2012

ASSESSMENT OF THE ROLE OF SOLUTE CARRIER DRUG TRANSPORTERS IN THE SYSTEMIC DISPOSITION OF FLUOROQUINOLONES: AN IN VITRO - IN VIVO COMPARISON

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ASSESSMENT OF THE ROLE OF SOLUTE CARRIER DRUG TRANSPORTERS IN THE SYSTEMIC DISPOSITION OF FLUOROQUINOLONES: AN *IN VITRO-IN VIVO* COMPARISON

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

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August, 2012

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all the people who helped me along the path to a successful completion of my Ph.D and made my dream come to life.
I would like to thank my advisor, Dr. Douglas H. Sweet, for not only helping me strengthen my concepts in the field of transporter research, but also for being a constant source of motivation and constructive criticism. His faith in my abilities, and passion for research and teaching has indeed inspired me to grow as a scientist.
I would also like to thank Dr. Jürgen Venitz for helping me understand the concepts in Pharmacokinetics and Pharmacodynamics. His commitment to research and teaching has indeed been a great source of inspiration.
I am grateful to my committee members – Dr. Patricia W. Slattum, Dr. Glen E. Kellogg, Dr. Philip M. Gerk, for their timely guidance and input towards the completion of my project.

I would also like to acknowledge Dr. Prajakta Badri for helping me understand the 'nuts and bolts' in kinetics research, as well as for her support and encouragement during my life as a student.
Thanks to all the Pharmaceutics faculty for their help in the course of my graduate studies, and suggestions in my research project through my 4 years as a graduate student. Also a special Thank you to Ms. Keyetta Tate and Ms. Laura Georgiadis for their help in ordering lab supplies and supporting all the departmental activities. I would also like to thank School of Pharmacy IT department for their co-operation and VCU School of Pharmacy for the financial support.

I would like to thank all my lab-mates Li Wang, Christine Farthing, and Xiaolei Pan for their advice in my research and for always brightening my days with all the 'lab fun'. Thank you to all my fellow and senior Pharmaceutics graduate students for always being there to help and advise me. A very special Thank you to my family in India and here in Richmond (Vasudha, Esha and Ancy), for always being there to hear my frustrations, and encouraging me unconditionally. A special thank you to my room-mates for helping me during the 'thesis writing' phase; this would have been an incomplete venture without their help. I would also like to thank my friends for all their love and support during my PhD.
Finally I would like to thank GOD for allowing all the things to fall in the right place at the right time, and giving me the determination to tread ahead through all peaks and troughs of this 4-year journey.

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LIST OF ABBREVIATIONS

FQ: Fluoroquinolone

PK: Pharmacokinetics

GI: Gastrointestinal

IV: Intravenous

CNS: Central nervous system

CYP450: Cytochrome P450

RPTC: Renal proximal tubule cell

Caco-2: Human colon carcinoma cell line

LLC-PK: porcine kidney cells

MDCK: Madin-Darby canine kidney cells

ABC: ATP-binding cassette

SLC: Solute carrier

MRP: Multidrug resistance protein

MDR1: Multidrug resistance gene (P-gp)

BCRP: Breast cancer resistance protein

OAT: Organic anion transporter

URAT: Urate transporter

OCT: Organic cation transporter

OATP: Organic anion transporting polypeptide

OCTN: Novel organic cation transporter

MATE: Multidrug and toxin extrusion transporter

AUC_{∞}: Area under the curve at time infinity

Vd_{ss}: Volume of distribution at steady-state

Vd_{ss}^{u}: Unbound volume of distribution at steady-state

$t_{1/2}$: Half-life in plasma

CL_{tot}: Total body clearance

CL_{tot}^{u}: Unbound total clearance

CL_{ren}: Renal clearance

CL_{ren}^{u}: Unbound renal clearance

CL_{nonren}: Nonrenal clearance

CL_{nonren}^{u}: Unbound nonrenal clearance

f_e: Fraction of the unbound (free) drug excreted unchanged in urine

f_u: Fraction of unbound (free) drug in plasma

$CL_{ren,\ tub}$: Estimated net renal tubular clearance

GFR: Glomerular filtration rate

K_i value: dissociation constant for an inhibitor of enzyme/transporter

IC_{50} value: Half maximal inhibition concentration

SNP: single-nucleotide polymorphism

ABSTRACT

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By Aditi Mulgaonkar, B. Pharmacy

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2012

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Fluoroquinolones (FQs) are broad-spectrum charged antimicrobials exhibiting excellent tissue/fluid permeation. Thus, FQ disposition depends essentially on active transport and facilitative diffusion. Although most early transporter studies investigating renal elimination of FQs have focused on apical efflux of FQs from renal proximal tubule cell (RPTC) into urine, their basolateral uptake mechanism(s) from blood into RPTC (i.e., first step to tubular secretion) has not yet been explored in detail. Renally expressed SLC22 members: organic anion (OATs)

and cation (OCTs) transporters are known to transport such small organic ionic substrates (molecular weight ~400 Da). Hence it is of interest to explore the role of these basolateral transporters in renal elimination of FQs, and to further quantitatively assess their impact in clinically observed FQ drug-drug interactions (DDI).

An initial systematic review of clinical literature for FQs (n=18) demonstrated substantial differences among their renal clearance (CL_{ren}~46-fold) and unbound renal clearance (CL_{ren}^{u}~20-fold), and suggested that tubular secretion and reabsorption could be major determinants of FQ half-life, efficacy, and DDIs. FQs (n=13) identified from the above review were investigated by *in-vitro* transport studies using stably transfected cell lines, for potential interactions with organic cation [human (h) OCT1, hOCT2 and hOCT3] and anion [mouse (m) and hOAT3, hOAT1; and hOAT4] transporters. Further, kinetic inhibition studies were conducted to determine inhibition potency (K_i/IC_{50} values) for those FQs exhibiting significant OCT/OAT inhibition in preliminary interaction experiments.

Gatifloxacin, moxifloxacin, prulifloxacin, and sparfloxacin were determined to be competitive inhibitors of hOCT1 with K_i = 250±18, 161±19, 136±33, and 94±8 µM, respectively. Moxifloxacin competitively inhibited hOCT3-mediated uptake, K_i = 1,598±146 µM. Enoxacin, fleroxacin, levofloxacin, lomefloxacin, moxifloxacin, prulifloxacin, and sparfloxacin exhibited competitive inhibition for mOat3 with K_i = 396±15, 817±31, 515±22, 539±27, 1356±114, 299±35, 205±12 µM, respectively. Fleroxacin and pefloxacin were found to inhibit hOAT1 with IC_{50} = 2228±84 and 1819±144 respectively. Despite expression in enterocytes, hepatocytes, and RPTC, hOCT3 does not appear to contribute significantly to FQ disposition. However, due to hepatic and potential RPTC expression, hOCT1 could play an

important role in elimination of these antimicrobials. Among renally expressed OATs in humans, hOAT1 and hOAT3 are likely to be involved in FQ elimination.

CHAPTER 1

ROLE OF RENAL SECRETORY AND REABSORPTIVE DRUG TRANSPORTERS IN SYSTEMIC DISPOSITION OF FLUOROQUINOLONES

Drawn from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69

1. A. INTRODUCTION

Recently, ciprofloxacin gained notoriety when it was used for the prophylaxis and treatment of Anthrax infection during the 2001 bioterrorist attacks which killed several people in the United States. However, as a class, quinolones have been employed in the treatment of bacterial infections for nearly 50 years. The first quinolone, nalidixic acid, was identified as an extremely effective agent in the treatment of urinary tract infections, but it suffered from poor oral absorption, short half-life, and its efficacy was limited to a narrow range of anaerobic gram-negative organisms (9, 141). Further intensive structure-activity relationship studies led to the development of successive generations of FQs which mainly improved their *in vitro* antimicrobial activity, i.e., being effective against a broader range of gram-negative microbes, some gram-positive organisms, and exhibiting higher potencies.

One of the earliest quinolone modifications was substitution of a hydrogen by a fluorine atom at position 6 of the 4-quinolone ring (Figure 1.1), resulting in these agents being referred to as fluoroquinolones (FQs), with flumequine being the first FQ (9, 141).

Second-generation FQs (e.g., ciprofloxacin, enoxacin, norfloxacin, ofloxacin) demonstrate increased activity against gram-negative bacteria, as well as Staphylococcus species, and improved tissue penetration, broadening their spectrum of use to include certain respiratory tract and soft-tissue infections. Third-generation FQs (e.g., grepafloxacin, levofloxacin, sparfloxacin) can be taken once daily – as a result of their prolonged half-life - and are also effective against some gram-positive organisms and atypical pathogens including species of Chlamydia, Haemophilus, Legionella, and Mycoplasma (9, 98, 141). Coupled with excellent oral bioavailability, their therapeutic indications were expanded to include treatment of conditions such as community-acquired pneumonia, acute bronchitis, pyelonephritis and prostatitis. Fourth-generation compounds (e.g., gatifloxacin, moxifloxacin, trovafloxacin) exhibit a further enhancement of activity against a still wider range of bacterial pathogens, expanding their therapeutic indications further, including penicillin- and cephalosporin-resistant pneumonias (9, 98, 141). However, despite this increased spectrum of activity and greater in vitro potency, FQs as a class have been associated with a number of significant adverse effects, which has resulted in a lack of FQ use as primary therapeutics for many indications (98, 101, 141, 153, 154). Currently, further structural modifications aimed at improving their pharmacokinetic (PK) properties and reducing adverse reactions are being investigated, and some later fourth-generation FQs (e.g., gemifloxacin) exhibit significant reductions in adverse effects (9).

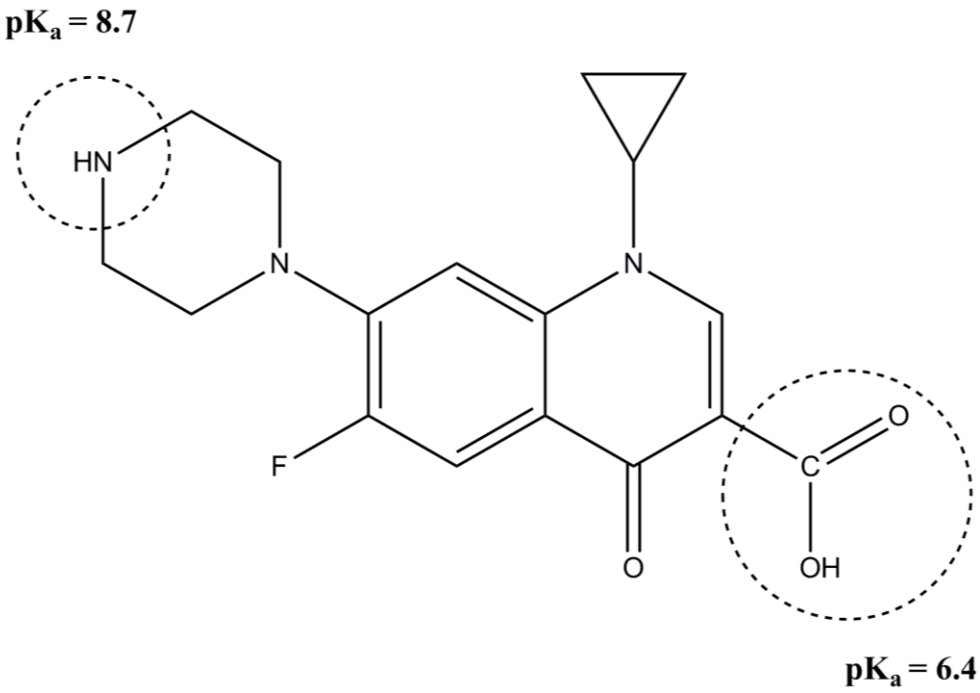

Figure 1.1. Prototypical fluoroquinolone structure

The structure of ciprofloxacin is shown indicating the two ionizable groups of FQs: the piperizinyl ring nitrogen (cation) and the carboxylic acid (anion) group. A fluorine atom (F) is shown at position 6. (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

1. B. ACTIVE TRANSPORT AND FLUOROQUINOLONE DISPOSITION

Despite being rapidly absorbed after oral administration, FQs exhibit a fairly broad range in oral bioavailability, from around 55% to greater than 90% (65, 121). Literature has suggested that FQs exist primarily as ionic species in the physiological pH range (Table 3.5, 3.6), including at the more acidic pH values as found in the gastrointestinal (GI) tract (29). Therefore, passive diffusion across the GI epithelium and other systemic tissue barrier epithelia should be a negligible component of their overall absorption, distribution, and elimination, making it likely that active transport and facilitated diffusion mechanisms are involved. Recently, a number of *in vivo* and *in vitro* studies have pointed towards the involvement of members of the ATP Binding Cassette (ABC) and Solute Carrier (SLC) transporter families in the handling of FQs (see Section 1.3) (4, 165, 186). Most FQs are eliminated primarily by renal excretion (Table 3.2), and secretion into the gastrointestinal lumen, hepatobiliary excretion, and hepatic metabolism represent important elimination routes for only a few of these agents (67, 121). Known hepatic metabolism involves CYP450-mediated oxidation (desmethylation and N-oxidation) and glucuronidation (4, 141). Hepatobiliary excretion of these metabolites can lead to enterohepatic recirculation, resulting in increased residence time and terminal elimination half-life, similar to the effect of co-administration of probenecid or cimetidine on renal FQ elimination (see Section 1.C). However, the circulating metabolites are thought not to contribute to the clinical antimicrobial efficacy, and no clinically significant metabolic drug-drug interactions have been identified.

1. C. EARLY *IN VIVO* AND *IN VITRO* DISPOSITION STUDIES INDICATING TRANSPORTER-MEDIATED FLUX OF FLUOROQUINOLONES

Investigations in humans with ciprofloxacin and temafloxacin, compounds exhibiting minor biliary excretion, found that after intravenous administration as much as 18% of the dose appeared in the feces, indicating an apparent intestinal secretory component (52, 151, 152). Later studies in rats confirmed intestinal secretion of parenterally administered ciprofloxacin, fleroxacin, and sparfloxacin, and demonstrated that co-administration of ciprofloxacin or pefloxacin significantly reduced the *in vivo* intestinal clearance of ofloxacin, suggesting a common transport system (132, 135). Subsequent inhibition of *in vivo* intestinal elimination of ofloxacin by verapamil and quinidine indicated possible involvement of the ABC transporter family, e.g., MDR1, in this process (132). *In vitro* studies conducted in Caco-2 cell monolayers, a model system for human intestine, demonstrated verapamil-sensitive secretion of grepafloxacin and sparfloxacin, also pointing to ABC transporter involvement in the intestinal secretion of FQs (23, 205). Such transporter-mediated secretion from the systemic circulation into the gut lumen may contribute to the therapeutic efficacy of certain FQs in the treatment of GI infections such as bacterial diarrhea.

A number of *in vivo* PK studies in humans have examined the effects of co-administration of cimetidine or procainamide, known inhibitors of the ‘classical’ renal organic cation transport system, on the renal secretion of FQs: Co-administration of cimetidine inhibited (~13-28%) the renal clearance of enoxacin, fleroxacin, gemifloxacin, and temafloxacin (1, 111, 146, 149). Conversely, concomitant administration of ciprofloxacin, levofloxacin, or ofloxacin decreased procainamide renal clearance (10, 103). Furthermore, it has been reported that co-administration of probenecid, the prototypical inhibitor of the ‘classical’ renal organic anion transport system,

significantly decreased (by ~25-60%) the renal clearance of ciprofloxacin, enoxacin, fleroxacin, gatifloxacin, gemifloxacin, levofloxacin, and norfloxacin in healthy volunteers (38, 42, 52, 75, 95, 119, 142, 144, 149, 159, 197, 198). This unique clinical footprint of interaction with both the renal organic cation and organic anion transport systems is thought to be due to the zwitterionic nature of these molecules (Figure 1.1 and Tables 3.5 and 3.6). Such drug-drug interactions have been confirmed in rats as well, where the renal clearance of ofloxacin, which exhibits 80-95% recovery in urine after oral dosing, was significantly reduced upon co-administration of either probenecid (~50%) or cimetidine (~70%) (39). Thus, despite renal secretion of FQs being well established as a major pathway for their elimination, the specific *in vivo* molecular mechanisms involved in their disposition have remained unclear.

Parallel *in vitro* studies conducted in renal cell lines also supported involvement of both organic cation and organic anion transporters in renal FQ handling: For example, the apical efflux from levofloxacin-loaded LLC-PK1 cells (derived from the porcine kidney) was significantly stimulated by an inwardly directed H^+ gradient, suggesting a role for the renal brush border H^+/organic cation antiport system in FQ elimination (124). However, cimetidine failed to inhibit basal uptake of levofloxacin in LLC-PK1 cells. On the other hand, in the opossum kidney cell model, enoxacin, grepafloxacin, and levofloxacin, each significantly inhibited the basal accumulation of para-aminohippurate, the prototypical organic anion transport system substrate (106). Furthermore, both levofloxacin and probenecid caused a significant inhibition of para-aminohippurate efflux across the apical membrane (106).

Recently, the cloning of hundreds of genes coding for transport proteins has made it possible to perform studies examining FQ interaction with known transporters selectively expressed in *in vitro* cell culture models; either by molecular identification of the transporters expressed in the

cell culture model (e.g., Caco-2 cells) or via establishment of transfected cell lines expressing specific transporters. Results from these types of studies investigating the involvement of ABC and SLC transporter family members are summarized in the following sections.

1. D. ATP BINDING CASSETTE (ABC) TRANSPORTERS AND DISPOSITION OF FLUOROQUINOLONES

The human ABC superfamily currently consists of 49 identified transporter proteins organized into 7 separate gene families (A-F; species differences do exist) (190). As their name implies, ABC transporters are able to directly utilize cellular energy by binding and hydrolyzing ATP, using the released energy to drive unidirectional transport (efflux) of substrate molecules across cell membranes (4). Due to their action as 'efflux pumps', a number of ABC transporters are linked to multidrug resistance. To date, members of the ABCB, ABCC, and ABCG families have been implicated in FQ disposition (Figure 1.2). In human RPTCs, there is evidence for protein expression and function for multidrug resistance protein 1 (MDR1, ABCB1; also known as P-glycoprotein), multidrug resistance associated protein 2 (MRP2, ABCC2), MRP4 (ABCC4), and breast cancer resistance protein (BCRP, ABCG2) (Figure 1.2). ABC transporters mediate the movement of a wide range of molecules including lipids, peptides, nucleosides, and xenobiotics ranging from less than 200 Da to about 1900 Da (140).

Concerning MRP1 (ABCC1), although transfection studies with polarized LLCPK-1 cells have demonstrated the basolateral membrane localization of human MRP1, its localization in human RPTCs has not been demonstrated (35, 85). However, if basolateral targeting is assumed, the efflux pump activity of MRP1 would potentially reduce the uptake of FQs from the systemic circulation and aid reabsorptive flux from the urine (Figure 1.2). In support of this hypothesis, ofloxacin was found to inhibit MRP1 activity in over-expressing human leukemia cells (178).

Further, the efflux of grepafloxacin was enhanced in MRP1 transfected LLCPK-1 cells (136). There are also a number of additional MRP family members for which renal mRNA expression has been reported, but no functional or protein expression data are available, and it is possible in the future that additional MRPs may be identified that could contribute to basal FQ efflux in RPTCs.

MDR1 is expressed in the apical membrane (Figure 1.2) and mediates the efflux of substrates into the urine (179). When its transport function was examined in polarized LLCPK-1 or MDCK (derived from canine kidney) cells transfected with MDR1, enhanced secretory transport and/or inhibition of transporter activity by grepafloxacin, levofloxacin, and sparfloxacin was observed (28, 74, 120). Further, studies utilizing Mdr1 knockout mice reported increased plasma concentrations and decreased urinary clearance of grepafloxacin, as well as significantly enhanced CNS permeation of sparfloxacin (28, 136). Apical expression of BCRP (Figure 1.2), coupled with increased inhibitable secretory flux of ciprofloxacin, grepafloxacin, norfloxacin, and ofloxacin across BCRP expressing MDCK cell monolayers, suggest a role for this transporter in renal FQ secretion (5, 69, 107). In support of this hypothesis, Bcrp knockout mice were found to have significantly elevated kidney tissue levels of ciprofloxacin and grepafloxacin as compared to wildtype, as well as significantly increased plasma concentration of ciprofloxacin, after both oral and intravenous dosing (5, 107). Both MRP2 and MRP4 have been localized apically in human RPTCs (Figure 1.2), but investigations into FQ handling by these two transporters have not been reported (139, 185). However, studies in Eisai-hyperbilirubinemia rats, which are naturally Mrp2 deficient, demonstrated decreased biliary excretion of grepafloxacin, suggesting a role for Mrp2 in FQ disposition (137). Further, experiments with a murine macrophage model found that significantly increased protein expression of Mrp2 and

Mrp4 correlated with ciprofloxacin-resistance, but only knockdown of Mrp4 expression resulted in reversal of the resistance phenotype. Thus, it is likely that MRP2 and MRP4 contribute to FQ secretion in human RPTCs.

