].

3. Third-generation inhibitors

These inhibitors, such as zosuquidar (LY335979) and tariquidar (XR9576), which are non-competitive inhibitors, as well as OC144-093, were **specifically designed to provide high specificity for ABCB1**, low toxicity, and no interaction with CYP3A4 [112,116]. These inhibitors are 10 times more effective in blocking ABCB1-mediated efflux compared to earlier generations [19,102].

Polymorphisms in the ABCB1 gene

The *ABCB1* gene is highly polymorphic. To date, 66 single nucleotide polymorphisms (SNPs) have been identified in the coding region [102]. Among these, 22 are synonymous SNPs and do not change the amino acid sequence, while 44 are nonsynonymous SNPs and result in amino acid change [102]. This might contribute to variability in substrate-drug PK and therapeutic response between patients. **Table I.3** summarizes some relevant coding SNPs affecting *ABCB1* and their functional impacts, with their approximate localization in **Figure I.17**.

rs Number	Nucleotide change/effect	Frequency (%) [*]	Functional impacts/ Summary of findings
rs2229109	c.1199G>A Missense variant: Ser400Asn	EUR: 3.28 SAS: 0.92 AFR: 0.30 EAS: 0.00	May alter substrate transport, potentially modifying intracellular accumulation. Functional effects remain substrate dependent. <i>In vitro</i> and <i>in vivo</i> studies have shown variable effects on drug accumulation, with changes depending on the drug investigated [117–120].
rs1128503	c.1236C>T Synonymous variant: Gly412Gly	EUR: 41.55 SAS: 58.69 AFR: 13.62 EAS: 62.70	May affect co-translational folding, potentially affecting substrate binding and transport efficiency [111]. <i>In vitro</i> studies suggest this SNP may modulate drug transport and intracellular accumulation. <i>In vivo</i> , clinical studies report controversial data but indicate a possible impact on drug transport, accumulation, and treatment response [121–123].

rs2032582	c.2677G>T/A Missense variant: Ser893Ala/Thr	EUR: 40.95/1.79 SAS: 59.20/5.01 AFR: 1.97/0.08 EAS: 39.78/13.39	May influence drug-induced ATPase activity, potentially altering substrate transport [111]. <i>In vitro</i> studies suggest an effect on drug transport and intracellular accumulation. <i>In vivo</i> , clinical studies report controversial data but suggest a potential role in modulating drug transport and treatment response [121,124,125].
rs1045642	c.3435C>T Synonymous variant: Ile1145Ile	EUR: 51.79 SAS: 57.46 AFR: 14.98 EAS: 39.78	May affect co-translational folding, possibly altering ATP binding and substrate interaction, with potential consequences on transport efficiency [111]. <i>In vitro</i> studies suggest an impact on drug transport and intracellular accumulation. <i>In vivo</i> , clinical studies report conflicting data but suggest a potential role in modulating treatment efficacy and resistance [121,126–129].

Table I.3. *ABCB1* relevant coding SNPs. *Allelic frequencies are from the 1000 Genomes project found at <http://www.pharmgkb.org/search/>. Eur: European, SAS: South Asian, AFR: African, EAS: East Asian.

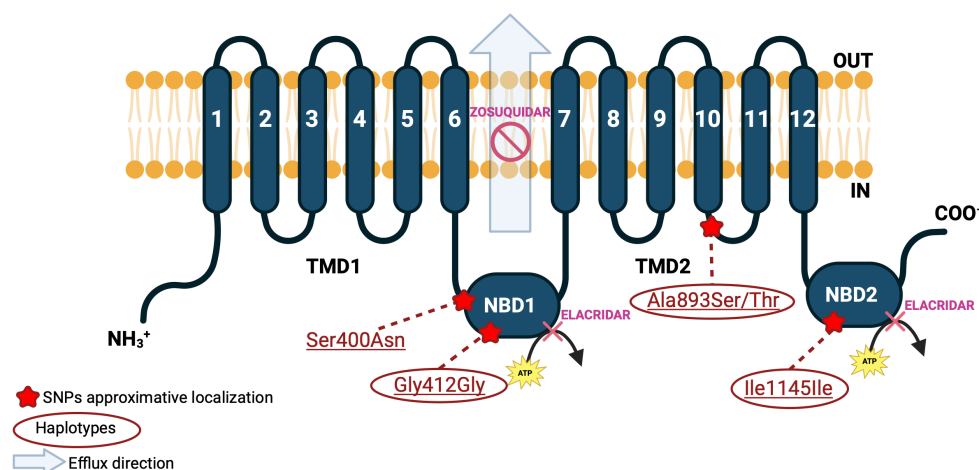


Figure I.17. *ABCB1* topology model. The structure includes approximate positions of relevant coding SNPs cited in **Table I.3**. The mode of action of inhibitors assessed in the thesis is also represented [130,131] (image created in Biorender.com, adapted from [19]).

The c.1199G>A polymorphism, located in exon 11, involves a guanine-to-adenine substitution at position 1199. At the protein level, this polymorphism results in a serine-to-asparagine substitution at position 400 within the sixth cytoplasmic loop, a domain critical for substrate recognition and ATP binding and hydrolysis [111,117]. This SNP affects transporter activity in a substrate-dependent manner (see Chapter III – article n°1).

The c.1236C>T polymorphism, located in exon 12, involves a cytosine-to-thymidine substitution at position 1236. At the protein level, this synonymous polymorphism is situated in the sixth cytoplasmic loop, between the critical A-loop and Walker-A domains [111]. While this SNP does not appear to alter protein expression or function, it may affect co-translational folding through ribosome stalling, potentially disrupting the folding of TMH6, a region essential for substrate binding [111].

The c.2677G>T/A polymorphism, located in exon 21, involves a guanine-to-thymidine/adenine substitution at position 2677. This polymorphism results in an alanine-to-serine or alanine-to-threonine substitution, with serine being more frequent [111]. At the protein level, it is located in the tenth cytoplasmic loop, near TMH9 and TMH10, regions critical for drug-induced ATPase activity and substrate transport. This variant alters substrate specificity and may impair the co-translational folding of TMH10, reducing transport efficiency and ATP hydrolysis [111].

The c.3435C>T polymorphism, located in exon 26, involves a cytosine-to-thymidine substitution at position 3435. This synonymous mutation, found in the twelfth cytoplasmic loop, does not change the encoded amino acid (isoleucine), a conserved hydrophobic residue in the second ATP-binding domain [111]. However, it has been associated with a two-fold reduction in ABCB1 expression in the duodenum [111].

This SNP may affect ribosome translation kinetics due to the use of a rare codon, causing ribosome pausing. This pausing, potentially caused by changes in mRNA secondary structure (stability) or reduced tRNA availability, could lead to misfolded proteins with impaired function [111]. Furthermore, this SNP may also affect key functional domains such as the Q-loop and Walker-A domain, essential for ATP binding and hydrolysis [111].

Linkages and haplotypes

These three polymorphisms are strongly linked, forming haplotypes [19,102]. The TTT haplotype predominates in the Asian population, while the CGC wild-type allele is more frequent in the African population. In Caucasians, the frequencies of the TTT and CGC haplotypes are approximately equal [111]. These SNPs may act synergistically to influence ABCB1 expression and function. It is therefore essential to study these SNPs collectively as haplotypes rather than individually, as their combined effects may result in additive and synergistic changes in ABCB1 activity [111].

b) ATP-Binding Cassette Sub-Family C

The ABCC subfamily, also referred to as the MRP family, is the largest gene family among ABC transporters, comprising 13 members [102]. This subfamily includes 12 functional transporters and one pseudogene (*ABCC13*) [102,108]. These transporters exhibit structural diversity, typically featuring two or three TMDs. Transporters with three TMDs (TMD0, TMD1, TMD2) have five segments in TMD0 and six segments each in TMD1 and TMD2 [19]. They also possess two NBDs, with the N-terminal end being extracellular and the NBDs located on the cytoplasmic side. However, in transporters with two TMDs, such as *ABCC4*, the N-terminal is intracellular, resembling the configuration of *ABCB1* [19].

ABCC transporters play a critical role in the development of multidrug resistance, actively expelling drugs from cells, like ABCB1 [102].

- **ABCC4: Gene location, structure, function, inhibition and polymorphisms**

Gene location, structure, and function

ABCC4 is encoded by the *ABCC4* gene located on chromosome 13q32 [103]. It encodes a protein of 1325 amino acids with a molecular weight of 149.5 kDa [132], making it the smallest member of the ABCC family [102]. A distinctive feature of ABCC4 is its dual localization in polarized cells. Depending on cell type and tissue specificity, ABCC4 may localize either to the apical or basolateral membrane of targeted cells [102]. For example, in hepatocytes, it localizes to the basolateral membrane, facilitating the transport of substrates into the bloodstream. However, in renal proximal tubule cells, ABCC4 is found on the apical side, contributing to the secretion of substrates into the urine [102]. Structurally, ABCC4 is distinguished by the absence of the N-terminal domain (TMD0) [112]. Functionally, as summarized in **Table I.2**, ABCC4 actively exports both endogenous and exogenous molecules, playing a significant role in cellular detoxification and homeostasis.

ABCC4 inhibitors

Most of these compounds have relatively low affinity and specificity for ABCC4. One example is MK571, which was initially developed as an LTD4 receptor antagonist. Despite its origin, MK571 has been shown to effectively inhibit ABCC1, ABCC2, and ABCC4 at a concentration exceeding 5 μ M. Other inhibitors, such as probenecid or gemfibrozil, have even less specificity and are considered general inhibitors of organic anion transporters [133].

Polymorphisms in the *ABCC4* gene

Although the *ABCC4* gene is highly polymorphic, only a few data are available regarding the functional implications of its variants [134]. In this thesis, we focus on discussing polymorphisms with clinical relevance based on both *in vitro* and *in vivo* data, selecting three coding variants for detailed analysis. These three selected polymorphisms are the most abundant in the Japanese population [135]. Below is a summary of these three polymorphisms, including their functional impacts (**Table I.4**) and their approximate localization in **Figure I.18**.

rs Number	Nucleotide change/effect	Frequency (%)*	Functional impacts/ Summary of findings
rs11568658	c.559G>T Missense variant: Gly187Trp	EUR: 3.08 SAS: 6.44 AFR: 0.00 EAS: 12.0	Alters the structural dynamics of the NBDs and TMDs, affecting conformational transitions essential for substrate transport [136]. <i>In vitro</i> studies suggest this SNP reduces efflux activity, impacting drug intracellular accumulation. <i>In vivo</i> , clinical studies indicate a potential impact on drug disposition and treatment-related side effects [137].
rs2274407	c.912G>T Missense variant: Lys304Asn	EUR: 8.05 SAS: 17.08 AFR: 20.95 EAS: 18.45	May alter splicing efficiency, potentially impacting mRNA maturation [138]. <i>In vivo</i> , clinical studies suggest a potential impact on drug toxicity and treatment outcomes [138,139].
rs3765534	c.2269G>A Missense variant: Glu757Lys	EUR: 0.89 SAS: 5.11 AFR: 0.08 EAS: 7.64	Possibly impairs cell membrane localization [140]. <i>In vitro</i> studies suggest this SNP may reduce transporter function and disrupt proper membrane localization. <i>In vivo</i> , clinical studies indicate a potential impact on treatment sensitivity and the risk of adverse effects [140,141].

Table I.4. *ABCC4* relevant coding SNPs. *Allelic frequencies are from the 1000 Genomes project found at <http://www.pharmgkb.org/search/>. Eur: European, SAS: South Asian, AFR: African, EAS: East Asian.

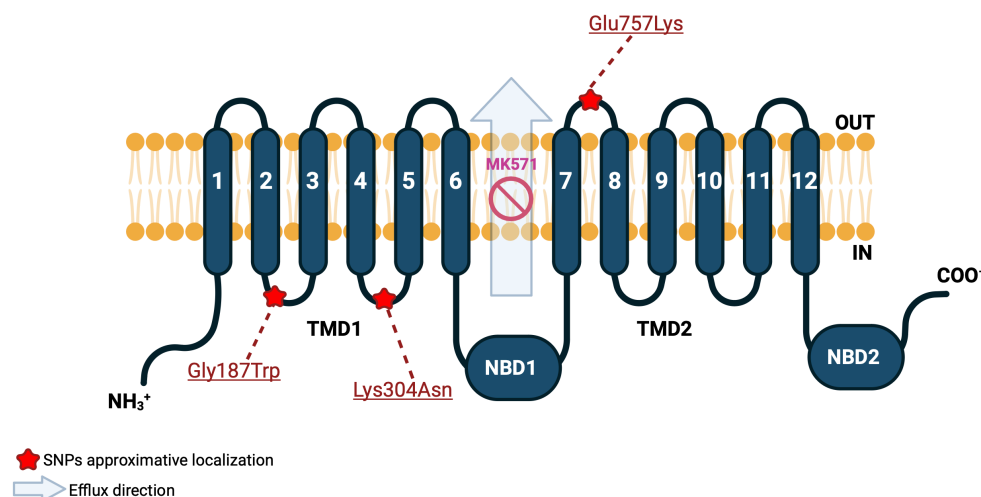


Figure I.18. ABCC4 topology model. The structure includes the approximate position of relevant coding SNPs cited in **Table I.4**. The mode of action of the inhibitor assessed in the thesis is also represented [142,143] (image created in Biorender.com, adapted from [19]).

The c.559G>T polymorphism in exon 5 of the *ABCC4* gene results in the substitution of glycine with tryptophan in the first intracellular loop, which is in direct contact with NBD1. The larger tryptophan residue introduces steric hindrance into the protein structure, leading to structural distortions and stronger electrostatic interactions with other residues. These changes reduce the flexibility of NBD1 and bring the two NBDs closer together, disrupting their normal positioning. Moreover, the SNP also affects TMD packing, causing deformation in TMD1 and increasing interactions between TMD1 and TMD2, making the conformational transition from inward-facing to outward-facing more energy-demanding. As a result, it impacts the ability of ABCC4 to transport substrate efficiently across the membrane [136].

The c.912G>T polymorphism in exon 8 of the *ABCC4* gene results in the substitution of lysine at position 304 with asparagine. This modification occurs in TMD1 between the fourth and fifth α -helices. While it is not clear whether this SNP directly influences transporter function, this region is critical for the structure and function of the ABCC4 protein.

Furthermore, this SNP is located at the 3' end of the splice acceptor site of exon 8, at the very first nucleotide of this exon in the ABCC4 pre-mRNA. The T allele may interfere with the splicing process, potentially causing errors in mRNA maturation and thus disrupting the correct expression or functionality of the encoded protein [138].

The c.2269G>A polymorphism in exon 18 of the ABCC4 gene results in the substitution of a glutamine to a lysine at the 757 position in the fourth extracellular loop, essential for proper localization of the protein on the cell membrane. This SNP seems to reduce ABCC4 function by impairing its cell membrane localization [140].

c) ATP-Binding Cassette Sub-Family G

The ABCG subfamily consists of six genes encoding five well-characterized “reverse half-transporters” [103]. A distinctive feature of this subfamily is the reversed orientation of their functional domain: they have a NBD at their N-terminus and a TMD at their C-terminus, in contrast to the configuration of all other ABC proteins [108,144]. These transporters become functionally active by forming either homodimers or heterodimers [102]. Among them, ABCG2 stands out as a key multidrug transporter, known for its significant role in conferring drug resistance to cancer cells [102].

- **ABCG2: Gene location, structure, function, inhibition and polymorphisms**

Gene location, structure, and function

ABCG2 is encoded by the *ABCG2* gene, located on chromosome 4q22, and is composed of 16 exons, encoding a protein of 655 amino acids with a molecular weight of 72 kDa [103]. Classified as a half-transporter, ABCG2 contains a single NBD and TMD. To be functionally active, ABCG2 requires homodimerization [109].

A notable structural feature of ABCG2 is the extended extracellular loop (EL3), located between TMH5 and TMH6, which is N-glycosylated, contributing to the protein's structural stability and functionality [144]. Functionally, ABCG2 plays a critical role in protecting cells from naturally occurring toxins, thereby ensuring cellular homeostasis [112]. But it also plays a critical role in the multidrug resistance, notably towards chemotherapeutic drugs and antibiotics (**Table I.2**).

Originally identified in the placenta, in mitoxantrone-resistant cell lines, and in multidrug-resistant breast cancer cell lines, ABCG2 was initially referred to by various names, including the placental ABC transporter (ABCP), mitoxantrone resistance-associated protein (MXP) [102,144], and BCRP [112].

ABCG2 inhibitors

A wide range of inhibitors has been identified for ABCG2, including specific, nonspecific, competitive, and natural compounds. These inhibitors are essential tools for overcoming ABCG2-mediated drug resistance and enhancing the bioavailability of therapeutic drugs.

The first **ABCG2-specific inhibitor** reported was fumitremorgin C (FTC), a mycotoxin produced by *Aspergillus fumigatus*. Although FTC demonstrated significant efficacy in inhibiting ABCG2-mediated drug efflux, its clinical application is limited due to its neurotoxicity [102,112]. To address this, structural analogs of FTC, Ko143 and Ko134, were developed [112]. These analogs exhibit reduced toxicity and potent inhibitory activity, making them promising candidates for preclinical and clinical investigations [102].

In addition to specific inhibitors, several **nonspecific inhibitors** targeting multiple ABC transporters, including ABCG2, have been identified. For instance, elacridar inhibits both ABCG2 and ABCB1 by interfering with ATP hydrolysis [102]. Similarly, cyclosporin A, reserpine, valspodar, and tariquidar have also demonstrated dual inhibitory effects on ABCG2 and other ABC transporters, generally by blocking

substrate-binding sites or disrupting ATP hydrolysis. Furthermore, **competitive inhibitors**, such as tyrosine kinase inhibitors (TKIs), inhibit drug efflux. These include gefitinib, imatinib, nilotinib, erlotinib, and sunitinib, which not only inhibit ABCG2-mediated drug efflux but also act as substrates themselves [102]. Finally, **natural compounds**, particularly flavonoids, have also been identified as ABCG2 inhibitors. This is the case of quercetin, hesperidin, and silymarin, which have demonstrated inhibitory effects on ABCG2-mediated efflux [102].

Polymorphisms in the *ABCG2* gene

To date, 45 polymorphisms of the *ABCG2* gene have been reported in the literature, of which 24 are considered clinically relevant [145]. Among these, two specific coding polymorphisms have been extensively studied due to their significant functional impact on ABCG2 protein functionality [145]. Below is a summary of these two polymorphisms, including their functional impacts (**Table I.5**) and their approximate localization in **Figure I.19**.

rs Number	Nucleotide change/effect	Frequency (%)*	Functional impacts/ Summary of findings
rs2231137	c.34G>A Missense variant: Val12Met	EUR: 6.06 SAS: 15.44 AFR: 6.28 EAS: 32.64	May disrupt proper transporter insertion into the plasma membrane [145]. <i>In vitro</i> studies suggest this variant may impair transporter activity. <i>In vivo</i> , clinical studies indicate a potential impact on drug accumulation and treatment-related adverse effects [146–148].
rs2231142	c.421C>A Missense variant: Gln141Lys	EUR: 9.44 SAS: 9.71 AFR: 1.29 EAS: 29.07	May affect folding and ATP-ase activity of the protein [145]. <i>In vitro</i> studies suggest this SNP may impact membrane localization. <i>In vivo</i> clinical studies indicate possible impacts on drug exposure, therapeutic response, and treatment-related side effects [149–153].

Table I.5. *ABCG2* relevant coding SNPs. *Allelic frequencies are from the 1000 Genomes project found at <http://www.pharmgkb.org/search/>. Eur: European, SAS: South Asian, AFR: African, EAS: East Asian.

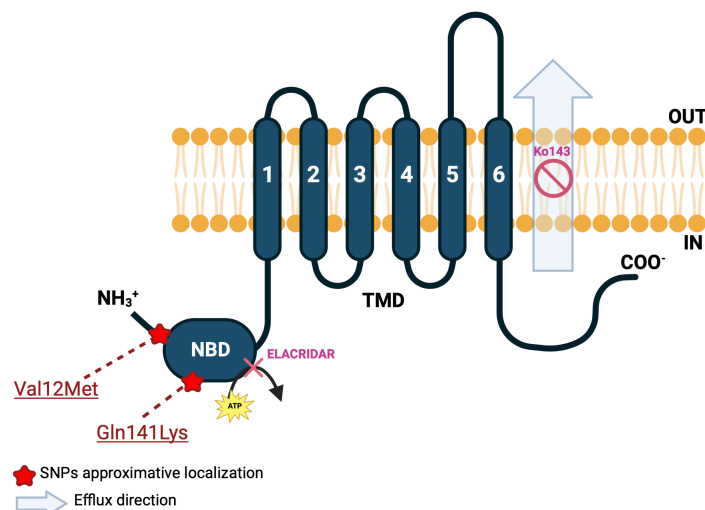


Figure I.19. ABCG2 topology model. The structure includes the approximate position of relevant coding SNPs cited in **Table I.5**. The mode of action of inhibitors assessed in the thesis is also represented [154] (image created in Biorender.com, adapted from [19]).

The c.34G>A polymorphism is located intracellularly near the N-terminal region of the ABCG2 protein, a region crucial for the insertion of ABCG2 into the plasma membrane. This polymorphism may disrupt the proper localization of the protein to the membrane, impairing its function and reducing its transport activity [145].

The c.421C>A polymorphism, located between the Walker A motif and the ABC signature region, involves the substitution of a neutral glutamine at position 141 with a positively charged lysine. This change may induce electrostatic repulsion between the lysine residue and nearby residues, specifically arginine 383 and lysine 382 in TMH1. This repulsion may impact the protein folding, leading to the degradation of the misfolded ABCG2 protein and consequently reducing the overall expression of the transporter [145]. Furthermore, the variant may also decrease ATPase activity, thereby diminishing the transport activity of ABCG2 [145]. This results in increased serum drug concentrations due to impaired drug efflux from the gut and other tissues, affecting drug PK, toxicity, and efficacy.

1.5.4 Impact of ABC polymorphisms on fluoroquinolone transport

Among the polymorphisms investigated, limited *in vivo* studies have demonstrated specific impacts on FQ transport, with a focus on *ABCB1*, *ABCG2*, and *ABCC4* polymorphisms.

For example, the ***ABCB1* 3435C>T** polymorphism has been associated with a decrease in sitafloxacin $C_{\max}$ [155]. Additionally, the ***ABCB1* 2677T>G/A** polymorphism has been linked to a 40% reduction in MXF prehepatic bioavailability in humans, resulting in a significant decrease in the AUC [156]. This suggests that the reduced function of these variants diminishes transporter-mediated efflux activity, leading to increased FQ cellular and tissue distribution. Such effects can be particularly pronounced in the intestine, liver, and kidneys, where *ABCB1* is highly expressed and plays a critical role in regulating drug disposition.

Furthermore, the ***ABCB1* haplotype (TTT)** and the ***ABCG2* 421C>A** polymorphism may influence susceptibility to neurological side effects during FQ therapy. A case study reported a patient developing seizures following treatment with levofloxacin, suggesting that polymorphisms in drug transporter genes at the BBB impair efflux activity, increasing drug concentration in the brain, which may contribute to neurological side effects [157].

Key insights

- **ABC transporters** are **ATP-powered pumps**, classified into subfamilies, using **conserved domains and motifs** to transport substrates.
- These transporters are **crucial in drug PK and resistance**, **translocating** a wide range of compounds, including **antibiotics**.
- This work focuses on **ABCB1, ABCC4, and ABCG2**, associated with CIP and MXF transport.
- Their **function** can be **influenced** by **inhibitors**, **post-translational modifications**, and **genetic polymorphism (SNPs)**.
- **Specific SNPs** in these transporters, with **clinical relevance**, are discussed in this section.
- Studies indicate that **SNPs in ABCB1 and ABCG2 impact FQ PK** and may increase **susceptibility to neurological side effects**.

I.6 Methods for testing fluoroquinolone activity on *Staphylococcus aureus*

This section discusses the two methods used in Chapter III – article n°2, to assess FQ activity on intracellular *S. aureus*. These complementary approaches offer distinct advantages. The static model, or **time-kill curves** (TKCs), provides preliminary data on bacterial activity under simplified and controlled conditions, allowing a direct assessment of the drug's efficacy. In contrast, the dynamic model, represented in my thesis by the **hollow fiber infection model** (HFIM), simulates clinical conditions, offering data that more accurately capture the complexities of real clinical scenarios.

I.5.1 Static model: Time-Kill Curves (TKCs)

TKCs are a widely used *in vitro* method to evaluate the bactericidal activity of antimicrobial agents against specific bacterial strains [158]. This method monitors bacterial viability at different time intervals, typically over a 24-hour period, under constant antibiotic exposure [159]. By plotting the results on a semi-logarithmic graph, the interaction between the antibiotic and the bacterial strain can be assessed, determining whether the antimicrobial effect is time- or concentration-dependent [158,160]. Indeed, TKC can evaluate microbial killing either as a function of time, when the antibiotic concentration remains stable, or as function of the antibiotic concentration, expressed as multiples of the MIC. This allows us to determine whether bacterial killing is influenced by the duration of exposure or if it increases with higher antibiotic concentrations [161].

Briefly, for extracellular bacteria, the TKC process involves adding known concentration(s) of (an) antimicrobial agent(s) into a bacterial culture suspended in

liquid medium, followed by incubation at 37°C. However, TKC can also be applied to infected cells when the aim is to evaluate the efficacy of the antibiotic against intracellular bacteria [162]. Then, samples are collected at predetermined time points (0 to 24 hours), cells are lysed in the case of intracellular bacteria to release intracellular bacteria, serially diluted, plated on agar, and incubated at 35°C-37°C for 24-48 hours to determine the number of colony-forming units (CFUs) [159]. Data are typically plotted on a graph with CFU/mL (or CFU per mg of protein for intracellular bacteria) on the Y-axis (logarithmic scale), generally expressed as the delta versus time 0, and time on the X-axis (arithmetic scale). This graphical representation allows for a clear differentiation between bacteriostatic and bactericidal effects. Bacteriostatic activity is defined as a reduction of less than 3 $\log_{10}$ CFU/mL during the 24-hour incubation period, while bactericidal activity corresponds to a reduction of at least 3 $\log_{10}$ CFU/mL (99.9% killing) over the same period [158,159] (**Figure I.20**).

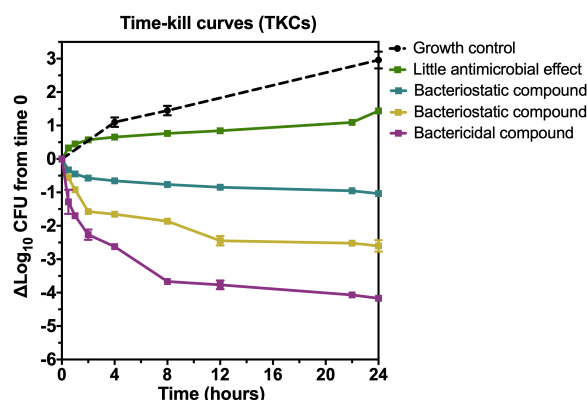


Figure I.20. TKC assay. Four test compounds were evaluated for their antimicrobial activity using TKC assays. Each compound was added to a medium containing a starting bacterial culture, alongside a growth control (negative control) for comparison. Bacterial load expressed as $\log_{10}$ CFU/mL, was measured at time 0 and at subsequent time points up to 24 hours. Each value represents the difference in bacterial load compared to the initial measurement at time 0. The green compound exhibited little antimicrobial effect, with bacterial growth levels comparable to the control. The blue and yellow compounds exhibited a bacteriostatic effect, reducing the initial bacterial load by less than 3 $\log_{10}$ CFU/mL. The purple compound exhibited a bactericidal effect, reducing the initial inoculum by more than 3 $\log_{10}$ CFU/mL.

TKCs are relatively simple and provide detailed quantitative kinetic data about the antimicrobial effect over time, making them a valuable tool to identify whether an antibiotic's efficacy is time- or concentration-dependent. However, TKCs also have limitations. Indeed, the use of constant antibiotic concentrations does not reflect the fluctuating PK observed *in vivo*, where drug absorption, distribution, metabolism, and excretion result in variable concentrations over time [163]. This may overestimate drug efficacy, especially for antibiotics with short $T_{1/2}$, as bacteria are continuously exposed to C_{max} *in vitro* [163]. Moreover, it could contribute to resistance development, as bacteria may be exposed to sub-inhibitory concentrations. Additionally, the absence of broth renewal limits the assay to 24 hours, which can prevent bacterial regrowth, potentially masking resistance development, clinical relapse, or the progressive antimicrobial activity needed for bacterial eradication over several days of treatment [163].

These limitations highlight the **need for complementary approaches, such as dynamic models**, to better simulate clinical conditions.

1.5.2 New approach: Dynamic model – Hollow fiber infection model (HFIM)

Currently, antibiotic activity against intracellular bacteria has primarily been evaluated under static conditions, such as TKC studies. The HFIM, widely considered the gold standard for *in vitro* PK/PD dynamic studies [164], has predominantly been applied to assess antibiotic activity against extracellular bacteria.

As a part of my doctoral research, I collaborated with the University of Poitiers to learn the technique for establishing a HFIM evaluating antibiotic (CIP) activity against extracellular bacteria (*S. aureus*). During a two-month internship with the team of Prof. W. Couet, in collaboration with Prof. S. Marchand and under the specific guidance of Prof. A. Chauzy, I acquired the expertise to model, in the HFIM,

the PK profile of CIP and test its activity against extracellular *S. aureus*. Upon returning to Belgium, I successfully adapted this model to study the activity of antibiotics, specifically CIP and MXF, against intracellular *S. aureus* infecting THP-1 monocytes (refer to Chapter III – article n°2).

In this section, I will provide a general overview of the HFIM and refer readers to the corresponding article for a detailed description of its development for intracellular bacteria and the results obtained.

a) Overview

- **Evolution from one compartment to a two-compartment model**

The one-compartment PK/PD model consists of a central reservoir containing the organism of interest, a diluent source, and a waste reservoir (**Figure I.21**). Drugs are introduced into the central reservoir via bolus infusion (C_{max}), and drug elimination is mimicked by adding drug-free diluent to reduce drug concentration over time [165]. However, while simple, this setup has limitations such as bacteria loss during the dilution process, complicating data interpretation. Furthermore, this open system increases the risk of contamination and exposure to pathogenic agents [164].

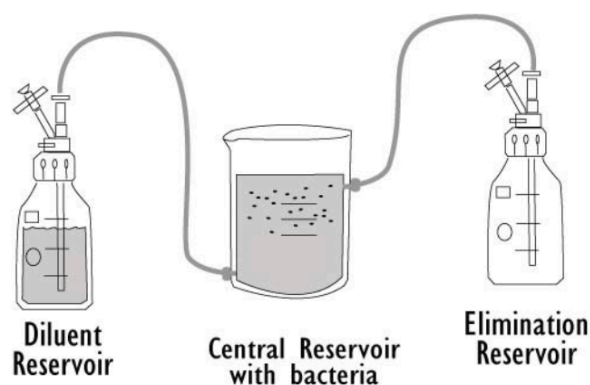


Figure I.21. Representation of the one compartment model (image from [166]).

In contrast, the two-compartment model (not to be confused with the classical two-compartment PK model), exemplified by the HFIM, addresses these limitations by confining bacterial populations within a specialized cartridge [164,165]. This design prevents bacterial loss during dilution, ensuring accurate sampling and more reliable data interpretations. Additionally, its closed design enhances biosafety by minimizing the risk of exposure to potentially harmful organisms [166].