These *in vitro* studies have provided considerable evidence to support the ABC transporter mediated flux of FQs in the body. However, for the purpose of this dissertation, the SLC-mediated transport of these agents will be highlighted further.

1. E. SOLUTE CARRIERS AND DISPOSITION OF FLUOROQUINOLONES:

1.E.1 Introduction to SLC mediated transport of ionic species:

SLCs are another class of membrane transporter proteins that mediate the movement of organic substrate molecules across barrier epithelia. The human SLC superfamily of transporters is currently proposed to be comprised of 55 separate gene families encompassing 362 identified transporter proteins (species differences do exist) (61). Presently, there is substantial evidence implicating members of the SLC22 (organic cation/anion/zwitterion transporters) and SLC47 (MATE) families in the renal handling of FQs. The SLC22 family (26 identified members) includes the organic cation transporters (OCTs and OCTNs), which handle mainly cationic and zwitterionic organic molecules, and the organic anion transporters (OATs), which mainly transport anionic and zwitterionic organic molecules (Figure 1.2) (164, 165, 186, 201). The SLC22 family members OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3), OAT1 (SLC22A6), and OAT3 (SLC22A8) are expressed in the basolateral membrane of RPTCs and *in vivo* serve to mediate the accumulation of substrate molecules from the blood into RPTCs (Figure 1.2) (91, 165, 186). In the apical membrane OCTN1 (SLC22A4) and OCTN2 (SLC22A5) likely mediate the efflux of substrate molecules from the RPTCs into the urinary space, whereas OAT4 (SLC22A11) and URAT1 (SLC22A12) have been proposed to mediate

reabsorption of substrates from the urine into RPTCs (Figure 1.2) (91, 165, 186). The SLC47 multidrug and toxin extrusion transporters (MATEs; 2 identified members) mostly interact with cationic and zwitterionic molecules (175, 177). While both MATE1 (SLC47A1) and MATE2 (SLC47A2) are found in the apical membrane of human RPTCs, only eight of the identified SLC22 family members are conclusively known to function in this tissue (Figure 1.2) (165, 175, 186).

The existence of the SLC superfamily of drug transporters was first surmised during the study of the physiological functioning of the primary elimination organs – liver and kidney (165). The renal elimination of visible charged organic molecules such as indigo carmine and phenol red following oral administration, led to exhaustive studies to comprehend the potential renal physiological mechanisms causing the removal of these species from the blood into the urine (165). This allowed generation of detailed renal proximal tubule cell (RPTC) models, explaining the potential processes resulting in secretion of organic anion and cations into the urine (165). It was later concluded that these processes may be mediated by specialized membrane proteins (165). Such initial physiological observations were the basic foundation for the subsequent cloning and functional characterization of individual transporter proteins within the different transporter families.

As all the barrier epithelia in the body, including the RPTC, are polarized, the driving forces governing the trans-cellular entry and exit of charged molecules are very different (165, 201). Thus understanding of these physiological mechanisms is important, in order to assess localization of the identified transporters in these barrier epithelial models, and furthermore, accurately determine the transport mechanisms involved in the overall flux of ionic species (e.g., renal secretion in case of RPTC) (165, 201). The progress in cloning of individual transporters in

the barrier epithelia, and intensive *in vitro* studies demonstrating their functions in heterologous expression systems, revealed that the earlier opinions regarding transcellular movement of ionic species were, in fact, oversimplified (165). It was demonstrated by such *in vitro* studies that multiple transporter paralogs of each gene family could mediate the cellular entry and subsequent exit of charged molecules (165). It was also revealed that transport pathways for organic anions and organic cations, were not mutually exclusive, and that some molecules such as zwitterionic substrates (e.g., FQs) could be transported by either OATs, OCTs or even both systems, to determine their overall flux (Figure 1.1 and 1.2) (93, 138, 165). Moreover, the identified transporters exhibited considerable overlap in substrate (xenobiotics and endogenous molecules) specificities, e.g., the organic anion/cation/zwitterion transporter (Slc22) family has overlapping substrate specificities with organic anion transporting polypeptide (OATP; Slc21), multidrug and toxin extrusion (MATE; Slc47), and the ATP-binding cassette (ABC; Abc) families of transporters (46, 165). With the developments in transport literature, experimental evidence has now confirmed that organic ionic substrates can potentially enter the cell via OCTs/OATs/OATPs and exit via OCTN/ MATE/ABC transporters: this has introduced a whole new concept of substrate 'crossover' (Figure 1.2) (46, 165). In addition to this, a number of single nucleotide polymorphisms (SNPs) in multiple transporter gene families (e.g., OCTs, OATPs), that affect their degree of activity, and subsequently substrate PK, have been identified (71, 173, 180). Such advancement in scientific knowledge concerning active transporters mediating *in vivo* flux of organic ionic xenobiotics and endogenous molecules, has indeed increased complexities in the physiological pathways governing their 'net' PK (46, 73, 165). This has thus introduced a new challenge for accurate prediction of biologically relevant (mechanistic) models demonstrating the 'net' transport of charged molecules.

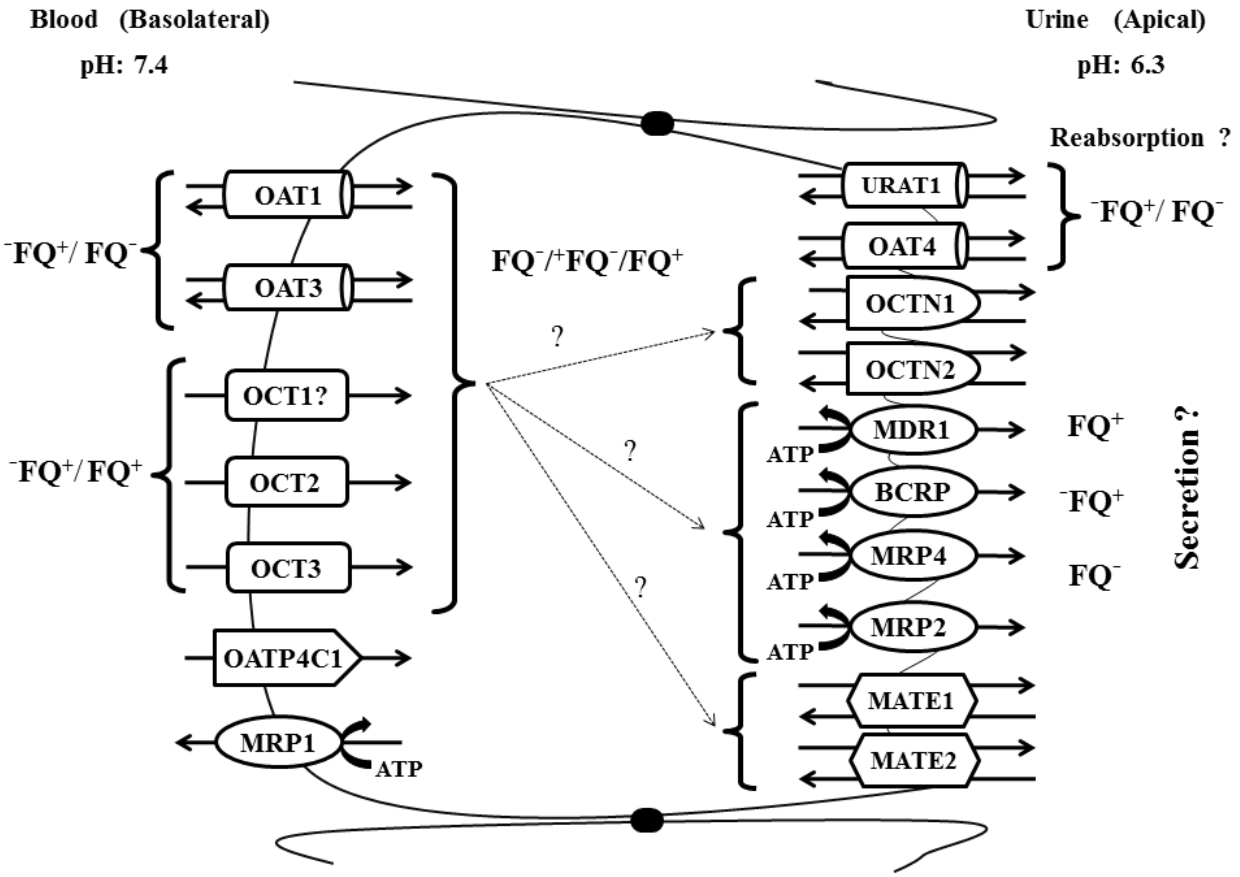

Figure 1. 2. Proposed renal proximal tubule cell model, illustrating drug transporters and pathways involved in fluoroquinolone disposition

The figure shows potential transport mechanisms and pathways involved in the renal elimination of FQs. FQs which exist predominantly as zwitterions and anions in blood (pH 7.4), may enter renal proximal tubule cells via the action of basolateral uptake transporters such as organic anion (OAT1 and 3) and organic cation (OCT1, 2 and 3) transporters. Cellular exit into the urinary space via apical efflux transporters may involve organic cation (OCTN1 and OCTN2), multidrug and toxin extrusion (MATE1 and MATE2), and/or ATP-binding cassette (MDR1, BCRP, MRP2, and MRP4) transporters. Finally, FQs may be excreted in the urine, or subjected to active reabsorption mediated by uptake transporters expressed in the apical membrane, such as the organic anion transporters OAT4 and/or URAT1. Note: Expression and basolateral localization of OCT1 in human RPTC is still controversial, although this has been confirmed for the rat ortholog of Oct1. FQ transport by organic anion transporting polypeptide 4C1 (OATP4C1) is currently unexplored; however, the related transporter OATP1A2 (which is not expressed in human RPTCs) has been implicated in intestinal FQ transport. The potential role of human MRP1 transport in the efflux of FQs from the cell back into the bloodstream (due to its basolateral localization) has yet to be explored. FQ^-, FQ^+, and $^-FQ^+$ denote anionic, cationic, and zwitterionic FQ microspecies, respectively. (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

1.E.2 SLC transporter family mediating disposition of fluoroquinolones:

SLC transport function is indirectly coupled to cellular energy, using the energy stored in concentration gradients and/or the membrane potential as driving force. Although hepatic expression of hOCT1 is well accepted, its renal expression and localization still remains controversial (80, 91, 180). However, localization of the rat Oct1 ortholog to the basolateral membrane in the RPTCs was demonstrated (80). Nevertheless, potential interactions of OCT1/Oct1 with the FQs have yet to be explored in detail. Due to its basolateral membrane targeting and membrane potential-sensitive mechanism of action, OCT2/Oct2 is established as an influx carrier mediating the movement of substrates from the renal circulation into the cytoplasm of RPTCs (115, 167, 170). Accordingly, the inhibition of hOCT2-mediated transport in transfected cells by grepafloxacin, levofloxacin, and moxifloxacin suggests that renal FQ elimination may be due to the action of this transporter (72, 125). While OCT3/Oct3 also has been demonstrated to function as a facilitated-diffusion carrier, mediating the RPTC accumulation of substrates from the blood, interaction of this transporter with FQs remains unexplored (82).

Both OAT1/Oat1 and OAT3/Oat3 are basolateral organic anion/dicarboxylate exchangers that utilize the outwardly directed endogenous α-ketoglutarate gradient to drive RPTC uptake of subtrates from the systemic circulation (20, 115, 166, 168, 169, 171). A recent study with stably-expressing cell lines demonstrated that ciprofloxacin is a substrate for mOat3, and has moderate interactions with hOAT3; while this FQ did not demonstrate significant interactions with hOAT1/mOat1 (187). Also, norfloxacin, ofloxacin, and gatifloxacin each exhibited a concentration-dependent inhibition of mOat3-mediated transport (187). Furthermore, experiments in Oat3 knockout mice using clinically relevant ciprofloxacin concentrations

demonstrated that the *in vivo* effect of transporter deletion is similar to the reported effect of concomitant probenecid administration on FQ disposition in humans (187).

It is of note that, although RPTC influx of FQs via hOATP4C1 (SLCO4C1) has not been reported, it should be investigated in the future (Figure 1.2). This is based upon a report identifying the related transporter, hOATP1A2 (SLCO1A2), as mediating accumulation of levofloxacin and likely being responsible for the high-affinity uptake component for levofloxacin identified in Caco-2 cells (100). hOATP1A2-mediated uptake of ciprofloxacin, enoxacin, gatifloxacin, lomefloxacin, and norfloxacin were also observed (100).

Apical RPTC membrane localization coupled with an organic cation/H^+ exchange mechanism properly situates OCTN1/Octn1 and OCTN2/Octn2 as potential efflux pathways for FQs (174, 203, 204). While direct OCTN1/Octn1-mediated transport of FQs has not been demonstrated, both levofloxacin and ofloxacin produced significant inhibition of tetraethylammonium transport in OCTN1-expressing cells, suggesting this transporter may play a role in renal FQ secretion (204). Evidence for OCTN2 interaction with FQs is also somewhat indirect, as it was investigated as inhibition of carnitine transport in a Caco-2 cell isolate found to express OCTN2, but not OCTN1 (62). In these Caco-2 cells, both levofloxacin and grepafloxacin produced significant inhibition of carnitine uptake, supporting involvement of OCTN2 in both intestinal absorption and RPTC efflux of FQs (62).

The apical efflux and reabsorption of small organic anions into and out of the urinary space still remains somewhat poorly understood. Early studies with apical membrane vesicles supported both a facilitated diffusion mechanism (efflux) and an anion exchange mechanism (uptake or efflux depending upon energetics) (164, 165, 186). Despite being immunolocalized to the apical RPTC membrane, the mechanism of hOAT4 action also remains clouded, as

conflicting data indicating it is a facilitated diffusion carrier and an exchanger were reported (15, 32, 57). Further complicating the issue is debate over whether the exchange mechanism drives efflux from the RPTCs or the reabsorption of compounds from the urinary space into RPTCs. Currently, there are no studies investigating the interaction of FQs with hOAT4 and its potential role in the secretion and/or reabsorption of FQs remains unknown. Finally, hURAT1/Urat1 localization to the apical membrane of RPTCs and its function as an organic anion/urate exchanger are consistent with its mediating the efflux of organic anions from RPTCs into the urine in exchange for certain substrate molecules such as urate (33). Whether FQs can substitute for urate and, thus, be actively reabsorbed from the urinary space by URAT1 remains uninvestigated.

The transporters hMATE1/Mate1 and hMATE2/Mate2 represent mammalian orthologs of bacterial transporters demonstrated to confer resistance to FQ therapy (13). Although MATE1 and MATE2 were initially identified in the same study, only MATE1 was functionally characterized and found to operate as an organic cation/H^+ exchanger targeted to the apical membrane of RPTCs (Figure 1.2) (127). The rat ortholog of MATE1 was reported to transport a number of FQs including ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, and norfloxacin (123). The function and membrane targeting of MATE2 has not been reported. However, what appears to be a kidney-specific splice variant of MATE2, sometimes referred to as MATE2-K, has been examined. This variant contains a 108 basepair deletion in Exon7 resulting in the loss of 36 amino acids in the length of the protein product, but it still shares 94% amino acid identity with the full-length MATE2 isolate (105). Functional analysis confirmed it operates as an organic cation/H^+ exchanger and is likely targeted to the apical membrane, however the antibody used to establish localization would recognize both the full-length and truncated MATE2-K

forms (105). Levofloxacin and ciprofloxacin were found to be potent inhibitors of the MATE2K variant (175).

1. F. FLUOROQUINOLONE-ASSOCIATED ADVERSE EFFECTS

Although the FQs have been used extensively for a wide array of infections down the years, there have been several mild-to-severe adverse events observed on their clinical use in patients. The most common adverse effects associated with these agents range from mild effects on the gastrointestinal tract such as nausea, vomiting, and diarrhea, to moderate or severe phototoxicity, to extremely serious CNS effects including seizures, anxiety, and toxic psychosis (98, 101, 141, 153, 154). A number of other rare adverse events have been reported including severe renal (crystalluria, interstitial nephritis, hemolytic-uremic syndrome, and acute renal failure) and hepatic toxicities, cardiac effects, hypoglycemia, and tendon rupture (98, 101, 141, 153, 154). These toxicities are generally associated with higher serum, tissue, and urinary FQ concentrations, resulting from their prolonged presence in the body. As a result, several FQs had to be withdrawn from the U.S. market. For example, temafloxacin was removed from the market soon after its approval due to high instances of hemolysis and renal failure (122, 154). Trovafloxacin was linked to severe hepatotoxicity in over 100 patients, sometimes resulting in hepatic necrosis and acute hepatic failure (154). Cardiac effects including tachycardia, prolongation of the QTc interval, and onset of torsades de pointes, including fatalities, have been observed with a number of FQs including levofloxacin, moxifloxacin, sparfloxacin, gatifloxacin, and grepafloxacin; this cardiac risk contributed to the withdrawal of the latter three (8, 141, 154). Ciprofloxacin, moxifloxacin, and pefloxacin have been associated with tendinitis and tendon rupture in patients of all ages (84, 101, 154). Incidences of tendon injury became so prevalent that in 2008 the FDA issued a class label change for a Boxed Warning for increased risk of

tendinitis and tendon rupture. Also more recently, in 2011, the FDA issued yet another class label change for a Boxed Warning for increased risk of FQ-associated exacerbation of Myasthenia Gravis. Although most FQ-associated adverse effects occur only rarely, FQs have been prescribed with caution. Due to these adverse events, and their known elimination routes, FQs need to be administered with caution, and suitable dosage adjustments need to be conducted for some FQs depending on the clinical scenario and patient kinetics (88, 155). Also, as most of these rare adverse events occur due to complex mechanisms which still have to be studied in detail, it has become essential to study the PK of these agents to prevent their accumulation due to any pre-existing condition (e.g. renal impairment), or any potential drug interaction. Thus, a more complete understanding of the molecular mechanisms underlying these adverse effects, including the potential contribution of transporter proteins to their PK behavior and target organ toxicities is critical.

CHAPTER 2

RESEARCH HYPOTHESES AND SPECIFIC AIMS

2. A HYPOTHESES:

2.A.1 For fluoroquinolones (FQ) excreted unchanged in urine, in addition to passive glomerular filtration, active transport systems are involved in their renal tubular secretion and/or reabsorption.

2.A.2 Due to the zwitterionic nature and small molecular size of these molecules, members of the Solute Carrier (SLC22) transporter family, i.e., organic anion (OATs) and organic cation (OCTs) transporters, are likely to be involved in renal elimination of the FQs.

2.A.3 For at least some SLC22 family members, the PK interactions with the FQs will prove to be clinically significant.

2. B SPECIFIC AIMS TO ADDRESS THE ABOVE HYPOTHESES:

2.B.1. SPECIFIC AIM 1.

A systematic review of FQ biomedical literature will allow identification of FQs demonstrating sufficient PK information to further evaluate their disposition mechanisms –

i) Systematic review: to compile and calculate the PK parameters from clinical literature focusing on 'healthy human adult' population for the FQs and assess their 'renal elimination' component; estimate the PK parameter: renal tubular clearance ($CL_{ren,tub}$).
ii) To conduct a statistical analysis of the PK parameters and evaluate for the existence of a trend explaining differences in the *in vivo* PK profiles of the FQs.
iii) From the above analysis, identify the net renal elimination processes responsible for the excretion of the FQs.
iv) To compile and analyze the physicochemical properties of the FQs and identify suitable physicochemical characteristics suggestive of interaction with members of the organic anion/cation/zwitterion transporter family (SLC22).

2.B.2. SPECIFIC AIM 2.

To test the hypothesis that members of the organic anion/cation/zwitterion (SLC22) transporter family impact the observed $CL_{ren,tub}$ for identified FQs –

i) Literature has suggested that organic anion (OATs: hOAT1/mOat1, hOAT3/mOat3) and organic cation (OCTs: hOCT1, hOCT2 and hOCT3) transporters of the SLC22 family are localized on the basolateral membrane of RPTC. Initial preliminary studies using stably transfected cell lines, will be conducted for selected FQs identified in the systematic review,

to detect any significant interactions of these agents with the transporters. Further, transporter based kinetic experiments will be conducted to examine inhibition constants (K_i values) or half maximal inhibition concentrations (IC_{50} values) of FQs for these transporters. These studies will involve an investigation of the role of these basolateral transporters in renal uptake of FQs from blood to RPTC, i.e., the first step to renal elimination of the FQs.

ii) Among the SLC transporters, hOAT4 is known to be localized on the apical membrane of RPTC. FQs identified in the systematic review will be tested in preliminary studies using stably transfected cell lines, to investigate any significant interaction with this transporter. This will be followed by conducting kinetic experiments to investigate the inhibition potencies of any strong inhibitors. These studies will aim to identify hOAT4 as a potential reabsorptive transporter for the FQs in the RPTC, i.e. mediator for the FQs to enter the RPTC back from the urinary space.

As a summary, for each transporter, the following transport studies will be conducted with stably transfected cell lines, using FQs as inhibitors:

a) Preliminary screening study of the FQs identified from Specific Aim 1 to detect any significant drug transporter interactions.
b) To determine the linearity of transport (time course), and conduct concentration-dependency studies to estimate the K_m of prototypical substrates for individual transporters, and IC_{50} values for the FQs demonstrating significant inhibition in preliminary inhibition studies.
c) To assess the mode of inhibition (competitive/non-competitive/uncompetitive) for the FQs, followed by determination of their inhibition constants (K_i value).

2.B.3. SPECIFIC AIM 3.

To study the impact of OATs and OCTs towards the observed *in vivo* renal clearance for the studied dataset of FQs:

The ratios of the 'unbound C_{max}/IC_{50} (or K_i)' for the individual FQs will be calculated for each transporter (OAT and OCT) according to the recommendation in the FDA's recent drug-drug interactions (DDI) guidance for assessing transporter impact in clinically relevant DDI with FQs will be further analyzed.

CHAPTER 3

QUANTITATIVE PHARMACOKINETIC SYSTEMATIC REVIEW OF FLUOROQUINOLONES ADMINISTERED IN HUMANS

Drawn from manuscript published in *Expert Opin Drug Metab Toxicol*. May 2012; 8(5): 553-69

3. A SYSTEMATIC REVIEW OF FLUOROQUINOLONES

The FQ antimicrobials possess very similar structural scaffolds and physicochemical characteristics; yet, they exhibit a wide range of pharmacokinetic (PK) properties. Historical clinical PK literature and recent *in vitro* disposition studies have implicated the involvement of active transport mechanisms in renal handling of FQs, which accounts for one of the primary elimination pathways of these antibiotics (see Chapter 1). Therefore, the intent of this systematic review was to compile published human *in vivo* PK properties for FQs and to assess any relationships between pertinent, biologically relevant systemic PK variables and possible renal active transport mechanisms.

The analysis involved initial identification and review of FQ-related biomedical literature, which was then refined to articles pertaining to PK and urinary excretion studies in healthy human subjects. Once the studies were identified, they were carefully examined according to the inclusion criteria set-up for the analysis (as described ahead). These encompassed studies

specifically focused on healthy adult human subjects (between 18-60 years of age) within the normal weight range, i.e., depending on their body mass index wherever specified. The study did not set specific inclusion criteria for gender of the subjects. The patients were evaluated for overall good health before the study on the basis of medical history, physical examination, and laboratory evaluation procedures. In some FQ studies including different patient treatment-groups, (e.g., healthy volunteer groups along with groups of patients suffering from renal or hepatic impairment, or specific bacterial infections), only data from the healthy volunteers was considered after a careful assessment of their age, weight and organ functioning. Specifically, in some studies where the kidney function of the volunteers was assessed by measuring creatinine clearance, only the PK data of groups showing creatinine clearance $\geq$ 80 ml/min was considered for the analysis. The patients were required to be non-smokers and non-alcoholics (these conditions were assumed whenever not mentioned in the studies).

This review for FQs preferably included PK studies with intravenous (IV) route of administration (Table 3.2). For some of the FQs where the IV studies were not available, oral studies were considered for the analysis. However, in these oral studies, only apparent systemic volume of distribution and systemic/total clearance values were available, which were influenced by individual bioavailabilities (F_{oral}) of the FQs, and hence were not compiled. Essentially only single-dose studies were considered for this analysis. Linear PK was the main assumption for interpretation of all compiled PK parameters for the identified FQs. To assess this assumption for the identified FQs, repeated dose escalation and multiple dose studies were compiled, and the 'PK metrics', namely, average concentrations achieved at steady state (C^{ss}_{ave}), maximum and minimum concentrations on the concentration-time curve (C_{max}) and (C_{min}), and area under the concentration-time curve from zero time-point to infinity (AUC_{∞}: usually calculated by

trapezoidal rule in the studies), were analyzed for dose-related change (7, 31, 40, 43, 49, 51, 56, 176, 191, 192, 196). In these studies, the concentrations, (i.e., C^{ss}_{ave}, C_{max}, C_{min}) and the AUC_{∞} were found to increase dose-proportionally, while, the volume of distribution at steady state (Vd_{ss}) essentially remained constant with increase in dose. These observations thus confirmed that FQs would follow linear PK in the clinically administered doses, and thus linear PK was assumed for further calculations of PK parameters, discussed ahead in this chapter (134). In all the studies, the urine as well as blood sampling schedules were critically evaluated to optimize the urinary excretion and concentration-time curves for further PK analysis (the extrapolated AUC from the last sample point to infinity was usually not more than 20 % of the total AUC_{∞}).

The final database for all the FQs encompassed representative compounds from the second (n=9), third (n=4), and fourth (n=5) generations. Systemic pharmacokinetic properties were compiled for both intravenous and oral studies of the FQs. Pharmacokinetic variables included total body clearance (CL_{tot}), renal clearance (CL_{ren}), non-renal clearance (CL_{nonren}, which was obtained for FQs with available intravenous data only), Vd_{ss}, terminal half-life ($t_{1/2}$) and fraction excreted unchanged in urine (f_e) (Table 3.1 and 3.2). When not provided in the original references, CL_{ren} was calculated by: $CL_{ren} = U_{\infty}/AUC_{\infty}$ (U_{∞}, amount excreted in urine from zero to infinity) (Table 3.1 and 3.2). If the studies did not report body weight (BW) corrected PK parameters, then the parameters were corrected for BW using mean BW of the subjects in the study. In cases where BW was not mentioned, a BW of 70 kg was assumed (41). The fraction unbound in plasma (f_u) was obtained from *in vitro* protein binding studies conducted using human plasma (Table 3.2) (64, 145, 161, 208). The plasma-protein-binding-corrected pharmacokinetic variables, namely unbound volume of distribution (Vd_{ss}^{u}) and unbound total (CL_{tot}^{u}), nonrenal (CL_{nonren}^{u}) and renal (CL_{ren}^{u}) clearances, were further calculated using f_u (Table

3.1 and 3.2). Finally, a new term - defined as 'net renal tubular clearance' ($CL_{ren,tub}$) - was calculated by: $CL_{ren,tub} = CL_{ren}^{u}$ – glomerular filtration rate (GFR, assumed to be 1.6 ml/min/kg); a negative value indicates net tubular reabsorption, while a positive value indicates net tubular secretion. This $CL_{ren,tub}$ variable, quantifying the contribution of renal tubular reabsorption and/or secretion, was used to categorize the FQs examined in this study (n=18) into three groups according to their differences in renal tubular handling (Figure 3.1, Tables 3.1, 3.2 and 3.4). Group 1 represents those FQs undergoing net tubular reabsorption (defined as $CL_{ren,tub} < -1$ ml/min/kg), Group 2 includes FQs identified as having little or no net tubular transport (defined as $-1 \leq CL_{ren,tub} \leq 1$ ml/min/kg), and Group 3 contains the FQs exhibiting net tubular secretion (defined as $CL_{ren,tub} > 1$ ml/min/kg) (Table 3.1 and 3.4).

Relevant physicochemical properties (Tables 3.5 and 3.6), such as molecular weight, hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), number of rotatable bonds (nRot), molar volume, logarithmic value of the FQ distribution coefficient (log D), pK_a and percent ionization were obtained for all the FQs (except Antofloxacin, as physicochemical data was unavailable through the software) using SciFinder Scholar (2010) and ACD/PhysChem Suite (Advanced Chemistry Development, Inc.).

Table 3.1. Calculated pharmacokinetic properties for the fluoroquinolones

PK property	Formula
Vd_{ss}^{u}	Vd_{ss} / f_u
CL_{tot}^{u}	CL_{tot} / f_u
CL_{ren}	U_∞/AUC_∞
f_e	$CL_{ren}*100 / CL_{tot}$
CL_{ren}^{u}	CL_{ren} / f_u
CL_{nonren}	$CL_{tot} - CL_{ren}$
CL_{nonren}^{u}	CL_{nonren} / f_u or $CL_{tot}^{u} - CL_{ren}^{u}$
$CL_{ren,tub}$	CL_{ren}^{u} – Glomerular filtration rate (assumed to be 1.6 ml/min/kg)
	$CL_{ren,tub} < -1$ = Net tubular reabsorption (Group 1)
	$CL_{ren,tub} \geq -1$, but ≤ 1 = No net tubular transport (Group 2)
	$CL_{ren,tub} > 1$ = Net tubular secretion (Group 3)

Vd_{ss}^{u}: unbound volume of distribution at steady-state; Vd_{ss}: volume of distribution at steady-state; CL_{tot}^{u}: unbound total clearance; CL_{tot}: total clearance; CL_{ren}: renal clearance; U_∞: amount excreted in urine from zero to infinity; AUC_∞: area under the concentration-time curve from zero to infinity; f_e: fraction of parent drug excreted unchanged in urine expressed as %; CL_{ren}^{u}: unbound renal clearance; CL_{nonren}: nonrenal clearance; CL_{nonren}^{u}: unbound nonrenal clearance; $CL_{ren,tub}$: net renal tubular clearance

3. B PHARMACOKINETIC PROPERTIES OF FLUOROQUINOLONES:

The newer generations of FQs exhibit wider systemic distribution characteristics and longer duration of action as compared to the older compounds (11, 29, 159, 208). This may partially be a consequence of increased plasma protein binding, resulting in decreased elimination. For example, the fourth-generation FQ, trovafloxacin, shows plasma protein binding of approximately 76% (f_u = 24%) and an elimination/terminal half-life ($t_{1/2}$) of 11.2 hours, while the second-generation FQ, ciprofloxacin, has plasma protein binding of only 40% (f_u = 60%) and a correspondingly shorter $t_{1/2}$ of 4.2 hours (Table 3.2). However, as discussed below, differences in renal excretion mechanisms ($CL_{ren,tub}$) are likely more important for their duration of action (Table 3.2 and 3.4, Figure 3.1).

The newer FQs also exhibit increased tissue penetration, allowing them to reach higher intracellular concentrations (159, 208). Systemically, this translates into significantly greater FQ levels in target organs such as the intestine, kidney, liver, lungs and prostate than in the plasma (68, 76, 147, 148). Bone stands out as a tissue in which FQ permeability is generally poor (44). FQ levels in secretions are inconsistent, with most FQs reaching concentrations in saliva, pleural fluid, and bronchial epithelial lining fluid that are above that measured in serum, but exhibiting considerable variation in sweat, tears, and blister fluids (29, 141, 147, 159). Terminal half-lives in the individual fluid secretions also vary, with $t_{1/2}$ in the saliva being shorter than in plasma, while it was similar to plasma in bronchial secretions, and slightly longer in sweat, tears, and blister fluids (29, 141, 147, 159). With the exception of meningococcal infections, FQ permeation of the cerebrospinal fluid is extremely limited (48).

Not surprisingly, urine and biliary FQ concentrations often greatly exceed those in plasma as a consequence of the excretory functions of the kidney and liver. Indeed, for many FQs, their

unchanged urinary levels are considerably higher than their minimum inhibitory concentrations for most urinary pathogens, explaining their therapeutic success in the treatment of urinary tract infections (121, 141, 149, 161, 193). Similarly, for those FQs which undergo extensive intestinal secretion or hepatic metabolism, the unchanged drug and metabolite concentrations in feces are high, rendering them effective in the treatment of many gastrointestinal infections (68, 141, 147). Thus, in instances where the FQs are excreted primarily unchanged by the kidneys (e.g., ofloxacin, levofloxacin), renal clearance is an essential component of their total body clearance (Table 3.2). On the other hand, in situations where FQs are removed to a large extent by hepatic elimination (e.g., moxifloxacin, rufloxacin), nonrenal clearance is an important determinant of their pharmacokinetics (Table 3.2).

Table 3.2. Compiled pharmacokinetic parameters from the systematic review for n=18 fluoroquinolones

IV studies	$t_{1/2}$ (hr)	f_u (%)	Vd_{ss}^u (L/kg)	f_e (%)	CL_{tot} (ml/min/kg)	CL_{tot}^u	CL_{ren}	CL_{ren}^u	CL_{nonren}^u	$CL_{ren,tub}$	Group	Refs.
Ciprofloxacin	4.2	60	3.9	50	10.3	17.0	5.1	8.4	8.6	6.80	3	(27, 64, 97, 199)
Ofloxacin	6.6	70	--	80	3.7	5.7	3.0	4.6	1.1	3.00	3	(99)
Enoxacin	4.3	60	3.3	56	5.1	8.6	2.9	4.8	3.8	3.17	3	(111)
Gatifloxacin	11.3	60	3.0	80	2.5	4.2	2.0	3.4	0.9	1.79	3	(43)
Moxifloxacin	14.2	45	4.5	20	2.5	5.4	0.6	1.3	4.1	-0.30	2	(145, 156, 158)
Lomefloxacin	6.4	85	2.2	56	3.3	5.6	1.9	2.2	3.4	0.59	2	(53, 160, 200)
Levofloxacin	7.1	69	1.7	62	2.1	3.5	1.3	1.9	1.6	0.28	2	(17, 19)
Fleroxacin	13.0	77	1.8	66	1.4	2.4	0.9	1.2	1.1	-0.39	2	(161)
Trovafloxacin	11.2	24	5.4	11	1.4	6.0	0.2	0.7	5.3	-0.92	2	(176, 192)
Antofloxacin	20.3	83	4.6	58	2.1	2.5	1.2	1.5	1.1	-0.12	2	(194)
Oral studies												
Gemifloxacin	7.1	35					3.3	9.5		7.94	3	(1, 2)
Norfloxacin	4.3	80					7.5	9.4		7.80	3	(30)
Amifloxacin	3.6	50					1.7	3.3		1.70	3	(21)
Temafloxacin	7.7	74					1.8	2.4		0.81	2	(50)
Grepafloxacin	12.2	72					0.5	0.7		-0.91	2	(31)
Rufloxacin	34.9	40					0.2	0.6		-1.01	1	(89, 131)
Sparfloxacin	20	55					0.3	0.5		-1.13	1	(37, 112)
Pefloxacin	8.6	75					0.4	0.5		-1.10	1	(114)

$t_{1/2}$: half-life in hr; f_u: fraction of unbound drug expressed as %; Vd_{ss}^u: unbound volume of distribution at steady-state; CL_{tot}: total clearance; f_e: fraction of parent drug excreted unchanged in urine expressed as %; CL_{tot}^u: unbound total clearance; CL_{ren}^u: unbound renal clearance; CL_{nonren}^u: unbound nonrenal clearance; $CL_{ren,tub}$: net renal tubular clearance, calculated as CL_{ren}^u - glomerular filtration rate (GFR assumed to be 1.6 ml/min/kg); Groups 1, 2 and 3 were assigned as defined in Table 3.4. For oral studies, true Vd_{ss}^u, CL_{tot}, and f_e could not be obtained because only apparent values, which were influenced by the individual bioavailabilities (F_{oral}), were available. Since in most instances CL_{nonren}^u was calculated as $CL_{tot}^u - CL_{ren}^u$, this value was also excluded from the oral studies dataset. For parameter estimates obtained from the systematic review, the values reported are the average of mean values; therefore, standard deviations have not been reported (Refer to Appendix I). (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69).

Evaluating the pharmacokinetic properties in the final database (n=18) reveals the fraction unbound in plasma (f_u) showed a limited, 3.5-fold difference (Table 3.3) amongst the FQs; overall, plasma protein binding was low. Both Vd_{ss}^{u} and CL_{tot}^{u} were fairly uniform, indicating that the systemic distribution and overall elimination – after correction for plasma protein binding – varied only moderately across FQs (Table 3.3). Generally, each FQ showed at least some extravascular/intracellular distribution [Vd_{ss}^{u} >> plasma (0.04 L/kg) and total body water volume (0.6 L/kg)], and their clearances were lower than hepatic blood flow (20 ml/min/kg), but exceeded GFR for some. Most notably, their CL_{ren}^{u} values showed a wide distribution with a 20-fold difference among the compounds, illustrating that - despite being similarly distributed throughout the body - other factors (e.g., ionization state, renal drug transporters, see section 3.C) significantly influence their renal handling. Determination of the renal tubular clearance ($CL_{ren,tub}$) provides further insight as to which tubular handling process (e.g., net secretion vs. net reabsorption) plays the greatest role in renal elimination of each individual FQ, which further allows classification into FQs in Groups 1, 2 and 3 (Figure 1.2 and 3.1, Table 3.2 and 3.4). For these FQ groups, there were no substantial differences between CL_{nonren}^{u}, Vd_{ss}^{u} and f_u, suggesting particular molecular properties/specific transporter interactions may account for differences in renal handling, but may not affect other PK properties (Table 3.3). Most importantly, it was found that the mean plasma half-lives ($t_{1/2}$) showed significant differences between the three groups (Table 3.4): The $t_{1/2}$ of Group 1 was significantly longer as compared to Group 3 ($p < 0.01$), with the FQs in Group 1 showing a 3 to 4-fold higher $t_{1/2}$ than those in Group 3, and ~2-fold higher $t_{1/2}$ than the FQs in Group 2 (Table 3.4). Values for Group 1 and Group 2, as well as Group 2 and Group 3 did not differ significantly. This suggests that the differences in renal tubular handling between the various FQs may be the major reason for their differences in

systemic half-life, i.e., the role/contribution of renal tubular drug transporters may be the main determinant for the duration of action for FQs.

The renal tubular clearance differences of FQs between Group 1 and Group 3 (Table 3.4) may be explained by significant interactions of FQs with renal tubular drug transporters: FQs in Group 1, wherein CL_{ren}^{u} is less than GFR, are likely to predominantly/efficiently interact with apically expressed transporters that mediate their (net) tubular reabsorption (Figure 1.2). Of course, interactions with basolateral and apical transporters in the blood to urine (secretory) direction in the RPTC, prior to being offset/overcome by the reabsorptive flux, are likely to occur as well. On the other hand, FQs in Group 3, wherein CL_{ren}^{u} exceeds GFR, are expected to be substrates for basolateral and apical transporters and to exhibit a substantial blood to urine secretory flux, i.e., net tubular secretion. Thus, the marked dispersion of CL_{ren}^{u} and $CL_{ren,tub}$ values among FQs could be attributed to carrier-mediated mechanisms existing in RPTC. Such transporter interactions should be critically evaluated, as this may also be one explanation for some reported *in vivo* drug-drug interactions, e.g., decreased clearance of FQs co-administered with cimetidine or probenecid (see Chapter 1, Section 1.C). Such interactions could significantly affect FQ disposition kinetics and hence alter their efficacy and/or toxicity profiles.