- **HFIM design and principle**

The HFIM is an advanced *in vitro* PD model designed to study interactions between bacterial populations and antibiotics under dynamic, clinically relevant conditions. A key component of this system is the hollow fiber cartridge, which contains thousands of semi-permeable tubular fibers (capillaries) [167], each approximately 200 microns in diameter [164]. These fibers delimit two distinct spaces: the intra-capillary space (ICS) within the fibers and the extra-capillary space (ECS) surrounding them (**Figure I.22**).

These semi-permeable fibers allow the free diffusion of small molecules, such as drugs, nutrients and metabolic waste, from the central reservoir through the ICS, while retaining larger entities, including bacteria, cells, parasites and viruses, within the ECS [164,167]. This ensures that bacteria remain confined within the ECS, where they can be precisely exposed to controlled drug concentrations over time. Indeed, the fibers allow rapid equilibration of drug molecules between the ECS and the central reservoir, mimicking the PK observed in patients. The high surface-area-to-volume ratio of the fibers ($> 150 \text{ m}^2/\text{mL}$ of volume) ensures rapid, uniform drug distribution and precise control over drug absorption and elimination [164]. This dynamic design allows the HFIM to simulate fluctuating drug concentrations, setting it apart from static models such as TKC.

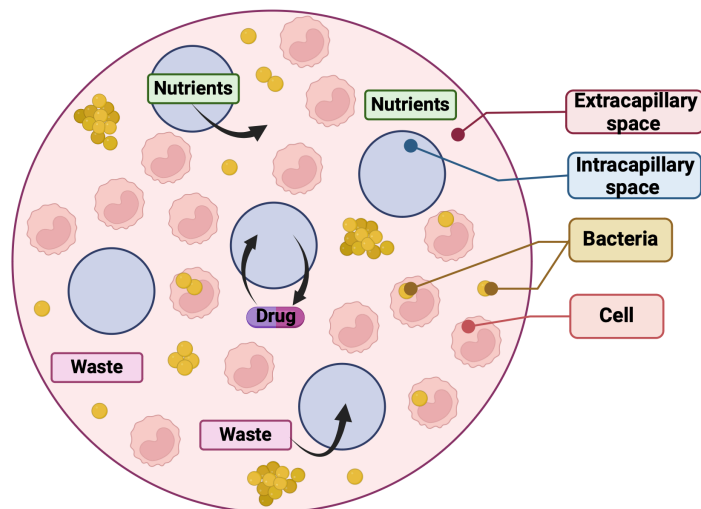


Figure I.22. Schematic representation of a cross-section in a hollow fiber cartridge (image created in Biorender.com, adapted from [164,168]).

The HFIM utilizes three main types of cartridges, chosen based on the properties of the drug being studied. The **polysulfone cartridge** is the first choice for most experiments due to its compatibility with a wide range of drug chemistries [166]. Polysulfone fibers are chemically stable and biocompatible, though they have general mechanical properties and relatively low molecular retention [167]. The **cellulosic cartridge**, on the other hand, is preferred for experiments involving polar drugs or when large volumes of solvents, such as DMSO or chloroform, are required [166]. While cellulose fibers are inexpensive and derived from many sources, they have low chemical resistance, low permeability, and limited selective separation performance [167]. The **polypropylene cartridge** is used when the drug exhibits strong hydrophobicity and requires dissolution in a high concentration of solvent or may have strong non-specific binding with polysulfone fibers [167]. Polypropylene fiber offers excellent chemical stability, high physical strength and a low price [167].

b) Advantages and limitations of the HFIM

Advantages	Limitations
- Dynamic in PK/PD simulations: Accurately mimics human PK/PD profiles for both antibiotics, antiviral drugs, antifungals... - Resistance studies: Allows detection of resistance emergence over extended durations. - Controlled environment: Provides precise control over drug absorption, elimination, and dosing kinetics. - Reproducibility: Closed, bio-safe system minimizes contamination risk, ensuring consistent and reproducible results. - High bacterial inocula: Supports high bacterial load (e.g., 10^8 CFU/mL) necessary for resistance studies, without ethical concerns. - Long-term experiments: Allows extended-duration experiments (weeks), impractical in animal models; repetitive sampling can be performed over time. - Ethical benefits: Reduces the reliance on animal testing, addressing ethical concerns. - Versatile applications: Supports both monotherapy and combination therapy studies, including investigations into different bacterial growth phases and interactions with cells. - Compact and efficient: Allows multiple cartridges to run simultaneously, reducing resource requirements compared to animal models.	- No immune system representation: Limits applicability to immunocompetent scenarios but is an advantage for antitubercular drugs. - Lack of standardized guidelines: Absence of comprehensive recommendations like the CLSI guidelines for TKC studies. - Host physiology limitations: Does not replicate host-specific factors such as metabolism, protein binding, or tissue distribution.

Table I.6. Advantages and limitations of the HFIM [164,165,167].

In conclusion, while the HFIM is not designed to replace static susceptibility testing, animal studies, or clinical trials, it serves as a validated and complementary tool in drug development [166]. Indeed, regulatory agencies such as the European Medicine Agency (EMA) and the U.S. Food and Drug Administration (FDA) recognize its value in generating preclinical data to support drug development. By bridging the gap between *in vitro* studies and clinical applications, the HFIM plays a pivotal role in antibiotic research, helping optimize dosing strategies, exploring combination therapies, and better understanding of resistance mechanisms [166].

c) Parameter simulations

To perform simulations within the HFIM, we rely on clinical PK data reported in the literature. These clinical parameters: C_{max} , $T_{1/2}$, T_{inf} (infusion duration), and τ (dosing duration) are essential for configuring the system. They inform critical settings, including pump flow rates, the antibiotic solution concentration to prepare, and the diluent volume needed to mimic drug elimination. This system operates under first-order kinetics, meaning that drug elimination is directly proportional to its concentration. This simplifies modeling while maintaining clinical relevance; it needs some adjustments to account for limitations such as the absence of protein binding and tissue distribution, which cannot be mimicked in this mono-compartmental model. To do the calculations, we used an application developed by the University of Poitiers [169], which employs well-established PK equations to calculate the necessary experimental parameters (**Table I.7**). Once the clinical parameters are entered into the system (**Figure I.23**), the application provides a detailed table with the input parameters (highlighted in green) and the calculated parameters (highlighted in red). This allows users to determine the pump settings and solution volumes and concentrations to replicate the desired PK profile accurately (**Figure I.24**).

CHAPTER I – INTRODUCTION

The screenshot shows a software interface with four main panels for parameter input:

- Pump characteristics:**
 - Minimum Pump Flow (mL/min): 0,01
 - Maximum Pump Flow (mL/min): 150
 - Step of pump (mL/min): 0,1
- Experimental system characteristics:**
 - Central volume (mL): 300
 - Flow rate central to cartridge (mL/min): 60
 - Cartridge volume (mL): 60
 - ☒ Is volume constant?
- Drug characteristics:**
 - Name of the drug: Ciprofloxacin
 - Half life of drug (hours): 1.8
 - Peak concentration after 1st dose (mg/L): 2.8
 - Experiment duration (hours): 24
- Dosing characteristics:**
 - Type of injection: ☒ Bolus, ☐ Infusion, ☐ Loading dose + Infusion
 - Infusion duration (hours): 1
 - Infusion volume (mL): 5
 - Dosing interval (hours): 12
 - Total number of doses: 2

Figure I.23. Screenshot of the application interface [169]. This interface allows the user to input clinical PK parameters such as C_{max} , $T_{1/2}$, T_{inf} and τ .

Parameter	Value
Drug	
Drug Name	Ciprofloxacin
Cmax central after 1 dose ($\mu\text{g/mL}$)	2.8
Infusion solution concentration ($\mu\text{g/mL}$)	242.901
Half life central (h)	1.8
Volume	
Central volume (mL)	300
Cartridge volume (mL)	60
Spent volume diluant to central (mL)	3327.106
Waste volume (mL)	3327.106
Infusion	
Infusion volume for 1 dose (mL)	5
Total number of doses	2
Dosing interval (h)	12
Total infused volume (mL)	10
Experiment	
Duration of experiment (h)	24
Flow pump diluant to central (mL/min)	2.31
Flow pump central to waste (mL/min)	2.31
Flow pump central to cartridge (mL/min)	60
Flow infusion pump (mL/h)	5

Figure I.24. Key settings calculated by the application [169]. Red-highlighted parameters are calculated values; green-highlighted ones are user inputs derived from the literature adjusted for protein binding and tissular diffusion.

Below are the key parameters calculated by the application with their associated formulas (**Table I.7**).

Parameters	Formula	Description
Infusion solution concentration C_{perf} (mg/L)	$C_{\text{perf}} = \frac{C_{\text{max}} \times V_d \times k_e \times T_{\text{inf}}}{1 - \exp(-k_e \times T_{\text{inf}})} \times \frac{1}{V_{\text{perf}}}$	The concentration of the solution to be infused. C_{max} is the predicted peak concentration after the first injection (mg/L). V_d is the volume of distribution (L) (=volume central + volume cartridge). T_{inf} is the infusion time. V_{perf} is the volume injected at each dose (L). k_e is the elimination rate constant ($k_e = \frac{\ln(2)}{t_{1/2}}$) (h).
Peristaltic pump flow diluent rate CL (L/h)	$CL = k_e \times V_d$	The flow rate of the peristaltic pump that introduces diluent into the central reservoir to simulate the elimination of the antibiotic (L/h).
Diluent volume required for the entire experiment V_{dil} (L)	$V_{\text{dil}} = CL \times \text{Total experiment duration}$	The total volume of diluent required to simulate the elimination process throughout the entire duration of the experiment (h).

Table I.7. Formulas used by the application to determine parameters.

d) Applications of the HFIM

As explained before, the HFIM offers several important applications in the study and development of antimicrobials, including evaluation of drug effects, the study of drug resistance, and the assessment of combination regimens.

- **Evaluation of drug effects**

HFIM allows for the **optimization of drug dosing**, reducing the need for extensive animal testing and providing valuable insights for guiding clinical trials [165,166]. HFIM also aids in **determining PK/PD parameters** driving the efficacy of drugs [165,167]. For example, in the study of Noel et al., they showed that the dominant PD index driving fosfomycin activity against *S. aureus* were C_{max}/MIC and AUC/MIC , while $T>MIC$ was poorly related [170]. The model is capable of **testing a wide variety of drugs**, allowing researchers to identify the most effective treatments for a specific bacterial strain [167]. This has been successfully applied to **antibiotics, antivirals and antifungals**, helping to optimize treatment strategies. For example, in the study by Meletiadiis et al., human PK of antifungal drugs (amphotericin B, voriconazole, and caspofungin) were simulated, revealing significant differences in their activity against three clinical isolates of *Aspergillus* [171]. Additionally, HFIM can be used to study the activity of drugs against **biofilm-forming bacteria** and **intracellular infections**. For example, in the study by Patel et al., they evaluated the PK/PD parameters of a novel PolC DNA polymerase inhibitor, CRS0540, against intracellular *Listeria monocytogenes* [172].

- **Study of drug resistance**

By adjusting the antibiotic concentration and inoculum density, researchers can simulate conditions that promote the emergence of drug-resistant bacteria.

The model allows for the **detection of the mutant selection window**, providing a predictive framework for when bacterial resistance might emerge under specific conditions [167]. Furthermore, this system helps **identify optimal dosing regimens to minimize the amplification of resistance** [167]. For example, the study of Vanscoy et al. evaluated the relationship between ceftolozane-tazobactam dosing and resistance development. They demonstrated that more intensive dosing regimens prevented resistance amplification and sterilized the system, while intermediate-intensive regimens promoted resistance development of resistant *E. coli* subpopulations [173]. It can also **mimic extreme conditions of resistance (antibiotic concentration or density of bacteria)**, offering data that can be used to develop strategies to counteract resistance in clinical settings [167]. For example, in the study of Tam and al., they evaluate the capacity of meropenem to suppress resistance to *Pseudomonas aeruginosa*, inoculated at a high density (10^8 CFU/mL) [174].

- **Evaluation of combination regimen**

By simulating various drug concentrations and dosing schedules, the model helps to **determine optimal therapeutic regimens for treating infections** [166]. This flexibility allows researchers to **test multiple combinations to identify synergistic effects** that might improve clinical outcomes [167]. For example, Broussou et al. demonstrated that in *S. aureus* biofilm-associated infections, the combination of vancomycin and amikacin offered limited additional efficacy compared to amikacin monotherapy. However, this combination could reduce possible resistance to amikacin and is a relevant strategy to prevent the selection of antibiotic-resistant bacteria during treatments [175]. Additionally, HFIM **replicate pathophysiological conditions**, such as augmented renal CL, which significantly impacts antibiotics PK in specific patient populations, guiding more personalized and effective treatment

strategies [167]. In this context, Bergen et al. demonstrated that individualized dosing regimens achieving piperacillin concentrations of at least 5xMIC are essential for maximizing bacterial killing and suppressing resistance development in *Pseudomonas aeruginosa*. Their study emphasized that in critically ill patients, altered PK, such as augmented renal CL, can lead to suboptimal antibiotic concentrations, promoting bacterial regrowth and resistance emergence [176].

Key insights

- **TKC** is a **static *in vitro*** method that evaluates the **bactericidal or bacteriostatic** activity of antibiotics but is **limited by constant drug concentrations and low duration (24 hours)**, failing to reflect dynamic *in vivo* conditions.
- **HFIM** is an advanced **two-compartment *in vitro* model**, allowing precise **control of drug concentrations** and **simulating dynamic *in vivo* conditions**.
- HFIM is a **complementary tool in drug development**, not a replacement for animal models or clinical trials.
- HFIM allows the **evaluation of drug efficacy**, the study of **drug resistance**, and the assessment of **combination regimens**.
- This model has a **wide range of applications**, including **testing for intracellular bacteria**.

CHAPTER II – CONTEXTUALIZED OBJECTIVES

Problematic

Intracellular infections caused by *S. aureus* represent a major public health challenge due to the bacterium's ability to invade and persist within host cells. Inside these cells, *S. aureus* evades immune defenses and the activity of many antibiotics, complicating its eradication and often leading to chronic or recurrent infections. This presents a critical **pharmacodynamic (PD)** challenge, as the intracellular environment influences bacterial susceptibility to antibiotics. Additionally, subtherapeutic drug concentrations achieved within host cells may lead to the emergence of antibiotic resistance, further complicating treatment. This highlights the importance of understanding the **pharmacokinetics (PK)** of antibiotics at the cellular level, specifically their ability to reach and maintain effective concentrations.

Among antibiotics, FQs, due to their broad activity spectrum and ability to penetrate and accumulate inside cells, are promising candidates for treating intracellular infections. However, their intracellular activity can be influenced by several factors, including efflux transporters, such as ABC transporters, which can limit FQ accumulation in host cells. Furthermore, **genetic polymorphisms** in the genes coding for these transporters might contribute to interindividual variability in drug PK, thereby potentially affecting therapeutic outcomes and explaining differences observed between patients. Finally, physiological fluctuations in drug extracellular levels, mainly driven by dosing frequency and ADME mechanisms, further modulate intracellular drug concentration and, therefore, the drug's activity, posing an additional challenge for optimizing therapy.

Hypothesis

We hypothesize that ABC transporters and their genetic polymorphisms (e.g., single nucleotide polymorphisms, SNPs) influence the cellular disposition of FQs, such as ciprofloxacin (CIP) and moxifloxacin (MXF). Specifically, suboptimal cellular concentrations of these antibiotics, mediated by transporter activity, genetic polymorphisms and physiological extracellular drug concentration fluctuations, could modulate their efficacy against intracellular *S. aureus*.

Contextualized objectives

Main objective

To investigate the PK, PD, and pharmacogenetic parameters influencing the intracellular activity of fluoroquinolones, particularly CIP and MXF, against intracellular *S. aureus*.

Specific objectives

(1) Pharmacokinetics (PK): to evaluate the implication of ABC transporters in modulating the cellular disposition of CIP and MXF; **(2)** Pharmacogenetics: to assess the impact of common polymorphisms in ABC transporters on their ability to transport CIP and MXF at the cellular levels; **(3)** Pharmacodynamics (PD): to investigate the intracellular (and extracellular) activity of CIP and MXF against *S. aureus* under dynamic conditions mimicking systemic drug exposure fluctuations, compared with conventional static conditions (i.e., constant drug exposure).

Research strategy

The research strategy is divided into two main parts, each corresponding to a specific experimental model and to distinct scientific articles (presented in Chapter III).

1. In vitro models using HEK293 cell lines (Chapter III – article n°1)

HEK293 cell lines overexpressing efflux ABC transporters of interest (ABCB1, ABCG2, and ABCC4) were used, including variants with functional SNPs:

- a. *ABCB1*: c.1199G>A (rs2229109) and the common haplotype c.1236C>T (rs1128503), c.2677G>T/A (rs2032582) and c.3435C>T (rs1045642);
- b. *ABCG2*: c.421C>A (rs2231142).

These models allowed for the *in vitro* evaluation of ABC transporters impact on the cellular disposition of CIP and MXF, as well as the impact of specific genetic polymorphisms on transporter activity. The use of specific inhibitors allowed for confirm the contribution of transporter activity in FQ disposition.

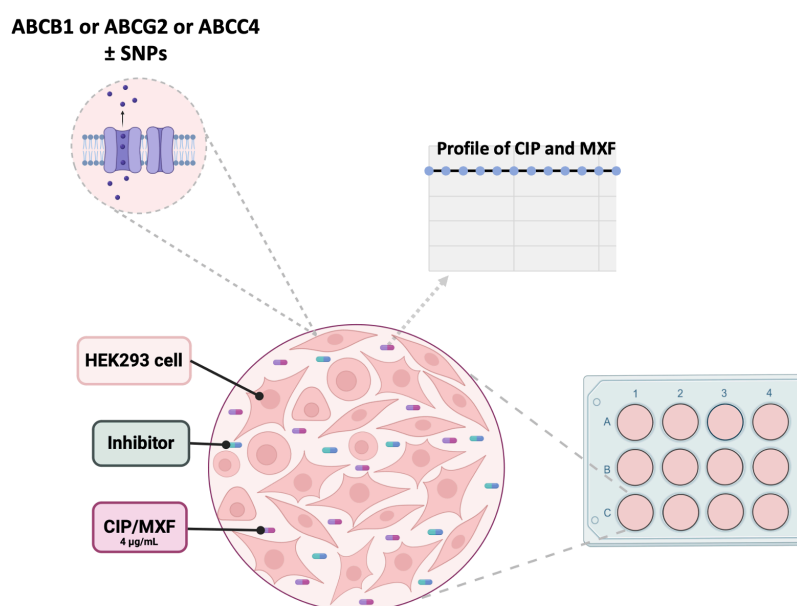


Figure II.1. Illustration of the *in vitro* model using HEK293 cell lines. The diagram represents recombinant HEK293 cell models overexpressing proteins of interest. These cells were used to evaluate the implication of transporters in the cellular disposition of CIP and MXF. Experiments were conducted at their C_{max} , in the presence or absence of transporter-specific inhibitor(s).

2. *In vitro* dynamic model (PK/PD model) (Chapter III – article n°2)

A hollow fiber infection model (HFIM) was developed to simulate CIP and MXF fluctuations in plasma concentrations as generally observed in patients and to assess their impact on both intracellular and extracellular activity against *S. aureus*. By closely mimicking real-time concentrations, the HFIM provided insights about the interplay between fluctuating drug concentrations, intracellular accumulation, and antibacterial efficacy, offering a realistic framework for understanding drug behavior and therapeutic outcomes.

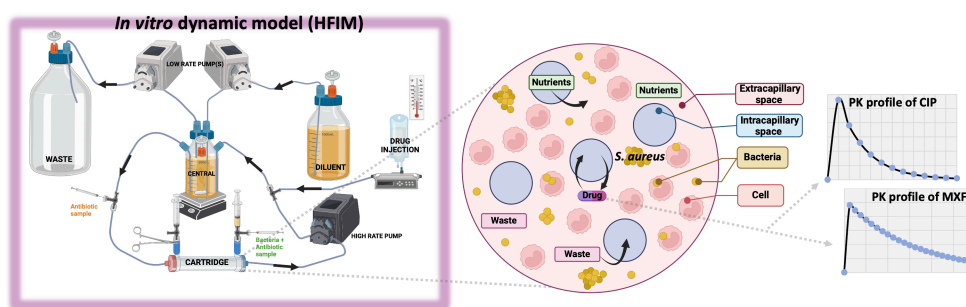


Figure II.2. Illustration of the HFIM. The diagram first depicts the complete setup of the system, including the interconnection between compartments. A detailed view of the cartridge, used as a PK/PD compartment, is presented, showing the environment where intracellular and extracellular *S. aureus* viability are monitored over time, under fluctuating CIP or MXF concentrations.

CHAPTER III – PERSONAL RESEARCH WORK

III.1 *Impact of ABC transporters and genetic polymorphisms on fluoroquinolone accumulation*

III.1.1 Considerations on basal transporter expression

For the experiments detailed in article n°1, we used human embryonic kidney (HEK293) cell lines, a widely employed model for studying the *in vitro* impact of ABC transporters. These models were specifically designed to stably overexpress the transporter of interest along with its associated SNP(s) [117,126]. However, before interpreting the results, key considerations must be addressed.

Endogenous transporters are present at basal levels in control HEK293 cells, and their expression can vary between cell batches. This variability can influence experimental outcomes; endogenous transporter activity may interfere with or mask the functional effects of the overexpressed protein, complicating data interpretation. Batch-to-batch differences in mRNA expression, further confirmed at the protein levels by Western blot, have been linked to inconsistencies in results, particularly regarding substrate efflux. For instance, CIP was identified as a substrate of ABCB1 in one transfected cell line but not in another, despite both expressing the same wild-type (WT) ABCB1 transporter.

To address these challenges, **this thesis recommends systematic validation of transporter expression in all experimental controls.** Ensuring consistency in baseline expression is crucial to accurately assess the contribution of an overexpressed transporter while minimizing potential interference from endogenous transporters.

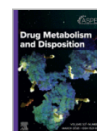
III.1.2 Article n°1



Drug Metabolism and Disposition

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Article

In vitro assessment of ATP-binding cassette transporters and their functional genetic polymorphisms on fluoroquinolone accumulation in human embryonic kidney 293 recombinant cell lines

Gwenaëlle Mahieu^{1,2}, Vincent Haufroid^{3,4}, Françoise Van Bambeke¹⁺ , Laure Elens^{2,3+}

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***In vitro* assessment of ATP-binding cassette transporters and their functional genetic polymorphisms on fluoroquinolone accumulation in human embryonic kidney 293 recombinant cell lines**

Gwenaëlle Mahieu, Vincent Haufroid, Françoise Van Bambeke⁺, Laure Elens⁺

⁺These authors share equal contribution as last authors.

Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium (M. G., F. V. B.) ; Integrated PharmacoMetrics, pharmacoGenomics and Pharmacokinetics (PMGK) Research group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium (M. G., L. E.) ; Louvain centre for Toxicology and Applied Pharmacology, Institut de recherche expérimentale et clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium (V. H., L. E.) ; Department of Clinical Chemistry, Cliniques Universitaires Saint-Luc, Brussels, Belgium (V. H.).

Running Title: Transporters and SNPs affecting fluoroquinolone disposition

Corresponding Author: Françoise Van Bambeke, Prof. Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute (LDRI). Université catholique de Louvain (UCLouvain). Avenue Emmanuel Mounier 73, B1.73.05, 1200, Brussels, Belgium. Tel: +32-2-764.73.78. e-mail: francoise.vanbambeke@uclouvain.be

Non-standard Abbreviations: ABC, ATP-Binding Cassette; ABCB1, ATP-Binding Cassette subfamily B member 1; ABCC2,4, ATP-Binding Cassette subfamily C member 2, 4; ABCG2, ATP-Binding Cassette subfamily G member 2; ANOVA, Analysis Of Variance; BCRP, Breast Cancer Resistance Protein; CIP, Ciprofloxacin;

C_{max}, maximum plasma Concentration; FITC, Fluorescein isothiocyanate; HEK293, Human Embryonic Kidney 293; HRP, horseradish peroxidase; MAF, Minor Allele Frequency; MRP, Multidrug Resistance Protein; MRP4, Multidrug Resistance Protein 4; MXF, Moxifloxacin; P-gp, P-glycoprotein; PheA, Pheophorbide A; qRT-PCR, quantitative Real-Time Polymerase Chain Reaction; Rh123, Rhodamine 123; SNP(s), Single Nucleotide Polymorphism(s); TBS-T, Tris-Buffered Saline with 0.02% Tween; WT, Wild-Type.

Significance Statement

This study demonstrates that moxifloxacin and ciprofloxacin are substrates of ABCB1, with ciprofloxacin also transported by ABCC4. Specific *ABCB1* polymorphisms (CGT and TTT haplotypes) reduce the ABCB1 transport capacity towards fluoroquinolones. These findings highlight the importance of considering ABCB1 and ABCC4 inducers or inhibitors, which may affect fluoroquinolone disposition in tissues and cells, as well as *ABCB1* polymorphisms that could explain interindividual variability in pharmacokinetic profiles.

a) Abstract

Fluoroquinolone tissue distribution and cellular accumulation are hindered by efflux transporters, including ABCB1, ABCG2, and ABCC4. Genetic polymorphisms (single nucleotide polymorphisms) can impact transporter activity, leading to interindividual variability in systemic and cellular pharmacokinetics of their substrates. This study assesses the impact of these transporters on moxifloxacin and ciprofloxacin cellular accumulation *in vitro*, and the effect of common single nucleotide polymorphisms in *ABCB1* (c.1199G>A (rs2229109); common haplotype c.1236C>T (rs1128503), c.2677G>T/A (rs2032582), and c.3435C>T (rs1045642)) and in *ABCG2* (c.421C>A (rs2231142)). Recombinant human embryonic kidney cell lines overexpressing wild-type or variant transporters were generated via stable plasmid transfection. The impact of transporter overexpression on fluoroquinolone cell disposition was assessed through accumulation experiments in the presence of specific inhibitors to establish the link between transporter expression and differential accumulation. Results indicated that ABCB1 overexpression reduced moxifloxacin cellular concentration by 30% but inconsistently that of ciprofloxacin, and that zosuquidar or elacridar reversed these effects. ABCG2 had no impact. ABCC4 markedly reduced ciprofloxacin accumulation by 25%, even at a basal level, an effect reversed by MK517. Contrary to the wild-type and the c.1199A carriers, *ABCB1* CGT and TTT variants did not reduce antibiotic accumulation. In conclusion, moxifloxacin and ciprofloxacin are substrates of the wild-type and 1199G>A ABCB1, while CGT and TTT haplotypes had a marginal impact on fluoroquinolone transport by ABCB1. Ciprofloxacin is a preferential ABCC4 substrate. Due to the large body distribution of these transporters, our findings may help rationalize their role and the impact of their polymorphisms in fluoroquinolone disposition in tissues and cells.

b) Introduction

The fluoroquinolones moxifloxacin (MXF) and ciprofloxacin (CIP) are widely used in clinical practice due to their broad spectrum of activity, high bactericidal activity [9,10,49], ability to accumulate in phagocytic and non-phagocytic cells [15,177,178], and wide tissue distribution [49]. As they can easily reach infection sites in various locations and accumulate therein, they exhibit useful activity against several intracellular bacteria thriving in different subcellular compartments [11–14].

However, this accumulation is controlled and possibly limited by efflux transporters that affect their body disposition and intracellular accumulation in infected cells, impacting their activity against intracellular bacteria [16–18,67,87]. Transporter genes represent an important proportion of the human genome, as transporters exert vital roles in the bioavailability and cellular trafficking of endogenous and exogenous compounds. The ATP-binding cassette (ABC) superfamily is composed of 48 efflux transporters classified into seven subfamilies, designated A to G, based on their sequence homology [19,103]. Located in the plasma membrane, these ATP-dependent active transporters unidirectionally translocate a wide range of substances (lipids, bile acids, xenobiotics, and peptides) from the intracellular to the extracellular compartments [19]. By reducing the cellular concentration of potentially toxic compounds, they play a key role in body and tissue protection and detoxification [112,179].

Several lines of evidence indicate that certain efflux transporters may impact fluoroquinolone cellular accumulation. A potential role of ABCB1 (P-glycoprotein, P-gp) for MXF [96,97] and CIP [98,180] and of ABCG2 (breast cancer resistance protein, BCRP) [93,99] and ABCC4 (multidrug resistance protein 4, MRP4) [17,67,87,99] for CIP has been demonstrated.

However, these data have been generated using different cell models (J774 macrophages, Calu-3 epithelial cells, Madin-Darby canine kidney II (MDCKII) cells, and intestinal Caco-2 cells) or experimental procedures (transepithelial transport), rendering comparison difficult or impossible. Furthermore, these studies did not consider the potential influence of genetic polymorphisms.

In this study, we have evaluated the effect of these three ATP-binding cassette (ABC) transporters on MXF and CIP transport activity using comparable *in vitro* models of recombinant human embryonic kidney 293 (HEK293) cell lines. We also assessed the potential functional impact of the most common coding single nucleotide polymorphisms (SNPs) in ABCB1 and ABCG2 (see Table S1 for a summary of the frequency of these SNPs in different ethnic groups). The *ABCB1* c.1199G>A coding SNP, located in exon 11 (rs2229109), has a reported minor allelic frequency (MAF) of around 6% in Caucasians and is associated with a serine to asparagine substitution at position 400 (Ser400Asn) in the sixth cytoplasmic loop of the ABCB1 transporter, a domain involved in substrate recognition [117]. The three most common variants in the *ABCB1* coding region (rs1128503 (1236C>T, Gly412Gly), rs2032582 (2677G>T/A, Ala893Ser/Thr) and rs1045642 (3435C>T, Ile1145Ile)) are located in the sixth, tenth, and twelfth cytoplasmic loops, respectively. Among these, rs1128503 and rs1045642 are synonymous, while rs2032582 is nonsynonymous. These variants are commonly found in strong linkage disequilibrium, with a MAF of about 50% for the variant haplotype (TTT) in the Caucasian population [19,115]. Lastly, the *ABCG2* c.421C>A coding SNP located in exon 5 (rs2231142) has a reported MAF of approximately 5-10% in Caucasians [181] and is associated with a glutamine-to-lysine substitution at position 141 (Gln141Lys) [19] in the first cytoplasmic loop. Recent studies have associated this polymorphism with protein folding and trafficking default, partial degradation, and reduced overall and cell surface expression [182].

c) Materials and Methods

Drugs and chemicals

Moxifloxacin-HCl (MXF) and ciprofloxacin-HCl (CIP) were provided as microbiological standards by Bayer AG (Leverkusen, Germany); rhodamine 123 (Rh123), LY335979 (zosuquidar HCl), GF120918 (elacridar), MK571 NaCl, and Ko143 hydrate were obtained from Sigma-Aldrich (St-Louis, MO). Dulbecco's modified eagle medium, fetal bovine serum, penicillin/streptomycin (10,000 U mL⁻¹) and enzyme-free cell dissociation buffer were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA); geneticin (G418) and hygromycin B were purchased from Roche (Basel, Switzerland), and pheophorbide a (PheA) was purchased from Sanbio (UDEN, The Netherlands). All flow cytometry antibodies (ABCB1 antibodies (Fluorescein isothiocyanate (FITC) mouse anti-human CD243, clone 17F9, reference 557002; and FITC mouse IgG2b κ isotype control, clone 27–35, reference 555742); ABCG2 antibodies (Peridinin Chlorophyll-Cyanine 5-5-a (PerCP-CyTM5.5) mouse anti-human CD338, reference 561460; and PerCP-CyTM5.5 mouse IgG2b κ isotype control, reference 558304)) were purchased from BD Biosciences (Franklin Lakes, NJ). All chemicals used in drug quantification were of analytical grade.