Table 3.3. Summary of systemic pharmacokinetic and physicochemical properties of fluoroquinolones in the systematic review

	Mean	Range	Fold difference
Pharmacokinetic property			
Vd_{ss}^{u} (L/kg)	3.4	1.7-5.4	3.1
f_u (%)	57	24-85	3.5
CL_{tot}^{u} (ml/min/kg)	6.6	2.4-17.0	7.1
CL_{ren} (ml/min/kg)	1.9	0.2-7.5	46.2
CL_{ren}^{u} (ml/min/kg)	3.1	0.5-9.5	20.2
CL_{nonren}^{u} (ml/min/kg)	3.1	0.8-8.6	10.1
Physicochemical property			
Molecular weight (Da)	369.6	319-462	1.4
Molar volume (cm^3)	254.3	203-300	1.5
Log D (pH = 7.4)	-0.2	-1.1-1.1	--

Vd_{ss}^{u}: Unbound volume of distribution at steady-state; f_u: Fraction of unbound drug in plasma; CL_{tot}^{u}: Unbound total body clearance; CL_{ren}: Renal clearance; CL_{ren}^{u}: Unbound renal clearance; CL_{nonren}^{u}: Unbound nonrenal clearance: Da: Daltons; Log D: Logarithmic value of distribution co-efficient. (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

Table 3.4. Characteristics used to define the groups as reported in Table 3.2 and Figure 3.1 based on renal tubular handling and associated plasma half-lives

$CL_{ren, tub}$ (ml/min/kg)	Net renal handling process	Group	$t_{1/2}$ (hr)
Negative (< -1)	net tubular reabsorption	1	21.2 ± 13.1 (n=3)
≥ -1 and ≤ 1	little/no net tubular transport	2	11.5 ± 4.6 (n=8)
Positive (> 1)	net tubular secretion	3	5.9 ± 2.7 (n=7)

$CL_{ren,tub}$: estimated net renal tubular clearance; $t_{1/2}$: half-life in plasma (reported as mean ± standard deviation). (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

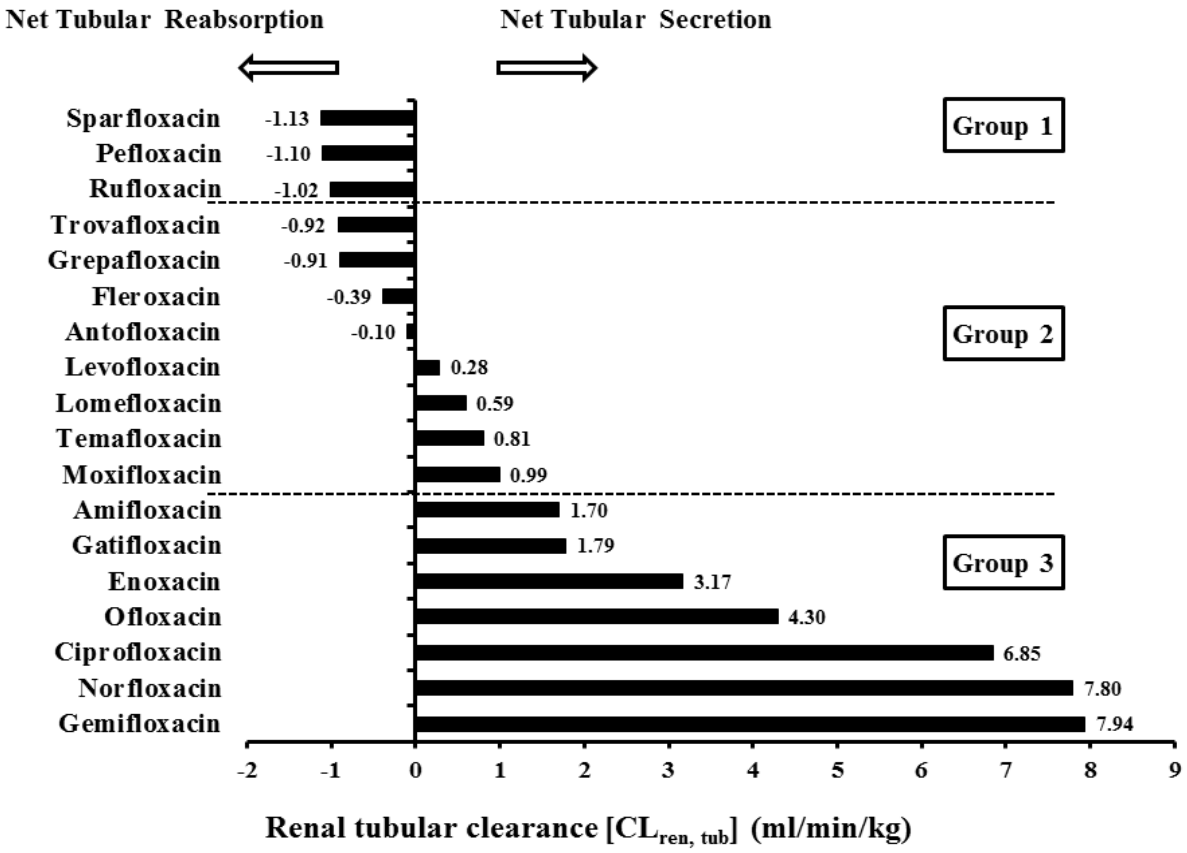

Figure 3.1. Categorization of fluoroquinolones based on renal tubular clearance ($CL_{ren,tub}$)

FQs were separated into three groups based upon their $CL_{ren,tub}$ obtained from the pharmacokinetic systematic review. FQs were assigned to groups according to the criteria listed in Table 3.4. (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

3. C. PHYSICOCHEMICAL PROPERTIES OF FLUOROQUINOLONES

As FQs have evolved through successive generations of drug discovery, their molecular structures have been modified to some extent. Molecular size, molecular weight, LogD, pK_a, molar volume, number of hydrogen bond donors, number of hydrogen bond acceptors, number of rotatable bonds, and microspecies (ionization) profiles at physiological pHs were estimated for individual FQs (Tables 3.3 and 3.4). The values reported herein were restricted to those eighteen FQs for which sufficient data were obtained in the systematic review to estimate unbound renal clearance (CL_{ren}^{u}) and renal tubular clearance ($CL_{ren,tub}$) (See Table 3.2). No significant trends were detected on comparing the hydrogen bond donors, hydrogen bond acceptors, number of rotatable bonds, LogD (at pH 7.4), molecular weights, or molar volumes across the three groups (Refer Appendix II and Table 3.3). LogD values (at pH 7.4) for the FQs were predominantly low, indicating their largely hydrophilic nature. FQs in the final database had similar structural scaffoldings with two pK_a values, an acidic pK_a between 5.19 and 6.44 and a basic pK_a between 6.30 and 10.63 (Table 3.3; notably, the software was unable to accurately predict acidic pK_a values for fleroxacin, lomefloxacin, norfloxacin and pefloxacin). The close proximity of the two pK_a values dictates that these FQs exist predominantly as zwitterions in the physiological pH range due to the dissociation of a carboxyl group at the 3-position of the quinolone ring and the protonation of the piperizinyl ring nitrogen (Figure 1.1) (9, 29). However, Table 3.5 suggests that ionization profiles demonstrate considerable differences between FQs in their proportion of anionic (A), cationic (C), neutral (N) and zwitterionic (Z) species at physiologically relevant pH values. For example, gemifloxacin is predicted to exist completely as a zwitterion at the blood pH of 7.4, while rufloxacin would show a minor zwitterionic component (17%) and be primarily anionic (82%), and ciprofloxacin would be

predominantly zwitterionic (82%), with co-existing cationic (10%) and anionic (7%) microspecies. However, at the urinary pH of 6.3 it is predicted that gemifloxacin will exhibit both zwitterionic (82%) and cationic (18%) microspecies, rufloxacin will become primarily a zwitterion (67%) with some anionic (21%) and cationic (5%) microspecies, and ciprofloxacin will be largely cationic (60%) with some zwitterionic (39%), but no anionic species. In general, for the FQs listed in Table 3.5, zwitterionic and anionic species are most prevalent at pH 7.4, whereas at pH 6.3 zwitterionic and cationic species dominate. Regardless, as FQs exist as charged molecules in blood and urine, their absorption, distribution, and elimination are likely to be influenced by active transport mechanisms in addition to passive diffusion and glomerular filtration.

Prulifloxacin was specially included into the dataset for analysis of physicochemical properties of FQs (Tables 3.5 and 3.6), as it is the only prodrug FQ, which is currently marketed (alatrofloxacin, the prodrug of trovafloxacin was withdrawn due to hepatotoxicity) (81, 122). This prodrug is absorbed mainly from the upper small intestine, followed by hepatic first-pass metabolism by an α-esterase (paraoxonase) to being converted into the active FQ: ulifloxacin (12, 81). Hence, it was of interest to study the *in vitro* OCT-FQ interactions, potentially mediating prulifloxacin's GI absorption and hepatic metabolism for conversion into its active metabolite, ulifloxacin. This analysis predicted prulifloxacin to exist predominantly as an anion at the pH values of 7.4 (95%A, 3%Z) and 6.3 (55%A, 6%C, 23%N, 17%Z) (Table 3.6). Due to absence of *in vivo* studies matching our inclusion criteria for this FQ prodrug (detected as ulifloxacin *in vivo*), as well as due to unavailability of the active metabolite ulifloxacin for *in vitro* studies, no analysis has been demonstrated for ulifloxacin. However, the prodrug was pursued further for *in vitro* testing (See Chapters 4 and 5).

The information summarized in Tables 3.2-3.5 and Figure 3.1 may allow prioritization of *in vitro* studies designed to identify the active transporters that contribute to the renal tubular secretion and/or reabsorption of FQs: For example, at blood pH, sparfloxacin exists almost completely as zwitterionic (80%) and cationic moieties (11%), with only a small anionic component (8%). Furthermore, the pharmacokinetic literature indicates that co-administration of probenecid does not inhibit its renal clearance (143). Taken together, this suggests that a basolateral OCT pathway (e.g., OCT1, OCT2 and/or OCT3) mediates its accumulation in human RPTCs from the blood and its subsequent efflux into the urine (perhaps via an OCTN or MATE) (Figure 1.2). However, at urinary pH, sparfloxacin exists as cationic (63%) and zwitterionic moieties (36%), with no anionic species. Coupled with the lack of inhibition by co-administered probenecid, this suggests an unidentified apical organic cation uptake transporter mediates its tubular reabsorption (95, 143). Similarly, moxifloxacin, whose renal handling is unaffected by probenecid (157) and has ionization profiles of 93%Z, 7%A, 0%C at pH 7.4 and 51%Z, 0%A, 49%C at pH 6.3, may cross the basolateral membranes of human RPTCs via OCT1, OCT2 and/or OCT3 and exit across the apical membrane via any one or all of the identified efflux transporters (Figure 1.2). On the other hand, for compounds predominately anionic in the blood such as fleroxacin (86%A, 13%Z, 0%C at pH 7.4) and levofloxacin (62%A, 37%Z, 0%C at pH7.4), whose renal excretion is known to be inhibited by co-administration of probenecid (38, 81, 142), basolateral RPTC uptake is likely mediated by OAT1 and/or OAT3, followed by apical ATP Binding Cassette transporter mediated efflux. Hence these structural parameters and ionization profiles of FQs, may potentially aid in prediction of renal transport mechanisms likely to mediate the *in vivo* renal disposition of these antimicrobials (See section 1.C).

Table 3.5. Physicochemical properties of the fluoroquinolones in the systematic review

Drug:	MW (Da)	Log D (pH = 7.4)	HBD	HBA	nRot	Molar Volume (cm^3)	pK_a (acidic)	pK_a (basic)
Amifloxacin	334	-0.7	2	7	3	231.7	6.2 ± 0.4	7.4 ± 0.4
Ciprofloxacin	331	-0.3	2	6	3	226.8	6.4 ± 0.4	8.7 ± 0.1
Enoxacin	320	-0.6	2	7	3	230.7	6.0 ± 0.7	8.2 ± 0.1
Fleroxacin	321	-0.3	1	6	4	262.1	--*	7.2 ± 0.4
Gatifloxacin	375	-0.2	2	7	4	270.8	6.4 ± 0.5	8.7 ± 0.4
Gemifloxacin	389	-0.7	3	9	6	236.3	6.0 ± 0.7	9.2 ± 0.3
Grepafloxacin	359	0.6	2	6	3	263.0	6.4 ± 0.5	8.7 ± 0.4
Levofloxacin	361	-0.4	1	7	2	244.0	5.2 ± 0.4	7.4 ± 0.4
Lomefloxacin	351	0.04	2	6	3	261.6	--*	8.6 ± 0.4
Moxifloxacin	401	0.3	2	7	4	285.0	6.4 ± 0.5	10.6 ± 0.2
Norfloxacin	319	-0.7	2	6	3	237.4	--*	8.7 ± 0.1
Ofloxacin	361	-0.4	1	7	2	244.0	5.2 ± 0.4	7.4 ± 0.4
Pefloxacin	333	-0.2	1	6	3	252.5	--*	7.4 ± 0.4
Rufloxacin	363	-0.3	1	6	2	234.9	5.2 ± 0.2	7.3 ± 0.4
Sparfloxacin	392	0.8	4	7	4	273.2	6.4 ± 0.5	8.6 ± 0.6
Temafloxacin	417	-0.9	2	6	3	292.5	6.0 ± 0.4	8.7 ± 0.4
Trovafloxacin	416	-1.1	3	7	4	258.3	5.8 ± 0.7	7.9 ± 0.2
Prulifloxacin	462	1.1	1	9	4	283.6	5.9 ± 0.4	6.3 ± 0.7

*The software was unable to accurately predict acidic pK_a values for fleroxacin, lomefloxacin, norfloxacin and pefloxacin;
MW: molecular weight; Log D (pH = 7.4): logarithmic value for the distribution coefficient at pH 7.4; HBD: number of hydrogen bond donors; HBA: number of hydrogen bond acceptors; nRot: number of rotatable bonds; pK_a (acidic): most acidic pK_a value; pK_a (basic): most basic pK_a value. Antofloxacin was excluded in this analysis as physicochemical could not be obtained for this FQ by the software. (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

Table 3.6. **Ionization profiles for fluoroquinolones in systemic (pH 7.4) and urinary (pH 6.3) compartments**

	Ionization profile (%) at pH 6.3				Ionization profile (%) at pH 7.4			
Drug:	**A**	**C**	**N**	**Z**	**A**	**C**	**N**	**Z**
Amifloxacin	4	59	13	23	57	5	13	25
Ciprofloxacin		60		39	7	10		82
Enoxacin		20		78	14			84
Fleroxacin	30	7	3	60	86			13
Gatifloxacin		49		51	7	7		86
Gemifloxacin		18		82				97
Grepafloxacin		61		39	6	10		83
Levofloxacin	11	7		82	62			37
Lomefloxacin		13		86	10			88
Moxifloxacin		49		51	7			93
Norfloxacin		17		82	8			90
Ofloxacin	11	7		82	62			37
Pefloxacin	9	14		75	60			39
Prulifloxacin	55	6	23	17	95			3
Rufloxacin	25	5		67	82			17
Sparfloxacin		63		36	8	11		80
Trovafloxacin	6	14		80	47			52
Temafloxacin		41		59	7	5		88

Molecular microspecies: A (anionic), C (cationic), N (neutral), and Z (zwitterionic). Determined using ACD/PhysChem Suite Version 12 (Advanced Chemistry Development, Inc). *Antofloxacin was excluded in this analysis as physicochemical could not be obtained for this FQ by the software. (Adapted from manuscript published in *Expert Opin Drug Metab Toxicol.* May 2012; **8**(5): 553-69)

CHAPTER 4

HUMAN ORGANIC CATION TRANSPORTERS 1 (SLC22A1), 2 (SLC22A2), 3 (SLC22A3) AS DISPOSITION PATHWAYS FOR FLUROQUINOLONE ANTIMICROBIALS

(draft of a manuscript submitted to the journal: *Antimicrobial Agents and Chemotherapy*)

4. A. INTRODUCTION

Through decades of clinical advancement, the quinolones, now known as fluoroquinolones (FQ), have been widely popular as broad-spectrum antimicrobials, in human as well as veterinary medicine (6, 66, 141). They are utilized for infections of the soft-tissue, skin, bone, meninges, respiratory tract, gastrointestinal tract, and genitourinary tract (66). For example, ciprofloxacin and ofloxacin undergo considerable hepatobiliary elimination resulting in high concentrations in the feces and, hence, are preferentially indicated for treating gastrointestinal infections (66, 68, 96). Whereas, e.g., ciprofloxacin, levofloxacin, norfloxacin, and ofloxacin, are known to be renally eliminated as the ‘unchanged drug’, resulting in urinary concentrations of parent drug that are much higher than their minimum inhibitory concentrations for most infectious bacteria, thus rendering them as important antimicrobials for genitourinary tract infections (9, 47).

The development of newer FQs has enabled improved efficacy and therapeutic duration of action. However, this pharmacological benefit of higher systemic and tissue concentrations has resulted in a number of FQs demonstrating mild to severe toxicities, eventually leading to withdrawal from the pharmaceutical market for some (98). Moreover, all currently marketed FQs have been mandated by the FDA to carry labeled warnings associated with their use, due to side effects like tendinitis (in 2008) and exacerbation of Myasthenia Gravis (in 2011). With the existence of such broad toxicities associated with the use of FQs, there is an increased need to elucidate the underlying biochemical mechanisms driving their overall kinetics and target organ disposition. Such knowledge should aid a priori identification of potential drug-drug interactions, as well as future drug design strategies.

Considering that renal excretion is one of the major elimination pathways for most FQs after entering the systemic circulation (121, 126), investigations regarding the mechanisms governing their flux across renal proximal tubule cells (RPTC), i.e., renal basolateral uptake (removal from the blood into RPTC), apical efflux (from the RPTC into the urinary space), and potential reabsorption (from the urinary space back into the RPTC), are warranted. Further, as the basic structural scaffold of FQs has essentially remained unchanged (198), all FQs are predicted to exist predominantly as ionized molecules in the physiological pH range; having co-existent cationic, anionic, and electroneutral (zwitterionic and/or neutral) species (116). Due to their mostly ionic nature, passive diffusion should account for a negligible component of their movement across cell membranes, leaving active transport and facilitated diffusion mechanisms likely to govern the overall kinetics of these agents in the body (116, 165).

Recently, we conducted a systematic review of clinical literature reporting *in vivo* pharmacokinetic properties of FQs and correlated this data with available *in vitro* studies

examining FQ interactions with transporters (116, 165). This allowed identification of a subset of FQs (ciprofloxacin, enoxacin, fleroxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, pefloxacin, prulifloxacin, rufloxacin and sparfloxacin) with high potential to interact with members of the SLC22 (organic cation/anion/zwitterion transporters) family, which are known to be expressed in the RPTC and to mediate RPTC flux of such charged species (116, 165). For example, a number of clinical studies have documented significant changes in FQ (e.g., enoxacin, fleroxacin, and levofloxacin) pharmacokinetics upon concomitant administration with cimetidine, a well-characterized substrate of human (h)OCT1 (SLC22A1) and hOCT2 (SLC22A2), and inhibitor of hOCT3 (SLC22A3) (91, 172, 181). A significant decrease in the renal (CL_{ren}) and total (CL_{tot}) clearances (each ~13-28%) of the FQs was observed, with an accompanying increase in the area under the concentration curve from zero time-point to infinity ($AUC_{0\text{-}inf}$) by ~28% for enoxacin and levofloxacin (42, 111, 149). Similarly, co-administration of ciprofloxacin, levofloxacin, or ofloxacin with procainamide, a class I antiarrhythmic agent and known inhibitor of the hOCTs, significantly reduced CL_{ren} and increased $AUC_{0\text{-}inf}$ of procainamide and its metabolite, N-Acetylprocainamide (NAPA) in patients (10, 58, 103, 104, 110, 202). Levofloxacin induced the greatest effect, decreasing the CL_{ren} of procainamide by ~26% and of NAPA by ~20% (10).

In accordance with this 'clinical footprint' for hOCT involvement in renal FQ disposition, recent *in vitro* studies using stably transfected cell lines have demonstrated inhibition of hOCT2, a membrane potential sensitive facilitated diffusion carrier targeted to the basolateral membrane of RPTC, by grepafloxacin (K_i value = 10.4 µM), levofloxacin (IC_{50} = 127 ± 27 µM) and moxifloxacin (72, 91, 125). However, potential FQ interactions with hOCT1 and hOCT3 have not been investigated. Thus, the objective of this work was to kinetically characterize the

potency of interaction of the identified subset of FQs with hOCT1, hOCT2 and hOCT3 and then apply this information to quantitatively assess the clinical relevance of any such interaction via calculation of the drug-drug interaction index (unbound C_{max}/IC_{50} or K_i).

4. B. MATERIALS AND METHODS

4.B.1. Chemicals and reagents

Unlabeled tetraethylammonium (TEA) bromide and 1-methyl-4-phenylpyridinium (MPP^+) iodide were purchased from Sigma Aldrich (St. Louis, MO). Quinine monohydrochloride dihydrate was purchased from Fisher Scientific (Waltham, MA). Ciprofloxacin hydrochloride, norfloxacin and ofloxacin hydrochloride were purchased from MP Biomedicals (Solon, OH). Enoxacin, fleroxacin, gatifloxacin, levofloxacin hydrochloride, lomefloxacin hydrochloride, moxifloxacin hydrochloride, pefloxacin mesylate, prulifloxacin, rufloxacin hydrochloride, and sparfloxacin were purchased from LKT Laboratories, Inc. (St. Paul, MN). Radiolabeled [^{14}C]TEA and [^{3}H]MPP^+ were obtained from PerkinElmer (Waltham, MA). Dulbecco's Modified Eagle's Medium with high glucose (DMEM) and Serum Supreme were purchased from Fisher Scientific (Waltham, MA). Penicillin/streptomycin and G418 (geneticin) were purchased from Invitrogen Life Technologies (Grand Island, NY) and VWR International (Radnor, PA), respectively.

4.B.2. Cell line maintenance and transport assay

The human embryonic kidney (HEK293) cell lines stably-expressing hOCT1, hOCT2, or hOCT3, and the corresponding empty vector transfected background control line (HEK293-EV), were developed as described previously (54, 55). Cell lines were maintained in DMEM

containing 10% Serum Supreme, 1% penicillin/streptomycin, and G418 (100 µg/ml) at 37ºC with 5% CO_2.

Accumulation assay protocols were adapted from our previously published methods (187, 195). Briefly, cells were seeded in 24-well tissue culture plates (250,000 cells/well) and grown in the absence of antibiotics for 2 days under suitable cell culture conditions (37°C and 5% CO_2). On the day of the experiment, the culture medium was removed and cells were equilibrated for 10 min with transport buffer (Hank's balanced salt solution containing 10 mM HEPES, pH 7.4; Sigma-Aldrich, Saint Louis, MO). Next, this transport buffer was removed and replaced with 500 µl of transport buffer containing either 1-30 µM unlabeled TEA with [^{14}C]TEA (0.25 µCi/ml) added as tracer for hOCT1 and hOCT2, or 1-30 µM unlabeled MPP^+ with [H^3]MPP^+ (0.25 µCi/ml) added as tracer for hOCT3 in presence/absence of 0.1-2,000 µM FQs or 200 µM quinine (as detailed in figure legends). Following incubation, buffer was removed and the cells were immediately rinsed three times with excess ice-cold transport buffer, lysed in 200 µl of 1 M NaOH, neutralized with 250 µl of 1 M HCl plus 200 µl of 0.1 M HEPES. Aliquots were assayed for radioactivity by liquid scintillation counting and for total protein content using a Bradford protein assay kit (BioRad, Hercules, CA). Uptake was reported as picomoles per milligram total cell protein. All experiments were performed at least three times in triplicate (i.e., three wells/treatment repeated at least three times).

4.B.3. Kinetic and statistical analyses

All data used in kinetic determinations were corrected for background accumulation in HEK293-EV cells prior to analysis. Dose-response curves were analyzed by nonlinear regression using GraphPad Prism® software version 5.04 (GraphPad, San Diego, CA). Prior to

determination of inhibition constants (K_i value), the Michaelis Menten constants (K_m values) for TEA and MPP^+ were validated with those previously published for hOCT1 and hOCT3 (91, 102). Further, the type of inhibition was evaluated using mixed model inhibition analysis (22). This model uses the following equations to assess the mode of inhibition:

$$V_{max}^{App} = \frac{V_{max}}{1 + \frac{I}{\alpha * K_i}} \quad \text{and} \quad K_m^{App} = K_m * \frac{1 + \frac{I}{K_i}}{1 + \frac{I}{\alpha * K_i}}$$

With the final equation being,

$$Y = V_{max}^{App} * \frac{X}{K_m^{App} + X}$$

where 'Y' is the substrate (in this case TEA/MPP^+) uptake rate observed, 'X' and 'I' are the substrate and inhibitor (FQ) concentrations respectively, V_{max} is the maximum transporter velocity in absence of the inhibitor, K_m value is the Michaelis-Menten constant of the substrate and K_i value is the inhibition constant generated from the experimental dataset. The type of inhibition is defined by the 'alpha value' (α) obtained. Inhibition is identified as competitive, if α is a large number ($\alpha > 1$), as non-competitive, if $\alpha = 1$, or as uncompetitive, if α is small, but greater than zero ($0 < \alpha < 1$) (22). Subsequently, K_i values for the FQs were calculated using the appropriate model based upon the identified inhibition mechanism.

Data are reported as mean ± S.E.M. Statistical significance was determined using one-way analysis of variance (ANOVA) with Dunnett's pairwise comparison post hoc test to measure significant differences. The value for significance was set at 0.05.

4. C. RESULTS

4.C.1. Characterization of fluoroquinolone interaction with human OCT1

TEA uptake in HEK293-hOCT1 cells (25.41 ± 2.3 pmol/mg protein/15 min) was ~18 fold higher than that measured in HEK293-EV cells (1.44 ± 0.18 pmol/mg protein/15 min), which exhibited a consistent quinine-insensitive (data not shown) background accumulation of ~5-6% (Figure 4.1). Addition of 200 µM quinine (vs. 1 µM TEA) reduced TEA accumulation in the HEK293-hOCT1 cells to the background level observed in HEK293-EV cells. In order to grossly identify which, if any, of the FQs of interest exhibited inhibition of hOCT1 transport strong enough to warrant further kinetic analysis (≤ ~60% inhibition), they were each independently tested at a concentration of 1 mM (Figure 4.1). Under these conditions, enoxacin failed to produce significant inhibition of hOCT1, while ciprofloxacin (~33%), fleroxacin (~20%), levofloxacin (~38%), lomefloxacin (~43%), norfloxacin (~24%), ofloxacin (~38%), pefloxacin (~40%) and rufloxacin (~47%) exhibited significant, but weak inhibition. Only gatifloxacin (~77%), moxifloxacin (~85%), prulifloxacin (~75%), and sparfloxacin (~75%) produced inhibition greater than 60% (level of inhibition established as the cut-off value under these preliminary test conditions for further kinetic analysis).

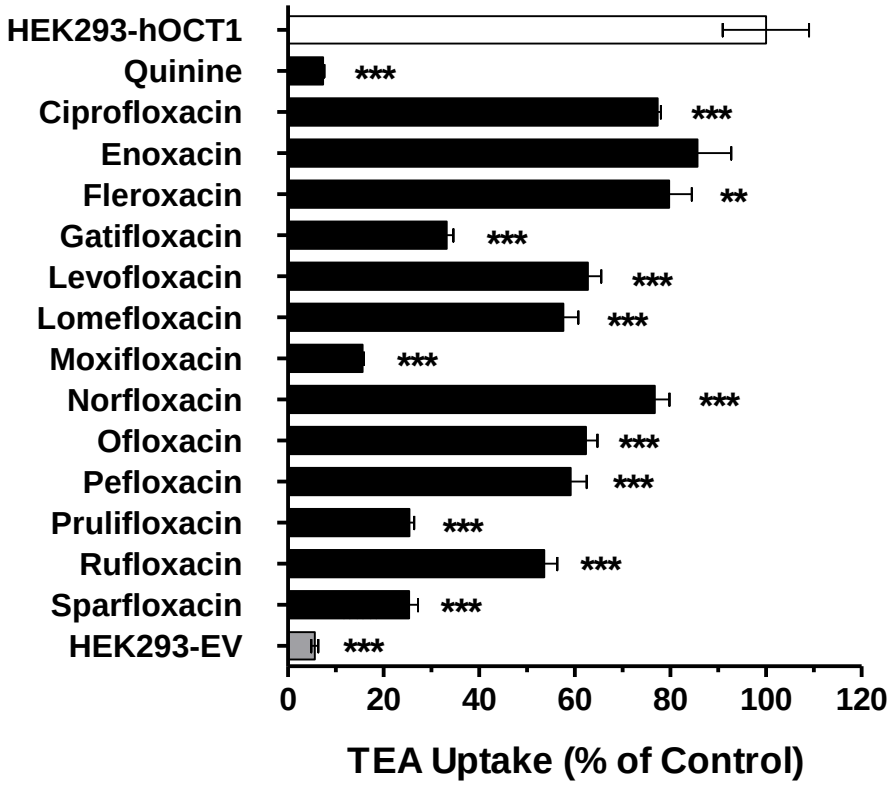

Figure 4.1. Inhibition of human OCT1-mediated transport by fluoroquinolones

Uptake of 1 µM [^{14}C]TEA was measured for 15 min in HEK293 cells stably expressing hOCT1 (open bar) in presence of unlabeled FQs (1 mM) or quinine (200 µM), a prototypical hOCT inhibitor, (black bars). The mock-vector transfected HEK293-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Uptake is expressed as a percentage of the positive control (HEK293-hOCT1). Values are expressed as mean ± S.E.M. and significant differences between HEK293-hOCT1 and treatments were analyzed using one-way ANOVA analysis followed by Dunnet's post hoc test (*** $p<0.001$).

To identify the proper model(s) to utilize for determination of inhibition potencies (K_i values) for gatifloxacin, moxifloxacin, prulifloxacin, and sparfloxacin on hOCT1, experiments were conducted to identify the mode of inhibition produced by each compound. Previous work found hOCT1-mediated TEA accumulation in HEK293 cells to be linear through at least 2 min and reported a Michaelis-Menten constant (K_m) value of 229 µM for TEA, and we obtained similar results in our laboratory (55, 91). Based on these parameters we performed independent saturation analysis experiments using an accumulation time of 1 min and a TEA concentration of 1 µM in the absence and presence of two concentrations of each FQ; gatifloxacin, moxifloxacin, and prulifloxacin each at 200 µM and 500 µM and sparfloxacin at 150 µM and 350 µM. The mode of inhibition was then identified by nonlinear regression analysis of background-corrected data using the 'mixed-model inhibition' analysis in GraphPad Prism. The α values obtained were all much greater than 1, indicating these four FQs are competitive inhibitors of hOCT1 (Table 4.1).

Finally, the strength of FQ inhibition on hOCT1 was quantitated (K_i values) by concentration-dependency studies (Figure 4.2). Inhibition of hOCT1-mediated TEA uptake by increasing FQ concentrations (0.1 - 2,000 µM) was analyzed by nonlinear regression selecting competitive inhibition. K_i values were estimated as 250 ± 18 µM for gatifloxacin, 161 ± 19 µM for moxifloxacin, 136 ± 33 µM for prulifloxacin, and 94 ± 8 µM for sparfloxacin (Figure 4.2 and Table 4.1).

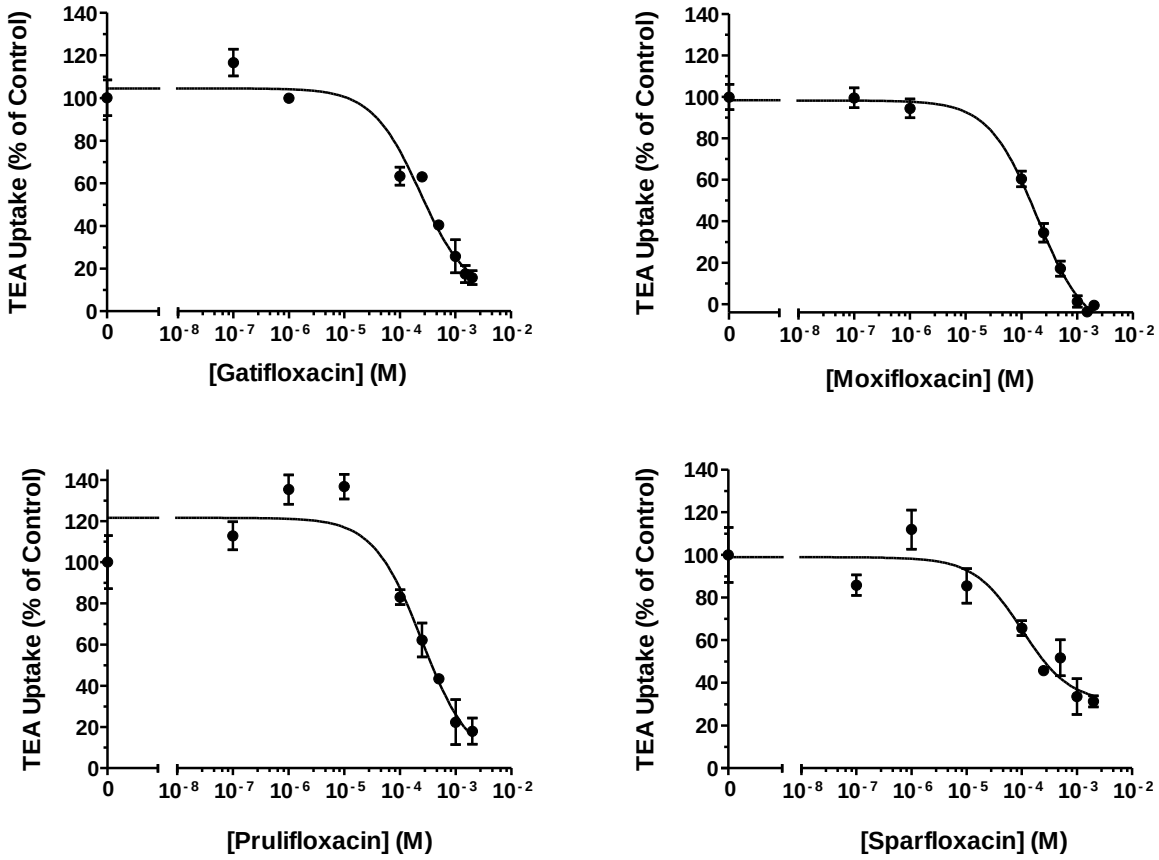

Figure 4.2. Determination of binding affinities (K_i values) for gatifloxacin, moxifloxacin, prulifloxacin and sparfloxacin on human OCT1

One minute [^{14}C]TEA (1 μM) uptake was measured in the absence or presence of 0.1-2,000 μM FQs in HEK293-hOCT1 cells. Uptake was corrected for non-specific background accumulation in HEK293-EV cells and expressed as percent of control. Data are presented as mean ± S.E.M. The K_i values were determined by non-linear regression selecting competitive mode in GraphPad Prism. All experiments were conducted at least 3 times in triplicate and the graphs are single representative experiments.

4.C.2. Characterization of fluoroquinolone interactions with human OCT2

TEA uptake in HEK293-hOCT2 cells (136.58 ± 1.74 pmol/mg protein/15 min) was ~60 fold higher than that detected in HEK293-EV cells (2.24 ± 0.37 pmol/mg protein/15 min = quinine-insensitive (data not shown) background accumulation of ~1-2%) (Figure 4.3). Addition of 200 µM quinine (vs. 1 µM TEA) inhibited the TEA accumulation in HEK293-hOCT2 cells by ~80%. As described above, we first sought to identify those FQs (1 mM) capable of producing strong inhibition of hOCT2-mediated TEA (1 µM) uptake (Figure 4.3). In marked contrast to hOCT1, none of the examined FQs significantly inhibited hOCT2 under these conditions. In fact, enoxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, and sparfloxacin appeared to stimulate TEA uptake under these conditions (Figure 4.3). In the absence of inhibition, no further kinetic analysis was performed.

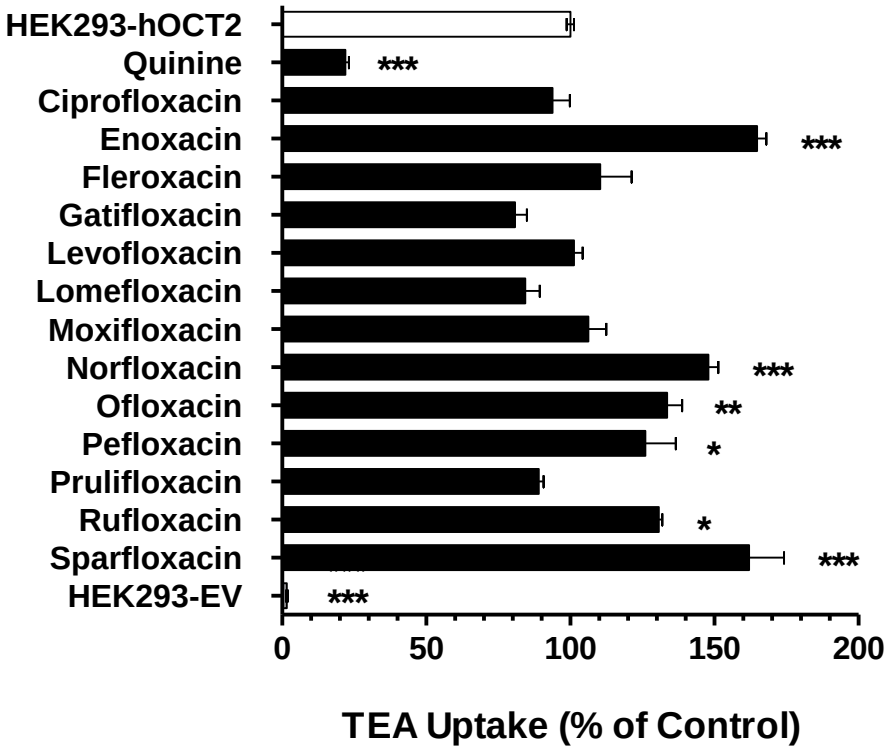

Figure 4.3. Effect of fluoroquinolones on human OCT2-mediated transport

Uptake of 1 µM [^{14}C]TEA was measured for 15 min using HEK293 cells stably expressing hOCT2 in absence (open bar) or presence of 1 mM unlabeled FQs or 200 µM quinine (black bars). The mock-vector transfected HEK293-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Uptake is shown as percent of control (HEK293-hOCT2). Values are given as mean ± S.E.M. and significant differences between HEK293-hOCT2 and treatments were analyzed using one-way ANOVA analysis followed by Dunnet's post hoc test (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

4.C.3. Characterization of fluoroquinolone interactions with human OCT3

To track hOCT3 transport activity, MPP^+ (1 µM) was used as substrate (Figure 4.4). MPP^+ accumulation in HEK293-hOCT3 expressing cells (84.09 ± 4.29 pmol/mg protein/15 min) was ~21 fold greater than that obtained in the control HEK293-EV cells (4.01 ± 0.17 pmol/mg protein/15 min). Accumulation of MPP^+ in the HEK293-EV cells was insensitive to addition of 200 µM quinine (data not shown), however accumulation in the HEK293-hOCT3 cells in the presence of quinine was dampened to a level similar to that measured in the control cells. Similar to what was observed for hOCT2, none of the FQs (1 mM), with the exception of moxifloxacin (~30%), inhibited hOCT3-mediated transport under test parameters (Figure 4.4). Again, apparent stimulation of transport activity occurred in the presence of some FQs, namely fleroxacin, levofloxacin, lomefloxacin, ofloxacin, and pefloxacin. Other than ofloxacin and pefloxacin each being associated with increased substrate uptake by hOCT2 and hOCT3, no consistent pattern of inhibition or stimulation was noted across transporters or substrates (Refer Appendix III).

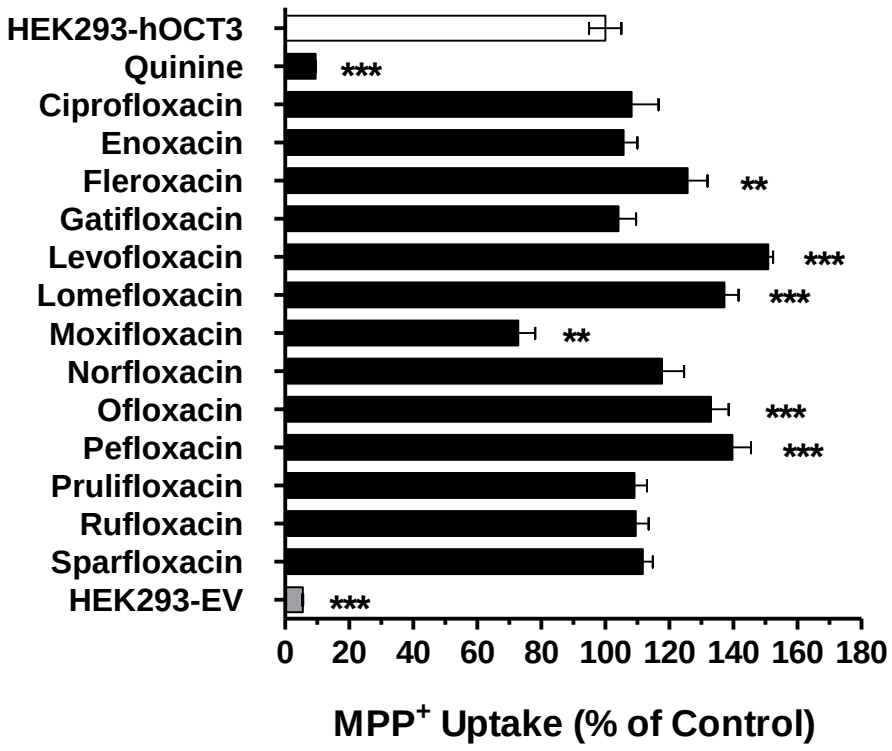

Figure 4.4. Effect of fluoroquinolones on human OCT3-mediated transport

Uptake of 1 µM [^{3}H]MPP$^+$ was measured for 15 min in HEK293 cells stably expressing hOCT3 in absence (open bar) and presence of 1 mM unlabeled FQs or 200 µM quinine (black bars). The mock-vector transfected HEK293-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Uptake on was plotted as a percentage of the positive control (HEK293-hOCT3). Values are expressed as mean ± S.E.M. and significant differences between HEK293-hOCT3 and treatments were analyzed using one-way ANOVA followed by Dunnet's post hoc test (**$p<0.01$ and ***$p<0.001$).

Although the inhibition produced by moxifloxacin was somewhat weak, since it was the only FQ to produce any significant inhibition of hOCT3 and it was one the strongest inhibitors of hOCT1, we performed kinetic analysis of this compound on hOCT3 to allow for comparison. Previous work reported hOCT3-mediated MPP^+ accumulation in HEK293 cells to be linear through at least 2 min with a K_m value of ~40-50 μM, and we confirmed similar results in our laboratory (54, 91, 102). Nonlinear regression analysis (using 'mixed-model inhibition') of background-corrected saturation data (accumulation time of 1 min with 1 μM MPP^+) collected in the absence and presence of moxifloxacin at 500 μM and 1,000 μM yielded an α value much greater than 1, indicating competitive inhibition (Table 4.1). Subsequent dose-dependence experiments using 0.1 - 2,000 μM moxifloxacin to inhibit MPP^+ uptake yielded a K_i estimate of 1,598 ± 146 μM (Figure 4.5 and Table 4.1).