Cell lines and cell culture

All HEK293 cells were grown in Dulbecco's modified eagle medium with 4.5 g L⁻¹ glucose, L-glutamine, and sodium pyruvate supplemented with 10% (v/v) of fetal bovine serum and 1% (v/v) of penicillin/streptomycin solution at a temperature of 37 °C in a 5% CO₂ atmosphere. For subculture, cells were detached using enzyme-free cell dissociation buffer. Cells were used between the second and the 13th passage, with verification of the transporter expression level before the first ad after the last experiment.

HEK293 cells overexpressing the ABCC4 transporter were kindly supplied by Elaine M. Leslie from the University of Alberta (Canada) and cultured in the presence of G418 ($600 \mu\text{g mL}^{-1}$) to select stable recombinant cells [183].

Generation of ABCB1 recombinant cells

The generation of stable recombinant cell lines has been described previously [117,126]. In short, HEK293 cells were transfected with a pcDNA3.1 vector with cDNA encoding either the ABCB1 wild-type (WT), variant, or an empty vector using Lipofectamine (Thermo Fisher Scientific). Stable recombinant cells were selected by incubating the transfected cells with G418 (1 mg mL^{-1}) 48 h after transfection. For ABCB1_{1199G > A}, three recombinant cell lines were generated: HEK_{control} (pcDNA3.1 empty vector), HEK_{1199G} (wild-type), and HEK_{1199A} (variant), and designated as CTL, 1199G, and 1199A in this study. For ABCB1_{1236C>T - 2677G>T - 3435C>T}, four cell lines were used: HEK_{control}, HEK_{1236C-2677G-3435C} (wild-type), and two different variant combinations, HEK_{1236C-2677G-3435T} and HEK_{1236T-2677T-3435T}, referred to as CTL, CGC, CGT, and TTT, respectively.

Generation of ABCG2 recombinant cells

Details of the model development have been described previously [184]. Briefly, HEK293 cells were transfected by Lipofectamine (Thermo Fisher Scientific) with a pCMV3 vector containing cDNA encoding either the ABCG2 WT (421C), the variant (421A), or a pCMV3 empty vector. Stable recombinant cells were selected by incubating the transfected cells with hygromycin B (0.5 mg mL^{-1}) 48 h after transfection. Three recombinant cell lines were generated, consisting of cell lines stably transfected with the pCMV3 empty vector (HEK_{control}), the WT vector (HEK_{421C}), or the variant vector (HEK_{421A}). These will be further referred to as CTL, 421C, and 421A.

Characterization of transfected cell lines

Characterization of transporter expression

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify the number of *ABCB1*, *ABCG2*, *ABCC2*, and *ABCC4* gene copies in the different cell lines. Briefly, RNA was extracted from cells (5×10^6 cells) using the RNeasy Mini Kit from Qiagen (Hilden, Germany) and reverse transcribed into cDNA following the GoScript Reverse Transcription Mix, Oligo(dT) Protocol (Promega, Madison, US) according to manufacturer instructions. qRT-PCR was carried out with SYBR-green (Bio-Rad, Hercules, CA) using the CFX96 Touch Real-Time PCR detection system (Bio-Rad). *18S* expression was used to normalize the expression of *ABCB1*, *ABCG2*, *ABCC2*, and *ABCC4*. The primer sequences for *18S*, *ABCB1*, *ABCG2*, *ABCC2*, and *ABCC4* were as follows: (forward) 5'-CGG CTA CCA CAT CCA AGG AA -3' (reverse) 5'-ATA CGC TAT TGG AGC TGG AAT TAC C -3' ; (forward) 5'-AGC AAA GGA GGC CAA CAT AC -3' (reverse) 5'-GGC TTC ATC CAA AAG CAA AA -3' ; (forward) 5'-CAG GAC TCA ATG CAA CAG GA-3' (reverse) 5'-TTC AGG TAG GCA ATT GTG AGG-3' ; (forward) 5'-CCT CAT TCA GAC GAC CAT CC-3' (reverse) 5'-ATT CTC AAT GCC AGC TTC CT-3' and (forward) 5'-AAA ATG TAC GCC TGG GAA AA-3' (reverse) 5'-GGT GAA GGT CAC AAA CAC GA-3'.

Western blot

The expression of transporter proteins was further characterized through western blot, according to a previously published method with certain modifications [117,185]. Cell pellets were harvested using an adapted Radio ImmunoPrecipitation Assay lysis buffer (sodium deoxycholate 1%, tetraethylammonium bromide 100 mM, NaCl 300 mM, IGEPAL (Sigma-Aldrich) 2%, SDS 0.2%, n-dodecyl- β -D-maltoside

0.4%, pH 7.2-7.5) supplemented with a complete protease inhibitor cocktail (Roche) and incubated for 15 minutes on ice. Following this incubation, the cell lysates were then vortexed for 30 seconds and further diluted in LDS Sample Buffer (Invitrogen, Thermo Fisher Scientific) containing 25% of sample reducing agent (Invitrogen, Thermo Fisher Scientific) and water to achieve total protein concentrations of 12, 6, 3 μg for ABCB1; 10 μg for ABCG2 and ABCC4 in a final volume of 20 μL per sample. Each sample (20 μL) and 4 μL of protein ladder (PageRuler Prestained, Thermo Fisher Scientific) were loaded onto a 4-12% Bis-Tris gel (NuPAGE, Thermo Fisher Scientific). The electrophoretic migration and the transfer of proteins onto a polyvinylidene difluoride transfer membrane (Thermo Fisher Scientific) were then achieved using a blotting pad (VWR, Leuven, Belgium) at an initial voltage of 175 V for 60 minutes followed by a reduced voltage of 30 V for 60 minutes. Blots were blocked with bovine serum albumin 1% in Tris-Buffered Saline with 0.02% Tween (TBS-T) pH 8 for at least 60 minutes. After three washing steps with Tris-Buffered Saline with 0.02% Tween (TBS-T) during 5 minutes, membranes were incubated overnight at 4°C with a monoclonal primary antibody depending on the targeting protein: anti-PgP (ab170904, rabbit, 1:10000 dilution, Abcam) for ABCB1; anti-MRP4 (ab233382, rabbit, 1:1000, Abcam) for ABCC4; anti-BCRP (ab207732, rabbit, 1:1000, Abcam) for ABCG2; and the monoclonal anti- β -Actin antibody (A1978, mouse, 1:2000 dilution, Sigma-Aldrich) for β -actin, diluted in Tris-Buffered Saline with 0.02% Tween (TBS-T) with milk powder (150 mg 5 mL⁻¹ of TBS-T). The next day, after three steps of 5 minutes washing in TBS-T, membranes were incubated for 60 minutes with a secondary antibody diluted in TBS-T with milk powder (Anti-rabbit IgG horseradish peroxidase (HRP) (A27036, 1:2500 dilution, Invitrogen) for ABCB1 and ABCG2; Anti-Rabbit IgG H&L (HRP) (ab205718, 1/20 000 dilution, Abcam) for ABCC4; and Anti-mouse IgG (H+L) (A27025, 1:2500 dilution, Invitrogen) for β -Actin).

Subsequently, after two additional 5-minute washing steps in TBS-T and one washing step in Tris-Buffered Saline, protein detection was accomplished through chemiluminescence using HRP substrate (Immobilon Classico, Millipore, Burlington, MA).

Flow cytometry

ABCB1 and ABCG2 expression were evaluated after one week of growth in the presence of the selection antibiotics (G418 and Hygromycin B, respectively). The cell surface expression protein for ABCB1 and ABCG2 was evaluated by flow cytometry as previously described [186]. Briefly, for each cell line, 1×10^6 cells were collected by centrifugation and washed three times with ice-cold FACS buffer (PBS supplemented with 1% decompemented FBS and 1 mM EDTA [Thermo Fisher Scientific]). Cells were then incubated for 45 min on ice in the dark with buffer containing the following antibodies (1×10^6 cells/condition): (A) anti-ABCB1 antibody (1:10 dilution in 100 μ L of buffer) or anti-ABCG2 antibody (1:20 dilution in 100 μ L of buffer); (B) with the corresponding isotype control (diluted 1:10 in 100 μ L of buffer for ABCB1 or isotype control diluted 1:5 for ABCG2 in 100 μ L of buffer); (C) in 100 μ L of buffer with no antibody. After incubation, the cells were washed with ice-cold buffer, centrifuged, and resuspended before being analyzed using a BD FACSVerse flow cytometer with BD FACSuite software. Additional data analysis and plotting were carried out in FlowJo (version 10.6.1).

Characterization of transporter functionality using fluorescent substrates

For ABCB1, the functionality of the overexpressed transporter was assessed using the reference substrate Rh123 and two different ABCB1 inhibitors, namely zosuquidar [117,131,187] and elacridar [130], which differ by their mechanism of inhibition. Elacridar modulates ATPase activity, inhibiting ATP hydrolysis, essential for transporter activity [130].

By contrast, zosuquidar directly docks in the substrate pocket of the transporter, blocking the change of conformation required for substrate efflux [131]. The functionality of ABCG2 was assessed using PheA, a specific ABCG2 fluorescent substrate [188], and the ABCG2 inhibitor Ko143.

For these functional assays, 350,000 cells were plated in 12-well plates in complete medium one day before the experiment was performed. The next day, cells were incubated either for 120 minutes at 37 °C in a 5% CO₂ atmosphere in the dark with 5 µM Rh123 alone or in combination with either 0.2 µM zosuquidar or 5 µM elacridar, or for 60 minutes with 5 µM PheA alone or in combination with 1 µM Ko143. At the end of the incubation, the supernatants were discarded, the cells were washed twice with PBS at 4 °C, and lysed by adding 200 µL of water per well followed by overnight agitation in the dark at 4 °C. After 10 min of centrifugation at 20,817 RCF, the fluorescence of Rh123 and PheA in the supernatant were measured, using a Spectramax M3 fluorimeter (Molecular Devices, San José, CA) with excitation and emission wavelengths set at 485/530 nm and 410/600 nm, respectively.

Fluoroquinolone accumulation experiments

One day before the experiment, 350,000 cells were seeded in 12-well plates in 2 mL of complete medium. The next day, the medium was removed and replaced with fresh complete medium containing either MXF or CIP at 4 µg mL⁻¹ (9.13 µM and 10.36 µM respectively), corresponding to the maximum plasma concentration (C_{max}; total drug concentration) observed in patients undergoing conventional therapy [189,190], alone or combined with ABCB1 inhibitors (zosuquidar 0.2 µM or elacridar 5 µM), ABCG2 inhibitor (Ko143 1 µM), or a general Multidrug Resistance Protein (MRP) inhibitor (MK571 25 µM) to assess the role of ABCC4 [142]. After 120 minutes of incubation at 37 °C in a 5% CO₂ atmosphere, the medium was discarded,

cells were washed twice with PBS at 4 °C, and lysed by adding 200 µL of glycine-HCl buffer (pH 3, exalting fluoroquinolone fluorescence for quantification [17,67]) followed by overnight agitation in the dark at 4 °C. The next day, both cells and supernatant were transferred to a 1.5 mL Eppendorf (Hamburg, Germany) and centrifuged at 20,817 RCF for 10 minutes at 4 °C. Quantification of MXF and CIP was performed in the supernatant and proteins in the cell pellets for normalization.

Quantification of cell-associated fluoroquinolones

The cellular concentrations of MXF and CIP were quantified by fluorometry using a Spectramax M3 fluorimeter with excitation and emission wavelengths set at 298/504 nm and at 275/450 nm, respectively [17,67]. Calibration curves for both MXF and CIP were established within a concentration range of 10 ng mL⁻¹ to 300 ng mL⁻¹ in glycine-HCl buffer (pH 3) and quantified in a similar fashion. For each experiment, the absolute amounts of MXF and CIP recovered from the cell extracts were normalized to the amount of proteins measured using a DC (detergent compatible) Protein Assay from Bio-Rad (Hercules, CA) according to the manufacturer's instructions.

Statistics and data analysis

All accumulation data are mean ± standard error of the mean (SEM) expressed as a percentage of the CTL value (100%) of *n* experiments repeated at least three times (*N* = 3) in independent triplicates (*n* = 3 different wells), using GraphPad Prism version 8.0 (GraphPad software, San Diego, CA). Two-way analysis of variance (ANOVA) was performed under the null hypothesis that the means of the groups compared were equal. When the differences between the means were significant (*P* < 0.05), Tukey's multiple comparison tests were performed.

d) Results

Characterization and validation of the recombinant models

Transporter expression

The expression of the different transporters of interest was quantified by qRT-PCR across all models, revealing marked increases in recombinant transporter transcripts compared to control cells (100-fold for *ABCB1* (Figure 1A), 1000-fold for *ABCG2* (Figure 1B), and 36-fold for *ABCC4* (Figure 1C)). At the protein level, western blot analysis showed stronger signals in recombinant models than in controls (Figures S1-S2-S3, supplemental data). Flow cytometry analysis of membrane transporter expression indicated that over 92% of the recombinant cell populations expressed the transporter, whereas control cells were negative for transporter expression (Figure S4, supplemental data for flow cytometry profiles of *ABCB1* and *ABCG2*; no data for *ABCC4*). The expression of *ABCC2*, also described as a multidrug transporter, was similar and negligible (<0.1%) in all cell lines (Figure S5, supplemental data).

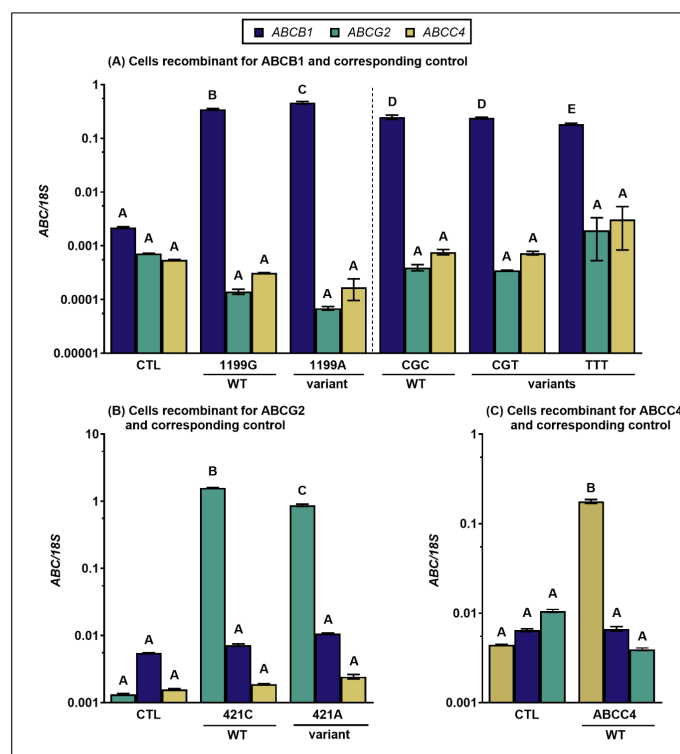


Figure 1. ABC transporter RNA transcripts. ABC transporter RNA expression levels normalized to the 18S expression level (housekeeping gene) in (A) HEK_{control} (CTL), HEK_{1199G>A} (1199G, 1199A) and HEK_{1236C>T-2677G>T-3435C>T} (CGC, CGT, TTT) cell lines transfected to overexpress ABCB1 WT or its variants; (B) HEK_{control} (CTL) and HEK_{421C>A} (421C, 421A) cell lines transfected to overexpress ABCG2 WT or its variants; (C) HEK_{control} (CTL) and HEK_{ABCC4} (ABCC4) cell lines transfected to overexpress ABCC4 WT. The HEK_{control} (CTL) in each panel corresponds to the clone used to overexpress the corresponding transporter and transfected by the corresponding empty vector. Results are reported as mean $\pm$ SD (n = 3). Statistical analysis: 2-way ANOVA with Tukey post-hoc test, comparison between all transporters in all cell lines. Values with different letters are statistically different from each other ($P < 0.05$).

Transporter functionality

The functionality of ABCB1 and ABCG2 was evaluated by measuring their capacity to export specific fluorescent substrates, Rh123 and PheA, respectively. The results (Figure 2A, 2B) show significantly lower intracellular Rh123 fluorescence in cells overexpressing ABCB1 compared to control (CTL) cells, with a reduction of approximately four-fold ($P < 0.0009$) and, with no differences in Rh123

accumulation between WT (1199G, CGC) and variant (1199A, CGT, TTT) cell lines, indicating no impact of the studied polymorphisms on Rh123 accumulation. The addition of the specific ABCB1 inhibitor zosuquidar (Zo; $K_i = 59$ nM) [187] slightly increased the accumulation of Rh123 in control cells, which express a low but detectable basal level of *ABCB1* RNA transcripts (Figure 1A). Most evidently, both zosuquidar and the non-specific ABCB1 inhibitor elacridar (Ela; $K_i = 1.16$ nM) [191] restored Rh123 cellular accumulation in ABCB1-transfected cells, bringing its concentration back to levels closer to those measured in the corresponding CTL cells.

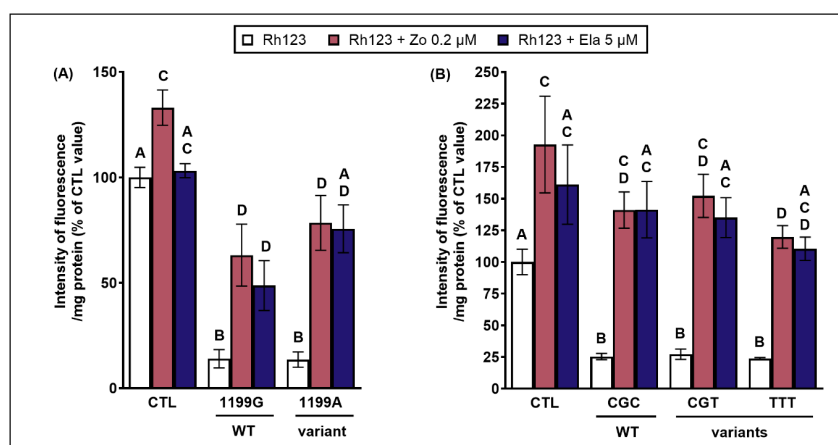


Figure 2. Impact of ABCB1 transporter on the cellular accumulation of rhodamine 123 (Rh123). Cell-associated fluorescence of Rh123 (5 μ M) after 2 h of incubation in control conditions or in the presence of the ABCB1 inhibitors zosuquidar (Zo; 0.2 μ M) or elacridar (Ela; 5 μ M) in (A) HEK_{control} (CTL), HEK_{wild type} (1199G), HEK_{variant} (1199A) and (B) HEK_{control} (CTL), HEK_{wild type} (CGC), and HEK_{variant} (CGT, TTT) cell lines. The HEK_{control} (CTL) in each panel corresponds to the clone used to overexpress the corresponding transporter and transfected by the corresponding empty vector. Intensity of fluorescence is reported in arbitrary units. Results are reported as mean $\pm$ SEM (n = 9) expressed as a percentage of the CTL value (100%). Statistical analysis: 2-way ANOVA with Tukey post-hoc test, comparison between all conditions in all cell lines. Values with different letters are statistically different from each other ($P < 0.05$).

Similar findings were obtained for the ABCG2 transporter and the PheA substrate. As shown in Figure 3, PheA fluorescence levels were lower in recombinant ABCG2 cells (421C, 421A) compared to control cells (CTL), with a reduction of approximately 50% ($P < 0.0026$). The addition of the ABCG2 specific inhibitor Ko143 ($K_i = 25.9$ nM) [154,192] restored PheA cell-associated fluorescence in all recombinant cells. No effect of the 421C>A ABCG2 polymorphism was observed.

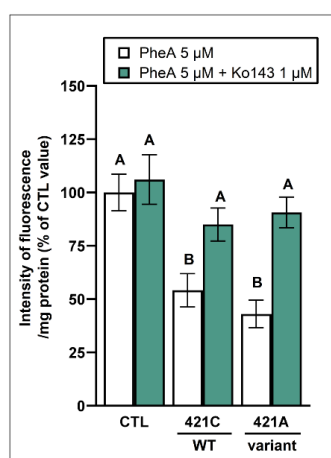


Figure 3. Impact of ABCG2 transporter on the cellular accumulation of pheophorbide a (PheA). Cell-associated fluorescence of PheA (5 μ M) after 1 h of incubation in control conditions or in the presence of the ABCG2 inhibitor Ko143 (1 μ M) in HEK_{control} (CTL), HEK_{wild type} (421C) and HEK_{variant} (421A). The HEK_{control} (CTL) in each panel corresponds to the clone used to overexpress the corresponding transporter and transfected by the corresponding empty vector. Intensity of fluorescence is reported in arbitrary units. Results are reported as mean $\pm$ SEM (n = 9) expressed as a percentage of the CTL value (100%). Statistical analysis: 2-way ANOVA with Tukey post-hoc test, comparison between all conditions in all cell lines. Values with different letters are statistically different from each other ($P < 0.05$).

Effect of ABCB1 overexpression and polymorphisms on cellular fluoroquinolone concentrations

To evaluate the effect of transporters and inhibitors under clinically relevant conditions, experiments were conducted using fluoroquinolone concentrations corresponding to their C_{max}.

1199G>A polymorphism

A reduction of approximately 30% in the cellular concentration of MXF was observed in cell lines expressing either WT (1199G) or mutant (1199A) ABCB1, compared to the control (CTL) cells (Figure 4A1) ($P < 0.0040$). No significant difference in MXF concentration was observed between cells expressing the WT or the variant protein. The addition of zosuquidar or elacridar abolished the effect of the overexpressed transporter, resulting in cellular concentrations similar to those observed in the CTL cell line, while Ko143 and the non-specific inhibitor MK571 [191] were ineffective. In contrast, CIP showed the same level of cellular concentration in all cell lines, unaffected by Ko143, but significantly increased by MK571 in the CTL cell line ($P < 0.0001$) or by zosuquidar and elacridar in both transfected cell lines (Figure 4A2) ($P < 0.0470$).

1236C>T - 2677G>T - 3435C>T polymorphisms

Here also, the cellular concentration of MXF was significantly lower in the cell line overexpressing WT ABCB1 (CGC) compared to control cells (CTL) (Figure 4B1), showing a reduction of approximately 30% ($P < 0.0003$). Additionally, MXF concentration tended to be higher in CGT and TTT cells when compared to the WT haplotype (CGC) and no more different from that measured in CTL cells, suggesting that these two SNP haplotypes have a reduced effect on MXF transport. Regarding inhibitors, zosuquidar and, to a larger extent, elacridar increased MXF concentration in cells expressing the WT haplotype, but not significantly in cells expressing ABCB1 polymorphisms. As observed in the experiments conducted with the 1199G>A polymorphism, Ko143 and MK571 had no significant effect on MXF cellular disposition in any cell line. Regarding CIP, a small reduction in its cellular concentration was observed in the wild-type vs. CTL cells, contrary to what was reported in panel A2. It is attributed to the fact these are two different clones of wild-type cells, with possibly variable basal expression levels for this transporter.

Notably, CIP cellular concentration tended to be higher in cells overexpressing CGT or TTT variants than the WT transporter, reaching values similar to those measured in control cells. Elacridar, and to a lower extent zosuquidar, increased its accumulation in cells overexpressing the ABCB1 WT transporter or the CGT variant, but not the TTT variant (Figure 4B2). Here also, MK571 significantly increased CIP cellular disposition in CTL cells by approximately threefold ($P < 0.0001$), while Ko143 had no effect on CIP disposition in any cell line.

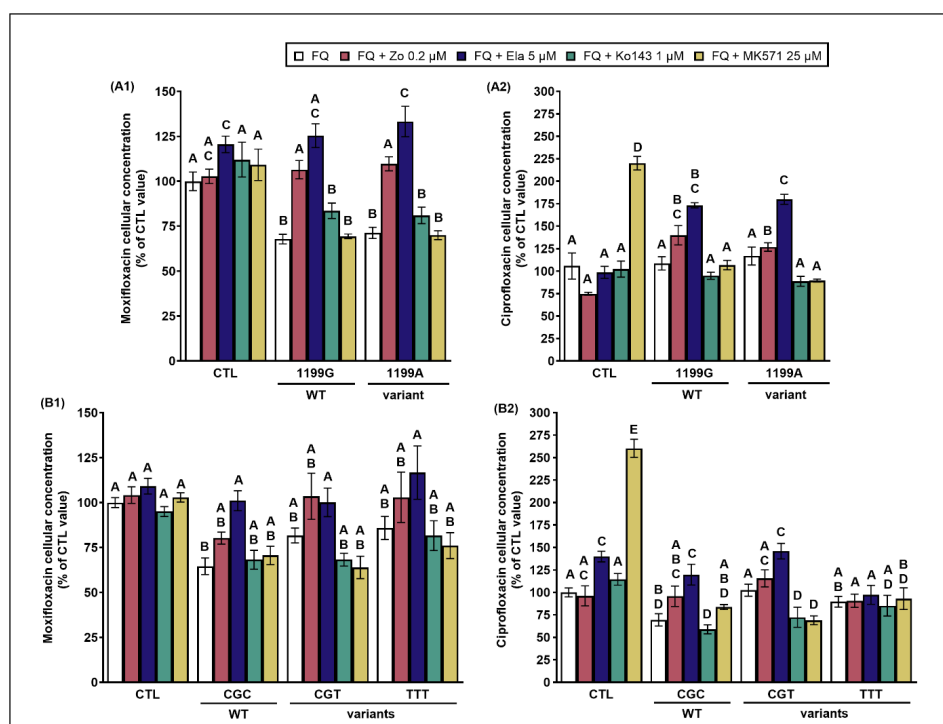


Figure 4. Impact of ABCB1 and its polymorphisms on MXF and CIP cellular accumulation. Cellular accumulation of the fluoroquinolones (FQ) MXF and CIP ($4 \mu\text{g mL}^{-1}$) after 2 h of incubation in control conditions or in the presence of zosuquidar (Zo; $0.2 \mu\text{M}$); elacridar (Ela; $5 \mu\text{M}$); Ko 143 ($1 \mu\text{M}$), or MK571 ($25 \mu\text{M}$) in **(A1 and A2)** HEK_{control} (CTL), HEK_{wild type} (1199G), HEK_{variant} (1199A), and **(B1 and B2)** HEK_{control} (CTL), HEK_{wild type} (CGC), HEK_{variant} (CGT, TTT). The HEK_{control} (CTL) in each panel corresponds to the clone used to overexpress the corresponding transporter and transfected by the corresponding empty vector. Results are reported as mean $\pm$ SEM ($n = 9$) expressed as a percentage of the CTL value (100%). Statistical analysis: 2-way ANOVA with Tukey post-hoc test, comparison between all conditions in all cell lines. Values with different letters are statistically different from each other ($P < 0.05$).

Effect of ABCG2 overexpression and 421C>A polymorphism on cellular fluoroquinolone concentrations

Given that ABCG2 is known to facilitate the transport of CIP, we investigated the effect of ABCG2 overexpression and the impact of the 421C>A polymorphism on the cellular accumulation of MXF and CIP (Figure 5A, 5B). Both WT (421C) and variant (421A) ABCG2 cell lines showed similar cellular concentrations of MXF or CIP as compared to control cell lines that express the transporter to a very low basal level (Figure 1B). Consistently, the addition of Ko143 had no effect on the MXF or CIP accumulation in any ABCG2 overexpressing cell lines. Furthermore, the addition of elacridar and MK571 increased the accumulation of MXF and CIP, respectively, in CTL cell lines ($P < 0.0001$).

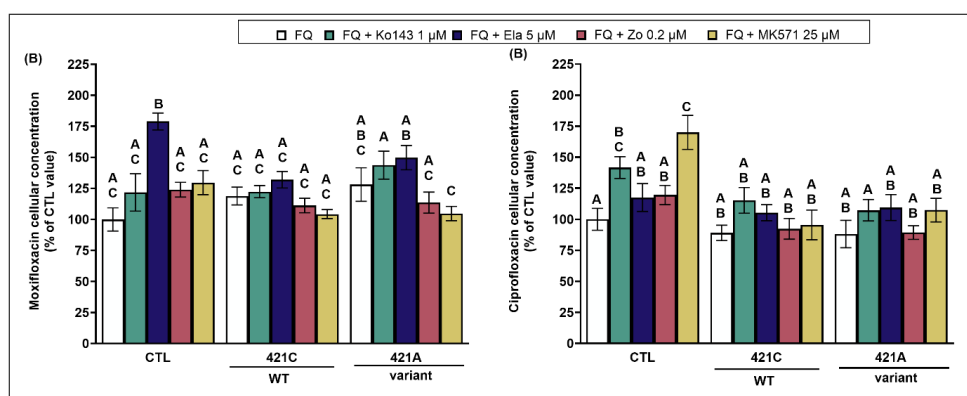


Figure 5. Impact of ABCG2 and polymorphisms on MXF (A) and CIP (B) cellular accumulation. Cellular accumulation of the fluoroquinolones (FQ) MXF (A) and CIP (B) ($4 \mu\text{g mL}^{-1}$) after 1 h of incubation in control conditions or in the presence of Ko143 (1 μM); elacridar (Ela; 5 μM); zosuquidar (Zo; 0.2 μM) or MK571 (25 μM) in HEK_{control} (CTL), HEK_{wild type} (421C), or HEK_{variant} (421A). The HEK_{control} (CTL) in each panel corresponds to the clone used to overexpress the corresponding transporter and transfected by the corresponding empty vector. Results are reported as mean $\pm$ SEM ($n = 9$) expressed as a percentage of the CTL value (100%). Statistical analysis: 2-way ANOVA with Tukey post-hoc test, comparison between all conditions in all cell lines. Values with different letters are statistically different from each other ($P < 0.05$).

Effect of ABCC4 overexpression on cellular fluoroquinolone concentrations

To elucidate our observation that MK571 increases CIP accumulation in both CTL cell lines, we evaluated the impact of the ABCC4 transporter on MXF and CIP accumulation in a HEK293 recombinant model overexpressing ABCC4. ABCC4 was specifically considered rather than other ABCC multidrug transporters, based on previous data indicating that neither ABCC1 nor ABCC2 were involved in CIP transport [67,193]. The results presented in Figures 6A and 6B indicate that the ABCC4 transporter had no impact on MXF accumulation but significantly reduced that of CIP, with a reduction of approximately 25% ($P < 0.0222$). Furthermore, MK571 enhanced CIP accumulation in recombinant cells expressing ABCC4 and in CTL cells ($P < 0.0001$), consistent with our previous experiments. This effect of MK571 on CIP accumulation was concentration-dependent in recombinant cells but not in CTL cells.

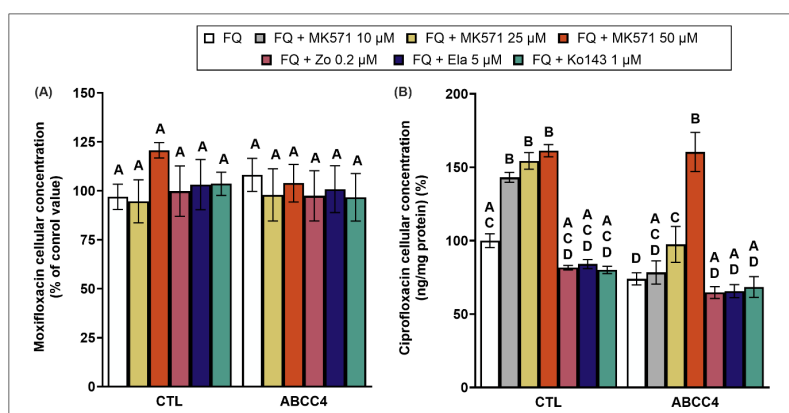


Figure 6. Impact of ABCC4 on MXF and CIP cellular accumulation. Cellular accumulation of the fluoroquinolones (FQ) MXF (A) and CIP (B) ($4 \mu\text{g mL}^{-1}$) after 1 h of incubation in control conditions or in the presence of MK571 (10 μM , 25 μM and 50 μM), zosuquidar (Zo; 0.2 μM); elacridar (Ela; 5 μM); Ko143 (1 μM) in HEK_{control} (CTL) and HEK_{ABCC4} (ABCC4). Results are reported as mean $\pm$ SEM ($n = 9$) expressed as a percentage of the CTL value (100%). The HEK_{control} (CTL) in each panel corresponds to the clone used to overexpress the corresponding transporter and transfected by the corresponding empty vector. Results are reported as mean $\pm$ SEM ($n = 9$) expressed as a percentage of the CTL value (100%). Statistical analysis: 2-way ANOVA with Tukey post-hoc test, comparison between all conditions in all cell lines. Values with different letters are statistically different from each other ($P < 0.05$).

e) Discussion

In this study, we assessed the functional relevance of ABCB1, ABCG2, and ABCC4 transporters and of key genetic polymorphisms in *ABCB1* and *ABCG2* (1199G>A; 1236C>T-2677G>T-3435C>T and 421C>A) for MXF and CIP cellular disposition. Stable HEK293 cell lines overexpressing WT (1199G and CGC for *ABCB1*; 421C for *ABCG2*) or variant alleles (1199A, CGT, and TTT for *ABCB1*; 421A for *ABCG2*) were generated.