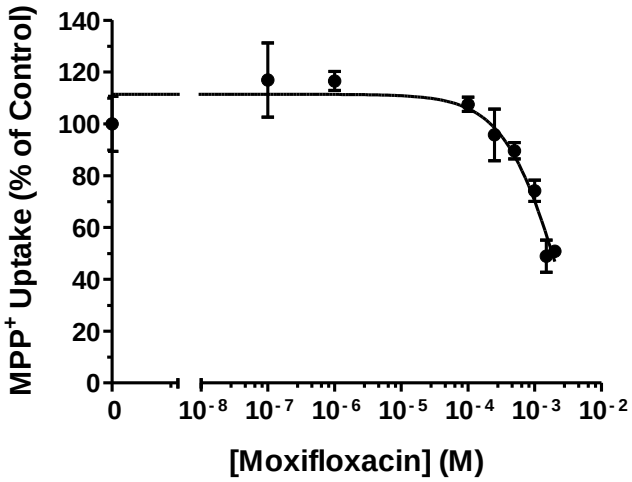

Figure 4.5. Determination of the binding affinity (K_i value) for moxifloxacin on human OCT3

[^{3}H]MPP$^+$ uptake was measured for 1 min in the absence or presence of 0.1-2,000 μM moxifloxacin in HEK293-hOCT3 cells. Uptake was corrected for non-specific background accumulation in HEK293-EV cells and expressed as percent of control. Data are presented as mean ± S.E.M. The K_i value was determined by non-linear regression selecting competitive mode in GraphPad Prism. The experiment was conducted 3 times in triplicate and the graph is a single representative experiment.

Table 4.1. Kinetic parameters, unbound C_{max}, and calculated drug-drug interaction indices for human OCT1 and OCT3.

	α value	K_i (μM)	Unbound C_{max} (μM)	Drug-drug Interaction Index	References (for unbound C_{max})
hOCT1					
Gatifloxacin	1.66×10^{11}	250 ± 18	3.54 - 12.22 (200-800 mg IV dose)	0.01-0.05	(43, 119)
Moxifloxacin	7.16×10^{25}	161 ± 19	3.79 - 4.06 (400 mg IV dose)	0.02-0.03	(156, 157)
Prulifloxacin	2.62×10^{20}	136 ± 33	---		
Sparfloxacin	6.88×10^{22}	94 ± 8	1.81 - 2.79 (200-800 mg oral dose)	0.02-0.03	(112, 113)
hOCT3					
Moxifloxacin	9.27×10^{16}	1,598 ± 146	3.79 - 4.06 (400 mg IV dose)	0.002-0.003	(156, 157)

K_i: inhibition constant; Unbound C_{max}: unbound maximum plasma concentration obtained from human pharmacokinetic studies conducted in healthy adults (age: 18-45 y) after correction for plasma protein binding (concentrations are expressed as a range for the different doses administered); Drug-drug Interaction Index: calculated as unbound C_{max}/K_i; IV: intravenous; ---: only the active metabolite of prulifloxacin, ulifloxacin, is detected in the systemic circulation.

4. D. DISCUSSION

Fluoroquinolones are one of the most commonly prescribed and efficacious antimicrobials for many infections. However, many of the newer broad-spectrum FQs are often not used as primary therapeutics purportedly due to cost-effectiveness, concern about development of bacterial resistance, risk to special populations, and a variety of mild-to-severe toxicities observed in many patients (47, 70, 129, 206). Clearer understanding of the biochemical pathways governing their kinetics in the body, drug interactions, and associated organ toxicities should aid development of clinical strategies to circumvent these issues, as well as support discovery of more efficacious FQs. As the clinical pharmacokinetics of FQs are known to be affected by identified renal organic cation transport system inhibitors and substrates, and FQs are predominantly zwitterionic in nature in the gastrointestinal environment and the systemic circulation, they have been explored for potential interactions with organic cation/zwitterion transport systems (116). Within the Amphiphilic Solute Carrier (SLC) superfamily, members of the SLC22 (organic cation/anion/zwitterion transporters) and SLC47 (multidrug and toxin extrusion transporters) families are known to transport organic cations, and have been preliminarily examined with respect to FQ interaction (Figure 4.6). Inhibition of hOCT2 (SLC22A2), hOCTN1 (SLC22A4), and hOCTN2 (SLC22A5) by both levofloxacin and grepafloxacin has been observed (62, 72, 125, 204). Ciprofloxacin and levofloxacin were reported to inhibit hMATE1 (SLC47A1) and hMATE2K (SLC47A2), with IC_{50} values of 231 ± 57.3 μM and 38.2 ± 11.8 μM, respectively, for hMATE1, and 98.7 ± 14.1 μM and 81.7 ± 23.1 μM, respectively, for hMATE2K (175). Thus, members of these transporter families may be important determinants of FQ disposition *in vivo*.

Human OCT1 (SLC22A1), hOCT2, and hOCT3 (SLC22A3) are facilitated diffusion carriers that mediate cellular uptake of substrates (91, 165). Human OCTN1 and hOCTN2 are antiporters, with hOCTN1 mediating H^+/organic cation or organic cation/organic cation exchange, whereas hOCTN2 has been linked to carnitine uptake via carnitine/organic cation exchange as well as organic cation/organic cation exchange (91, 165). Thus, they can mediate cellular entry or exit, depending upon membrane localization and substrate gradients. In the enterocyte (Figure 4.6), hOCT3, hOCTN1, and hOCTN2 are expressed in the apical/luminal membrane and may mediate FQ absorption (91, 165). hOCT1 and hOCT2 are also found in enterocytes, however, they are localized to the basolateral membrane and would therefore mediate FQ uptake from the systemic circulation into the enterocyte. In hepatocytes (Figure 4.6), hOCT1 and hOCT3 are known to be expressed in the basolateral/sinusoidal membrane and may influence hepatic FQ influx (91, 165). Finally, in the RPTC (Figure 4.6), hOCT2 and hOCT3 are basolateral and may participate in FQ accumulation from the blood, whereas, hOCTN1 and hOCTN2 are targeted to the apical/brush border membrane and represent potential FQ efflux and/or reabsorptive pathways (91, 165). Renal expression and targeting of hOCT1 remains controversial, with conflicting localization reports in the literature, however, the rat Oct1 ortholog has been immunolocalized to the basolateral membrane of RPTCs (80). For the SLC47 family, the transporter hMATE1 (SLC47A1) has been localized to the apical membrane of hepatocytes and RPTCs, and hMATE2K (SLC47A2) is targeted to the apical membrane of RPTCs, with potential roles in FQ entry into bile and/or urine (16, 91, 92, 127). Thus, a number of transporters belonging to the SLC superfamily are known to be expressed in the intestine, liver, and kidney and are poised to contribute to the absorption, distribution, and excretion of FQ antimicrobials.

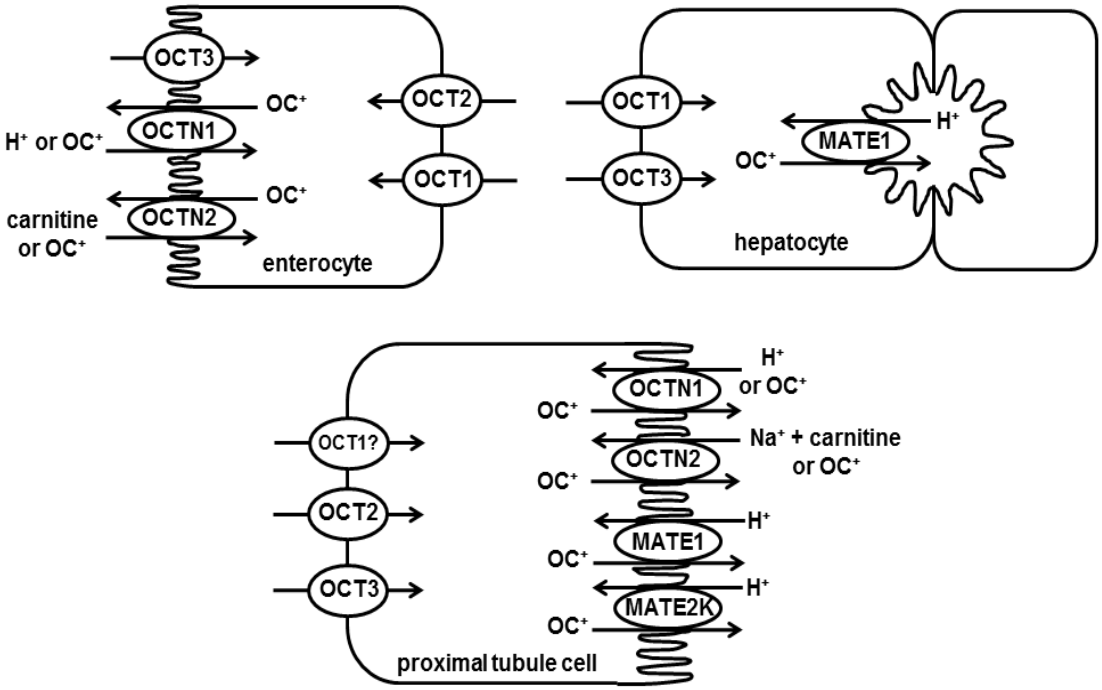

Figure 4.6. Models depicting membrane targeting of transporters discussed in this study in enterocytes, hepatocytes, and renal proximal tubule cells

The results reported herein suggest that, at least for the examined set of 13 FQs, only hOCT1, and not hOCT2 or hOCT3, is likely to be involved in FQ disposition (Figures 4.1, 4.3 and 4.4). Thus, although there is high amino acid sequence homology between hOCT1, hOCT2, and hOCT3 (~50-70%), there is a stark difference between FQ specificity of these transporter paralogs (3, 165). The mode of inhibition and concentration-dependency experiments enabled calculation of inhibition constants (K_i values) for hOCT1, which indicated potential for FQ inhibition of this transporter's activity with a rough potency hierarchy of sparfloxacin ≥ prulifloxacin ≅ moxifloxacin ≥ gatifloxacin (Table 4.1). Human OCT1 expression in the basolateral membranes of enterocytes, hepatocytes, and perhaps RPTC, indicates hOCT1-mediated accumulation from the systemic circulation may play a role in the disposition of these FQs (Figure 4.6 and Table 4.1). Moxifloxacin was found to be a relatively weak inhibitor of hOCT3 with a K_i value of ~1,600 µM. However, given a high luminal GI tract concentration of moxifloxacin after oral dosing, hOCT3 may represent an important pathway for moxifloxacin absorption due to its localization in the apical membrane of enterocytes and facilitated diffusion mechanism of action. Further, hepatic metabolism accounts for ~80% of moxifloxacin elimination (91, 145), and abundant hepatocyte expression of hOCT1 in the sinusoidal membrane combined with the modest K_i value of 161 µM, may indicate a role for hOCT1 in the hepatic uptake of moxifloxacin from the systemic circulation, facilitating the metabolism and elimination of this FQ. Also, in case of the prodrug prulifloxacin, a similar K_i value of 136 µM for hOCT1 coupled with high pre-systemic concentrations (following GI absorption, >> K_i value for hOCT1), may suggest an important role of this OCT in mediating the first-pass metabolism of this prodrug FQ to its active metabolite (ulifloxacin) (See Chapter 3, Section 3.C.) (12, 81). Lack of interaction of prulifloxacin with hOCT3, suggests that the GI absorption of this prodrug

may not be mediated by this transporter (Figure 4.4); on the other hand, hOCT1 may potentially be involved in the uptake of prulifloxacin from the blood back into the enterocyte thus slowing down its hepatic metabolism and resultant conversion to its active FQ (Figure 6, See Chapter 3, Section 3.C). Nevertheless, these postulations are based on the assumption that the aforesaid FQs are not only inhibitors for the OCTs, but are also being transported by these OCTs (substrates). The complete lack of interaction of levofloxacin with hOCT2 (Figure 4.3) is in discrepancy with a previously published study which found a relatively potent inhibition (IC_{50} for hOCT2 = 127 ± 27 µM) (125). However, the substrate used in the published study was creatinine, which has a ~9-10 fold lower affinity for hOCT2 as compared to TEA (K_m for creatinine = 4,000 µM vs. K_m for TEA = 431 ± 87 µM), and the laboratory settings as well as the experimental design were different (182, 183). Moreover, the results obtained herein concur with another study that found no effect of 2.5 mM levofloxacin on hOCT2-mediated transport (182).

Recently, a quantitative method to assess the potential clinical relevance of such transporter interactions, based on the kinetic parameters obtained through *in vitro* assays, referred to as the drug-drug interaction index (DDI index), has been suggested (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf) (73). The DDI index is defined as the ratio of the unbound maximal plasma concentration (unbound C_{max}) of the drug divided by the *in vitro* K_i or IC_{50} value determined for a particular transporter. A value ≥ 0.1 indicates potential for clinically relevant DDIs in instances of co-administration/poly-pharmacy with other drugs identified as inhibitors or substrates of the transporter in question. The implication for the pharmaceutical industry being that *in vivo* drug interaction studies would have to be conducted prior to obtaining FDA approval (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/u

cm292362.pdf). Consequently, we compiled human *in vivo* pharmacokinetic and protein binding studies for gatifloxacin, moxifloxacin, prulifloxacin, and sparfloxacin, and calculated DDI indices for hOCT1 and hOCT3 (Table 4.1). As indicated, all DDI index values were found to be < 0.1 over prescribed dosing ranges, suggesting these interactions have low potential to result in clinically relevant DDIs on hOCT1 or hOCT3. However, such individual transporter interactions could be of considerable importance in situations where FQs would be ideally prescribed, e.g., in acute exacerbation of chronic bronchitis, in patients with complicated urinary tract infections (UTI), or trimethoprim-sulfamethoxazole resistance during the treatment of UTIs (47, 66). In addition to such scenarios, FQ based interactions may gain importance where there exists an inter-patient variability associated with enzymatic and/or transporter-based single nucleotide polymorphisms (SNPs), affecting the kinetics and resultant pharmacodynamics of the interacting drugs (10, 83, 108). For example, the levofloxacin-cimetidine interaction was suggested to be clinically important in patients who were 'slow acetylators' (acetylation being an important metabolic step in procainamide elimination), as renal elimination would become the primary excretion pathway for procainamide in such a scenario (10).

Unexpectedly, some FQs produced significant stimulation of hOCT-mediated TEA/MPP^+ uptake (Figures 4.3 and 4.4). However, this effect varied considerably among the hOCTs for individual FQs with no readily identifiable pattern or association with FQ structural features. For example, ofloxacin and pefloxacin significantly inhibited hOCT1, yet significantly stimulated hOCT2- and hOCT3-mediated uptake (Figures 4.1, 4.3 and 4.4). In contrast, ciprofloxacin, gatifloxacin, and prulifloxacin each inhibited hOCT1, but were without effect on hOCT2 or hOCT3. Further, norfloxacin, rufloxacin, and sparfloxacin inhibited hOCT1, stimulated hOCT2 activity, but were without effect on hOCT3 activity; while fleroxacin, levofloxacin, and

lomefloxacin inhibited hOCT1, were without effect on hOCT2 activity, but stimulated hOCT3. Such sporadic transporter stimulation/inhibition by FQs has been previously reported in the literature, e.g., ciprofloxacin caused stimulation of hOAT1, but inhibition of hOAT3 (187) and sparfloxacin was described as a 'borderline stimulator' for MRP2 (130). In fact, such *in vitro* stimulation of transporter activity actually has been observed for a variety of drug classes in addition to FQs, including steroids, anticancer chemotherapeutics, and non-steroidal anti-inflammatory drugs (87, 130, 187). It was postulated that such effects are due to interaction with an allosteric binding site(s), causing a conformational change to the transport protein and consequently modulating the kinetics of substrate molecules (87, 130). The stimulatory behavior by some FQs observed herein for hOCT2 and hOCT3 is consistent with such an allosteric binding mechanism. However, considering the varied response among the hOCTs (Figures 4.1, 4.3, and 4.4), the fact that several of these FQs were demonstrated to be competitive inhibitors of hOCT1 (Table 4.1), the high degree of sequence homology between hOCT1-3 (50-70%), and their similar predicted membrane topologies, the location and make-up of such unique allosteric binding sites remains unclear and requires much further investigation.

Finally, whether such stimulatory effects of FQs on hOCTs are observed *in vivo* is an important novel question. In contrast to inhibitory DDIs, where victim drug pharmacokinetics are characterized by decreased elimination and increased terminal half-life, such stimulatory DDIs could result in increased elimination and shortened terminal half-life of victim drugs resulting in marked loss of efficacy. For example, hOCT2 has recently been identified as a key mediator in renal elimination of metformin, an important therapeutic in the treatment of type 2 diabetes (86, 109, 180). Thus, concomitant use of, e.g., ofloxacin, pefloxacin, or sparfloxacin, might stimulate renal metformin elimination mediated by hOCT2, potentially reducing metformin's efficacy and

duration of action. If so, in situations where FQ therapy might be called for in a diabetic patient on metformin therapy, use of ciprofloxacin might be a more prudent clinical strategy. Future *in vitro* studies aimed at unraveling the mechanistic basis of such stimulatory effects of FQs on hOCTs, and clinical studies designed to assess the potential impact of such effects *in vivo*, will be important next steps.

CHAPTER 5

EVALUATION OF ORGANIC ANION TRANSPORTERS 1 (SLC22A6), 3 (SLC22A8), AND 4 (SLC22A11) AS POTENTIAL RENAL ELIMINATION PATHWAYS FOR FLUOROQUINOLONE ANTIMICROBIALS

5. A. INTRODUCTION

Amongst the large array of antibacterial agents used in human and veterinary medicine, FQs continue to be prescribed as potent broad-spectrum antibiotics (6, 66, 126). The development of newer FQs has resulted in agents with wider systemic distribution characteristics, longer durations of action and a resultant improvement in therapeutic efficacies (116, 126, 159). Subsequently, FQs have been indicated for treatment of aerobic as well as anaerobic bacterial infections, with therapeutic applications being governed by their *in vivo* pharmacokinetics (PK) and tissue/fluid concentrations (66, 126). The newer FQs like gemifloxacin and moxifloxacin, achieve higher concentrations in the respiratory tract tissues and fluids, and hence are primarily indicated for treatment of infections like acute exacerbation of chronic bronchitis, community acquired pneumonia and sinusitis (66, 88). While some of the earlier FQ molecules like ciprofloxacin, levofloxacin, norfloxacin, and ofloxacin are known to be excreted primarily as the unchanged drug in urine, with concentrations much higher than their

minimum inhibitory concentrations for most pathogens; this has enabled their use in the treatment of urinary tract infections (9, 47). However, owing to increasing concern associated with development of bacterial resistance, special populations (pregnant women, geriatrics, pediatrics), and variety of observed adverse events during therapy, FQs therapy has been limited for most indications (47, 70, 129, 206).

With the development of newer FQs, there have been occurrences of mild-to-severe adverse events associated with their use, e.g., convulsions and anxiety, torsades de pointes, phototoxicity, tendinitis, hypoglycemia etc. (98). Such adverse events have eventually resulted in withdrawal of many FQs from the worldwide pharmaceutical market (116). The existence of such varied toxic events has made it essential to identify the biochemical mediators which would govern the overall *in vivo* kinetics of FQs, and furthermore, aid in designing more efficient tools in antimicrobial therapy.

FQs are small molecular weight (~400 Da) compounds, which predominantly exist as charged, i.e., coexisting cationic, anionic, and zwitterionic species, throughout the physiological pH range (116, 198). Due to this, it is more likely that *in vivo* PK of the FQs would be primarily driven by active transport and facilitated diffusion mechanisms, while passive diffusion would account for only a negligible component in their movement across membrane barriers (116, 165). Earlier studies have demonstrated that FQs are well absorbed systemically following oral administration, with moderate to excellent bioavailability (126). Subsequently, for most systemically absorbed FQs, renal excretion is one of the primary pathways, along with hepatic metabolism and minor biliary excretion (4, 116, 126). Although specific transport and metabolic pathways mediating the nonrenal (hepatic metabolism and biliary excretion) elimination of FQs have been identified, more studies have to be conducted to elucidate detailed mechanisms

governing overall renal flux of these agents across the renal proximal tubule cells (RPTC) (4, 5, 26, 128). This would include basolateral uptake (from the circulation into RPTC), apical efflux (from the RPTC into the urinary space) and/or potential reabsorption (from the urinary space back into the RPTC), before being eliminated into the urine (4, 5, 26, 128). In fact, such processes (net renal tubular secretion/reabsorption) have been predicted to occur for an identified subset of FQs like ciprofloxacin, enoxacin, fleroxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, pefloxacin, prulifloxacin, rufloxacin and sparfloxacin, based on a systematic review of their clinical PK literature conducted in healthy humans (116). Furthermore, existence of charged species of the FQs at physiological pH has suggested the potential for their transport by members of the Solute Carrier (SLC) family, which mediate transport of charged (anionic/cationic/zwitterionic) species and are known to be expressed in the RPTC (116, 165).