While membrane localization of the transporters was previously demonstrated in our models [117,126,184,194], we tested the functionality of the overexpressed proteins by analyzing the accumulation of the ABCB1 and ABCG2 substrates [117,188] and assessing the effects of specific transporter inhibitors. We observed that Rh123 accumulation was fourfold lower in all ABCB1-transfected cell lines compared to CTL cells. These findings align with previous studies using the same cell model and further validate our experimental setting [117,126]. Furthermore, we demonstrate that Rh123 accumulation in CTL cells was slightly affected by the presence of the ABCB1 inhibitor zosuquidar (consistent with low ABCB1 basal expression in this model) and sensitive to both zosuquidar and elacridar in transfected cell lines. The concomitant use of the specific, competitive ABCB1 inhibitor zosuquidar [187] and of the non-specific, inhibiting ATP-hydrolysis, inhibitor elacridar enables more comprehensive ABCB1 inhibition by targeting different mechanisms [130,131]. It allows comparison of their efficacy, assessment of interactions with *ABCB1* polymorphisms, and prevents compensatory drug efflux via other transporters like ABCG2, also inhibited by elacridar ($K_i = 12.3$ nM) [192]. For the ABCG2 transporter, a similar conclusion can be drawn as PheA accumulation was twofold higher in CTL cells compared to those overexpressing ABCG2. Moreover, the ABCG2 specific inhibitor Ko143, which has no effect on PheA

accumulation in CTL cells that express *ABCG2* to very low levels, restores it to CTL values in transfected cell lines.

Collectively, these experiments validate that both recombinant proteins (ABCB1 and *ABCG2*) and their variants are overexpressed, functional, and could be effectively inhibited by their respective inhibitors in this model.

The results obtained with CIP and MOX on these functional models are summarized in a table for clarity (see supplemental Table S2). Consistent with other *in vitro* studies [96,97], our findings (reduced accumulation in recombinant cell lines, reversed by specific inhibitors) confirm the ABCB1 role in the active transport of MXF. Likewise, the specific effects of ABCB1 inhibitors on CIP accumulation confirm that this transporter also plays a role in CIP cellular disposition, though to a lesser extent than for MXF [98,180]. ABCB1 transports various substrates that do not necessarily share structural similarities [195], making it difficult to develop theories based on substrate structure only, even for drugs within the same pharmacological class. In this respect, it is worth mentioning that MXF is more lipophilic than CIP and essentially present in zwitterionic form at neutral pH [67]. This might explain why MXF is better transported, as ABCB1 substrates are mainly hydrophobic and amphipathic compounds [195].

Strikingly, we observe that the non-specific MRP transporter inhibitor MK571 ($K_i < 2 \mu\text{M}$) [192] increases by approximately threefold in CIP accumulation in all CTL clones. This effect may be due to ABCC4 basal expression in these cells. Confirming this hypothesis, and in accordance with previous observations in J774 macrophages overexpressing ABCC4 [87,88], CIP accumulation is reduced by approximately 25% in HEK cells overexpressing ABCC4, and higher MK571 concentrations (50 μM) are needed to restore CIP accumulation in these cells. Consequently, our hypothesis is

that ABCC4 basal expression in all HEK293 cell lines reduces CIP accumulation and, hence, the expected role of ABCB1 and ABCG2 [93] in transfected cells. These results highlight the importance of carefully considering the basal expression of transporters in a given cell line, as they can have a non-negligible impact on drug substrate accumulation, leading to erroneous interpretation of the overexpressed transporter's real impact in the same cell line. In the present case, the effects of non-specific and, to a lesser extent, specific ABCB1 inhibitors remain observable in cells overexpressing ABCB1 or its variants, but it might have been different in cells that did not express ABCC4 and accumulated more CIP.

Lastly, we did not find any significant effect of ABCG2 or its genetic polymorphisms on CIP and MXF cellular accumulation. To the best of our knowledge, MXF transport by ABCG2 has not been reported so far, and its role in CIP transport does not seem predominant in the presence of other efflux transporters [93,99].

We did not detect any significant effect of certain SNPs investigated. Specifically, MXF or CIP cellular accumulation in cells overexpressing *ABCB1* 1199A or *ABCG2* 421A variant alleles did not differ from that measured in cells overexpressing the corresponding WT transporter. Previous studies have demonstrated that the 1199G>A SNP influences the transporter activity in a substrate-dependent manner. For instance, the 1199A variant exhibited reduced ABCB1 activity towards tacrolimus in recombinant HEK293 and K512 cell lines, while it enhanced ABCB1 activity towards vinblastine in K562 recombinant cell lines [117]. However, this variant did not significantly affect cyclosporin A, Rh123 (HEK293 and K562), doxorubicin (K562), or rivaroxaban (HEK293) [196], darunavir (HEK293) [186] or bictegravir (HEK293) transport [197]. Similarly, the *in vitro* effect of the 1199G>A SNP was less pronounced for nilotinib compared to imatinib (HEK293), a better ABCB1 substrate [118]. Given its localization in the sixth cytoplasmic loop involved

in substrate recognition, the 1199G>A polymorphism may selectively influence the binding of certain substrates while leaving others unaffected. Two distinct binding sites have been identified in ABCB1, namely H- and R-sites. H-site is involved in highly hydrophobic substrate recognition, while R-site recognizes smaller substrates and facilitates ATP hydrolysis, both essential for ABCB1 transport [198]. At the present time, however, it is unknown if 1199G>A ABCB1 polymorphism might impact substrate interaction with these sites and in which site MXF or other substrates bind in this pocket, making it difficult to rationalize how it may affect various drugs [199]. In contrast to the WT ABCB1 protein, the TTT and CGT haplotypes either do not, or only minimally reduce the cellular accumulation of MXF and CIP. Both haplotypes share the variant at the third nucleotide (c.3435C>T), suggesting its possible causative role. To our knowledge, this is the first *in vitro* study that has investigated the role of these polymorphisms on fluoroquinolone disposition. Limited *in vivo* studies have shown specific impacts on fluoroquinolone pharmacokinetics. For example, the ABCB1 3435C>T (CGT) polymorphism is associated with a decrease in the sitafloxacin C_{max} [155]. It introduces a rare codon that causes a ribosome translational pause, which affects protein folding kinetics by chaperones, thereby altering ABCB1 function [200]. Additionally, the ABCB1 2677T>G/A (rs2032582) polymorphism correlates with a 40% reduction in MXF prehepatic bioavailability in humans, leading to a decrease in the area under the curve [156]. These data suggest that the reduced function of this variant increases fluoroquinolone cellular and tissue distribution, supported by our *in vitro* data, which further identifies the c.3435C>T SNP within the haplotype as the causative variant.

As for the TTT variant haplotype, although there is ongoing debate about the functional implications of this haplotype, literature indicates a trend towards lower impact of this SNP on drug transport compared to the wild type. For example, the

ABCB1 TTT variant haplotype is linked to a lower decrease in the accumulation of imatinib compared to *ABCB1* CGC [126], also in line with our own findings with MXF and CIP.

In summary, *ABCB1*, which is predominantly expressed in epithelial cells, plays a pivotal role in the absorption, distribution, and excretion of various substrates, including antibiotics. This study has demonstrated that this transporter significantly influences fluoroquinolone cellular concentration *in vitro*, with a more pronounced effect on MXF than on CIP and highlights the impact of specific polymorphisms. These findings may help explain the limited *in vivo* data suggesting that *ABCB1* genetic polymorphisms can affect fluoroquinolone disposition. Noteworthy, the 3435TT genotype is more common in white populations, while the CC genotype is more frequent in Africans [201], indicating the potential value of comparing fluoroquinolone pharmacokinetics in these populations.

Conversely, *ABCC4*, expressed in phagocytic cells [87], reduces by approximately 25% the intracellular CIP concentration, potentially impacting its efficacy against intracellular pathogens [202]. The consequences of these modulations need to be addressed in terms of efficacy, risks of resistance selection, or toxicity in well-designed *in vivo* or clinical studies.

Despite these important findings, some limitations of this work should be acknowledged. Indeed, using a cell model overexpressing a specific transporter may not accurately reflect its role in the body, where its expression could be lower, and multiple transporters may interact to modulate drug disposition. However, these models remain valuable in pharmacology for understanding substrate specificity and comparing the impact of genetic polymorphisms. A model incorporating all relevant transporters may nevertheless usefully complement this

work to assess the individual contribution and relative importance of each transporter in MXF and CIP disposition. Additionally, while we confirm the overexpression of ABCC4 in the recombinant cell lines, we could not sort exclusively recombinant cells that express ABCC4 for technical reasons, which could introduce variability in our results. Secondly, only key polymorphisms of *ABCB1* and *ABCG2* were explored, but the study would need to be extended to others in the future. *ABCC4* polymorphisms could also be investigated, although they are rare in the population and not associated so far with clinically-significant changes in drug pharmacokinetics [203]. Lastly, the *in vitro* nature of the study does not capture the complexity of pharmacokinetic processes like tissue distribution or hepatic or renal elimination, which involve transporters *in vivo*. Nevertheless, our work may help understand interindividual variations in fluoroquinolone pharmacokinetics among patients.

To conclude, in HEK293 recombinant cell models, our study indicates that *ABCB1* plays a significant role in MXF transport, while *ABCC4* is more crucial for CIP transport. Additionally, we show that *ABCB1* CGT and TTT haplotypes seem to reduce *ABCB1* efflux ability towards these compounds. Importantly also, our work highlights the need to consider the interfering role of the basal expression of transporters when studying drug transport in recombinant cell lines to correctly interpret the resulting data.

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CHAPTER III – PERSONAL RESEARCH WORK

Authorship contribution

Participated in research design: G. M., F. V. B. and L. E.

Conducted experiments: G. M.

Contributed new reagents or analytic tools: V. H.

Performed data analysis: G. M. and F. V. B.

Wrote and contributed to the writing of the manuscript: G. M., V. H., F. V. B. and L.E.

Footnote

G.M. is a research fellow (FC43775), and F.V.B. is a research director from the Belgian Fonds de la Recherche Scientifique (FRS-FNRS).

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The authors declare no conflict of interest.

SUPPLEMENTAL DATA

Table S1: Frequency of the SNPs investigated in different ethnic groups.

		Frequency (%)*			
		European	South Asian	African	East Asian
ABCB1	1199G>A	3.28	0.92	0.30	0.00
	1236C>T	41.55	58.69	13.62	62.70
	2677G>T/A	40.95/1.79	59.20/5.01	1.97/0.08	39.78/13.39
	3435C>T	51.79	57.46	14.98	39.78
ABCG2	421C>A	9.44	9.71	1.29	29.07

*Allelic frequencies are from the 1000 Genomes projects found in <http://www.pharmgkb.org/search/>.

Table S2: Summary of the main inhibitor effects on ciprofloxacin (CIP) and moxifloxacin (MXF) cellular concentration.

Transporter involved	Polymorphisms	Substrate (Conc.)	Inhibitors (Concentration)	Key observations (vs. CTL)
ABCB1	1199G (WT)	MXF (9.13 μ M)	Zosuquidar (0.2 μ M)	Increase cellular concentration (Significant effect)
			Elacridar (5 μ M)	Increase cellular concentration (Significant effect)
			Ko143 (1 μ M)	No significant effect
			MK571 (25 μ M)	No significant effect
	1199A (Variant)	CIP (10.36 μ M)	Zosuquidar (0.2 μ M)	Increase cellular concentration (Significant effect)
			Elacridar (5 μ M)	Increase cellular concentration (Significant effect)
			Ko143 (1 μ M)	No significant effect
			MK571 (25 μ M)	No significant effect
	1199A (Variant)	CIP (10.36 μ M)	Zosuquidar (0.2 μ M)	Increase cellular concentration (Significant effect)
			Elacridar (5 μ M)	Increase cellular concentration (Significant effect)

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			Ko143 (1 μ M) MK571 (25 μ M)	Increase cellular concentration (Significant effect) No significant effect No significant effect
	Haplotype CGC 1236C ; 2677G ; 3435C (WT)	MXF (9.13 μ M)	Zosuquidar (0.2 μ M)	Increase cellular concentration (No significant effect)
			Elacridar (5 μ M)	Increase cellular concentration (Significant effect)
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect
		CIP (10.36 μ M)	Zosuquidar (0.2 μ M)	Increase cellular concentration (No significant effect)
			Elacridar (5 μ M)	Increase cellular concentration (Significant effect)
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect
	Haplotype CGT 1236C ; 2677G 3435T (Variant)	MXF (9.13 μ M)	Zosuquidar (0.2 μ M)	No significant effect
			Elacridar (5 μ M)	No significant effect
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect
ABCG2	421C (WT)	MXF (9.13 μ M)	Zosuquidar (0.2 μ M)	No significant effect
			Elacridar (5 μ M)	No significant effect
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect
		CIP (10.36 μ M)	Zosuquidar (0.2 μ M)	No significant effect
			Elacridar (5 μ M)	No significant effect
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect
	Haplotype TTT 1236T ; 2677T 3435T (Variant)	MXF (9.13 μ M)	Zosuquidar (0.2 μ M)	No significant effect
			Elacridar (5 μ M)	No significant effect
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect
		CIP (10.36 μ M)	Zosuquidar (0.2 μ M)	No significant effect
			Elacridar (5 μ M)	No significant effect
			Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect

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	421A (Variant)	MXF (9.13 μ M)	Zosuquidar (0.2 μ M) Elacridar (5 μ M) Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect No significant effect No significant effect
		CIP (10.36 μ M)	Zosuquidar (0.2 μ M) Elacridar (5 μ M) Ko143 (1 μ M) MK571 (25 μ M)	No significant effect No significant effect No significant effect No significant effect
ABCC4	WT	MXF (9.13 μ M)	Zosuquidar (0.2 μ M) Elacridar (5 μ M) Ko143 (1 μ M) MK571 (25 μ M) MK571 (50 μ M)	No significant effect No significant effect No significant effect No significant effect No significant effect
		CIP (10.36 μ M)	Zosuquidar (0.2 μ M) Elacridar (5 μ M) Ko143 (1 μ M) MK571 (10 μ M) MK571 (25 μ M) MK571 (50 μ M)	No significant effect No significant effect No significant effect No significant effect Increase cellular concentration (Significant effect) Increase cellular concentration (Significant effect)

Figure S1. ABCB1 expression analysis by western blot. Cells transfected with empty plasmid (HEK_{control} [CTL]) and cells transfected with vector cDNA encoding the ABCB1 transporter (HEK_{wild type} [1199G], HEK_{variant} [1199A]). Dilutions (from the left to the right): 12, 6, 3, and 1.5 μ g of proteins.

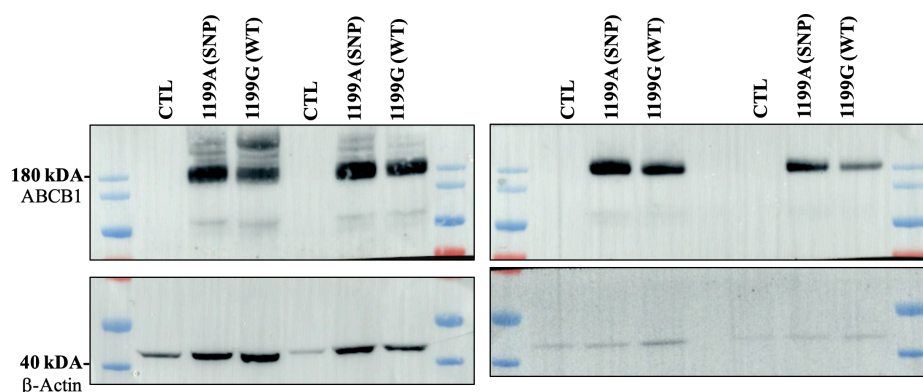


Figure S2. ABCC4 expression analysis by western blot. Cells transfected with empty plasmid (HEK_{control} [CTL]) and cells transfected with vector cDNA encoding the ABCC4 transporter (HEK_{ABCC4} [ABCC4]).

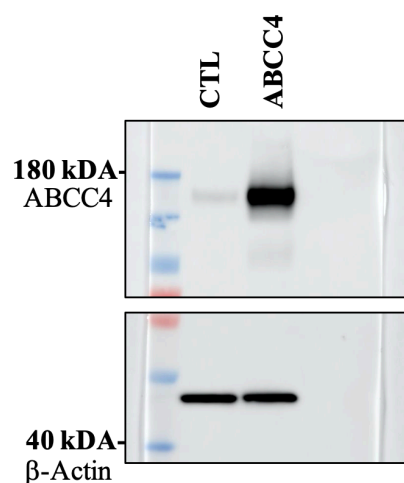


Figure S3. ABCG2 expression analysis by western blot. Cells transfected with empty plasmid (HEK_{control} [CTL]) and cells transfected with vector cDNA encoding the ABCG2 transporter (HEK_{variant} [421A], HEK_{wild type} [421C]).

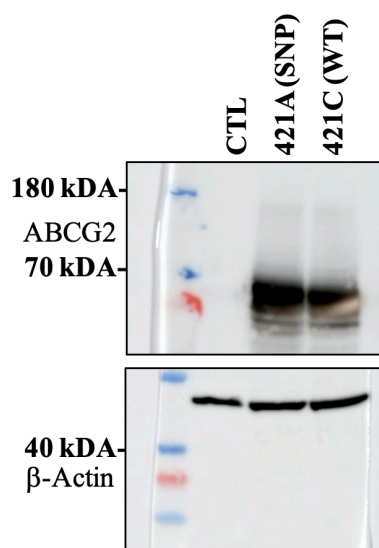
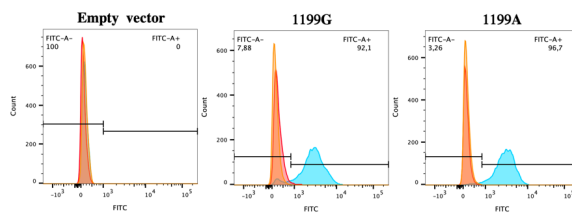
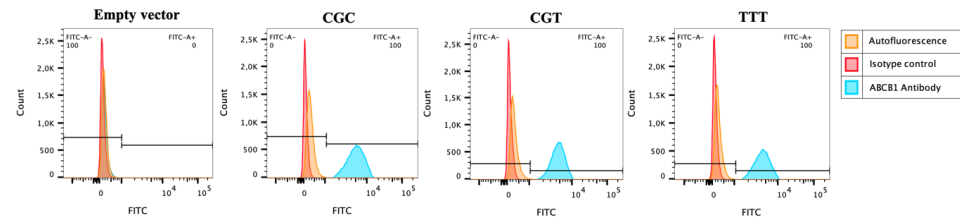


Figure S4. (A, B, C) ABCB1 and ABCG2 cell surface expression assessed by flow cytometry of HEK293 transfected cells stained with ABCB1 antibody, isotype control, and no staining (autofluorescence). (A) Empty pcDNA3.1 vector, ABCB1_{1199G}, ABCB1_{1199A}. (B) Empty pcDNA3.1 vector, ABCB1_{CGC}, ABCB1_{CGT}, ABCB1_{TTT}. FITC, Fluorescein isothiocyanate. (C) Empty pcMV3 vector, ABCG2_{421C}, ABCG2_{421A}, PerCP (Peridinin chlorophyll)-Cy5-5-a (cyanine).

(A)



(B)



(C)

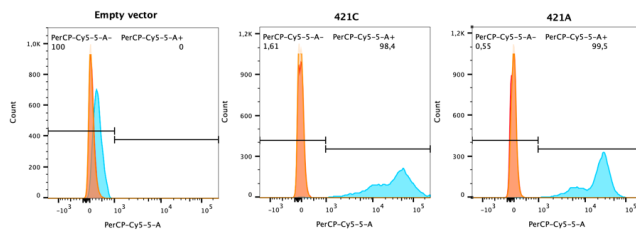
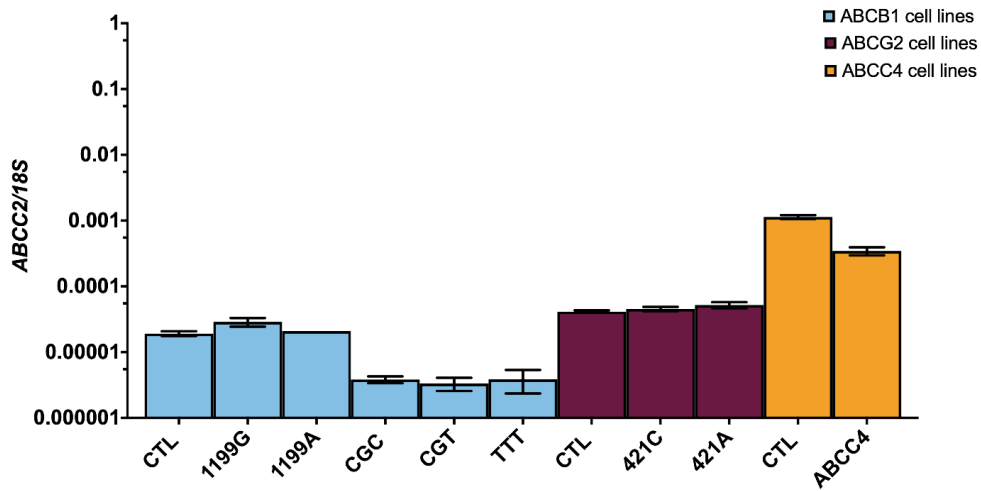


Figure S5. ABCC2 transporter RNA transcripts. ABCC2 transporter RNA expression levels normalized to the 18S expression level (housekeeping gene) in HEKcontrol (CTL), HEK1199G>A (1199G, 1199A) and HEK1236C>T-2677G>T-3435C>T (CGC, CGT, TTT) cell lines; HEKcontrol (CTL) and HEK421C>A (421C, 421A) cell lines; HEKcontrol (CTL) and HEKABCC4 (ABCC4) cell lines.



III.2 Hollow fiber infection model for antibiotic activity against *Staphylococcus aureus*

III.2.1 Technical considerations on the model

a) Setting up the HFIM

The central reservoir is a critical component of the HFIM, equipped with five functional ports, numbered 1 to 5 in **Figure III.1**:

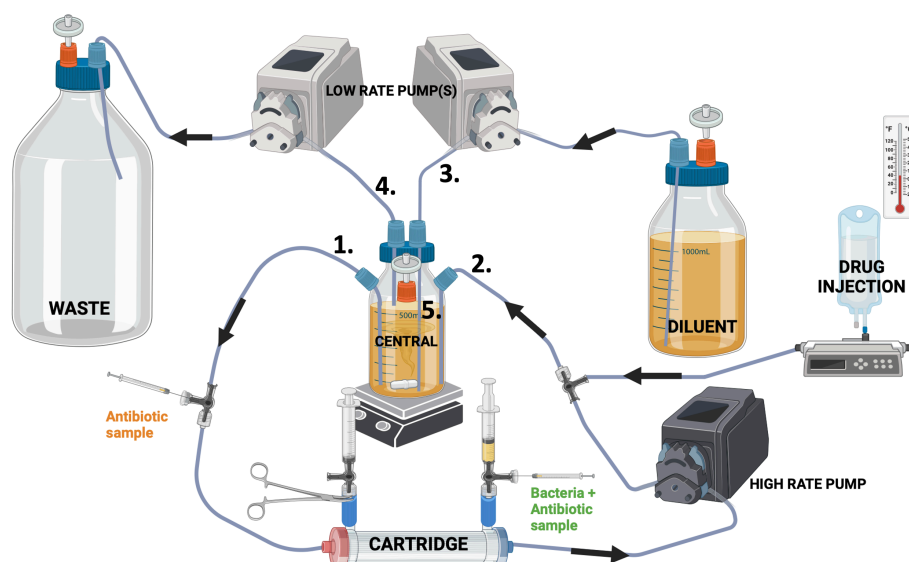


Figure III.1. Schematic representation of the HFIM for the study of the activity of a single antibiotic (image created in Biorender.com).

1. and 2. Recirculation ports: These two ports connect the central reservoir to the cartridge, allowing continuous medium circulation between the central reservoir and the cartridge, ensuring that the drug concentration remains in equilibrium between the two compartments.

3. Diluent reservoir port: This port introduces drug-free diluent into the central reservoir, simulating the process of drug elimination.

4. Waste reservoir port: This port removes excess medium from the central reservoir after diluent is added, maintaining a constant volume in the central reservoir.

5. Sterile filter port: This port acts as a vent, preventing pressure buildup in the central reservoir.

The medium circulation between these components is managed by a dual peristaltic pump system, which ensures the proper function of the HFIM. One **high flow rate pump (60 mL/min)** recirculates the medium between the cartridge and central reservoir, maintaining drug concentration equilibrium. The **second pump(s)** introduce(s) fresh medium into the central reservoir while simultaneously removing an equal volume to the waste container, precisely simulating drug elimination. This design ensures that the drug concentration in the central reservoir is equal to the concentration in the ECS of the cartridge. Indeed, the relatively small central reservoir volume (300 mL) allows for rapid changes in drug concentrations while maintaining a constant overall volume [167].

The drug can be added to the central reservoir in various ways, such as through bolus injection, constant infusion, or simulated oral/IV dosing, depending on the experimental design. This is achieved using an **additional pump** connected directly to the central reservoir. The system, particularly the central compartment and the cartridge, is put into a fixed incubator to simulate the environment suitable for the growth of bacteria and/or cells.

b) Sampling for PK/PD analysis

Samples can be collected at multiple time points, allowing studies to extend over several weeks if needed [166]. Sampling is carried out through two dedicated ports (**Figure III.1**), allowing precise analysis of both the central reservoir and the cartridge compartment for PK/PD evaluations. A sampling port provides direct access to the central reservoir to measure drug concentrations for PK studies analysis. For the cartridge, syringes attached to its top allow the collection of bacterial samples from the ECS for PD analysis by counting CFU. Before sampling, the ECS is flushed 3-4 times to ensure thorough mixing [166]. These samples also allow for the measurement of drug concentrations within the cartridge for PK analysis. This dual sampling ensures precise and reliable data collection, allowing simultaneous evaluation of bacterial responses to fluctuating drug concentrations.

c) Previous studies using HFIM with intracellular bacteria

Previous studies have used the HFIM to assess the efficacy of drug regimens against various intracellular pathogens, including *Mycobacterium* species and *Listeria monocytogenes*.

For example, Ruth et al. [204] used the HFIM to simulate the human PK of azithromycin, ethambutol, and rifampicin in both plasma and epithelial lining fluid, the latter being more representative of the site of infection. In their experiments, THP-1 cells infected by *Mycobacterium avium* were exposed to a triple-drug regimen for 21 days to evaluate its efficacy. The study analyzed both intracellular and extracellular bactericidal effects, considering drug-protein binding, and equilibrated the system for 24 hours before starting treatment. PK was assessed over the first 24 hours, while PD analysis was conducted on days 0, 2, 7, 14, and 21.

Similarly, Srivastava et al. [205] explored the potential of ceftriaxone, combined with avibactam, for treating disseminated tuberculosis in children. The goal was to overcome resistance mechanisms associated with β -lactamase production by *M. tuberculosis*. This experiment, also conducted with THP-1 cells infected with *M. tuberculosis*, lasted for 28 days, during which bacterial populations were monitored. The study assessed the percentage of time the drug concentration remained above the MIC and its correlation with bacterial killing to establish the Emax. The PK profile was evaluated for the first 24 hours, and PD was measured every 3 days during the first 14 days, with additional assessments at day 21 and 28. However, this study only addressed the effects on intracellular bacteria and did not provide information regarding system equilibration before initiating the experiments.

In another study, researchers developed a HFIM to investigate the dose-effect and dose-scheduling of MXF against *M. kansasii*. The experiments used THP-1 cells infected with *M. kansasii* and compared the effects of MXF to a three-drug regimen (isoniazid, ethambutol, and rifampicin) over 14 days. The AUC/MIC ratio was used to predict the effectiveness of MXF. Similar to the other studies, the PK profile was assessed for the first 24 hours, and PD was evaluated on days 3, 7, 10, and 14. However, this study also lacked information on system equilibration times and the protein binding of MXF.

Finally, the HFIM model was adapted for another intracellular pathogen, *Listeria monocytogenes*, to assess the effect of CRS0540, a thiadiazol urea compound targeting the PolC DNA polymerase in Gram-positive bacteria [172]. CRS0540 was administered one hour after infection of THP-1 cells and continued for seven days. The study focused on the evaluation of free drug concentrations during the first 24 hours, and PD was assessed daily throughout the 7-day period.

III.2.2 Article n°2

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A hollow fiber infection model to study intracellular and extracellular antibiotic activity against *Staphylococcus aureus*

Gwenaëlle Mahieu,^{1,2} Laure Elens,^{2,3} Noémie Prebonnaud,^{4,5} Alexia Chauzy,⁴ and Françoise Van Bambeke^{1,6,*}

¹Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium

²Integrated Pharmacometrics, Pharmacogenomics and Pharmacokinetics (PMGK) Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium

³Louvain Centre for Toxicology and Applied Pharmacology (LTAP), Institut de Recherche expérimentale et clinique (IREC), Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium

⁴Université de Poitiers, INSERM U1070, PHAR2, 86000 Poitiers, France

⁵Laboratoire de Toxicologie-Pharmacocinétique, CHU de Poitiers, Poitiers, France

⁶Lead contact

*Correspondence: francoise.vanbambeke@uclouvain.be

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Author list

Gwenaëlle Mahieu^{1,2}, Laure Elens^{2,3}, Noémie Prebonnaud^{4,5}, Alexia Chauzy⁴,
Françoise Van Bambeke^{1,6*}

Affiliations

¹ Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium.

² Integrated PharmacoMetrics, pharmacoGenomics and Pharmacokinetics (PMGK) Research group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium.

³ Louvain centre for Toxicology and Applied Pharmacology (LTAP), Institut de recherche expérimentale et clinique (IREC), Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium.

⁴ Université de Poitiers, INSERM U1070, PHAR2, 86000 Poitiers, France.

⁵ Laboratoire de Toxicologie-Pharmacocinétique, CHU de Poitiers, Poitiers, France.

⁶ Lead contact.

*Correspondence: francoise.vanbambeke@uclouvain.be

ORCID ID: G. Mahieu 0009-0000-5835-5857

ORCID ID: L. Elens: 0000-0002-0039-3583

ORCID ID: N. Prébonnaud 0009-0001-4631-3542

ORCID ID: A. Chauzy: 0000-0001-6443-5888

ORCID ID: F. Van Bambeke: 0000-0002-0052-7991

Highlights

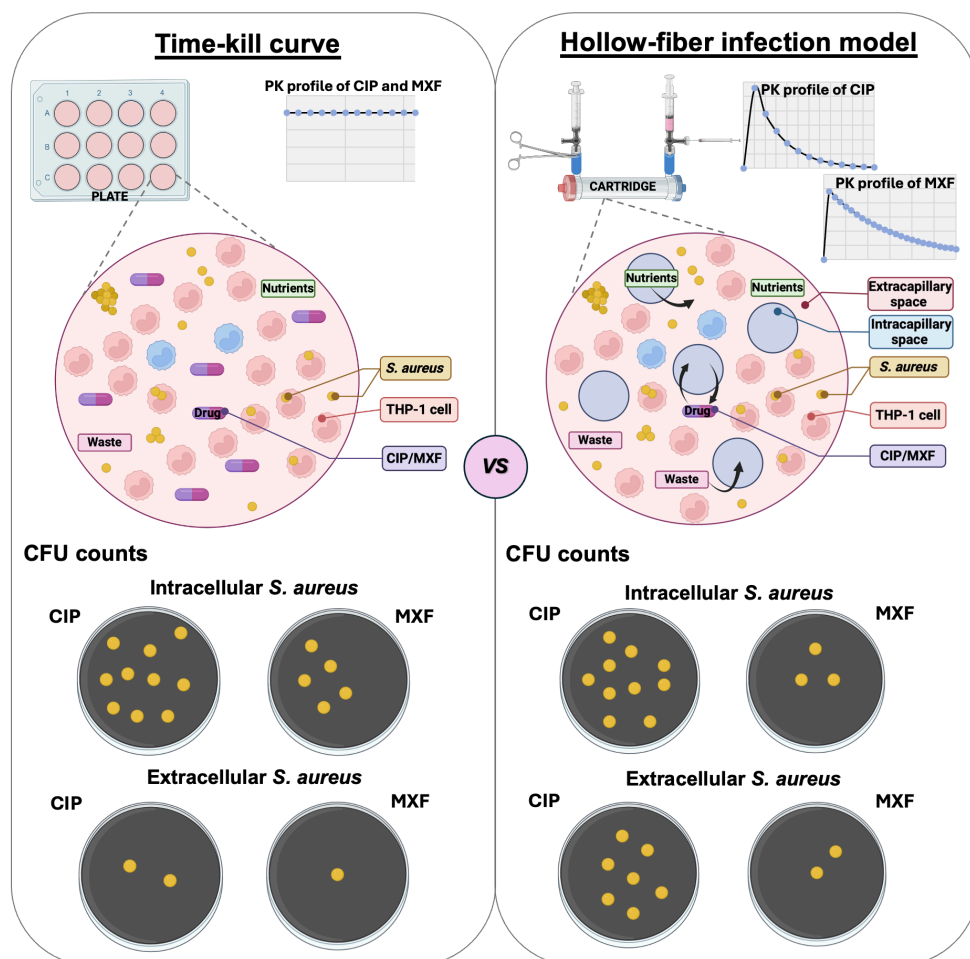
- Antibiotic efficacy is influenced by fluctuations in concentrations over time
- Hollow fiber dynamic models (HFIM) allow to mimic these fluctuations in vitro
- HFIM has been adapted here to fluoroquinolones against intracellular *S. aureus*
- Activity differed in dynamic vs static conditions, illustrating the model's relevance

a) Summary

Antibiotic activity against intracellular pathogens is commonly evaluated in static models that do not reproduce plasma concentration fluctuations. However, efficacy is influenced by exposure conditions related to the drug's pharmacokinetic profile. This study developed and validated an intracellular pharmacodynamic model using the hollow fiber system, the gold standard for evaluating extracellular antibiotic activity. The activity of fluoroquinolones, i.e., bactericidal antibiotics with intracellular tropism, was studied against intracellular *Staphylococcus aureus*, involved in the persistence/recurrence of infections. In this model, moxifloxacin was more effective than in static conditions (0.87 log₁₀ killing gain), while ciprofloxacin's kill rate was slower (18 vs. 12 h to achieve 1 log₁₀ killing). These differences were linked to the C_{max}/MIC ratio, which was 2.5-fold higher for moxifloxacin but 3.4-fold lower for ciprofloxacin in dynamic vs. static conditions. This model could be applied to other drugs, cell types, or pathogens, offering a tool for optimizing dosing schemes and considering intracellular reservoirs.

Keywords: fluoroquinolone, *Staphylococcus aureus*, intracellular infection, pharmacodynamics, pharmacokinetics, hollow fiber infection model

b) Graphical abstract



c) Introduction

Staphylococcus aureus, a Gram-positive bacterium, is a human commensal organism, colonizing 20-30% of individuals on the skin or mucous membrane, such as the nose [206,207]. However, it can also act as an opportunistic pathogen, causing severe infections, including osteomyelitis, pneumonia, or endocarditis that are associated with a risk of mortality [26,207]. Infections caused by *S. aureus* represent a major public health concern, particularly due to the pathogen's ability to develop resistance to antibiotics, as best exemplified by methicillin-resistant *S. aureus* (MRSA) [1,2]. On top of that, *S. aureus* is able to invade and persist within mammalian host cells, including phagocytic cells [7,207,208]. These intracellular niches contribute to the persistence and/or recurrence of the infection due to their reduced responsiveness to antibiotics [6,7]. Many previous studies, including some from our laboratory, indicate that this loss of efficacy occurs regardless of their capacity to accumulate within the host cells or colocalize with bacteria in infected subcellular compartments [11,82,84]. However, all these studies exposed infected cells to constant antibiotic concentrations, which do not mimic the fluctuations of antibiotic concentrations over time that bacteria encounter *in vivo*, depending on the drug's pharmacokinetic profile (PK).

Pharmacodynamic (PD) studies clearly demonstrate that the influence of concentration over time is a crucial determinant of antibiotic efficacy, which varies among antibiotic classes. For time-dependent antibiotics, such as β -lactams, efficacy is primarily driven by the duration that bacteria are exposed to concentrations above the MIC. In contrast, concentration-dependent antibiotics, like aminoglycosides, depend on the ratio of the peak concentration (C_{max}) to MIC. Many other classes show an intermediate profile, where efficacy is influenced by the global exposure (Area Under the Curve; AUC), with varying impacts of C_{max} and

exposure time [209]. This is the case for fluoroquinolones, which are considered antibiotics of choice against intracellular bacteria due to their ability to accumulate in both phagocytic and non-phagocytic cells and their proven activity against intracellular bacteria residing in different subcellular compartments (cytosol or vesicles of the phagolysosomal apparatus) [41]. To accurately apprehend the impact of concentration vs. time of exposure on their intracellular activity, a model capable of simulating their clinical pharmacokinetic profile is therefore critically needed.

The hollow fiber infection model (HFIM) consists of a hollow fiber cartridge supplied with culture fluid that is renewed over time at a predefined rate. To study the efficacy of a given antibiotic under dynamic conditions, the cartridge can be infected, and the fluid supplemented with the antibiotics being studied. The flow rate is then carefully adjusted to simulate the antibiotic pharmacokinetic profile. Samples are collected over time to ensure the simulated profile matches actual drug concentrations and, in parallel, to monitor the number of surviving bacteria. This model is considered the gold standard for studying antibiotic pharmacodynamics *in vitro* [164]. However, to the best of our knowledge, it has only been anecdotally adapted to evaluate antibiotic activity against intracellular bacteria, namely *Mycobacteria* [204,210] and more recently, *Listeria monocytogenes* [172]. The adaptation is more challenging when dealing with more cytotoxic species like *S. aureus*.

In this work, we successfully established a HFIM of intracellular infection of THP-1 monocytes by the *S. aureus* reference strain ATCC29213. We fully validated the model for bacterial and cell proliferation as well as for cell viability. We then used it to evaluate the activity of two fluoroquinolones, namely ciprofloxacin, a first-generation molecule with modest activity against Gram-positive bacteria, and

moxifloxacin, one of the most potent and widely used fluoroquinolones against *S. aureus* on the market [49,211]. Finally, we compared the activity observed in the dynamic hollow fiber system with that obtained in a conventional static model of time-kill-curves (TKC), where infected cells are exposed to constant antibiotic concentrations over time.

d) Results

Minimum inhibitory concentrations

The MIC of ciprofloxacin and moxifloxacin were $0.125 \mu\text{g mL}^{-1}$ and $0.06 \mu\text{g mL}^{-1}$, respectively, before hollow-fiber experiments and remained stable throughout the study.

Setting up the hollow fiber infection model (HFIM) experiments

Before testing antibiotic activity, we conducted a series of preliminary experiments aiming (1) at evaluating the capacity of the cells to adapt in the HF cartridge and (2) at determining the right MOI to allow at the same time equilibration of the infected cells and adequate infection. Optimization of these conditions was critical for ensuring the validity and reliability of the model.

Determination of the MOI allowing 12-hour pre-equilibration of the HF system

Initially, we aimed at determining the optimal MOI that would allow a 12-hour equilibration and adaptation period for *S. aureus* infected cells within the cartridge before starting antibiotic treatment [204]. Our goal was to achieve an intracellular inoculum of approximately 10^6 CFU per mg of total cell protein at the end of this preincubation period. This target post-phagocytosis inoculum is typically reached in static experiments using a MOI of 4 bacteria per cell, which is considered adequate for ensuring sufficient detection of surviving bacteria even after exposure to bactericidal antibiotics [11], while maintaining infected cells viable. To this end, we infected THP-1 cells with two different MOI of *S. aureus* (0.001 and 0.0001) and measured the intracellular and extracellular inoculum after 12 hours under two distinct conditions: a 'HF condition' where the cells were infected and incubated in the HF cartridge (closed system with no medium renewal) and a 'well condition' where the cells were infected and incubated in wells.

Both inocula were allowed to reach the desired bacterial load intracellularly (Figure 1A), with the cartridge containing the higher inoculum (MOI = 0.001) showing slightly, but not significantly, higher values compared to the other condition (Log_{10} CFU counts at 12 h: 7.28 ± 0.28 in the cartridge and 6.44 ± 0.57 in the well for MOI = 0.001 [$P \geq 0.05$]; 7.17 ± 0.77 in the cartridge and 6.17 ± 0.04 in the well for MOI = 0.0001 [$P \geq 0.05$]). In the absence of antibiotics, extracellular contamination developed over time, as indicated by the presence of extracellular bacteria in Figure 1B (Log_{10} CFU counts at 12 h: 6.43 ± 0.05 in the cartridge and 5.89 ± 0.49 in the well for MOI = 0.001 [$P \geq 0.05$]; 6.42 ± 0.12 in the cartridge and 6.06 ± 0.38 in the well for MOI = 0.0001 [$P \geq 0.05$]). No significant differences were observed between intracellular and extracellular counts for each inoculum tested.

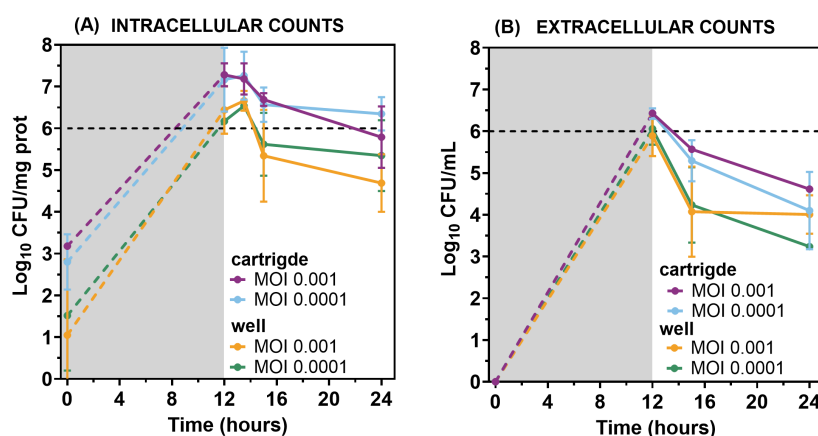


FIGURE 1. Determination of the MOI allowing a 12-hour equilibration before reaching the target inoculum of 10^6 CFU per mg cell protein (intracellular) or per mL (extracellular). The graphs show the change in the number of colonies forming units (CFU) (ΔLog_{10} CFU per mg of cell protein in THP-1 monocytes **(A)** or per mL of culture medium **(B)** over 24 hours of incubation using an initial MOI of 0.001 or 0.0001. Dotted line, target inoculum at 12h, i.e. the end of the equilibration phase that will correspond to time 0 (addition of antibiotics) in further experiments. The gray zone corresponds to this preconditioning phase. Data are means $\pm$ SD ($N = 1$, $n = 3$).

Cell viability of non-infected vs. infected cells in the HF cartridge vs. well

We then determined whether non-infected and infected THP-1 cells could adapt to a new environment in the cartridge by comparing their viability with that observed in a static well environment (Figure 2). Cell counts and viability were assessed for a MOI of 0.0001 after 12 hours of preincubation. When comparing the growth of non-infected cells in both environments, no difference was observed, with a cell count of approximately 1.5×10^6 cells mL⁻¹ reached after 3 days of culture (panel A) at statistically similar growth rates ($k = 0.004571$ h⁻¹ (cartridge) [95% CI, 0.0002950 to 0.009079] and 0.004356 h⁻¹ (well) [95% CI, 0.0005632 to 0.008325]). For longer incubation times, cell numbers started to decline, indicating cell death. Cell mortality was then followed over a 24-hour period after the 12 h preconditioning phase, with the same MOI. In the cartridge (panel B), mortality of non-infected cells increased linearly over time, at a rate of 0.55 h⁻¹ [95% CI, 0.47 to 0.64]. In contrast, infected cells remained viable during 12 h, after which mortality increased rapidly, at a rate of 13.07 h⁻¹ [95% CI, 10.54 to 15.61]. Importantly, when antibiotics were added to the cartridge at their C_{max}, cell viability was maintained over time, yielding mortality rates slightly higher than those observed for non-infected cells ($k = 1.39$ h⁻¹ [95% CI, 1.10 to 1.69] with ciprofloxacin and 0.71 h⁻¹ [95% CI, 0.50 to 0.93] with moxifloxacin). Similar experiments conducted in wells (panel C) demonstrated that there was no significant difference in cell mortality between the cartridge and well environments in the presence of antibiotics, with $k = 1.28$ h⁻¹ [95% CI, 0.79 to 1.77] and 0.65 h⁻¹ [95% CI, 0.42 to 0.89] for ciprofloxacin and moxifloxacin, respectively.

Based on these findings, a MOI of 0.0001 was selected for further HFIM and TKC experiments, as this was the lowest inoculum allowing to reach the desired initial infection load with acceptable cell mortality in antibiotic-treated cells during the projected duration of further pharmacological experiments.

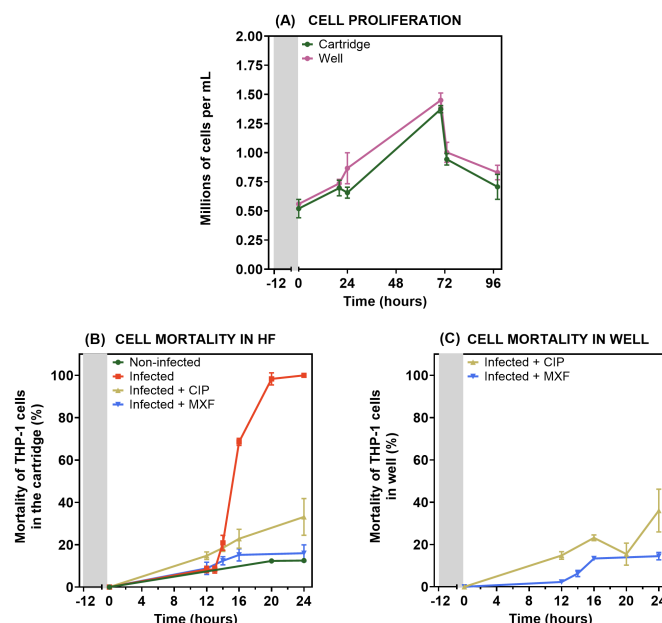


FIGURE 2. Cell proliferation and survival in the extracapillary space (ECS) of the HF cartridge vs. in the well. **(A)** Proliferation of THP-1 over time, with the number of cells expressed in millions per mL of culture medium. **(B, C)** Mortality of THP-1 over time in HF cartridge and well, respectively, expressed as a percentage (%) of dead cells. The gray zone corresponds to the 12 h preconditioning phase. Each data point represents the mean $\pm$ SD (N=1, n=3).

Pharmacokinetic profiles in HFIM

PK profiles simulating the IV administration of ciprofloxacin (400 mg twice-daily) and moxifloxacin (400 mg once-daily) were established based on literature data [189,190] and these simulations aimed at reproducing protein-unbound drug concentrations. Experimental concentration-time profiles were then checked in the HF model. The observed and targeted PK parameters, including C_{max}, half-life ($T_{1/2}$), time to maximum concentration (T_{max}), and AUC, are summarized in Table 2 with PK profiles shown in Figure 3. The observed profiles generally matched the planned values, although for ciprofloxacin, C_{max} values were 25% lower and $T_{1/2}$ 28% longer than expected, while for moxifloxacin, C_{max} and AUC values were 10% higher and $T_{1/2}$ 10% shorter than expected in the cartridge.

These variations are, however, smaller than interindividual variabilities reported in the clinics [212,213] and within the 20% error assumed for simulated data. Moxifloxacin concentrations were also measured intracellularly, but close to the limit of quantification (undetectable for ciprofloxacin). The data confirm that moxifloxacin accumulates to high levels in the cells and suggest that higher cellular concentrations are reached in dynamic conditions than in static conditions with, however, a delay of 8 h before reaching the maximum (approx. $100 \mu\text{g mL}^{-1}$ of cellular volume, considering a cell volume of $5 \mu\text{L}$ per mg of cell protein).

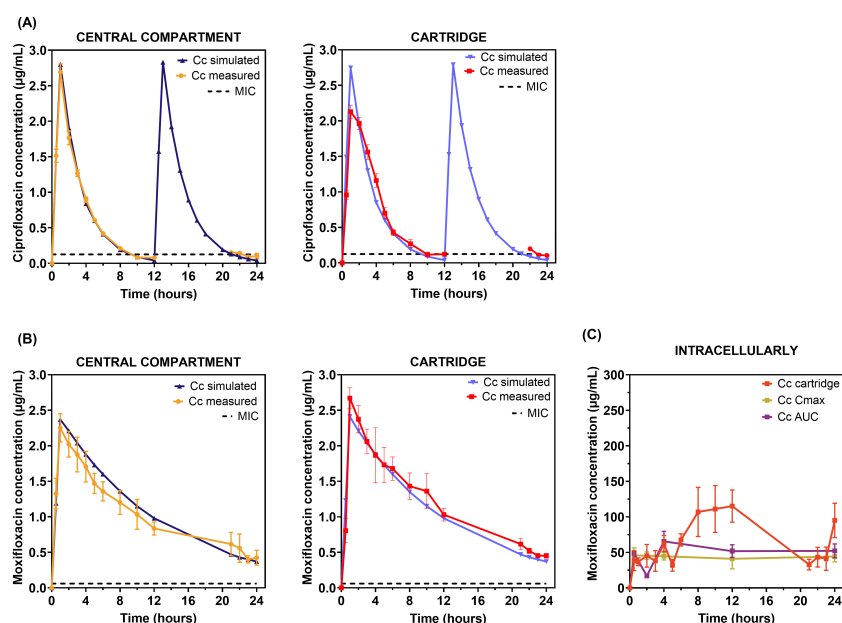


FIGURE 3. Fluoroquinolone concentration-time profile (PK) in the hollow fiber system model and intracellularly. (A) PK profile of ciprofloxacin for 400 mg twice-daily dosing schedule by IV route. **(B)** PK profile of moxifloxacin for a 400 mg once-daily dosing schedule by IV route. Data are shown for the fluids sampled in the central compartment and in the cartridge, respectively. Simulated values were obtained with the HF-App. Measured values are shown as means $\pm$ SEM of three independent replicates (when non-visible, error bars are smaller than the symbol size). Dashed line, MIC of the antibiotic against the studied strain. **(C)** Moxifloxacin concentration was measured inside the THP-1 cells, either for cells collected from the cartridge or from wells exposed to concentrations mimicking free Cmax or AUC. Cellular concentrations are expressed in $\mu\text{g mL}^{-1}$ of cell volume, considering a cell volume of $5 \mu\text{L}$ per mg cell protein [11]. Limit of quantification in fluids or cell lysates (LOQ) for CIP = $0.040 \mu\text{g mL}^{-1}$ (not detected in cell lysates) and LOQ for MXF = $0.015 \mu\text{g mL}^{-1}$.

TABLE 2.**Target and measured pharmacokinetic parameters in the HFIM experiment**

		CIPROFLOXACIN (CIP)		MOXIFLOXACIN (MXF)	
PARAMETERS ^a		Target ^b	Actual	Target ^b	Actual
C_{max} ($\mu\text{g mL}^{-1}$)	<i>Central</i>	2.80	2.69 $\pm$ 0.12	2.41	2.25 $\pm$ 0.20
	<i>Cartridge</i>	2.80	2.12 $\pm$ 0.091	2.41	2.67 $\pm$ 0.15
T_{1/2} (h)	<i>Central</i>	1.80	1.45 $\pm$ 0.81	8.49	7.95 $\pm$ 3.37
	<i>Cartridge</i>	1.80	2.31 $\pm$ 1.46	8.49	7.80 $\pm$ 2.04
T_{max} (h)	<i>Central</i>	1.00	1.00	1.00	1.00
	<i>Cartridge</i>	1.00	1.00	1.00	1.00
AUC₀₋₂₄ (mg.h L^{-1})	<i>Central</i>	17.31	17.21 $\pm$ 0.25	26.41	24.79 $\pm$ 1.75
	<i>Cartridge</i>	17.31	18.00 $\pm$ 0.70	26.41	28.50 $\pm$ 1.42
AUC₀₋₂₄/MIC	<i>Cartridge</i>	138.48	144 $\pm$ 5.6	440.17	475 $\pm$ 29.17
C_{max}/MIC	<i>Cartridge</i>	22.24	16.96 $\pm$ 0.73	39.83	44.5 $\pm$ 2.5

^aC_{max}, maximum serum concentration; T_{1/2}, elimination half-life; T_{max}, time at which C_{max} is reached; AUC₀₋₂₄, area under the 24-h concentration-time curve. All observed results are the mean of 3 independent experiments (N = 3) $\pm$ SEM.

^bAn error margin of $\pm$ 20% is assumed for these values.

Pharmacodynamic measurements in HFIM and comparison with TKC experiments

The antibacterial activity of ciprofloxacin and moxifloxacin was then examined against *S. aureus* under both dynamic (HFIM) and static conditions (TKC).

In the dynamic HFIM, antibiotic concentrations fluctuate over a 24-hour period, simulating *in vivo* pharmacokinetics. In contrast, the static TKC model uses fixed antibiotic concentrations over a 24-hour period. Here, we selected a concentration equivalent (a) to human free C_{max} that simulates the highest concentration to which bacteria could be exposed extracellularly but overestimates the 24-h exposure, or (b) to free AUC that mimics adequately the global exposure over time but not the peak effect considered as important for fluoroquinolones. In both models, antibacterial activity was assessed separately for intracellular bacteria infecting THP-1 and for extracellular bacteria escaping from cells in the culture medium.

In the absence of antibiotics, intracellular bacteria proliferated significantly in both models (Figure 4). Intracellular bacteria gained 1.1-1.3 log₁₀ CFU per mg of total cell proteins after 5 h in both models, and approximately 2 to 3 log₁₀ CFUs per mg of total cell proteins in the HFIM (panels A, B) or the TKC model (panels C, D), respectively, over the 24-hour period.

Both antibiotics could reduce bacterial counts of extracellular and intracellular bacteria, with an initial rapid killing phase followed by a plateau. In both the dynamic and static models, the two antibiotics were more active against extracellular than intracellular bacteria, and moxifloxacin achieved a faster and more pronounced reduction in bacterial counts compared to ciprofloxacin. Table 3 provides a detailed comparison of the pharmacodynamic parameters characterizing their activities. We calculated Minimal Durations for Killing (MDK) for different reductions in inoculum, a parameter usually used to characterize antibiotic tolerance [214] but also adequate to compare kill rates, especially when showing a biphasic profile, as observed here. This biphasic killing, already observed in previous reports, has been attributed to the presence of a tolerant subpopulation of bacteria, described as persisters [82,214]. We also estimated E_{max}, i.e., the maximal killing effect, corresponding to the plateau value of the exponential decay equation fitted to the data.

In the context of the present study, the comparison of each drug in the dynamic (HFIM) and the static (TKC) models is particularly important (see also Figure S2). Ciprofloxacin was equally effective (similar E_{max}) against intracellular bacteria in the HFIM and TKC models exposed to human free C_{max} during 24 h. However, it was less effective in static conditions simulating the human free AUC_{24h} (Figure 4 panels A and C; Figure S2 panel C). Furthermore, ciprofloxacin exhibited slower bacterial killing (longer MDK₉₀) in the HFIM than in static conditions (Table 3).

Extracellularly, ciprofloxacin demonstrated faster (shorter MDK₉₀) and more pronounced (more negative Emax) killing at free human Cmax in TKC than in the HFIM or in static conditions mimicking AUC (Table 3; Figure 4 panels A and C; Figure S2 panel E). The two latter conditions of exposure achieved a similar Emax, although faster killing was observed in the HFIM (Table 3).

Moxifloxacin caused significantly more pronounced killing intracellularly in the dynamic model than in static conditions, but MDK₉₀ or MDK₉₉ (when it was achieved) values were short and similar in both types of models (Figure 4, panels B and D; Figure S2 panel D; Table 3). Extracellularly, killing was slightly more pronounced (more negative Emax, limit of significance based on overlap of CI) and rapid (shorter MDK values) in static conditions compared to dynamic conditions (Table 3; Figure 4, panels B and D; Figure S2 panel F).

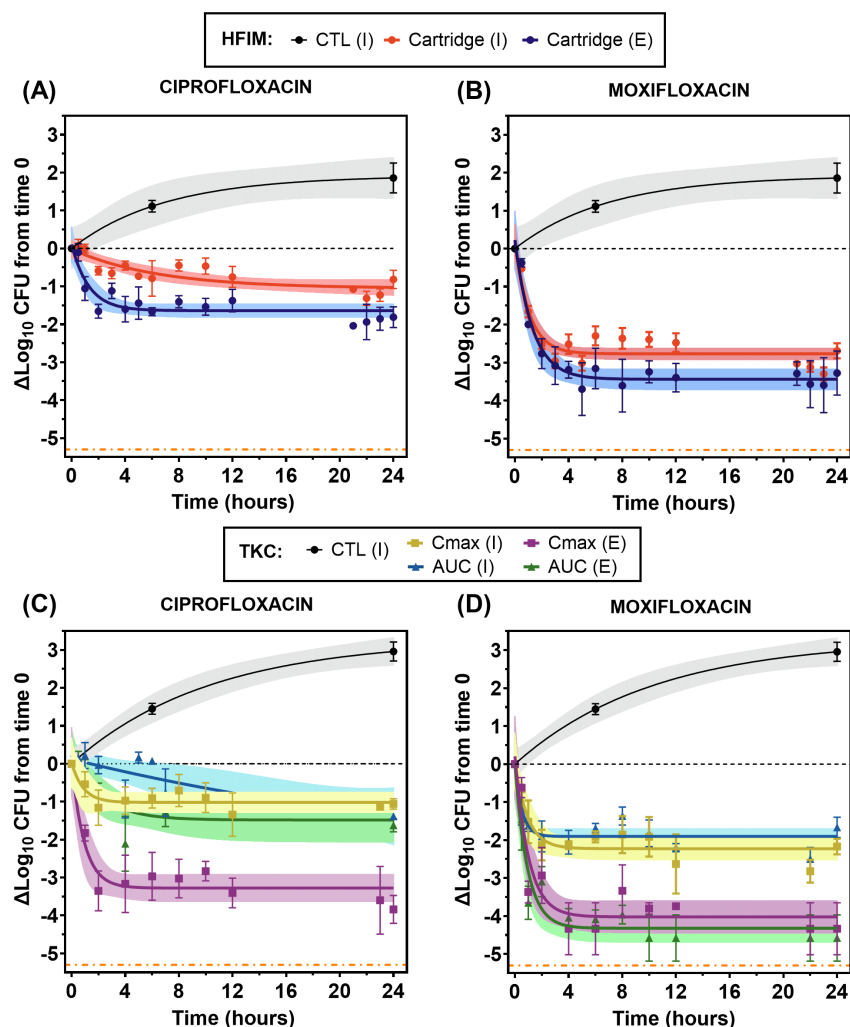


FIGURE 4. Fluoroquinolone activities in dynamic (HFIM) and static (TKC) conditions against extracellular and intracellular forms of *S. aureus*. The graphs show the change in the number of colonies forming units (CFU) ($\Delta \log_{10}$ CFU) per mL of culture medium (extracellular fraction; [E]) or in THP-1 monocytes (intracellular; [I]) per mg of cell protein over the time of incubation as compared to the initial inoculum. HFIM: ciprofloxacin (A) and moxifloxacin (B) concentrations fluctuate over time in the cartridge to mimic human PK profiles for unbound drugs (see Figure 3). TKC: ciprofloxacin (C) and moxifloxacin (D) were added at their maximal serum concentration (Cmax, free drug: $2.8 \mu\text{g mL}^{-1}$ for ciprofloxacin and $2.4 \mu\text{g mL}^{-1}$ for moxifloxacin) or at $0.7 \mu\text{g mL}^{-1}$ for ciprofloxacin and $1.1 \mu\text{g mL}^{-1}$ for moxifloxacin to simulate the free 24 h area under the concentration-time (AUC) reached in humans. Black dotted line: apparent static effect. Orange dotted line: theoretical limit of detection (1 CFU/plate). Data are means $\pm$ SEM with 95% CI; N = 2-3, n = 1. See also Figure S2.

TABLE 3.

Comparison of minimal duration of killing (MDK)^a and maximal efficacy (Emax)^b values of fluoroquinolones against extracellular and intracellular *S. aureus*, under dynamic (HF) and static (TKC) conditions.