Early drug interaction studies conducted in humans have suggested involvement of organic anion transporters (OATs: SLC22 family) in renal elimination of FQs (anionic species), on concomitant administration of drugs like probenecid and furosemide (38, 42, 59, 75, 78, 95, 119, 142, 144, 149, 162, 197). These studies demonstrated a significant decrease in renal clearance (CL_{ren}) (by ~25-60%) and in most cases, an increase in the terminal half-life and area under the concentration curve from zero to infinity (AUC_{∞}) for ciprofloxacin, enoxacin, fleroxacin, gatifloxacin, gemifloxacin, levofloxacin, and norfloxacin, in presence of probenecid, a uricosuric agent and known inhibitor of OATs (38, 42, 75, 95, 119, 142, 144, 149, 197). However, co-administration of probenecid did not affect the kinetics of sparfloxacin and moxifloxacin, for which CL_{ren} of the 'parent drug' accounts for a small fraction in their overall clearance (high hepatic metabolism) (143, 157). For lomefloxacin and furosemide (a loop

diuretic known to be transported by OATs) interaction, yet again, the CL_{ren} was found to decrease by ~33%, with ~12% increase in $AUC_{0\text{-}12h}$ (59, 162).

These clinical findings have been further characterized by *in vitro* experiments using stably transfected cell lines expressing these transporters (116, 187). While earlier literature, as well as our study (See Chapter 4) has demonstrated *in vitro* evidence for FQ handling by organic cation/zwitterion transporter members (OCTs, OCTNs, MATEs) of the SLC superfamily, the role of OATs in renal disposition of these antimicrobials is still relatively uninvestigated (116). A recent study using stably transfected cell lines demonstrated ciprofloxacin to be a substrate for mouse (m)Oat3 [Michaelis Menten constant (K_m) value = 70 ± 6 µM], and not mOat1 (187). Moreover, knockout mice experiments using clinically relevant concentrations of ciprofloxacin showed that the deletion of mOat3 resulted in *in vivo* kinetics mimicking those seen on co-administration of probenecid with FQs in humans (187). Furthermore, concentration-dependent studies using cell lines have demonstrated norfloxacin, ofloxacin, and gatifloxacin to be moderate inhibitors of mOat3 with inhibition constants (K_i) of 558 ± 75 µM, 745 ± 165 µM, and 941 ± 232 µM, respectively (187). For hOATs, only ciprofloxacin and gatifloxacin exhibited moderate inhibition of hOAT3-mediated ES uptake, but no additional characterization has been conducted for these FQ interactions. Thus, the objective of this work was to identify and characterize the potency of interactions of a selected subset of FQs with mOat1, hOAT1, mOat3, hOAT3 and hOAT4; and to further quantitatively assess the impact of OATs on clinically relevant FQ-drug interactions.

5. B. MATERIALS AND METHODS

5.B.1 Chemicals and reagents

Unlabeled paraaminohippuric acid (PAH) and estrone-3-sulfate (ES) and probenecid were purchased from Sigma Aldrich (St. Louis, MO). Ciprofloxacin hydrochloride, norfloxacin and ofloxacin hydrochloride were purchased from MP Biomedicals (Solon, OH). Enoxacin, fleroxacin, gatifloxacin, levofloxacin hydrochloride, lomefloxacin hydrochloride, moxifloxacin hydrochloride, pefloxacin mesylate, prulifloxacin, rufloxacin hydrochloride, and sparfloxacin were purchased from LKT Laboratories, Inc. (St. Paul, MN). Radiolabeled [^{3}H]PAH and [^{3}H]ES were obtained from PerkinElmer (Waltham, MA). Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1), DMEM with high glucose and Serum Supreme were purchased from Fisher Scientific (Waltham, MA). Eagle's minimum essential medium alpha modification (EMEM) was purchased from Sigma Aldrich (St. Louis, MO). Penicillin/streptomycin and hygromycin B were purchased from Invitrogen Life Technologies (Grand Island, NY). G418 (geneticin) was purchased from VWR International (Radnor, PA).

5.B.2 Cell line maintenance and transport assay.

The human embryonic kidney 293 (HEK293) Flp-In and chinese hamster ovary (CHO) Flp-In cell lines stably-expressing hOAT3 and mOat1 respectively, along with the corresponding empty vector transfected background control line (HEK293-EV and CHO Flp-In-EV respectively) were developed as described previously (189). The CHO cell lines stably-expressing hOAT1 along with the corresponding empty vector transfected background control lines (CHO-EV), were developed as described previously (63). The CHO Flp-In cell lines stably-expressing mOat3 along with the corresponding empty vector transfected background control

lines (CHO Flp-In-EV) were developed as described previously (188). The CHO-pro5 cell lines stably-expressing hOAT4 along with the corresponding empty vector transfected background control lines (CHOpro5-EV), were developed as described previously hOAT4 (207). The CHO Flp-In and CHO cell lines were maintained in DMEM/F12 containing 10% Serum Supreme, and 1% penicillin/streptomycin, with hygromycin (125 µg/ml) and G418 (500 µg/ml) as the selection antibiotics, respectively. The HEK293 Flp-In cell lines were maintained in DMEM with high glucose containing 10% Serum Supreme, 1% penicillin/streptomycin and hygromycin (50 µg/ml). The CHO-pro5 cell lines were maintained in EMEM containing 10% Serum Supreme, 1% penicillin/streptomycin and G418 (500 µg/ml). All the cell lines were cultured at 37ºC with 5% CO_2.

Accumulation assay protocols were adapted from our previously published methods (187, 195). Briefly, cells were seeded in 24-well tissue culture plates (250,000 cells/well) and grown without antibiotics for 2 days under suitable cell culture conditions (37°C and 5% CO_2). On the day of the transport study, culture medium was removed and cells were equilibrated for 10 min with transport buffer (Hank's balanced salt solution containing 10 mM HEPES, pH 7.4; Sigma-Aldrich, Saint Louis, MO). The transport buffer was then removed and replaced with 500 µl of transport buffer containing either 1-30 µM ES with [^{3}H]ES (0.25 µCi/ml) added in trace amounts for mOat3, hOAT3 and hOAT4, or 1-30 µM PAH with [H^3]PAH (0.25 µCi/ml) added in trace amounts for mOat1 and hOAT1, depending on the experiment, in presence/absence of 0.1-2,000 µM FQs or 1 mM probenecid (as detailed in figure legends). Following incubation for 1 or 15 min (as detailed in figure legends), the buffer was removed and the cells were instantly rinsed three times with excess ice-cold transport buffer. This was followed by lysis in 200 µl of 1 M NaOH, neutralization with 250 µl of 1 M HCl plus 200 µl of 0.1 M HEPES. The aliquots were

then assayed for radioactivity by liquid scintillation counting, and cellular accumulation was normalized for total protein content using a Bradford protein assay kit (BioRad, Hercules, CA). Uptake was reported as picomoles per milligram total cell protein. All experiments were performed at least three times in triplicate (i.e., three wells/treatment repeated at least three times).

5.B.3 Kinetic and statistical analyses

Prior to analysis, all data used in kinetic determinations were corrected for background accumulation in the corresponding empty vector control cells for each transporter-expressing cell line. The inhibition dose-response curves were analyzed by nonlinear regression using GraphPad Prism® software version 5.04 (GraphPad, San Diego, CA).

For the kinetic studies to determine the half maximal inhibition concentration for the FQs (IC_{50}), type of inhibition, and K_i values, the accumulation time of 1 min and substrate concentrations for ES and PAH (1-30 µM) were chosen based on previously determined Michaelis-Menten constant values in the cell lines: K_m value for ES in mOat3 = 12.2 ± 4.8 µM and the K_m value for PAH in hOAT1 = 15.4 µM (63, 187). Further, for the determination of K_i values, the type of inhibition was assessed using mixed model inhibition analysis as described previously (See Chapter 4; 22). The type/mode of inhibition was defined by the 'alpha value' (α) obtained. Inhibition is identified as competitive, if α is a large number ($\alpha > 1$), as non-competitive, if $\alpha = 1$, or as uncompetitive, if α is small, but greater than zero ($0 < \alpha < 1$) (See Chapter 4; 22). Subsequently, K_i values for the FQs were calculated using the appropriate model based upon the identified inhibition mechanism.

Data are reported as mean ± S.E.M. Statistical significance was determined using one-way analysis of variance (ANOVA) with Dunnett's pairwise comparison post hoc test to measure significant differences. The value for significance was set at 0.05.

5. C. RESULTS

5. C. 1. Interactions of fluoroquinolones with mouse Oat3

The ES uptake in CHO-mOat3 cells (46.3 ± 4.9 pmol/mg protein/15 min) was ~15 fold higher than that detected in CHO Flp-In-EV cells (3.1 ± 0.8 pmol/mg protein/15 min; Figure 5.1), demonstrating a consistent probenecid-insensitive (data not shown) background accumulation of ~6%. In presence of 1 mM probenecid (vs. 1 µM ES) the ES accumulation in CHO-mOat3 cells was reduced to the background level as observed in CHO Flp-In-EV cells. The FQs were tested independently for interaction with mOat3 at the high concentrations of 1 mM, wherein all of them exhibited a significant inhibition of mOat3 mediated uptake (Figure 5.1), while they showed a similar degree of uptake in the CHO Flp-In-EV cells (data not shown). Thus, the CHO Flp-In-EV cells represent a valid background correction method for the experiments. In order to characterize the individual inhibition potencies of the FQs for mOat3, only those FQs identified to produce strong inhibition (≥ 50%) of mOat3 mediated uptake were considered for further kinetic analysis. The FQs producing ≥ 50% inhibition were ciprofloxacin (~54%), enoxacin (~53%), fleroxacin (~70%), gatifloxacin (~70%), levofloxacin (~70%), lomefloxacin (~70%), moxifloxacin (~93%), ofloxacin (~50%), prulifloxacin (~85%) and sparfloxacin (~80%). Relatively weak (< 50%), but significant inhibition was exhibited by norfloxacin (~40%), pefloxacin (~44%) and rufloxacin (~44%). Previous kinetic studies have already characterized the K_i values of ciprofloxacin, gatifloxacin, norfloxacin and ofloxacin for mOat3 (187).

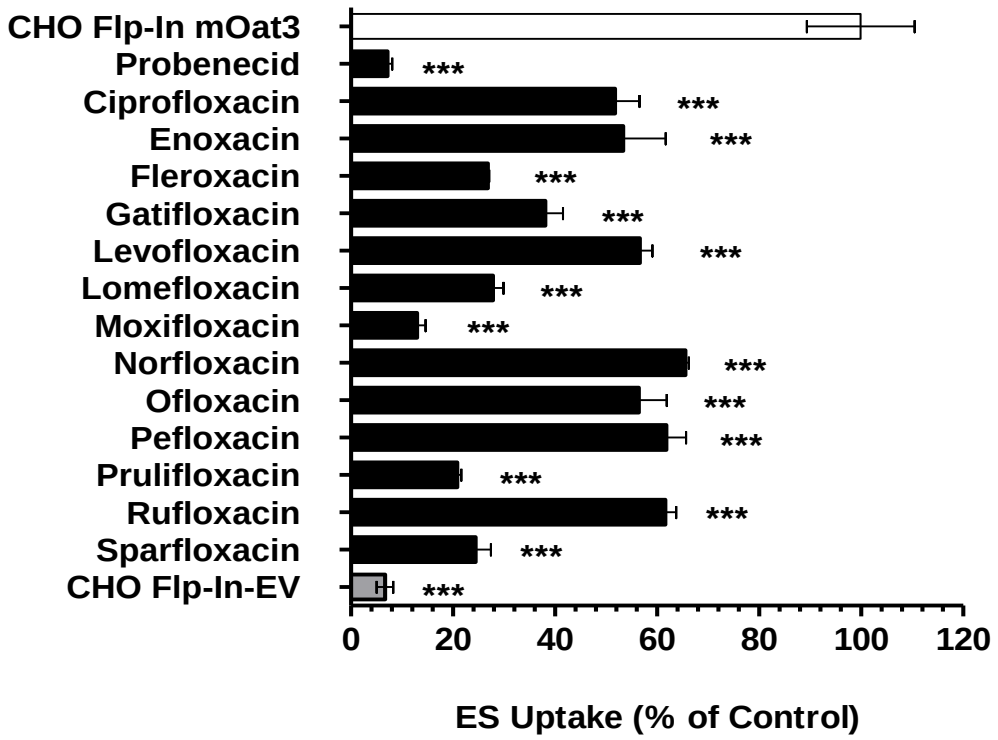

Figure 5.1. Inhibition of mouse Oat3-mediated transport by fluoroquinolones

Uptake of 1 µM [^{3}H]ES was measured at room temperature for 15 min using CHO Flp-In cells stably expressing mOat3 in presence of 1 mM unlabeled FQs (black bars). The inhibition by probenecid (1 mM), a prototypical inhibitor of OATs, was utilized in the experiments as negative control (black bar). The mock-vector transfected CHO Flp-In-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Uptake on the X-axis is expressed as a percentage of the positive control (open bar). Values are expressed as means ± S.E.M. and the significant differences are analyzed between the positive control treatment (with no inhibitor) and treatments in presence of FQs and probenecid, using one-way ANOVA statistical analysis followed by Dunnet's *post hoc* test in GraphPad Prism® version 5.04 (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments.

Further experiments were conducted to identify the type of inhibition for mOat3 by the FQs, enoxacin, fleroxacin, levofloxacin, lomefloxacin, moxifloxacin, prulifloxacin and sparfloxacin, in order to identify the appropriate model for the determination of K_i values. Previously mOat3-mediated ES accumulation was found to be linear through at least 5 min with K_m value of 12.2 ± 4.8 µM, and similar results were replicated in our laboratory (187). Thereafter, the saturation analysis was performed in the absence and the presence of two concentrations of the FQs: 200 and 450 µM enoxacin, 400 and 750 µM fleroxacin, 500 and 1000 µM levofloxacin, 350 and 750 µM lomefloxacin, 500 and 1000 µM moxifloxacin, 350 and 750 µM prulifloxacin, 250 and 500 µM sparfloxacin, using 1 min as accumulation time and ES concentration of 1 µM. Nonlinear regression analysis was then conducted with background corrected data, and the type of inhibition was identified for each FQ by using the 'mixed-model inhibition' analysis in GraphPad Prism. The resultant α values obtained were all much greater than 1, indicating these seven FQs are competitive inhibitors of mOat3 (Table 5.1).

Finally, concentration-dependency studies were conducted to quantify the strength of inhibition of each FQ for mOat3 by determining the K_i values (Figure 5.2, Table 5.1). The inhibition of mOat3-mediated ES uptake was analyzed in the presence of increasing FQ concentrations (0.1 - 2,000 µM) and K_i values were determined by nonlinear regression, selecting the competitive model for inhibition. The K_i values for the different FQs were estimated as follows: 396 ± 14.6 µM for enoxacin, 817 ± 31.3 µM for fleroxacin, 515 ± 22.2 µM for levofloxacin, 539 ± 27.1 µM for lomefloxacin, 1356 ± 114 µM for moxifloxacin, 299 ± 35 µM for prulifloxacin and 206 ± 11.6 µM for sparfloxacin (Figure 5.2 and Table 5.1).

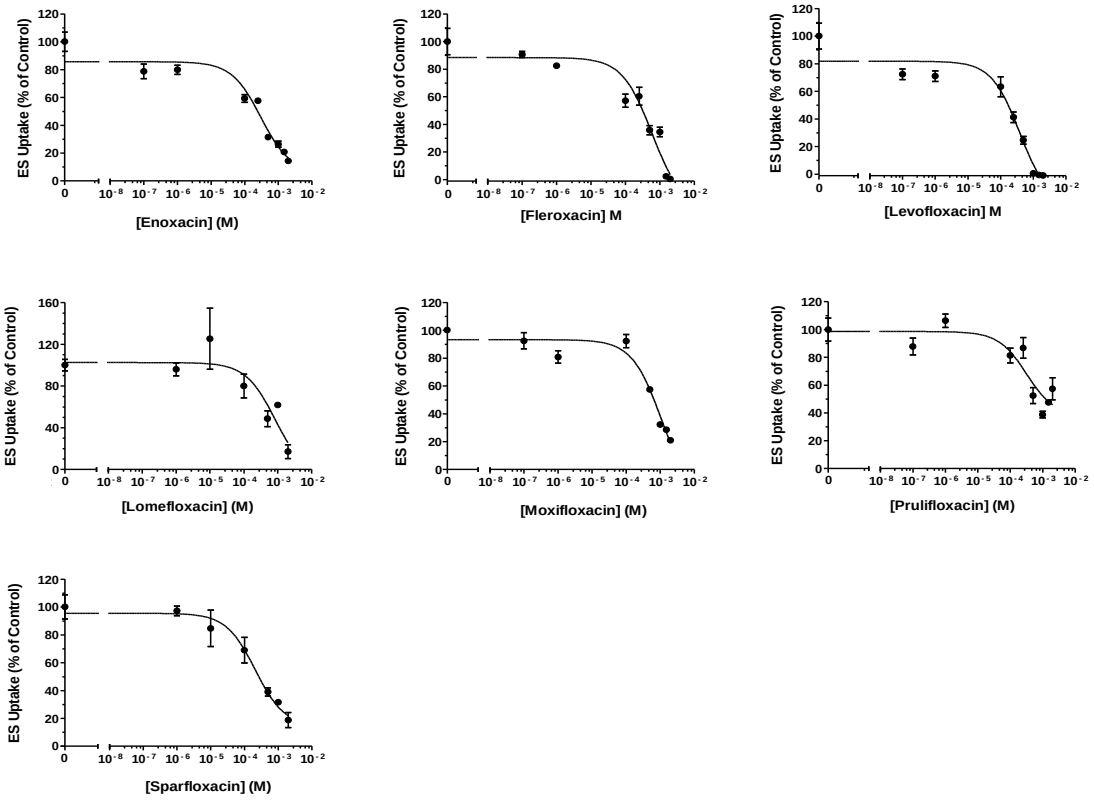

Figure 5.2. Determination of binding affinities (K_i) for enoxacin, fleroxacin, levofloxacin, lomefloxacin, moxifloxacin, prulifloxacin and sparfloxacin on mouse Oat3

[^{3}H]ES uptake was measured for 1 min at 0 - 2000 μM for each FQ in CHO-mOat3 cells; Uptake on the Y axis is expressed as a % of the positive control (in absence of inhibitor, normalized to 100%) and all points on the curves are expressed as means ± S.E.M. The % inhibitions for all the tested FQs were calculated after correcting for nonspecific accumulation in the empty-vector transfected cells, i.e., in CHO Flp-In-EV cells. The type of inhibition was identified using 'mixed model inhibition' in GraphPad Prism® version 5.04 and K_i values were determined from non-linear regression (inhibition curves) model using competitive inhibition. The K_m value for [^{3}H]ES ~12.2 μM in CHO-mOat3 cells was verified with earlier published literature (data not shown), and was subsequently used to calculate K_i values for the tested FQs. All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments.

Table 5.1. Kinetic parameters, unbound C_{max}, and calculated drug-drug interaction indices for mouse Oat3

	α value	K_i (μM)	Unbound C_{max} (μM)	Drug-drug Interaction Index	References (unbound C_{max})
Ciprofloxacin		198 ± 39[a]	7.3 - 9.6 (0.1 – 0.2 mg IV)	0.04 – 0.05	(187)
Norfloxacin		558 ± 75[a]	0.94 (1.3-1.6 mg oral)	0.002	(117)
Fleroxacin	1.6×10^{17}	817 ± 31	---[c]		
Levofloxacin	5.3×10^{17}	515 ± 22	---[c]		
Lomefloxacin	4.6×10^{13}	539 ± 27	---[c]		
Enoxacin	1.1×10^{19}	396 ± 15	4.98 (1.3-1.6 mg oral)	0.013	(117)
Moxifloxacin	9.6×10^{17}	1356 ± 114	2.24 (0.27 mg oral)	0.002	(145)
Prulifloxacin	1.0×10^{21}	299 ± 35	---[b]		
Sparfloxacin	1.5×10^{13}	205 ± 12	0.35 (0.15 mg oral)	0.002	(118)

[a]: Values are published results in (187); [b]: As only the metabolite levels are detected systemically, the studies have not been included; [c]: *In vivo* studies in mice could not be obtained for these FQs; α value: A constant value obtained using 'mixed model inhibition' analysis in GraphPad Prism; K_i value: Inhibition constant expressed as Mean ± SEM; Unbound C_{max}: Unbound maximum plasma concentration obtained from preclinical pharmacokinetic studies in mice, after correction for plasma protein binding. Drug-drug Interaction Index: calculated as unbound C_{max}/IC_{50} or K_i.