Cond.	Applied treatment	MDK ₉₀ (1 Log)	MDK ₉₉ (2 Log)	MDK _{99.9} (3 Log)	MDK _{99.99} (4 Log)	Emax
Dynamic (Intra)	CIP	18 h	N/A ^c	N/A	N/A	-1.05 log₁₀ [95% CI, -1.33 to -0.77]
	MXF	0.5 h	1.5 h	N/A	N/A	-2.77 log₁₀ [95% CI, -2.93 to -2.61]
Dynamic (Extra)	CIP	1.5 h	N/A	N/A	N/A	-1.64 log₁₀ [95% CI, -1.83 to -1.45]
	MXF	0.5 h	1 h	3 h	N/A	-3.44 log₁₀ [95% CI, -3.72 to -3.16]
Static (Intra)	CIP C_{max}	3 h	N/A	N/A	N/A	-1.02 log₁₀ [95% CI, -1.30 to -0.74]
	CIP AUC	12 h	N/A	N/A	N/A	N/A^d
	MXF C_{max}	0.75 h	2 h	N/A	N/A	-2.23 log₁₀ [95% CI, -2.53 to -1.93]
	MXF AUC	0.5 h	N/A	N/A	N/A	-1.90 log₁₀ [95% CI, -2.12 to -1.69]
Static (Extra)	CIP C_{max}	0.5 h	1 h	2.5 h	N/A	-3.28 log₁₀ [95% CI, -3.65 to -2.91]
	CIP AUC	2.5 h	N/A	N/A	N/A	-1.44 log₁₀ [95% CI, -2.09 to 0.87]
	MXF C_{max}	0.5 h	0.75 h	1.5 h	6 h	-4.02 log₁₀ [95% CI, -4.45 to -3.59]
	MXF AUC	0.25 h	0.5 h	1 h	2.5 h	-4.32 log₁₀ [95% CI, -4.70 to -3.94]

^a Estimated as the time needed to achieve 90% (1 log₁₀), 99% (2 log₁₀), 99.9% (3 log₁₀) or 99.99% (4 log₁₀) killing based on the equation of the exponential decay curve describing activity over time. ^b Value of the plateau in the equation of the exponential decay curve describing activity over time. These values are expressed as log₁₀ reduction from the initial (i.e. before the drug addition) inoculum (values in log₁₀ CFU per mg cell protein (Intra) or mL (Extra) for control of ciprofloxacin and moxifloxacin experiments [mean ± SD] are respectively: Dynamic, intra: 6.98 ± 0.35 and 6.93 ± 0.07; Dynamic, extra: 5.63 ± 0.47 and 5.21 ± 0.61; Static, Intra: 6.24 ± 0.49 and 5.59 ± 0.22; Static, extra: 5.26 ± 0.59 and 4.33 ± 1.18). ^c N/A Not Achieved. ^d Plateau value not reached in this condition.

e) Discussion

Due to the challenge of treating *S. aureus* intracellular infections with current antibiotics, there is an urgent need to optimize their use to ensure bacterial killing and the prevention of resistance. In this study, we successfully developed a dynamic *S. aureus* intracellular infection model, representing a significant advancement in simulating the interaction between antibiotics and bacteria within host cells.

While similar approaches have previously been applied to other intracellular pathogens such as *Listeria monocytogenes* [172] and *Mycobacterium spp.* [204,210], adapting the HFIM to intracellular *S. aureus* presented a unique challenge, particularly due to its ability to escape from the host cells by killing them in order to disseminate in the tissues [215]. To our knowledge, this is the first study using the HFIM model to assess the activity of antibiotics over 24 hours on both intracellular and extracellular *S. aureus*.

Previous HFIM studies evaluating antimicrobial activity against extracellular *S. aureus* have primarily focused on MRSA and biofilm-related infections. Some of these studies assessed the efficacy of specific antimicrobial agents in reducing MRSA virulence [216]. Others compared antibiotic combinations against biofilm and planktonic bacteria [175]. Additionally, other works have examined moxifloxacin [217] and ciprofloxacin [218] efficacy against extracellular, and not intracellular, *S. aureus* in the HFIM, but they did not consider the unbound fraction of the antibiotics, which is required to accurately assess drug activity.

In the present study, key parameters were optimized in order to adapt the model to intracellular *S. aureus*. These include the inoculum size, taking into account the need for an equilibrium time in the cartridge, and the proliferation and viability of

THP-1 cells within the cartridge. These optimizations, not described in the previously published models of intracellular HFIM, ensured that antibiotic effects were accurately measured without interference from confounding factors such as the stress caused by the infection process or the system itself. The 12-hour equilibrium period was specifically used to promote the proliferation of the intracellular inoculum, achieving a bacterial density of approximately $6 \log_{10}$ CFU after 12 hours, previously defined as adequate for assessing antibiotic activity [11,219,220]. During this period, some intracellular bacteria were released from THP-1 cells due to natural cell death, forming an extracellular population that was also exposed to fluctuating drug concentrations, further enhancing the physiological relevance of the model. When antibiotics were introduced, this contamination was modest, and the intracellular inoculum was well controlled, allowing experiments to be conducted for up to 24 hours while maintaining acceptable levels of cell mortality. Moreover, we showed that non-infected THP-1 cells survived and proliferated at rates comparable to standard conditions (in wells), confirming that the system environment does not negatively affect cellular health. These results validate the robustness and physiological relevance of our model for studying intracellular and extracellular forms of *S. aureus*.

In this optimized model, our findings highlight the limited activity of ciprofloxacin against intracellular *S. aureus*, which aligns with its established efficacy profile, primarily targeting Gram-negative bacteria such as *Escherichia coli* [221]. In contrast, moxifloxacin demonstrates strong intracellular activity against *S. aureus*, though complete eradication was not observed, consistent with existing literature [11,82].

Notably, moxifloxacin demonstrated higher intracellular efficacy in the dynamic HFIM system as compared to traditional time-kill curve (TKC) experiments.

Comparing these two systems (see Figure S2 for a direct comparison of different experimental conditions for each drug) is novel for intracellular bacteria, as previous studies with *L. monocytogenes* [172] or *Mycobacteria* spp. [204,210] did not perform TKC experiments in parallel. The differences observed between HFIM and TKC may be attributed to variations in the experimental setup. We observed higher accumulation of moxifloxacin inside THP-1 cells in the HFIM, potentially due to improved medium renewal in the dynamic system. This medium refreshment could reduce the accumulation of toxic cell debris and ensure sustained nutrient availability, supporting cell viability better than a well environment. Conversely, the intracellular activity of ciprofloxacin in the HFIM was lower (vs. TKC at C_{max}) or similar (vs. TKC simulating AUC) as compared to static models. We were unable to detect ciprofloxacin intracellularly in the HFIM to confirm this difference, but previous experiments in static models indicate that ciprofloxacin accumulates at lower levels and at a slower rate than moxifloxacin [17]. A delay in accumulation is observed for moxifloxacin in the HFIM despite its almost instantaneous accumulation in static conditions [67]. We may therefore suspect a similar delay occurs for the less diffusible ciprofloxacin, explaining its slower killing in the HFIM.

Regarding extracellular activity, both fluoroquinolones exhibited high efficacy, which can be attributed to their favorable PK/PD parameters. Notably, the AUC_{0-24h}/MIC ratio was well above 125 and the C_{max}/MIC ratio was also above 10 for both antibiotics in the HFIM. These threshold values are correlated with positive clinical and microbiological outcomes *in vivo* and in the clinics [49,55]. Moreover, the higher AUC_{0-24h}/MIC ratio (475 vs 144 h^{-1}) and C_{max}/MIC ratio (44 vs. 17) justify the faster and better response observed with moxifloxacin compared to ciprofloxacin. In static conditions at C_{max} , the AUC_{0-24h}/MIC ratio for both drugs is even higher, which explains the better efficacy observed, especially for ciprofloxacin in these conditions (AUC_{0-24h}/MIC ratios of 960 and 538 h^{-1} for

moxifloxacin and ciprofloxacin, respectively; i.e., values 2 to 3-fold higher than in dynamic conditions). Since fluoroquinolones are C_{max}/MIC and AUC_{0-24h}/MIC -dependent antibiotics, we also evaluated their activity in static conditions mimicking AUC_{0-24h} . In these conditions, the C_{max}/MIC ratio decreased from 40 to 18.33 for moxifloxacin and from 22.4 to 5.6 for ciprofloxacin. This reduction does not affect the activity of moxifloxacin but diminishes ciprofloxacin's efficacy (Figure S2), as it no longer meets the required threshold value of 10 for the C_{max}/MIC parameter, predictive of high efficacy [49,55].

f) Conclusion

In conclusion, the HFIM adapted to intracellular infection by *S. aureus* developed in this study provides a valuable tool for optimizing treatment strategies with conventional drugs and evaluating the efficacy of new compounds in targeting *S. aureus* intracellular infections. Although it does not fully replicate all aspects of the clinics, this model offers several advantages. First, it could be extended to other drugs or other strains, or adapted for specific purposes such as the detection of rare resistant mutants, which is sometimes challenging or non-feasible with *in vivo* models for ethical reasons [165]. Second, it allows for repetitive sampling, like blood sampling in patients, facilitating the monitoring of drug PK and of the infection dynamics over time. Lastly, the system accurately mimics the PK profiles observed in patients, making it a robust and reliable tool to establish optimized dosing regimens of already available or newly developed drugs, considering the intracellular component of the infection.

g) Limitations of the study

Despite its novelty, this study has some limitations. First, THP-1 cells, a permissive monocytic cell line, do not fully mimic the diversity and functionality of primary immune cells [222]. Further adaptation of the model could incorporate peripheral blood mononuclear cells (PBMCs), or other cell types, including bronchial epithelial cells, keratinocytes, or osteoblasts, where *S. aureus* is also known to survive [4]. However, this represents an even more complex challenge, as adherent cells may clog the pores of the fibers. Second, the experiments were conducted in a simplified *in vitro* environment, lacking a complete immune response involving other cell types and immune defense mechanisms. Third, the short duration of the experiments, limited to 24 hours, restricts the ability to observe the global effect of a full course treatment of 5 to 14 days [223]. Fourth, highly sensitive methods are needed to quantify intracellular antibiotics, as only small volume aliquots can be taken out of the system to maintain its equilibrium. Lastly, a single reference strain has been used to develop the model. A similar setup can be used to adapt it to relevant clinical isolates, starting with those already used in the past to successfully infect THP-1 cells [224].

Nevertheless, by simulating *in vivo* exposure, the HFIM may also help to better mimic human PK profiles, especially the free drug concentrations, than animal models. Protein binding is indeed variable among species, as exemplified by the lower protein binding of ciprofloxacin (10%) in mice [225]. Finally, another advantage is that the dynamic conditions of this newly developed intracellular HFIM may provide data of better predictive value compared to static assays (TKC). In particular, we noticed significant differences in efficacy or kill rates between the two models.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Françoise Van Bambeke (francoise.vanbambeke@uclouvain.be)

Materials availability

This study did not generate new unique reagents.

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AUTHOR CONTRIBUTION

G.M, F.V.B and L.E designed the research; G.M conducted the experiments; A.C. and N.P shared their expertise in HFIM models; G.M and F.V.B. wrote the manuscript; all authors reviewed and approved the final version of the document.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors utilized Co-PILOT to improve the readability and language of the manuscript. Following the use of these tools, the entire manuscript was thoroughly reviewed and edited by the authors.

h) Star methods text

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Drugs and chemicals

Moxifloxacin-HCl (MXF) and ciprofloxacin-HCl (CIP) were provided as microbiological standards by Bayer AG (Leverkusen, Germany); gentamicin sulfate was purchased from Medix Biochemica (St-Louis, MO); Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA); cation-adjusted Mueller-Hinton broth (CA-MHB) and Mueller-Hinton agar (MHA) were obtained from Sigma-Aldrich (St-Louis, MO).

Bacterial strain and cells

Staphylococcus aureus ATCC 29213 reference strain was acquired from the American Type Culture Collection (Manassas, VA) and freshly cultured before each experiment at 37 °C in CA-MHB with shaking at 130 rpm. MICs were determined by microdilution in CA-MHB at physiological pH according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). THP-1 cells, a human myelomonocytic cell line derived from the blood of a 1-year-old boy that displays macrophage-like activity [226], were maintained in our laboratory as previously described and freshly cultured before each experiment in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) at 37 °C in a 5% CO₂ atmosphere [162]. The model being set up for non-adhering cells, they were not further differentiated in macrophages.

METHOD DETAILS

Intracellular infection

Intracellular infection was conducted as previously described [162,219,220] with specific adaptations required for the performance of the experiments in the hollow fiber system (see results for justification of these changes). Briefly, the cells (5×10^5 cells mL⁻¹) were maintained as a loose suspension in RPMI 1640 medium supplemented with 10% FBS at 37 °C under a 5% CO₂ atmosphere. The day before the infection, *S. aureus* culture was freshly prepared in 10 mL of CA-MHB (37 °C, 130 rpm agitation) to reach a stationary-phase culture. The day of the experiment, bacteria were diluted at a ratio of 1:50 in fresh CA-MHB for 1h30 to ensure that the population was in log-phase growth. Bacteria were then opsonized during 45 min (37 °C, 130 rpm) using non-decomplemented human serum diluted 1:10 in serum-free culture medium (RPMI 1640). Phagocytosis was subsequently allowed at a low bacterium-macrophage ratio (MOI, multiplicity of infection) of 0.0001:1 for 45 min. Elimination of the non-phagocyted bacteria was achieved by incubation for 1 hour with gentamicin (25 µg mL⁻¹; 50 x MIC), followed by three washing steps with phosphate-buffered saline (PBS) and centrifugation at 1,300 rpm for 7 min. Infected cells were then resuspended in prewarmed complete RPMI (10% FBS) during 12 h in order to reach an average infection index (MOI) of 4 bacteria per macrophage (MOI = 4:1) (as determined by counting the numbers of colonies forming units (CFU)). This protocol resulted in a final intracellular inoculum of approximately 10⁶ CFU mg cell protein⁻¹.

Hollow fiber infection model (HFIM)

The HFIM was used to assess bacterial responses to clinically relevant PK profiles. This system is composed of a dialysis cartridge linked to a central reservoir, which is further connected to two additional compartments: a waste reservoir and a diluent reservoir (see [164,227] for an overall review of this system).

Fluid movement between compartments is facilitated by tubing and controlled via peristaltic pumps. Antibiotic is administered through an additional pump connected to the central reservoir (see Figure S1 for system configuration). The set up of the model is detailed in the next paragraphs. The results were subsequently compared with those obtained from traditional TKC experiments [11,83].

Materials

All experiments were conducted using RPMI 1640 medium supplemented with a reduced concentration of 2% FBS [204]. The hollow fiber system was incubated at a temperature of 37°C under a 5% CO₂ atmosphere. Hollow fiber Helixone[®] cartridges were procured from Fresenius Medical Care (FX-PAED Helixone[®] dialyzer, Bad Homburg, Germany), with a Neocap[®] obturator (Asept InMed, Quint-Fonsegrives, France) placed on the top of the cartridge to facilitate the collection of samples for pharmacokinetic and pharmacodynamic (PK/PD) analysis within the cartridge. The extracapillary space (ECS) of each cartridge has a 60 mL capacity. The central reservoir was constituted of a 500 mL bottle, pre-filled with 300 mL RPMI 1640 with 2% FBS. It was equipped with a connection system featuring two PTFE (polytetrafluoroethylene) ports secured with GL14 PP (polypropylene) screw caps (VWR, Radnor, Pennsylvania, US), equipped with a 0.22 µM membrane filter to facilitate pressure equalization (VWR) and was continuously mixed using a magnetic stirrer.

The diluent medium, composed of RPMI complete medium (2% FBS), was prepared in a 5 L borosilicate glass bottle (VWR), closed using a connection system incorporating two PTFE ports with GL14 PP screw caps and also equipped with a 0.22 µM membrane filter to facilitate pressure equalization (VWR). The diluent medium was extended to the bottom of the media bottle using platinum-cured silicone tubing and secured with a GL14 screw cap insert. The medium was subsequently pumped into the central reservoir using a peristaltic pump.

To maintain a constant volume within the system, the addition of fresh medium to the central reservoir was balanced by the simultaneous removal of an equivalent volume to the waste reservoir. Antibiotics were added in the central reservoir via a Rythmic™ Perf + computerized perfusion pump (Micrel Medical Devices, Koropi, Attiki, Greece). A second obturator was positioned on a three-way stopcock between the central reservoir and the cartridge to enable sample collection for PK measurement in the central compartment. Rapid equilibration of antibiotic concentration between the central compartment and the cartridge was ensured through a high-flow Masterflex™ L/S™ peristaltic pump (VWR) operating at a rate of 60 mL min⁻¹. Prior to the assembly of the system, the medium was filtered through a 0.20 µm pore size vacuum filtration apparatus (VWR).

THP-1 human monocytes were concentrated to a density of 5 x 10⁵ cells mL⁻¹ and infected with *S. aureus* as described above before being inoculated into the 60 mL dialysis cartridge via a BD Plastipak syringe (50 mL BD Becton; Dickinson and Company, Franklin Lakes, New Jersey, US).

Set-up

The hollow fiber system, free from bacteria or cells, was set up and incubated at 37°C before the start of the experiment. Twelve hours before the addition of antibiotic (time point T0), *S. aureus* infected THP-1 cells were introduced into the cartridge, which was subsequently positioned within the system and incubated without renewal of the medium. Preliminary experiments showed that this step was critical for facilitating: 1) the proliferation of the initial inoculum to reach a high enough concentration to allow further bacteria growth but low enough to avoid killing the host cells (typically 10⁶ CFUs mg cell protein⁻¹), and 2) the adaptation of the infected cells to this new environment. After this preincubation period, the system was activated for 1 hour at 60 mL min⁻¹ to enable medium renewal within

the cartridge. An initial sample was taken to determine the initial inoculum at this time point, defined as (T0) for experiments where the antibiotic was added at that time. Each antibiotic was administered as a 5 mL 1-hour infusion, with ciprofloxacin dosed once every 12-hours at a concentration of $242.91 \mu\text{g mL}^{-1}$ and moxifloxacin dosed once every 24-hours at a concentration of $179.95 \mu\text{g mL}^{-1}$. Diluent medium was subsequently pumped into the central reservoir using a peristaltic pump at a flow rate of 2.31 mL min^{-1} for ciprofloxacin and 0.49 mL min^{-1} for moxifloxacin to mimic the pharmacokinetic (PK) profiles of the antibiotics. Experiments were conducted over 36 hours for each antibiotic tested, consisting of 12 hours of incubation, an additional 1 hour for medium renewal within the cartridge, and 24 hours for sampling to evaluate the activity of the antibiotics.

Simulation of PK profiles in the HFIM

In this study, we simulated the PK profiles of ciprofloxacin and moxifloxacin administered via the intravenous route (IV) at doses of 400 mg twice daily and 400 mg once daily, respectively. Key parameters such as maximum serum concentration (C_{max}), infusion time (T_{inf}), dosing interval (τ), half-lives ($T_{1/2}$) were obtained from the study of Lettieri et al. [190] for ciprofloxacin and of Stass et al. [189] for moxifloxacin (see Table 1). Both studies evaluated total plasma concentrations. As the serum concentrations in culture media were low (2%), the protein concentrations were low; thus, these values were adjusted to mimic protein-unbound pharmacokinetic profiles. A protein-bound fraction in plasma of 30% for ciprofloxacin [190] and 40% for moxifloxacin [189] were considered. The simulations of target profiles were conducted using the HF-app, a web application developed with R-shiny by Vincent Aranzana-Climent et al. (INSERM U1070, Poitiers, France) [169]. This tool was designed to streamline the calculations involved in setting up hollow-fiber experiments for both single-drug and combination therapies.

TABLE 1.

Moxifloxacin and ciprofloxacin unbound pharmacokinetic parameters simulated* in the HFIM

Drug	C _{max} (mg mL ⁻¹)	T _{inf} (hours)	τ (hours)	T _{1/2} (hours)	Cl (mL min ⁻¹)	Reference
Moxifloxacin	2.41	1	24	8.49	0.49	[189]
Ciprofloxacin	2.80	1	12	1.80	2.31	[190]

C_{max}, maximum serum concentration; T_{inf}, infusion duration; τ, dosing interval; T_{1/2}, elimination half-life; Cl, clearance.

* Simulated with the HF-app: https://varacli.shinyapps.io/hollow_fiber_app/

Pharmacokinetic (PK) measurements in HFIM

Sampling was performed as follows: the central compartment of the HF system was sampled (500 µL) just before the addition of the antibiotic (T₀) and at multiple time points post-administration of the antibiotic in order to validate its concentration-time profile. In parallel, the cartridge was sampled (1 mL) to measure the antibiotic concentrations directly in contact with bacteria to take into account the penetration of the antibiotic in the extracapillary space within the cartridge. This sample was also used for PD determination (see PD measurement section). For antibiotic intracellular concentrations, after cell lysis for PD measurements (see TKC protocol), an aliquot of 50 µL was taken for analysis. (see Figure S1 supplementary material).

Both drugs were assayed by HPLC-MS/MS. To establish calibration standards, stock solutions of antibiotics were prepared at 0.1 mg mL⁻¹ in distilled water. Working solutions for calibration curves were prepared at concentrations ranging from 5000 to 5 ng mL⁻¹ for ciprofloxacin and from 2000 to 5 ng mL⁻¹ for moxifloxacin. These dilutions were performed in water with 0.1% formic acid for the intracellular calibration curve and in RPMI + 10% FBS for the extracellular calibration curve. The working solutions corresponding to the intracellular calibration curve were individually spiked with dry THP-1 cell pellets at a density of approximately 2 x 10⁶

cells per pellet. Subsequently, aliquots of each sample and each solution from the calibration curves (50 μL) were transferred into a 1.5 mL centrifuge tube and mixed with 25 μL of IS ($[\text{}^2\text{H}_8]$ -Ciprofloxacin at a concentration of 0.4 $\mu\text{g mL}^{-1}$ in distilled water for ciprofloxacin, and ciprofloxacin at a concentration of 0.8 $\mu\text{g mL}^{-1}$ in distilled water for moxifloxacin). Deproteinization was then achieved by the addition of 200 μL of acetonitrile with 0.1% formic acid followed by vortexing for 5 s. All samples were further centrifuged at 14,000 rpm at 4°C for 10 min. Lastly, 180 μL of the supernatants were transferred to an HPLC vial containing 20 μL of water with 0.2% formic acid for analysis. For moxifloxacin, the extracted samples were diluted 3 times in acetonitrile with 0.1% formic acid to ensure being in the linear range of the calibration curve. Samples were stored at 4 °C until analysis.

Samples were analyzed by the Triple Quad LC-MS method (Shimadzu Corporation, Kyoto, Japan). Both ciprofloxacin and moxifloxacin separations were achieved at ambient temperature on a 150 mm x 2 mm, 5 μM Gemini® NX-C18 LC column (Phenomenex, Torrance, CA, US). The mobile phase consisted of water with 0.1% formic acid and acetonitrile with 0.1% formic acid in a ratio of 75:25, which was pumped into the chromatographic system under isocratic conditions at a flow rate of 0.2 mL min^{-1} . The injection volume of the sample was 20 μL , and the samples were maintained in a thermostatic autosampler (4°C). The total analysis time was 7.5 min, with elution of ciprofloxacin and $[\text{}^2\text{H}_8]$ -ciprofloxacin occurring at 1.93 min and moxifloxacin at 1.92 min. The transitions from precursor to product ions for CIP were 331.4 m/z to 314 m/z , 339.2 m/z to 321.1 m/z for $[\text{}^2\text{H}_8]$ -Ciprofloxacin, and 402.1 m/z to 384.1 m/z for moxifloxacin. The quantifiable ranges established by the methods were from 5 to 0.005 $\mu\text{g mL}^{-1}$ for ciprofloxacin and from 2 to 0.005 $\mu\text{g mL}^{-1}$ for moxifloxacin. The peak area ratios of ciprofloxacin and moxifloxacin relative to their respective IS across each standard solution were calculated and subsequently plotted as a function of drug concentrations.

The calibration curves were acceptable only when exhibiting correlation coefficients (r^2) equal to or exceeding 0.98. Data acquisition was processed using Shimadzu LabSolutions software version 5.109.

Pharmacodynamic (PD) measurements in HFIM

For each sample, the bacterial load was assessed extracellularly and intracellularly. The content of the cartridge was vigorously mixed using two 20-mL syringes (BD Becton), from which a 1 mL suspension was extracted through an obturator. These samples were then processed as described for TKC experiments.

Time kill curves (TKC)

The experiments were conducted concurrently with HFIM to evaluate the effects of antibiotics under static conditions (TKC) in comparison to dynamic conditions. TKC were performed as described earlier with some modifications [11,83]. Cells infected as described above were plated into 12-well plates (2 mL per well) and incubated for 12 hours (37 °C, 5% CO₂ atmosphere) to achieve a MOI of 4:1, *i.e.*, a value corresponding to that obtained using the originally described model of intracellular infection [11,219,220]. At that time, the antibiotic was added at its maximal unbound concentration in human serum (C_{max}; 2.8 µg mL⁻¹ for ciprofloxacin and 2.4 µg mL⁻¹ for moxifloxacin for an administered dose of 400 mg [189,190]) in each well or at concentrations allowing to mimic the unbound area under the curve (AUC) in human serum over 24 hours, corresponding to 1.1 µg mL⁻¹ for moxifloxacin (equivalent to an AUC_{24h} of 26.41 mg.h L⁻¹ [189]) and 0.7 µg mL⁻¹ for ciprofloxacin (equivalent to an AUC_{12h} of 17.31 mg.h L⁻¹ [190]). Samples of 1 mL were collected at various time points: 0 h (before the addition of the antibiotic) and 0.5, 1, 2, 4, 6, 8, 10, 12, 22, and 24 hours after the addition of the antibiotic (1 well per time point). After centrifugation at 1,300 rpm for 7 min, 100 µL of the supernatant was spread on MHA + charcoal (2%) agar plates (20 mL) after

appropriate dilutions in PBS for quantification of extracellular CFU counts. Charcoal addition aimed at adsorbing residual antibiotic and avoiding carry-over effect [81]. The extracellular bacteria detected at this stage reflect the bacterial inoculum released due to the natural death of infected THP-1 cells during the incubation period. The remaining supernatant was then carefully removed, and the pellet resuspended in 1 mL of PBS and centrifuged again at 1,300 rpm for 7 min to eliminate any remaining extracellular bacteria. PBS was removed, and the pellet was resuspended in 500 μ L of distilled water for 5 min to lyse the cells. A 100 μ L aliquot of lysate was plated on MHA + charcoal (2%) agar plates (20 mL) after appropriate dilutions in PBS for quantification of the number of viable intracellular bacteria through colony counting. Forty μ L were also used for total cell protein measurement using a DC (detergent compatible) Protein Assay from Bio-Rad (Hercules, CA) according to the manufacturer's instructions. Data were expressed as the number of CFUs per milligram of cell protein (CFUs mg cell protein⁻¹) for intracellular bacteria and as the number of CFUs per milliliter (CFUs mL⁻¹) for extracellular bacteria.

Cell viability

Cell viability was evaluated in wells (TKC) and HFIM for non-infected and infected cells using the Trypan blue exclusion assay according to the provider's instructions (Gibco, Thermo Fisher Scientific). After harvesting and washing the cells in PBS, they were stained at a 1:1 ratio with Trypan blue solution 0.4%. After mixing, 10 μ L of the suspension was pipetted into a Burker counting chamber, and cells were counted according to the manufacturer's instructions. The percentage of mortality was expressed as the ratio between the number of blue-stained cells (dead) and the total number of cells in a specified surface area.

QUANTIFICATION AND STATISTICAL ANALYSIS

Experiments to set up the model were performed in triplicates in a single experiment, but viability and MOI were checked again in all further experiments. Experiments to determine pharmacokinetic profiles and activity were performed in 2 or 3 independent replicates. Plotting and calculations were performed using GraphPad Prism version 8.0 (GraphPad software, San Diego, CA). Observed PK parameters were determined using the exponential decay model on GraphPad. Statistical analyses were performed to assess treatment efficacy, THP-1 viability, and to determine the right MOI, using 95% confidence intervals (CI) to assess the precision of our measurements. For each treatment group, CI were calculated using GraphPad to quantify the uncertainty around each mean estimate. These intervals were interpreted to determine both the reliability of the observed effects and the statistically significant effect between groups. When comparing group means, a non-overlapping CI was considered indicative of a statistically significant difference between treatment effects. For other comparisons between two groups, student t-tests for independent samples were performed (differences were considered significant if p values were < 0.05 [$P < 0.05$]). All details about the number of replicates and the way data are represented (with SD, SEM, or confidence interval) are indicated in the captions of the figures or footnotes to tables.

SUPPLEMENTAL INFORMATIONS

FIGURE S1: Schematic representation of the HFIM used in this study.

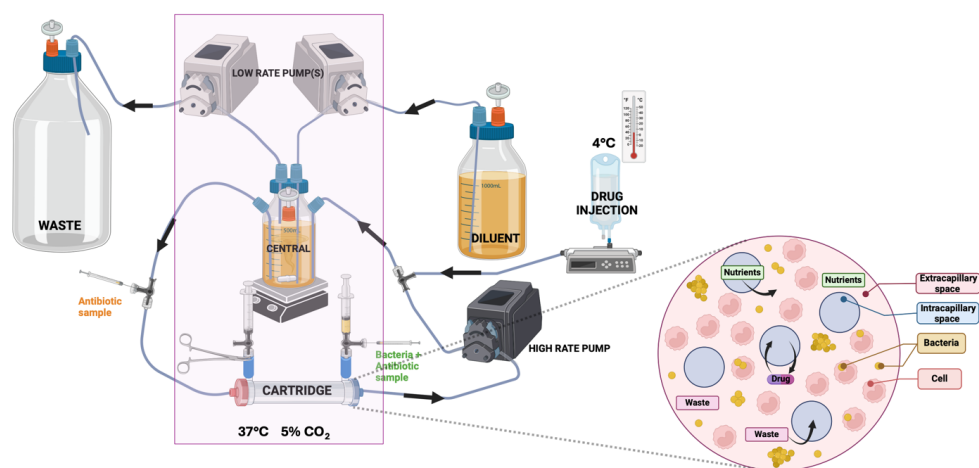
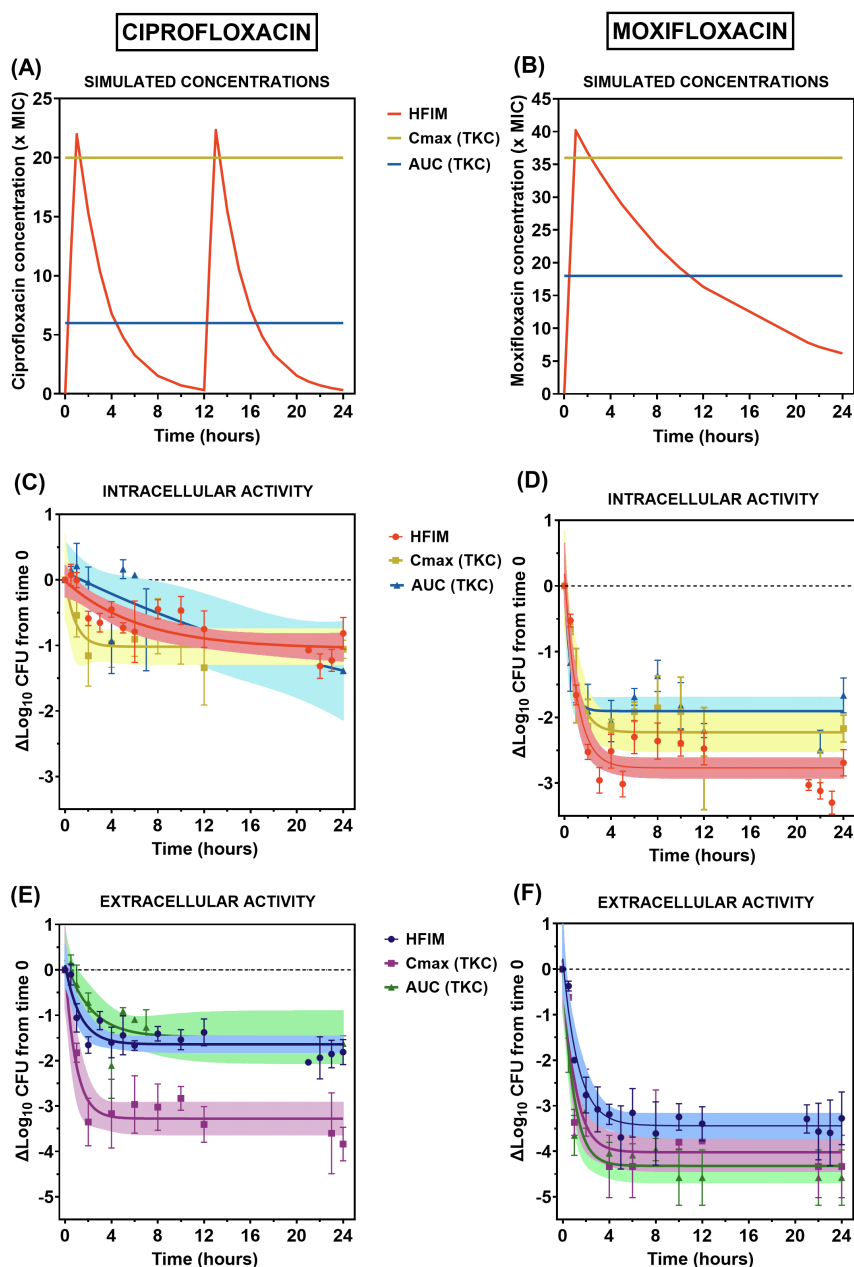


FIGURE S2: PK profiles vs. PD profiles for each fluoroquinolone. The top panels (A, B) reproduce the simulated antibiotic concentrations in the cartridge (HFIM) or in wells (Cmax and AUC) for TKC experiments, with concentrations expressed in multiples of the MIC of the antibiotic for the strain used. The middle (C, D) and bottom (E, F) panels compare the PD profile of the antibiotics intracellularly and extracellularly, respectively, in the same conditions. Related to Figure 4.



CHAPTER IV – GENERAL DISCUSSION & PERSPECTIVES

IV.1 Main achievements of this work

Intracellular *S. aureus* infections are challenging to treat. FQs are considered drugs of choice for such infections due to their ability to penetrate and accumulate within host cells (i.e., phagocytic cells), allowing activity against intracellular bacteria. However, they are not able to fully eradicate intracellular infections due to PK limitations, such as suboptimal distribution or suboptimal intracellular accumulation, as well as PD challenges. The latter represents the main obstacle to complete eradication, as the intracellular environment influences bacterial response to antibiotics.

To address these challenges, my thesis employed an integrative approach, combining PK, PD, and pharmacogenetic investigations to understand parameters impacting FQ efficacy in treating intracellular *S. aureus* infections. Specifically in this work: **(1) I investigated the role of ABC efflux transporters** expressed by host cells in mediating FQ efflux **and evaluated the impact of common genetic polymorphisms on transporter activity**; **(2) I learned to develop an *in vitro* dynamic model** to simulate plasma FQ concentrations over time, mimicking *in vivo* drug fluctuations; **(3) I implemented this model to study intracellular *S. aureus* infections**, allowing assessment of FQ efficacy in the context of fluctuating drug concentrations.

Through this work, I successfully achieved my initial goals, demonstrating that a deep understanding of PK/PD parameters, combined with the consideration of pharmacogenetic factors, is essential for optimizing the use of FQs, particularly for treating intracellular *S. aureus* infections.

(1) Study of the impact of ABC efflux proteins and their genetic polymorphism on FQ cellular pharmacokinetics as a possible reason for interindividual variability

My first focus was on the impact of ABC efflux proteins, particularly ABCB1, ABCG2, and ABCC4 transporters, on the cellular disposition of CIP and MXF (Chapter III – article n°1). These transporters, by unidirectionally translocating a wide range of substrates from intracellular to extracellular compartments, could influence both the PK of FQs (availability of antibiotics in infected compartment(s)), and, as a consequence, their PD (antibacterial activity within this/those compartment(s)). To enhance our translational perspective, I also investigated the functional impact of common SNPs in the genes coding for these proteins in order to identify variants that may affect the cellular and systemic disposition of FQs, potentially contributing to interindividual PK variability and in therapeutic response.

Comparable *in vitro* models of human recombinant HEK293 cell lines, stably overexpressing ABC transporters (wild-type or specific variants), were generated. HEK293 cells were transfected with an empty plasmid serving as a control. The polymorphisms investigated included the *ABCB1* variant c. 1199G>A (rs2229109) and the common haplotypes c.1236C>T (rs1128503), c.2677G>T/A (rs2032582) and c.3435C>T (rs1045642), as well as the *ABCG2* polymorphism c.421C>A (rs2231142). The results of this study showed that ABCB1 overexpression significantly reduces MXF and CIP cellular concentrations, which was reversed by the specific and the non-specific inhibitors zosuquidar and elacridar. ABCG2 had no impact on the accumulation of these antibiotics.

One possible explanation for the lack of ABCG2 effect on CIP accumulation in our model is the basal expression of ABCC4. Indeed, while ABCC4 did not affect MXF accumulation it significantly reduced CIP accumulation, even when expressed at basal levels. This effect was reversed by MK571, a non-specific ABCC inhibitor.

This suggests that ABCC4-mediated efflux may have reduced the actual cellular accumulation of CIP in the CTL cells, possibly masking the effect of potentially masking the effect of ABCG2 and potentially explaining the discrepancy with previous reports in the literature, where ABCG2 was identified as a relevant transporter for CIP. Additionally, contrary to the wild-type and the c.1199A carriers, *ABCB1* CGT and TTT haplotypes did not reduce antibiotic accumulation.

I confirmed that both MXF and CIP are substrates of the ABCB1 transporter, with CIP being a preferential ABCC4 substrate. Additionally, I demonstrated the importance of measuring basal transporter expression before conducting experiments, as endogenous transporters are present at low levels in HEK293 cells used for transfections. Moreover, their expression can vary significantly between different cell batches, potentially influencing the impact of an overexpressed transporter, especially when the substrate has a higher affinity for the endogenous transporter(s), necessitating careful interpretation of the results.

Furthermore, the ABCB1 model was used in a collaborative study that I have conducted to assess its efficacy with drugs from other pharmacological classes, such as rivaroxaban and tacrolimus (Chapter V – collaborative work).

(2) *In vitro* dynamic model adapted to intracellular infection by *S. aureus*

Antibiotic activity against intracellular bacteria is typically evaluated in static conditions, where infected cells are exposed to fixed drug concentrations over time. This approach does not mimic the *in vivo* situation, where antibiotic concentrations fluctuate over time. The HFIM, considered the gold standard for PK/PD dynamic studies *in vitro*, has essentially been used to evaluate antibiotic activity extracellularly. I learned the setup of this model during a two-month internship at the University of Poitiers (France) with the team of Prof. W. Couet, S.

Marchand, and especially with Prof. A. Chauzy. Specifically, I learned how to model the PK profile of CIP and test its activity against extracellular *S. aureus*. Back in Belgium, I adapted this model to intracellular *S. aureus* infecting THP-1 monocytes. Key optimizations included maintaining cell viability in the system and ensuring appropriate bacterial inoculum levels to prevent host cell toxicity.

Lastly, while the hollow fiber intracellular model has previously been used to study only a limited number of intracellular pathogens, such as *Mycobacterium avium*, *M. tuberculosis*, and *M. kansasii* in THP-1 cells for up to 28 days [204,205,210], as well as *L. monocytogenes* for 7 days in the same system [172], my study is the first to extend its application to intracellular *S. aureus*.

(3) Implementation of the HF model to study the intracellular and extracellular activity of FQ in dynamic conditions in comparison with static conditions

Using the HFIM, I replicated human PK profiles of CIP and MXF and compared their activity in these dynamic conditions (HF) with that obtained in static conditions (TKC assays), where antibiotic concentrations remain stable over time (Chapter III – article n°2). During these experiments, bacteria (*S. aureus*) were released from THP-1 cells due to the natural death of host cells, which allowed for a direct comparison of MXF and CIP efficacy against both the extracellular and intracellular forms of the bacteria, providing a more complete evaluation of antibiotic action in both compartments.

In both TKC and HFIM, I first demonstrated that MXF exhibited significantly superior efficacy compared to CIP in killing intracellular *S. aureus*. More conspicuously, I found that MXF was more effective in the dynamic model (HFIM) than in static conditions (TKC) against intracellular bacteria, while the CIP kill rate was decreased in the HFIM vs. TKC. These differences were attributed to more (MXF) or less (CIP)

adequate pharmacokinetic exposure in dynamic vs. static conditions. In both models, both antibiotics were more effective against extracellular than intracellular bacteria, with CIP showing superior efficacy under static conditions (TKC). These results suggest that antimicrobial activity may be over/underestimated in TKC assays and highlight the importance of the HFIM for simulating real clinical conditions, providing a more accurate assessment of antibiotic efficacy.

(4) Translational findings

Table (IV.1.) summarizes key translational findings on transporters that have shown an impact on CIP and MXF disposition in my studies, highlighting their possible consequences on *S. aureus* infections. It details the tissue and cellular localization of these transporters and their potential influence on antibiotic (AB) activity across various infection sites.

Transporter	Tissue/ Organ*	Specific cells*	Link with <i>S. aureus</i> infections
ABCB1	Brain	Endothelial cells of the BBB	Limits AB penetration into the CNS, potentially complicating the treatment of <i>S. aureus</i> meningitis or cerebral abscesses [228].
	Spleen	Immune cells (NK cells, B cells)	May influence the immune response to <i>S. aureus</i> infections [229] by affecting the distribution of immune mediators .
	Skin	Epithelial cells (outer root sheath cells, sebaceous gland cells)	May affect local AB transport [230], impacting the treatment of <i>S. aureus</i> -induced skin infections such as impetigo, infected wounds, abscesses... [231].
	Liver	Epithelial cells (hepatocytes, cholangiocytes)	Can affect hepatic clearance, renal excretion, and bioavailability [232] of AB targeting <i>S. aureus</i> infection.
	Kidney	Epithelial cells (glandular epithelial cells)	
	Intestine	Epithelial cells	Regulates intestinal absorption of orally administered AB, influencing

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			their bioavailability [232] and thus effectiveness against <i>S. aureus</i> infections.
	Other	Immune cells Macrophages	May affect AB accumulation in macrophage and thus its activity on intracellular <i>S. aureus</i> [4].
ABCC4	Liver	Epithelial cells (cholangiocytes, hepatocytes)	Can affect hepatic clearance, renal excretion, and bioavailability of AB targeting <i>S. aureus</i> infection.
	Kidney	Epithelial cells (glandular epithelial cells)	
	Lung	Epithelial cells (alveolar cells)	Potential role in modulating drug transport in <i>S. aureus</i> related pneumonia [233].
	Skin	Langerhans cells Epithelial cells (keratinocytes) Melanocytes	May affect local AB transport, impacting the treatment of <i>S. aureus</i> -induced skin infections such as impetigo, infected wounds, abscesses... [231].
	Heart	Mitotic cells	May affect AB distribution in <i>S. aureus</i> endocarditis [234].
	Other	Immune cells Macrophages	May affect AB accumulation in macrophage and thus its activity on intracellular <i>S. aureus</i> [4].

Table IV.1. Transporters impacting CIP and/or MXF disposition and their potential relevance to the treatment of *S. aureus* infections. *Specific cell expression (gene or protein) data was retrieved from the Human Protein Atlas (<https://www.proteinatlas.org>), highlighting the tissues and cell types with the highest expression levels. Only tissues and cell types associated with *S. aureus* infections have been selected.

IV.2 Discussion on limitations

I will here discuss limitations related to each achievement described in the previous section.

(1) Study of the impact of ABC efflux proteins and their genetic polymorphism on FQ cellular pharmacokinetics as a possible reason for interindividual variability

(1.a) HEK293 cells overexpressing single transporters

The use of HEK293 cells stably overexpressing individual ABC transporters is a widely employed model to investigate the role of specific transporters in drug disposition. This system offers a controlled *in vitro* environment to “isolate” the impact of a single transporter and assess the effect of functional genetic polymorphism(s) on drug transport. However, several limitations must be considered when extrapolating these findings to the complex physiological environments of human tissues.

Firstly, HEK293 cells are genetically modified to overexpress a single transporter at levels that exceed physiological expression. This “artificial” overexpression may amplify the transporter’s contribution in drug disposition, potentially leading to an overestimation of its role *in vivo*. Indeed, while FQs were tested at their $C_{\max}$ in this study, this concentration did not account for transporter overexpression, possibly skewing the observed transport dynamics. Additionally, *in vivo* human cells co-express multiple transporters with overlapping substrate specificities and compensatory mechanisms. By isolating the function of a single transporter, the HEK293 model fails to capture the interplay between multiple transporters, which is a crucial determinant of drug PK in physiological conditions. Moreover, CIP and MXF interact with various transporters (both influx and efflux) across different cell

types, including monocytes/macrophages, hepatocytes, and epithelial cells of the intestine and kidney. Each of these cell types exhibits a distinct transporter expression profile and an intracellular environment that cannot only be represented by the embryonic kidney-derived HEK293 cells. Consequently, findings obtained from HEK293 models may not fully reflect the transporter-mediated PK of FQ in all clinically relevant tissues. Another limitation is the low basal expression of endogenous transporters in HEK293 cells, as demonstrated in this study. This is particularly important because basal transporter activity can influence the observed effect of an overexpressed transporter (as demonstrated in Chapter III – article n°1). Lastly, the experimental conditions used with HEK293 cells involve static drug concentrations ($C_{\max}$ for 2 hours), which fail to replicate the fluctuating drug levels observed *in vivo*. Given that FQs are concentration-dependent antibiotics, these fluctuations are critical for understanding PK/PD relationships, as they can significantly affect the drug's antibacterial efficacy.

(1.b) Impact of polymorphisms on the efficacy/binding of inhibitors?

As discussed in this thesis, polymorphisms in ABC transporters (ABCB1, ABCG2, and ABCC4) can modulate the disposition of various substrates (Chapter I – section I.5). However, when considering their potential impact on the efficacy of inhibitors such as zosuquidar, elacridar, Ko143, and MK571, the evidence suggests that genetic polymorphisms have a negligible impact on inhibitor efficacy. Although it is theoretically possible that polymorphisms could influence inhibitor binding and efficacy, this effect has not been observed in practice for the inhibitor tested, at least in terms of efficacy. However, some ABCB1 inhibitors, such as cyclosporin A and verapamil, have been shown to be affected by haplotypes including the c. 3435C>T polymorphism, leading to reduced inhibitor susceptibility due to lower binding affinity [200]. In contrast, zosuquidar and elacridar exhibit higher affinity

and specificity for ABCB1, as they were specifically designed to act as potent inhibitors and are therefore less susceptible to being impacted by polymorphisms. Several key factors support this hypothesis:

- **Saturation of transporter binding sites**

A primary reason for which polymorphisms do not significantly affect inhibitor binding or efficacy is that these inhibitors are generally used at high concentrations, ensuring complete saturation of the transporter binding sites. Inhibitors such as zosuquidar, elacridar, and Ko143 are designed to have high potency, as reflected by their low inhibitory constant (K_i) values in the nanomolar range. In experimental settings, these inhibitors are added at concentrations that exceed their respective K_i values (micromolar range) (**Table IV.2**). As a result, even if polymorphisms alter binding affinity or transporter activity (either increasing or reducing it), the high inhibitor concentrations compensate for these variations, making any potential difference functionally irrelevant.

Inhibitor	Transporter	K_i values	Experimental concentration	x K_i
Zosuquidar	ABCB1	59 nM ¹	0.2 μ M	3.38
Elacridar	ABCB1	1.16 nM ²	5 μ M	4310
Ko143	ABCG2	25.9 nM ³	1 μ M	38
MK571	ABCC4	< 2 μ M ³	25 μ M	> 12.5

Table IV.2. Summary of inhibitors used in the study along with their target transporters, K_i values, experimental concentrations, and the calculated fold excess of experimental concentration relative to K_i values. ¹[187]; ²[191]; ³[192].

- **Lack of significant evidence of polymorphism effects on inhibitor efficacy**

While polymorphisms in ABC transporters have been shown to affect substrate transport, there is limited clinical or experimental evidence linking these polymorphisms to significant changes in inhibitor binding or efficacy.

Most polymorphisms have been studied in the context of substrate transport rather than their influence on inhibitor efficacy.

This highlights other limitations of *in vitro* models, which focus on the inhibition of a single transporter. Indeed, in physiological conditions, multiple transporters often work together to regulate drug disposition. If one transporter is genetically altered by a polymorphism or inhibited, other transporters with overlapping substrate specificities may compensate for the loss or gain of function. This phenomenon is reflected in article n°1, where ABCB1 plays a role in CIP disposition, but inhibition by zosuquidar or elacridar does not result in major changes in CIP transport, suggesting the presence of a compensatory transporter that mitigates the expected impact of inhibition.

Beyond inhibitors, ABC transporter activity can also be modulated by inducers, which may further complicate transporter-mediated drug disposition *in vivo*. Indeed, inducers can increase ABC transporter expression, potentially leading to reduced drug cellular concentration and thus reduced efficacy towards intracellular pathogens such as *S. aureus*. Such interactions may necessitate dose adjustment or careful monitoring to optimize antibiotic therapy. For example, ABCB1 inducers include rifampicin (antibiotic), apalutamide (antiandrogen), carbamazepine and phenytoin (antiepileptics), efavirenz (antiretroviral), dexamethasone (corticosteroid), and St. John's wort (herbal supplement with antidepressant properties) [102,235]. Additionally, NSAIDs have been shown to induce ABCC4. Moreover, rifampicin, glitazones, efavirenz, and dexamethasone have been demonstrated to induce ABCG2 expression [102].

(1.c) Transporter polymorphisms and their frequency

The selection of polymorphisms investigated in this thesis was based on practical considerations, including reported allelic frequency, their documented impact on other drugs, and the availability of necessary tools within the laboratory. However, this approach introduces certain limitations.

Firstly, the frequency of polymorphisms can vary significantly across populations (Chapter I – section I.5 for the polymorphisms investigated), which may result in distinct clinical consequences. This means that the findings of this work may not be entirely representative of all populations. Additionally, rare polymorphisms, although less common, can have significant effects on transporter function, potentially altering drug disposition, efficacy, or toxicity. These polymorphisms have not been prioritized in this study due to their low frequency and/or limited available data concerning drug impact.

(2) *In vitro* dynamic model adapted to intracellular infection by *S. aureus*

(2.a) HFIM not incorporating immune cells

For setting up the model, I used THP-1 cells, a widely used acute monocytic leukemia cell line. These non-adherent and permissive cells are ideal for studying antibacterial activity against intracellular infections [162]. However, they do not fully mimic the complexity and functionality of primary immune cells. Indeed, when used in isolation, the THP-1 cells represent an immunocompromised state, where the immune system is not fully functional. Therefore, using only THP-1 cells allows us to observe the direct action of antibiotics on bacteria, but it does not provide insight into how the immune system interacts and influences the antibiotic treatment.

To better mimic primary macrophages, THP-1 cells can be differentiated using phorbol-12-myristate-13-acetate (PMA) [222]. This differentiation process induces macrophage-like characteristics, such as enhanced phagocytosis and cytokine secretion, offering a more realistic representation of macrophage functions in the immune system. Alternatively, peripheral blood mononuclear cells (PBMCs), derived from human peripheral blood, provide a more physiologically relevant model. PBMCs consist of a diverse array of immune cells, including monocytes, lymphocytes, natural killer (NK) cells, and dendritic cells, reflecting more accurately the *in vivo* immune response [236]. **Table IV.3** summarizes the main advantages and limitations of using THP-1 cells (both undifferentiated and differentiated) and PBMCs.

Model	Advantages	Limitations
THP-1 monocytes (undifferentiated)	- Suspension cells, easy to handle and culture. - Simple to infect, making them widely used in intracellular studies. - Stable genetic background, ensuring reproducibility of findings. - Long-term viability; can be cultured for 3 months (25 passages) and stored for years. - Fast proliferation rate (doubling time: 35-50 hours). - Free of viruses and toxic metabolites, ensuring biosafety.	- Low phagocytic activity. - Lacks key surface markers and functional properties of mature macrophages (THP-1 differentiated) and primary macrophages (isolated from PBMCs).
THP-1 macrophages (differentiated)	- Enhanced macrophage-like functions, including increased phagocytosis and cytokine production (TNF-α and IL1-β). - Upregulation of key macrophage differentiation markers (CD14, CD36, CD11β). - Production of inflammatory cytokines (TNF and IL-1B).	- Adherent phenotype, requiring enzymatic detachment for passaging, is a challenge for the HF cartridge. - Loss of proliferative capacity upon differentiation.

	- Allows polarization into M1 (pro-inflammatory, antimicrobial, and cytotoxic, via INF- γ , LPS or TNF- α) or M2 (anti-inflammatory, tissue-repairing via IL-4, IL-10, TGF- β) phenotypes.	
PBMCs	- Derived directly from human peripheral blood, offering greater physiological relevance. - Heterogeneous immune cell population (monocytes, T cells, B cells, NK cells, dendritic cells), allowing for immune interaction studies. - Monocytes can be differentiated into macrophages or dendritic cells, closely mimicking <i>in vivo</i> responses.	- High donor variability, leading to experimental inconsistencies. - Time-consuming and demanding for the isolation process. - Short lifespan in culture. - Requires exogenous inflammatory stimuli (IL-1β, TNF-α or LPS to prevent apoptosis). - Not suitable for long-term storage in liquid nitrogen.

Table IV.3. Summary of advantages and limitations of THP-1 undifferentiated, differentiated, and PBMCs [222].

(2.b) Use of THP-1 cells does not capture all intracellular *S. aureus* infections

Another limitation of using THP-1 cells in this model is that they only allow the assessment of *S. aureus* infections within phagocytic cells, specifically within the acidic phagolysosomal compartment (~ pH 4.5 - 5) [237]. However, *S. aureus* is also known to infect non-phagocytic cells, such as epithelial cells, endothelial cells, and osteoblasts, where it resides in the cytosol. The cytosolic environment has a neutral pH (~7.2 - 7.4) [238], which can influence bacterial response and activity of antibiotics. Consequently, this model does not reflect the intracellular environment or the dynamics of drug efficacy in the cytosolic compartment. The findings are thus limited to conditions within phagocytic cells and cannot be extrapolated to infections involving non-phagocytic cells, limiting the broader applicability of the results.

(2.c) Short time duration

Another significant limitation of using THP-1 cells for *S. aureus* intracellular infection within this model is the short duration of cell viability under these conditions. The 24-hour time frame is insufficient to fully assess the efficacy of FQs, which require longer treatment durations (5 to 14 days) to demonstrate their bactericidal effects [223]. However, it is important to note that for other intracellular pathogens, such as *Mycobacterium spp.*, treatment durations of 14 [210] and 21 days [204] have been successfully employed in this model also using THP-1 cells, as these bacteria replicate more slowly within host cells. Similarly, for *Listeria monocytogenes*, treatment periods of seven days have been feasible [172], as this pathogen does not require host cell death to propagate throughout the body. In contrast, the rapid replication of *S. aureus*, combined with its need for host cell death to facilitate spread, significantly limits the available window to assess the full therapeutic potential of FQs.

IV.3 Perspectives and conclusion

1. Enhancing the physiological relevance of transporter studies

A key approach for improving transporter studies is the use of co-expression cell models that express multiple efflux transporters involved in CIP and MXF transport. Transporters do not function independently but rather in a coordinated manner, with compensatory mechanisms and varying substrate affinities. Therefore, using co-expression models would allow for a more precise evaluation of transporter interplay, providing a more physiologically relevant representation of drug disposition. For example, a previous study has successfully co-expressed ABCB1 and ABCG2 to assess their combined role in chemotherapeutic drug transport [239]. Additionally, a study from our laboratory developed co-expression models of ABCC1 and SLCO2B1 in HEK293 to investigate their impact on atorvastatin transport [184], further demonstrating the relevance of these models in understanding drug-transporter interactions [240] .

Following a similar methodology, I propose to co-express ABCB1 and ABCC4 at equivalent levels to evaluate their potential cooperative role in CIP disposition, thereby confirming my hypothesis (as discussed in Chapter III – article n°1). Additionally, the influx transporter of CIP and MXF should also be investigated in order to assess potential compensatory mechanisms and better approximate the real effect. For example, MXF and CIP have been shown to be substrates of SLCO1A2, potentially impacting their intestinal absorption, tissue distribution, and hepatic elimination [241].

Moreover, incorporating alternative cell models representing key organs involved in FQ ADME is crucial. For example, HepG2 cells (hepatocyte-like) are commonly used to model hepatic metabolism [242], while Caco-2 cells (enterocyte-like) are

ideal for studying intestinal absorption and cellular transport [243]. THP-1 cells, which can differentiate into macrophages, are useful for assessing intracellular drug accumulation, and MDCKII cells, which exhibit tight junctions and barrier properties similar to those of Caco-2 cells, can mimic renal transport dynamics and provide insights into drug permeability. Additionally, primary monocyte cultures could also be considered as a more physiologically relevant alternative to THP-1 cells for studying drug transport. As shown in **Table IV.4**, RNA expression levels of the transporters investigated in this thesis vary across different cell lines, closely resembling their variability observed in human tissues. This variability highlights the limitation of only using HEK293 cells.

	ABCB1	ABCG2	ABCC4
<i>Cell models</i>			
HEK293	19.1	1.8	11.3
Caco-2	52.0	26.9	34.4
Hep-G2	17.9	12.3	6.8
THP-1	0.0	0.0	10.0
<i>Human tissues</i>			
Intestine	94.5	132.8	10.1
Liver	27.1	34.9	1.3
Kidney	32.6	3.4	17.4
Macrophages	1.9	6.0	11.9

Table IV.4. RNA expression levels of transporters in various cell models and human tissues, presented as normalized transcript per million (nTPM), sourced from the Human Protein Atlas (<https://www.proteinatlas.org>).

In addition to the selection of appropriate cell models, polarization of cells is particularly relevant for studying the role of efflux transporters in FQ disposition. Caco-2 and MDCKII cell models, which exhibit polarized epithelial characteristics, allow for the differentiation of transporters expressed on either the apical or basolateral membrane, which is crucial for understanding directional transport and overall PK of drugs. Indeed, apical transporters play a significant role in drug excretion, while basolateral transporters facilitate drug absorption.

2. Investigating the impact of transporter regulation on drug cellular concentration

To further enhance the clinical relevance of these findings, future studies should investigate the impact of ABC transporter inducers to simulate clinical scenarios. This would allow simulating real clinical scenarios where transporter upregulation could alter drug cellular concentration. Indeed, inducer-mediated transporter upregulation could lead to reduced intracellular antibiotic concentrations, potentially compromising FQ antimicrobial potency against intracellular pathogens such as *S. aureus*. In this context, it would be interesting to verify ABC transporter expression before and after a 2-hours exposure to CIP and MXF in our cellular models to determine whether there is any potential induction of these transporters. This approach would be similar to previous studies conducted in our lab, where ABCC4 transporter overexpression was observed over a longer period of exposure of J774 mouse macrophages to supratherapeutic concentrations of CIP [88]. This assessment could provide a deeper understanding of whether an upregulation of ABC transporters, possibly triggered by FQ exposure, may contribute to a reduction in the intracellular concentration of these antibiotics. A deeper understanding of these interactions would be valuable for clinical recommendations, especially regarding dose adjustments or combination therapy strategies to counteract efflux-related drug resistance.

Furthermore, transporter silencing could be employed to confirm the specific contribution of individual transporters in drug efflux and to evaluate the impact of transporter downregulation on intracellular drug accumulation.

3. Expanding the study to other transporter polymorphisms

Lastly, further research should focus on the investigation of transporter polymorphisms, particularly ABCC4, which plays a significant role in CIP transport. As discussed in Chapter I – section I.5.3, some ABCC4 polymorphisms, due to their role in other substrate transport, should be investigated. Therefore, I propose to begin the experiments with the three polymorphisms that are frequent in the Japanese population (c.559G>T, c.912G>T and c.2269G>A), which are known to affect transporter efficacy, protein expression, and membrane localization, respectively. Indeed, despite being an ABCC4 substrate, CIP has not yet been evaluated in relation to these polymorphisms. Studying these polymorphisms is crucial for understanding interindividual variability in drug response and could provide valuable insights into personalized medicines, improving therapeutic outcomes based on individual genetic profiles.

4. Simulating drug diffusion in the HFIM

As explained in Chapter I – section 1.5.2, the $T_{1/2}$ parameter of both CIP and MXF has been adapted in the HFIM to mimic tissue diffusion and reproduce PK profiles. However, to better capture the complexity of *in vivo* drug distribution, I imagine an alternative strategy for future implementation.

This approach consists of **progressively modifying the $T_{1/2}$ of the drug in the system** to replicate the **multi-phasic elimination** observed in clinics, which is characterized by three distinct phases (**Figure IV.1**):

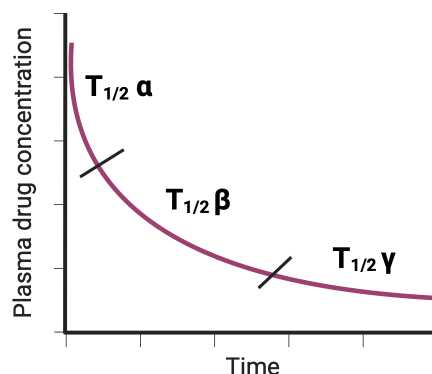


Figure IV.1. Representation of the multiphasic drug elimination following IV administration (created in Biorender.com).

- **$T_{1/2} \alpha$ (distribution phase):** A rapid initial decrease in plasma concentration due to drug distribution from the bloodstream into tissues;
- **$T_{1/2} \beta$ (elimination phase):** A gradual phase of elimination driven by metabolism and excretion;
- **$T_{1/2} \gamma$ (terminal or elimination phase):** A prolonged release from deep tissues, leading to drug persistence in the body.

These parameters, obtained from clinical studies, can be integrated into the HFIM by adjusting the pump flow rate delivering the diluent into the central compartment at three distinct time points corresponding to each $T_{1/2}$ phase. This sequential modulation could enhance the physiological relevance of the model by better simulating drug diffusion and elimination processes. For example, one study demonstrated that the $T_{1/2}$ of CIP were 0.04 ± 0.02 hours for $T_{1/2} \alpha$; 0.55 ± 0.28 hours for $T_{1/2} \beta$; 3.53 ± 0.49 hours for $T_{1/2} \gamma$ following a 200mg IV short infusion in healthy volunteers, based on a three-compartment model [244]. Additionally, this method improves reproducibility, as it eliminates the necessity for manual adjustments by the experimenter, particularly those related to tissue diffusion, making it easier to standardize.

5. Determination of the PK/PD parameters driving CIP and MXF intracellular activity

In this study, the PK/PD evaluation of CIP and MXF was initially based on parameters such as AUC/MIC and $C_{\max}$ /MIC predicting FQ efficacy against extracellular pathogens. However, given the significance of intracellular infections, it is essential to determine whether these same PK/PD parameters also drive intracellular antibacterial activity or if other factors play a more critical role. To achieve this, dose fractionation studies are necessary. These studies involve administering the same total drug exposure (AUC) but varying dosing regimens (e.g., modifying the peak concentrations ($C_{\max}$) and dosing interval) to identify which PK parameter(s) best correlate(s) with intracellular bacterial killing.

6. Evaluation of other clinical isolates of *S. aureus* in the HFIM

In my thesis, the HFIM was set up using ATCC 29213, one of the most commonly used strains in drug discovery research and quality control. This clinical isolate, also known as “Wichita”, is sensitive to a wide range of antimicrobials, including methicillin, and its whole genome is well characterized [245].

To expand the scope of this study, it would be valuable to explore additional clinical isolates to further assess the clinical efficacy of FQs. First, I propose evaluating **clinical isolates that differ in their persistence levels** to study their intracellular survival under dynamic conditions in the HFIM. Since persistence is a reversible state, fluctuating antibiotic concentrations could impact bacterial survival by reducing antibiotic pressure compared to static conditions. A promising approach would be to use previously studied clinical isolates tested for MXF efficacy in THP-1 monocytes under stationary culture conditions [224]. A comparative analysis of MXF-susceptible strains with low and high relative persister fractions (defined as

the percentage of inoculum surviving to 100x MIC moxifloxacin in stationary-phase culture for each isolate relative to ATCC 25923) could be conducted under conventional MXF therapy in the HFIM. If persistent strains fail to respond despite fluctuating drug exposure, genetic analysis could help identify potential FQ resistance-related mutations, such as *gyrA* and *parC* overexpression, which have been associated with high-persister phenotypes and enhanced bacterial survival within host cells [224]. Second, I propose examining **clinical isolates with increasing MICs** to determine whether intracellular susceptibility breakpoints align with extracellular measurements, particularly under fluctuating antibiotic concentrations, as previously studied in static conditions [81]. Finally, comparing the efficacy of MXF with other therapeutic options could help determine the most effective treatment strategy.

Lastly, the strain used in this study had a lower MIC for CIP (0.125 mg/L) than the modal MIC for CIP towards *S. aureus* (0.125-2 mg/L), which may have contributed to the observed activity of CIP. Using a strain with a modal MIC (0.5 mg/L for CIP) could have impacted the results, potentially leading to a reduced CIP efficacy and amplifying the differences between CIP and MXF intracellular activity.

7. Other cell type(s) to mimic *S. aureus* non-phagocytic infection

In future experiments, I aim to expand my investigations to other cell types, such as human keratocytes, osteoblasts, or bronchial epithelial cells, to further explore the broader applicability of my intracellular dynamic model. Each of these cell models provides unique insights into *S. aureus* intracellular infections. Keratinocytes are a relevant model for studying skin and soft tissue infections; osteoblasts and osteocytes can be used to study *S. aureus* infections in bone tissue, while bronchial epithelial cells are particularly important for investigating respiratory infections caused by *S. aureus*.

Human keratinocytes have already been used in the FACM lab to evaluate the activity of β -lactams and MXF against intracellular *S. aureus* infections, including MSSA and both hospital-acquired (HA) and community-acquired (CA) MRSA, under static conditions over a short 24-hour duration [81,246]. Similarly, a recent study assessed the efficacy of various antibiotics, including the FQs levofloxacin and ofloxacin, against intracellular *S. aureus* in osteocytes [247]. This study demonstrated that prolonged antibiotic exposure with a 7-day treatment was significantly more effective than a 1-day treatment. Furthermore, FQ activity was found to be pH-dependent, with greater efficacy at pH 7.2 (cytosol) compared to pH 5 (phagolysosome). These findings highlight the importance of prolonged antibiotic exposure and the need to account for intracellular pH variations when evaluating antibiotic effects against intracellular bacteria. In this context, it would also be interesting to monitor the evolution of intracellular pH during *S. aureus* infection and examine its impact on the activity of FQ transporters. This study is currently ongoing in our laboratory. Notably, similar research has been conducted in another model of *S. aureus* intracellular infection (bovine mammary epithelial cells). In this study, researchers demonstrated that *S. aureus* disrupts lysosomal function by alkalinizing the lysosomal environment. This alkalinization compromises the ability of lysosomes to degrade their contents, thus hindering the elimination of the bacteria [248]. If changes in pH occur during infection, they could modulate transport function by altering protein conformation, thereby affecting the overall transport efficiency of ABC transporters. This effect can also occur through the ionization of key residues within transmembrane domains, which are essential for substrate recognition and binding. Moreover, intracellular pH variations can modify the ionization state of transported molecules, such as FQs [89], potentially influencing their affinity for ABC transporters and thus altering

their intracellular concentration. However, to my knowledge, a direct impact on the activity of these transporters has not yet been demonstrated.

Additionally, another study from the FACM lab has investigated these three cell models (keratinocytes, osteoblasts, and bronchial epithelial cells) within the same study to evaluate the activity of radezolid (an oxazolidinone) over a 24-hour period [42]. This highlights the feasibility of using these models to evaluate antibiotic efficacy against intracellular *S. aureus*.

However, a key technical limitation of using these adherent cells in the HFIM is their strong attachment to the cartridge, making it difficult to collect samples for intracellular bacterial quantification at arbitrary time points. Unlike THP-1 cells, which are in suspension in the cartridge and could be easily sampled at different time points for intracellular quantification, adherent cells require enzymatic detachment. This process necessitates stopping the system, removing the medium from the cartridge, and enzymatic flushing, for example, with trypsin, to allow cell detachment.

To address this limitation, I think about assessing treatment efficacy by running the system for a predetermined period before sampling, rather than tracking bacterial killing over time. The duration of this period will be determined by preliminary cell viability tests to determine how long the system can be maintained before significant cell death occurs. Once this optimal timeframe is identified, I will apply the selected antibiotic treatment profiles (e.g., MXF) and allow the system to run under these conditions. At the end of the experiment, I will stop the system, detach adherent cells, and quantify intracellular bacteria by counting CFUs. This approach, inspired by the work of Zelmer et al., who evaluated antibiotic efficacy after one and seven days of treatment under static conditions [247], will allow us to assess whether the treatment is effective against intracellular infections in this model.

Furthermore, it will be essential to determine whether cell adhesion around the fibers in the cartridge could obstruct the flow of nutrients, waste, and antibiotics. However, these challenges have been successfully addressed in these 3D cell culture systems, which offer significant advantages over traditional flask cultures [168]. A 3D cell culture model refers to a system in which cells are grown in a three-dimensional environment that better mimics the physiological conditions found in the body. The hollow fiber system is an example of such a 3D model, allowing long-term, tissue-like cell culture. In this system, the fibers act as artificial capillaries, ensuring continuous nutrient perfusion and waste removal. The porous matrix of the fibers within the cartridge supports cell attachment, division, and reduces apoptosis, enhancing physiological relevance compared to conventional 2D cultures, where cells grow as a monolayer on plastic surfaces [168].

This 3D system has been used for virology research, using the HFIM to study influenza virus infection. In this model, virus-infected AX-4 cells (modified MDCK cells) were cultured around the fibers, and oseltamivir was continuously infused at different dosing regimens to evaluate its efficacy [168]. These findings suggest that the same system could also be adapted to investigate intracellular bacterial infections in adherent cells.

8. Other fluoroquinolones for comparison

Levofloxacin is a FQ antibiotic widely used in France due to its favorable safety profile compared to MXF, particularly regarding QT interval prolongation [249], making it the preferred option for patients at risk of cardiac complications. However, in Belgium, it is used less frequently as it is less potent than MXF against *S. aureus*. Delafloxacin, an anionic FQ available in France, differs from the other FQ by its enhanced efficacy in acidic environments, where it accumulates more effectively [43]. This characteristic makes it a promising candidate for treating

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intracellular infections. However, delafloxacin is not (yet) available in Belgium, which may limit treatment options in certain cases. Further research utilizing the hollow fiber infection model could provide valuable insights into the comparative efficacy of these antibiotics, helping to refine treatment strategies for challenging intracellular infections.

Conclusion

Through an integrative approach combining PK, PD, and pharmacogenomics, this thesis sheds light on the factors influencing MXF and CIP efficacy in intracellular *S. aureus* infections. My findings reveal the pivotal role of ABC efflux transporters in modulating cellular FQ disposition, with genetic polymorphisms possibly contributing to interindividual variability in drug response. By developing an *in vitro* dynamic model for intracellular *S. aureus*, I demonstrated that traditional static models (e.g., TKC) fail to fully capture the complexity of intracellular infection dynamics. The significant differences between static and dynamic conditions highlight the importance of considering physiological drug fluctuations when evaluating intracellular antibiotic activity. From a clinical perspective, integrating transporter genotyping and PK-guided dosing could enhance antibiotic efficacy while minimizing the risk of therapeutic failure. My results suggest a shift in preclinical antibiotic evaluation, favoring the dynamic models that better reflect physiological conditions, especially in *in vitro* testing. In the long term, these results could be incorporated into PBPK models² to enhance the understanding of FQ efficacy, particularly in acidic compartments. By integrating data on drug accumulation and activity under different physiological conditions, this research could help to refine dosing strategies and improve the prediction of therapeutic outcomes. However, further *in vivo* validation and clinical trials remain necessary to translate my findings into clinical practice (e.g., dosage adjustments). To conclude, this work bridges the gap between fundamental research and clinical applications, contributing to the optimization of antibiotic therapy for intracellular *S. aureus* infections.

²Physiologically based pharmacokinetics (PBPK) modeling is used to predict the ADME of chemicals in humans or animals, integrating anatomical, biochemical, and physiological parameters for drug development, toxicity assessment, and biological research [250].

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V.1 Collaborative work

The **ABCB1 transporter** plays a **crucial role** in mediating the **efflux of a wide range of substrates**, including **antibiotics**. As a part of my thesis, I conducted **collaborative work** to **validate** our **accumulation model** by examining the capacity of **ABCB1** to transport molecules from **other pharmacological classes: rivaroxaban** and **tacrolimus**. This research provided **valuable insights** on the **influence** of the **ABCB1 transporter on drug disposition** and further validates the **relevance of our model** in investigating potential **drug-drug interactions**.

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BRIEF REPORT

***In vitro* assessment of the risk of ABCB1-mediated drug-drug interaction between rivaroxaban and tacrolimus in human embryonic kidney 293 recombinant cell lines**

Gwenaëlle Mahieu^{1,2} | Anne-Laure Sennesael³ | Lionel Pochet⁴ |
Vincent Haufroid^{5,6} | Françoise Van Bambeke¹ ✉ | Anne Spinewine^{7,8} ✉ |
Laure Elens^{2,5}  ✉

¹Pharmacologie Cellulaire et Moléculaire, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium

²Integrated Pharmacometrics, Pharmacogenomics and Pharmacokinetics (PMGK) Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium

³Université catholique de Louvain (UCLouvain), Pharmacy Department, Namur Thrombosis and Hemostasis Center, Namur Research Institute for Life Sciences, Centre Hospitalier Universitaire UCL Namur, Namur, Belgium

⁴Namur Medicine & Drug Innovation Center (NAMEDIC - NARILIS), University of Namur, Namur, Belgium

⁵Louvain Centre for Toxicology and Applied Pharmacology, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium

⁶Department of Clinical Chemistry, Cliniques Universitaires Saint-Luc, Brussels, Belgium

⁷Clinical Pharmacy and Pharmacoepidemiology Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium

⁸Department of Pharmacy, Centre Hospitalier Universitaire (CHU) UCL Namur, Yvoir, Belgium

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Gwenaëlle Mahieu^{1,2}, Anne-Laure Sennesael³, Lionel Pochet⁴, Vincent Haufroid^{5,6}, Françoise Van Bambeke¹, Anne Spinewine^{7,8}, Laure Elens^{2,5}

Affiliations

1 Pharmacologie Cellulaire et Moléculaire, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium

2 Integrated PharmacoMetrics, PharmacoGenomics and Pharmacokinetics (PMGK) Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Brussels, Belgium

3 Université catholique de Louvain (UCLouvain), Pharmacy Department, Namur Thrombosis and Hemostasis Center, Namur Research Institute for Life Sciences, Centre Hospitalier Universitaire UCL Namur, Namur, Belgium

4 Namur Medicine & Drug Innovation Center (NAMEDIC - NARILIS), University of Namur, Namur, Belgium

5 Louvain Centre for Toxicology and Applied Pharmacology, Institut de recherche expérimentale et clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium

6 Department of Clinical Chemistry, Cliniques Universitaires Saint-Luc, Brussels, Belgium.

7 Clinical Pharmacy and Pharmacoepidemiology Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Belgium

8 Department of Pharmacy, Centre Hospitalier Universitaire (CHU) UCL Namur, Yvoir, Belgium

Correspondence: Laure Elens, PhD, Integrated PharmacoMetrics, PharmacoGenomics and Pharmacokinetics (PMGK) Research Group, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), Avenue Emmanuel Mounier 72, B1.72.02 Brussels, Belgium (E-mail: laure.elens@uclouvain.be).

V.2.1 Abstract

Background: In lung transplant patients, direct oral anticoagulants are often taken in combination with immunosuppressive drugs such as tacrolimus. Since tacrolimus is a substrate and inhibitor of the efflux protein ABCB1, also transporting direct oral anticoagulants, a possible drug-drug interaction mediated by competition for this transporter needs to be investigated.

Objectives: To determine the *in vitro* effect of tacrolimus on ABCB1-mediated rivaroxaban transport in order to support clinician practice.

Methods: Recombinant cell line models, based on human embryonic kidney 293 cells, were generated by a stable transfection process to overexpress ABCB1 or not (control cells). The impact of tacrolimus on ABCB1-mediated rivaroxaban transport was assessed by accumulation experiments.

Results: ABCB1 expression decreased the cellular accumulation of rivaroxaban and tacrolimus at their respective clinically relevant concentrations when compared with control cells. This confirms the involvement of ABCB1 in the active transport of tacrolimus and rivaroxaban. However, tacrolimus had no significant influence on rivaroxaban disposition at those clinically relevant concentrations.

Conclusion: Our study does not provide evidence for a possible interaction between tacrolimus and rivaroxaban when used together in practice.

Keywords: ABCB1, anticoagulants, drug interaction, HEK293 Cells, lung transplantation, rivaroxaban, tacrolimus

Essentials:

- Lung transplant patients are polymedicated and hence at risk of drug-drug interactions.
- ABCB1 expression reduces the cellular accumulation of rivaroxaban and tacrolimus.
- A clinically relevant concentration of tacrolimus has no impact on rivaroxaban disposition.

This result suggests no clinically significant interaction between tacrolimus and rivaroxaban.

V.2.2 Introduction

Direct oral anticoagulants (DOACs) such as apixaban, edoxaban, and rivaroxaban are frequently used in clinical practice. Their indications include stroke prevention in non-valvular atrial fibrillation as well as the treatment and secondary prevention of venous thromboembolism [251,252]. Both are common complications in lung transplant patients, with an incidence of 25% for nonvalvular atrial fibrillation [253] and 8% to 44% for venous thromboembolism [254–256]. Anticoagulant therapy is therefore often required in this population. Lung transplant patients are polymedicated and hence at risk of drug-drug interactions [252]. Immunosuppressive drugs are administered lifelong to transplant patients for the prophylaxis of allograft rejection, among which tacrolimus, a calcineurin inhibitor (CNI), which shares metabolic and transport pathways with DOACs [257,258]. Notably, tacrolimus is a substrate and inhibitor of ABCB1 (P-glycoprotein (P-gp)) [259], also known to transport DOACs [252,258]. Nevertheless, this potential interaction remains poorly investigated. While the US Food and Drug Administration and a prospective pilot study reported no interaction [260,261], the European Heart Rhythm Association calls for caution in case of concomitant use [262]. Consequently, this lack of sufficient data leads to heterogeneity in guidance and clinical practice [263]. This study therefore aimed to assess the *in vitro* impact of tacrolimus on ABCB1-mediated rivaroxaban transport in order to support clinician practice.

V.2.3 Materials and methods

Materials

Rivaroxaban and ($^{13}\text{C}_6$)-rivaroxaban-d5 (internal standard) were supplied by Alsachim; tacrolimus (TAC), rhodamine 123 (Rh123), and LY335979 (zosuquidar [Zo] HCl) were obtained from Sigma-Aldrich; and ascomycin (internal standard) was purchased from Enzo Life Sciences. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (PS), and an enzyme-free cell dissociation buffer were purchased from Gibco (Thermo Fisher Scientific); Geneticin (G418) was purchased from Roche.

Cell culture

Human embryonic kidney (HEK) 293 cells were grown in DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% volume per volume (v/v) of FBS and 1% (v/v) of PS solution at a temperature of 37 °C in a 5% CO₂ atmosphere. For subculturing, cells were detached with enzyme-free cell dissociation buffer.

Generation of ABCB1 recombinant cells

The generation of stable recombinant cell lines has been previously described [117,126]. In short, HEK293 cells were transfected by a pcDNA3.1 vector, with complementary DNA encoding either the ABCB1 wild-type (HEK_{ABCB1}) or an empty vector (HEK_{control}) using Lipofectamine (Thermo Fisher Scientific). Stable recombinant cells were selected by culture with G418 (1 mg/mL).

Characterization of ABCB1 cell lines

HEK293 cells show low endogenous levels of ABCB1 expression, and overexpression of the transporter in transfected cells was previously demonstrated using flow cytometry, immunofluorescence, and western blot [117,126].

The functionality of the overexpressed transporter was further assessed here using the reference substrate Rh123 and the specific ABCB1 inhibitor Zo [117,187]. The day before the experiment, 350,000 cells were plated in 12-well plates in complete medium (DMEM + 10% FBS). Cells were then incubated for 120 minutes at 37 °C in a 5% CO₂ atmosphere in the dark with 5 µM Rh123 alone or combined with 0.2 µM Zo. At the end of the incubation, supernatants were discarded, and cells were washed twice with phosphate-buffered saline (PBS) at 4 °C and lysed by addition of 200 µL of water per well and overnight agitation. The fluorescence of Rh123 was measured in the supernatant after centrifugation at 14,000 rpm for 10 minutes using a Spectramax M3 fluorimeter (Molecular Devices) with excitation and emission wavelengths set at 485 nm and at 530 nm, respectively. Quantitative real-time polymerase chain reaction (PCR) was also performed to quantify the number of *ABCB1* gene copies in the different cell lines. In brief, RNA was extracted from cells (5×10^6 cells) using RNeasy Mini Kit from Qiagen and reverse-transcribed in complementary DNA following the GoScript Reverse Transcription Mix, Oligo(dT) protocol (Promega). Real-time PCR was carried out with SYBR green (Bio-Rad) using the CFX96 Touch Real-Time PCR detection system (Bio-Rad). *18S* expression was used to normalize ABCB1 expression. The primer sequences for *18S* and *ABCB1* were respectively (forward) 5'-CGG CTA CCA CAT CCA AGG AA -3' and (reverse) 5'-ATA CGC TAT TGG AGC TGG AAT TAC C -3' and (forward) 5'-AGC AAA GGA GGC CAA CAT AC -3' and (reverse) 5'-GGC TTC ATC CAA AAG CAA AA -3', respectively.

ABCB1 expression was also characterized by western blot according to a previously published method, with some modifications [117]. The cell pellets were recovered with an adapted RIPA (Radio-Immunoprecipitation Assay) lysis buffer (sodium deoxycholate 1%, tetraethylammonium bromide 100 mM, NaCl 300 mM, IGEPAL (Sigma-Aldrich) 2%, sodium dodecyl sulfate (SDS) 0.2%, n-dodecyl-β-D-maltoside

(DDM) 0.4%, pH 7.2-7.5) supplemented with a complete protease inhibitor cocktail (Roche) and incubated for 15 minutes on ice. The cell lysates were then vortexed for 30 seconds and further diluted in LDS Sample Buffer (Invitrogen, Thermo Fischer Scientific) containing 25% of sample reducing agent (Invitrogen, Thermo Fischer Scientific) and water to obtain 15 µg of total protein in 20 µL of sample. Twenty microliters of sample and 4 µL of protein ladder (PageRuler Prestained, Thermo Fischer) were loaded on a 4 to 12% Bis-Tris gel (PageRuler Prestained, Thermo Fischer). Migration and transfer of proteins onto a polyvinylidene difluoride (PVDF) transfer membrane (Thermo Fischer Scientific) were then achieved using blotting pad (VWR) at 175 V for 60 minutes and 30 V for 60 minutes, respectively. Blots were blocked with Bovine Serum Albumin (BSA) 1% in Tris-Buffered Saline with 0.02% Tween (TBS-T) pH 8 for at least 60 minutes. After 3 washing steps with TBS-T during 5 minutes, membranes were incubated overnight at 4 °C with a monoclonal primary antibody depending on the targeting protein, namely anti-P-glycoprotein (ab170904, rabbit, 1:10,000 dilution, Abcam) for ABCB1 or monoclonal anti-β-actin antibody (A1978, mouse, 1:2000 dilution, Sigma-Aldrich) diluted in TBS-T with milk powder (150 mg/5 mL of TBS-T). The next day, after 3 steps of 5 minutes washing in TBS-T, membranes were incubated 60 minutes with a secondary antibody diluted in TBS-T with milk powder (antirabbit immunoglobulin G HRP; A27036, 1:2500 dilution, Invitrogen) for ABCB1 and antimouse immunoglobulin G (H+L) (A27025, 1:2500 dilution, Invitrogen) for β-actin. Finally, after 2 5-minute washing steps in TBS-T and 1 washing step in Tris-Buffered Saline (TBS), proteins were detected by chemiluminescence using horseradish peroxidase (HRP) substrate (Immobilon Classico, Millipore).

Rivaroxaban accumulation

One day before the experiment, 350,000 cells were seeded in poly-L-lysine-coated 24-well plates in 500 µL of complete medium. After 24 hours of culture, the medium

was removed and replaced by a fresh complete medium containing rivaroxaban alone or combined with tacrolimus or Zo. Rivaroxaban was used at 100 or 500 ng/mL to cover the range of plasma concentrations measured in treated patients [264], and tacrolimus was used at 80 nM (66 ng/mL) and 1 μ M (822 ng/mL), corresponding to peak plasma level [265] and a supratherapeutic concentration shown to inhibit ABCB1 in cell lines overexpressing the transporter, respectively [266]. Zo was used at 0.2 μ M, as ABCB1-specific inhibitor (K_i , 59 nM) [187]. After 120 minutes of incubation at 37°C in a 5% of CO₂ atmosphere, the medium was discarded, and cells were washed twice with 1 mL ice-cold PBS and detached with ice-cold PBS containing 0.2% EDTA. Cell pellets were stored at -80 °C until drug extraction and quantification by LC-MS/MS analysis.

Rivaroxaban quantification

Rivaroxaban was extracted and quantified by LC-MS/MS analysis according to a previously described and published method with a calibration curve ranging from 0.03125 ng/mL to 12.5 ng/mL of rivaroxaban [196]. For each experiment, the absolute amount of rivaroxaban recovered from the cell extracts was normalized to the amount of proteins measured with a DC (detergent compatible) Protein Assay from Bio-Rad according to the manufacturer's instructions.

Tacrolimus quantification

Tacrolimus was extracted and quantified from cell pellets according to a previously published LC-MS/MS method [267], and concentrations normalized to the concentration of proteins in the samples.

Statistical analysis

All experiments were replicated at least thrice ($N = 3$) in triplicate ($n = 3$). Results are presented as mean with SEM. Statistical analysis were performed using

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GraphPad Prism version 8.0 (Graphpad Software). Unpaired *t*-test (cell lines) and 2-way analysis of variance (cell lines + conditions) were performed under the null hypothesis that the means of the compared groups were equal. When the differences between means were significant ($P < .05$), Bonferroni's or Tukey's multiple comparison tests were carried out.

V.2.4 Results and discussion

Model validation

The HEK293 transfected cell model, which specifically overexpresses the transporter of interest, is commonly employed to investigate drug-drug interactions [268–270]. To validate the model, the ability of ABCB1 to export Rh123, a known fluorescent ABCB1 substrate [117], was assessed. The results (Figure 1A) demonstrate significantly lower fluorescence levels in cells overexpressing ABCB1 compared with control (CTL) cells, indicating higher Rh123 efflux in ABCB1 recombinant cells. Zo restored cellular Rh123 accumulation in ABCB1 transfected cells, eliminating the difference between both cell lines under these conditions. These results confirm that the difference in accumulation between CTL and ABCB1 transfected cells is due to the efflux activity driven by a functionally active ABCB1 in recombinant cells. Of note, Zo also significantly enhanced Rh123 accumulation in CTL cells. This observation could be attributed to a low but detectable basal expression of *ABCB1* in HEK293 cells (Figure 1B). Protein expression of ABCB1 in HEK293 cells was also evaluated by western blot analysis of total cell extracts (Figure 1C). The results confirmed that HEK_{ABCB1} recombinant cell line overexpressed ABCB1 protein while ABCB1 basal expression was undetectable in HEK_{control} cells.

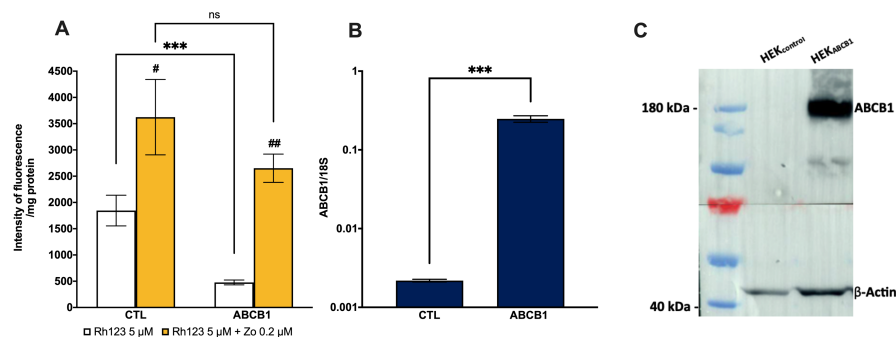


FIGURE 1. (A) Intracellular accumulation of rhodamine 123 (Rh123) is influenced by ABCB1 transporter. Intracellular accumulation of Rh123 (5 μ M) with (0.2 μ M) or without the ABCB1 inhibitor zosuquidar (Zo; $N = 3$; $n = 3$) in human embryonic kidney_{control} (CTL), and human embryonic kidney_{ABCB1} (ABCB1). Statistical analysis: comparison with CTL, *** $P < .001$; comparison between Rh123 alone and Rh123 + Zo in each cell line, # $P < .05$; ## $P < .01$. **(B) ABCB1 transporter RNA transcripts.** ABCB1 RNA expression levels in HEK_{control} (CTL) and HEK_{ABCB1} (ABCB1) normalized to the 18S expression level (housekeeping gene; $N = 1$; $n = 3$). Statistical analysis: ABCB1 compared with CTL: *** $P < .001$. **(C) ABCB1 expression analysis by western blot.** Cells transfected with empty plasmid (HEK_{control}) and cells transfected with vector complementary DNA encoding the ABCB1 transporter (HEK_{ABCB1}). ns, not significant.

Impact of tacrolimus on rivaroxaban ABCB1 mediated transport

First, I assessed the cellular disposition of rivaroxaban in CTL cells and cells overexpressing ABCB1 (Figure 2). The cellular accumulation of rivaroxaban increased proportionally to its extracellular concentration in the range investigated. In cells overexpressing ABCB1, it was reduced to 30% of its value in CTL cells. Rivaroxaban cellular accumulation was substantially increased by Zo, especially in ABCB1-overexpressing cells (3.5-fold increase in ABCB1 vs 1.7-fold increase in CTL cells). The fact that rivaroxaban concentrations are (i) decreased when ABCB1 is overexpressed and (ii) increased upon incubation with Zo, confirms that rivaroxaban is a substrate for ABCB1 [196,271].

Next, I tested whether tacrolimus could counteract the defeating effect of ABCB1 on rivaroxaban accumulation. At a low, clinically relevant concentration (80 nM (66 ng/mL)), tacrolimus did not modify rivaroxaban accumulation in any cell type. At a higher concentration (1 μ M (822 ng/mL)), tacrolimus significantly increased the

accumulation of rivaroxaban used at 100 ng/mL in both cell types, but only in cells overexpressing ABCB1 when the extracellular concentration of rivaroxaban was 500 ng/mL. This observation is coherent with a previous work coming to the same conclusion as the ABCB1 substrate mitoxantrone [266]. Furthermore, our data show that Zo is a more potent ABCB1 inhibitor than tacrolimus since it increases rivaroxaban concentrations to higher levels than tacrolimus even when used at lower concentration (0.2 μ M vs 1 μ M (822 ng/mL)). Lastly, rivaroxaban itself at high concentration (500 ng/mL) seems to inhibit the basal activity of ABCB1, blunting ABCB1 inhibition by tacrolimus in these conditions.

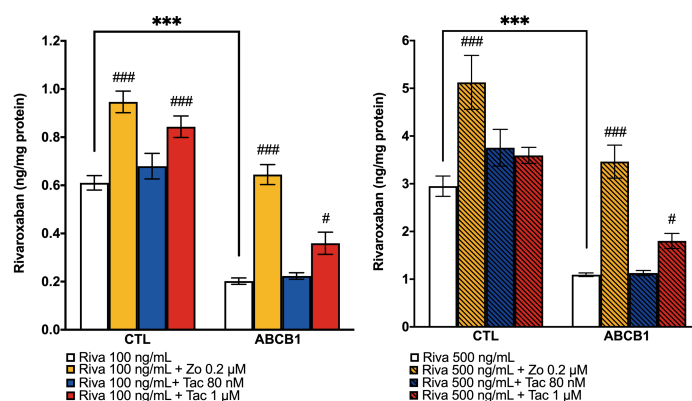


FIGURE 2. Tacrolimus (TAC) does not affect ABCB1-mediated rivaroxaban (Riva) transport at clinically relevant concentrations. Cellular accumulation of Riva after 120 minutes of incubation ($N = 3$; $n = 3$) with an extracellular concentration of 100 ng/mL (left) or 500 ng/mL (right) in human embryonic kidney_{control} (CTL) and human embryonic kidney_{ABCB1} (ABCB1) cells. Riva cellular concentrations are normalized to the total amount of cellular proteins. Statistical analysis: comparison with CTL cells, *** $P < .001$; comparison between Riva alone and Riva with inhibitor in each cell line, # $P < .05$, ### $P < .001$. Zo, zosuquidar.

Based on this last observation, I have measured tacrolimus cellular concentration (Figure 3). The accumulation was similar in the presence of low (100 ng/mL) or high (500 ng/mL) rivaroxaban concentrations. This is in line with a clinical study showing that rivaroxaban caused small but non clinically relevant changes in tacrolimus through values [272]. However, tacrolimus is a known substrate of ABCB1 [259] and, as already demonstrated, the cellular concentration of tacrolimus was

significantly decreased ($P < .05$) in recombinant cell line overexpressing ABCB1 in comparison to CTL cells, and this effect was also concentration- dependent [117].

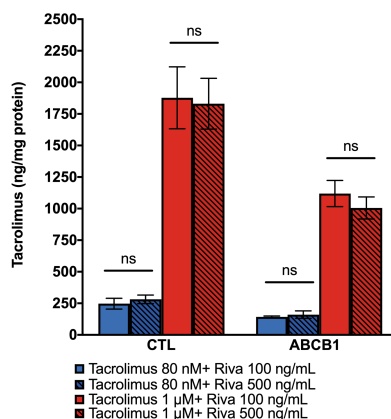


FIGURE 3. ABCB1-mediated tacrolimus (TAC) transport is not impacted by low (100 ng/mL) or high (500 ng/mL) concentrations of rivaroxaban (Riva). Cellular accumulation of tacrolimus after 120 minutes of incubation ($N = 3$; $n = 3$) with an extracellular concentration of 80 nM (blue) or 1 μM (red) in human embryonic kidney_{control} (CTL) and human embryonic kidney_{ABCB1} (ABCB1). Tacrolimus cellular concentrations are normalized to the total amount of cell proteins. Statistical analysis: comparison between tacrolimus + 100 ng/mL Riva and tacrolimus + 500 ng/mL Riva, $P > .05$. ns, not significant.

V.2.5 Conclusion

Our *in vitro* study confirms that ABCB1 expression reduces the cellular accumulation of rivaroxaban and tacrolimus at their respective clinically relevant concentrations, confirming that both drugs are ABCB1 substrates. However, tacrolimus does not seem to impact rivaroxaban disposition at clinically relevant concentrations, while the concentration of tacrolimus is not affected by the presence of low or high clinically relevant concentrations of rivaroxaban. In conclusion, our study does not provide evidence against the concurrent use of these 2 molecules in lung transplant patients, which is in the line of other studies showing no interaction between tacrolimus and rivaroxaban mediated by ABCB1 transport [261,273]. Pending further confirmation through a well-designed prospective clinical trial including therapeutic drug monitoring of both drugs, our work suggests to reconsider the cautious current recommendations of European scientific associations.

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Author contribution : G.M, A.L.S, F.V.B, A.S and L.E designed the research; V.H designed and provided the cell models ; G.M conducted the experiments; L.P and V.H conducted the high-performance LC/MS-MS analysis; G.M drafted the manuscript; all authors reviewed and approved the final version of the document.

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