5. C. 2. Interactions of fluoroquinolones with human OAT3

The ES uptake in HEK293 Flp-In-hOAT3 cells (7.94 ± 0.24 pmol/mg protein/15 min) was ~4.8 fold higher than that detected in HEK293 Flp-In-EV cells (1.64 ± 0.16 pmol/mg protein/15 min, probenecid-insensitive (data not shown) background accumulation of ~20%) (Figure 5.3). In presence of 1 mM probenecid (vs. 1 µM ES) the ES accumulation was inhibited in HEK293-hOAT3 cells by ~70%. As described above, the FQs were initially tested at the high concentration of 1 mM to identify those capable of producing strong inhibition of hOAT3-mediated ES (1 µM) uptake (Figure 5.3). At the test concentrations, the FQs demonstrated the same effect (negligible transport) on the HEK293 Flp-In-EV cells (data not shown); thus these cells represent valid background controls for these experiments. Ciprofloxacin was the only FQ which significantly inhibited ES uptake mediated by mOat3 as well as hOAT3 (by ~40%), while enoxacin, levofloxacin, moxifloxacin and prulifloxacin caused stimulation of ES uptake under the experimental conditions (Figure 5.3). Due to absence of any strong significant inhibition (≥ 50%, as discussed above) of hOAT3-mediated ES uptake in presence of the FQs, no further kinetic analysis was performed.

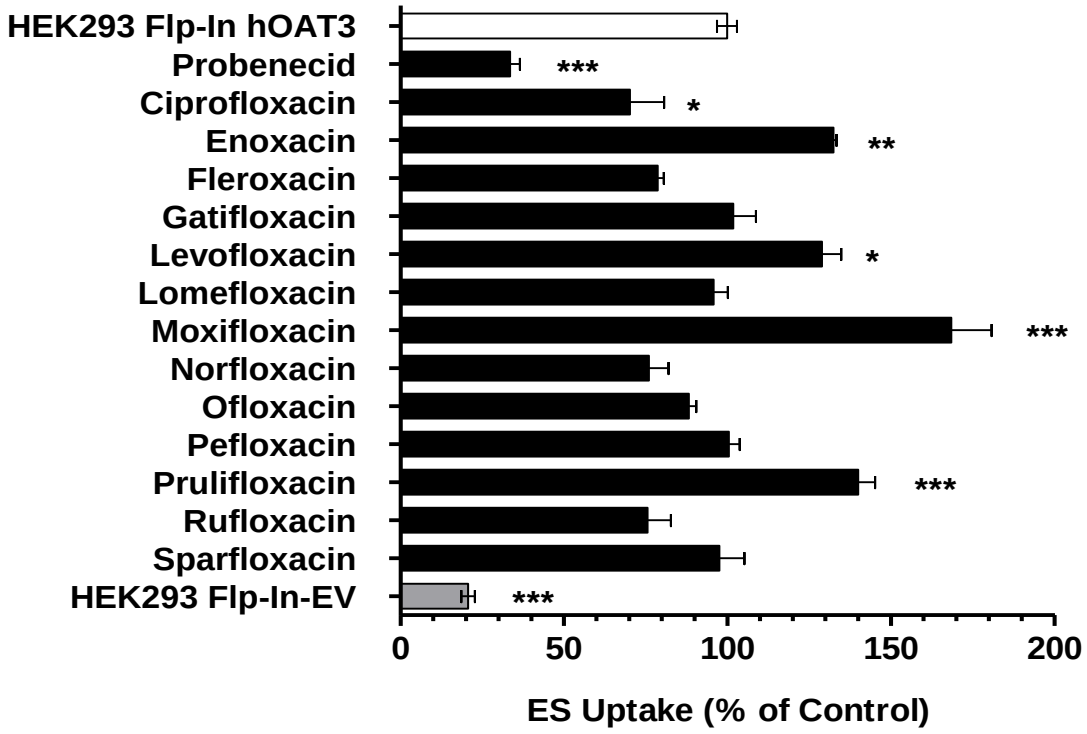

Figure 5.3. Interactions of fluoroquinolones with human OAT3

Uptake of 1 µM [^{3}H]ES was measured at room temperature for 15 min using HEK293 Flp-In cells stably expressing hOAT3 in presence of 1 mM unlabeled FQs (black bars). Probenecid (1 mM), a prototypical inhibitor of OATs, was used for the experiments as negative control (black bar). The empty-vector transfected HEK293 Flp-In-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Uptake on the X-axis is expressed as a percentage of the positive control (open bar). Values are expressed as means ± S.E.M. and the significant differences were analyzed between the positive control treatment (with no inhibitor) and treatments in presence of FQs and probenecid, using one-way ANOVA statistical analysis followed by Dunnet's *post hoc* test in GraphPad Prism® version 5.04 (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments.

5. C. 3. Interactions of fluoroquinolones with mouse Oat1

To study the mOat1 transporter interaction, PAH (1 µM) was used as the prototypical substrate (Figure 5.4). The accumulation of PAH in the CHO Flp-In mOat1 expressing cells (35.5 ± 5.11 pmol/mg protein/15 min) was ~21 fold greater than that obtained in the control CHO Flp-In-EV cells (2.2 ± 0.3 pmol/mg protein/15 min). This PAH accumulation in the CHO Flp-In-EV cells was found to be insensitive to the addition of 1 mM of probenecid (data not shown), however accumulation in the CHO Flp-In mOat1 cells in the presence of probenecid was decreased to the level similar to that obtained in the CHO Flp-In-EV cells (Figure 5.4). Unlike the observation in mOat3 expressing cells where all FQs at 1mM test concentrations, exhibited significant inhibition of ES uptake, only rufloxacin (~45%) and sparfloxacin (~45%) exhibited a significant inhibition of mOat1-mediated PAH uptake, among all the tested FQs (Figure 5.4). Again, ciprofloxacin, levofloxacin, lomefloxacin and ofloxacin demonstrated an apparent stimulation of the mOat1-mediated transport activity under these experimental conditions (Figure 5.4). Hence, as none of the FQs produced strong significant inhibition of mOat1-mediated PAH uptake (≥ 50%, as discussed above), no further kinetic analysis was performed for this transporter.

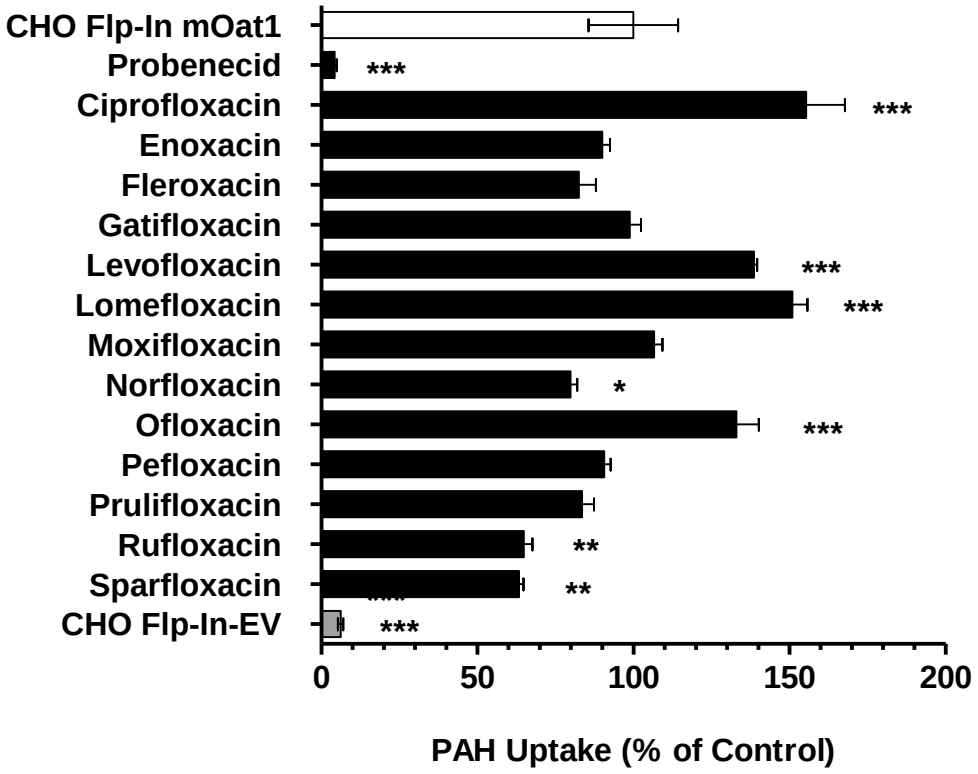

Figure 5.4. Effect of fluoroquinolones on mouse Oat1-mediated PAH transport

Uptake of 1 µM [^{3}H]PAH was measured at room temperature for 15 min using CHO Flp-In cells stably expressing mOat1 in presence of 1 mM unlabeled FQs (black bars). Probenecid (1 mM), the prototypical inhibitor of OATs, was used as a negative control for the experiments (black bar). The empty-vector transfected CHO Flp-In-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Uptake on the X-axis is expressed as a percentage of the positive control (open bar). Values are expressed as means ± S.E.M. and the significant differences have been analyzed between the positive control treatment (with no inhibitor) and treatments in presence of FQs and probenecid, using one-way ANOVA statistical analysis followed by Dunnet's *post hoc* test in GraphPad Prism® version 5.04 (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments.

5. C. 4. Interactions of fluoroquinolones with human OAT1

Similar to mOat1, PAH (1 µM) was used as substrate for hOAT1 (Figure 5.5). The accumulation of PAH in the CHO-hOAT1 expressing cells (10.1 ± 0.92 pmol/mg protein/15 min) was ~5.6 fold greater than that obtained in the control CHO-EV cells (1.78 ± 0.11 pmol/mg protein/15 min). The PAH accumulation in the CHO-EV cells was insensitive to the addition of 1 mM of probenecid (data not shown). In the presence of probenecid, the CHO-hOAT1 cell accumulation was decreased to the level similar to that obtained in the control cells (Figure 5.5). In these cell lines, rufloxacin (~90%), pefloxacin (~80%), fleroxacin (~70%), lomefloxacin (~50%), ofloxacin (~50%) and sparfloxacin (~50%) exhibited a significant inhibition of hOAT1-mediated PAH uptake (Figure 5.5). Ciprofloxacin, enoxacin, gatifloxacin, moxifloxacin and norfloxacin again demonstrated an apparent stimulation of mOat1-mediated transport activity under these experimental conditions. On comparing the species orthologs, it was observed that sparfloxacin and rufloxacin were the only two FQs which inhibited PAH uptake, while ciprofloxacin stimulated PAH uptake by mouse and human OAT1. Gatifloxacin, moxifloxacin and prulifloxacin were not found to interact with hOAT1 or mOat1.

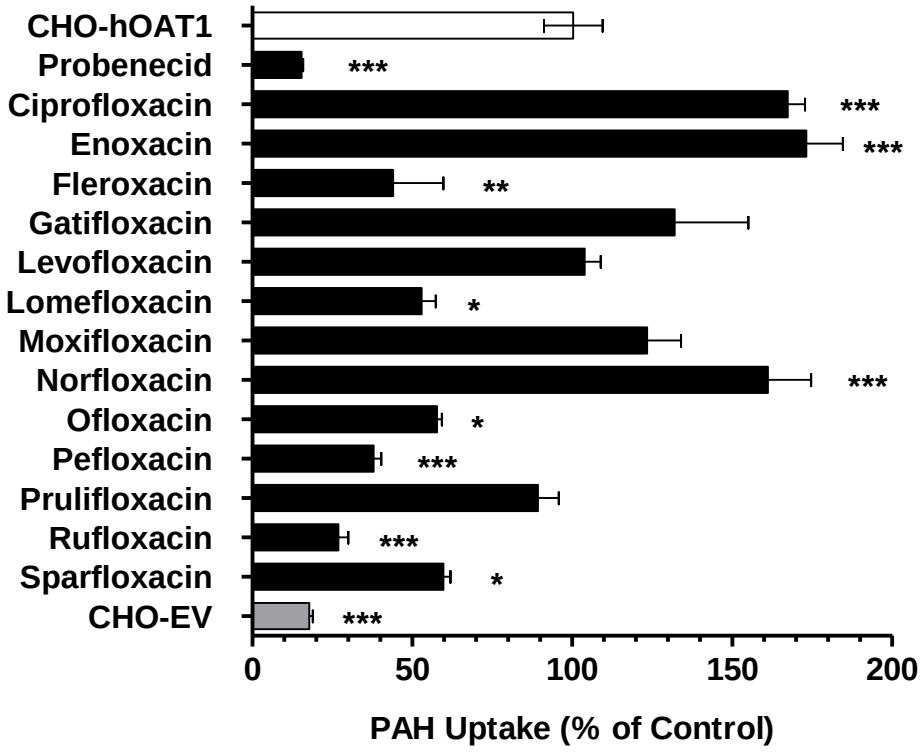

Figure 5.5. Inhibition of human OAT1-mediated transport by fluoroquinolones

The uptake of 1 µM [^{3}H]PAH was measured at room temperature for 15 min using CHO cells stably expressing hOAT1 in presence of 1 mM unlabeled FQs (black bars). Probenecid (1 mM), a prototypical inhibitor of OATs, was used for the experiments as negative control (black bar). The empty-vector transfected CHO-EV cells served as reference for nonspecific background substrate accumulation (grey bar). Uptake on the X-axis is expressed as a percentage of the positive control (open bar). Values are expressed as means ± S.E.M. and the significant differences were analyzed between the positive control treatment (with no inhibitor) and treatments in presence of FQs and probenecid, using one-way ANOVA statistical analysis followed by Dunnet's *post hoc* test in GraphPad Prism® version 5.04 (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments.

For the FQs demonstrating significant inhibition of >50%, further kinetic analysis was conducted to quantify the strength of inhibition. The concentration-dependency studies were conducted for hOAT1 with rufloxacin, pefloxacin and fleroxacin to determine the IC_{50} values (Figure 5.6, Table 5.2). The inhibition of hOAT1-mediated PAH uptake was analyzed in presence of increasing FQ concentrations (0.1 - 2,000 µM) and IC_{50} values were determined by nonlinear regression. Rufloxacin demonstrated stimulation of hOAT1-mediated PAH uptake at all lower concentrations (0.1, 1, 10, 100, 250, 500 µM) except 1 and 2 mM (~80-90% inhibition, similar to that seen above in Figure 5.6), and thus was not considered for further kinetic analysis (data not shown). The IC_{50} values for pefloxacin and fleroxacin were 2252 ± 135 µM and 2228 ± 84.3 µM respectively. As these FQs were found to be weak inhibitors of hOAT1, lomefloxacin, ofloxacin and sparfloxacin showing only ~50% inhibition in the preliminary testing, were not analyzed further.

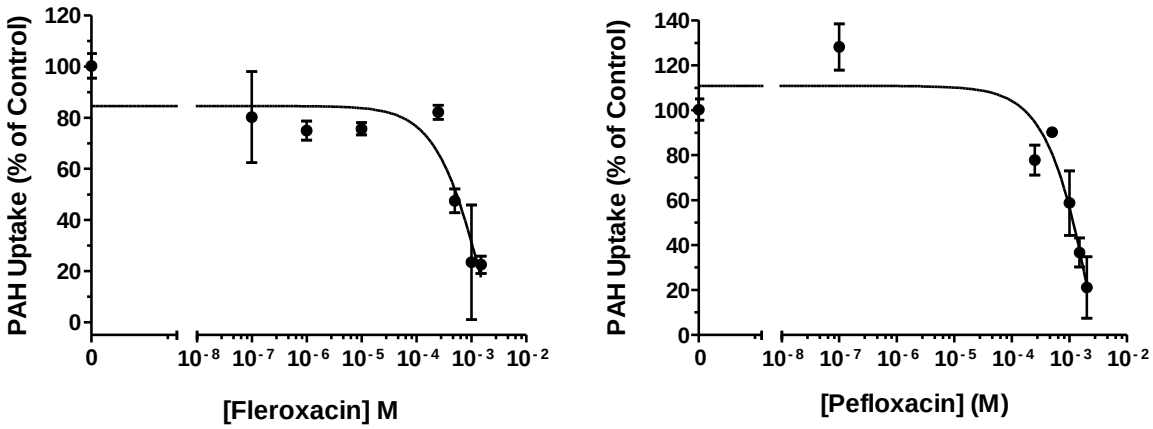

Figure 5.6. Determination of inhibition potencies (IC_{50}) for fleroxacin and pefloxacin on human OAT1

The uptake of [^{3}H]PAH was measured for 1 min at 0-2000 μM of fleroxacin and pefloxacin in CHO-hOAT1 cells; Uptake on the Y axis is expressed as a % of the positive control (in absence of inhibitor, normalized to 100%) and all points on the inhibition curves are expressed as means ± S.E.M. The % inhibitions for all the tested FQs were calculated after correcting for nonspecific accumulation in the empty-vector transfected cells, i.e., in CHO-EV cells. The IC_{50} values were determined from non-linear regression (inhibition curves) model using GraphPad Prism® version 5.04. All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments.

Table 5.2. Kinetic parameters, unbound C_{max}, and calculated drug-drug interaction indices for human OAT1

	IC_{50} (µM)	Unbound C_{max} (µM)	Drug-drug Interaction Index	References (unbound C_{max})
Fleroxacin	2228 ± 84	6.8 (100 mg, IV)	0.003	(161)
Pefloxacin	1819 ± 144	8.6 (400 mg, oral)	0.005	(40, 114)

IC_{50} value: Half maximal inhibitory concentration value of the FQ inhibitor for the transporter, expressed as Mean ± SEM; Unbound C_{max}: Unbound maximum plasma concentration obtained from preclinical pharmacokinetic studies in healthy humans, after correction for plasma protein binding. Drug-drug Interaction Index: calculated as unbound C_{max}/IC_{50} or K_i; IV: Intravenous route of administration.

5. C. 5. Interactions of fluoroquinolones with human OAT4

As hOAT4 is postulated to be a reabsorptive transporter localized to the apical membrane in the RPTC, other than the intracellular pH conditions of the RPTC, it is exposed to a lower pH of ~6.3, assumed to be the urinary pH conditions. It was predicted from our previous systematic review that some FQs could be potentially reabsorbed from the urinary space, back into the RPTC. Hence their interactions with hOAT4 were tested at the physiological (pH = 7.4) as well as urinary (pH = 6.3) pH conditions (Figure 5.7).

In order to study the interaction of FQs with hOAT4, the apically localized transporter in the RPTC, ES (1 µM) was used as a substrate (Figure 5.7). The accumulation of ES (positive controls at pH 7.4 and 6.3) in CHO-hOAT4 expressing cells (32.9 ± 3.9 pmol/mg protein/15 min at pH 7.4, 30.8 ± 2.5 pmol/mg protein/15 min at pH 6.3) was ~5.6 fold and ~3 fold greater than that obtained in the control CHO-EV cells (5.9 ± 0.3 pmol/mg protein/15 min at pH 7.4, 10 ± 3.6 pmol/mg protein/15 min at pH 6.3) at the pH conditions 7.4 and 6.3 respectively. This background accumulation by the CHO-EV cells was found to be probenecid-insensitive (~18%) (data not shown). In the presence of probenecid, the accumulation of ES in the CHO-hOAT4 cells was decreased by ~40%. At pH = 7.4, none of the FQs exhibited a significant inhibition of hOAT4-mediated ES uptake (Figure 5.7). Also, as seen earlier with other OATs, some FQs like moxifloxacin, pefloxacin and prulifloxacin demonstrated a significant stimulation of ES uptake by hOAT4. At pH = 6.3, deemed to be more physiologically relevant for the working of hOAT4, the ES uptake was found to increase by ~35% as compared to the ES uptake at pH = 7.4 (Figure 5.7). Moreover, at pH 6.3, in addition to moxifloxacin, pefloxacin and prulifloxacin (stimulated ES uptake at pH 7.4), fleroxacin, ofloxacin and rufloxacin also demonstrated stimulation of

hOAT4-mediated ES uptake. Due to absence of any strong inhibition of the hOAT4-mediated uptake at this pH = 6.3 (≥ 50%, as discussed above), no further kinetic analysis was performed.

On comparing the FQ effect with hOAT4 at the two pH values (Figure 5.7, Panel C), it can be seen that only the interaction of ciprofloxacin, ofloxacin, pefloxacin, and prulifloxacin were not sensitive to changes in pH of the extracellular medium (no significant change in ES uptake with pH change). All the other FQs significantly increased the hOAT4-mediated ES uptake at pH 6.3 versus 7.4 (Figure 5.7, Panel C).

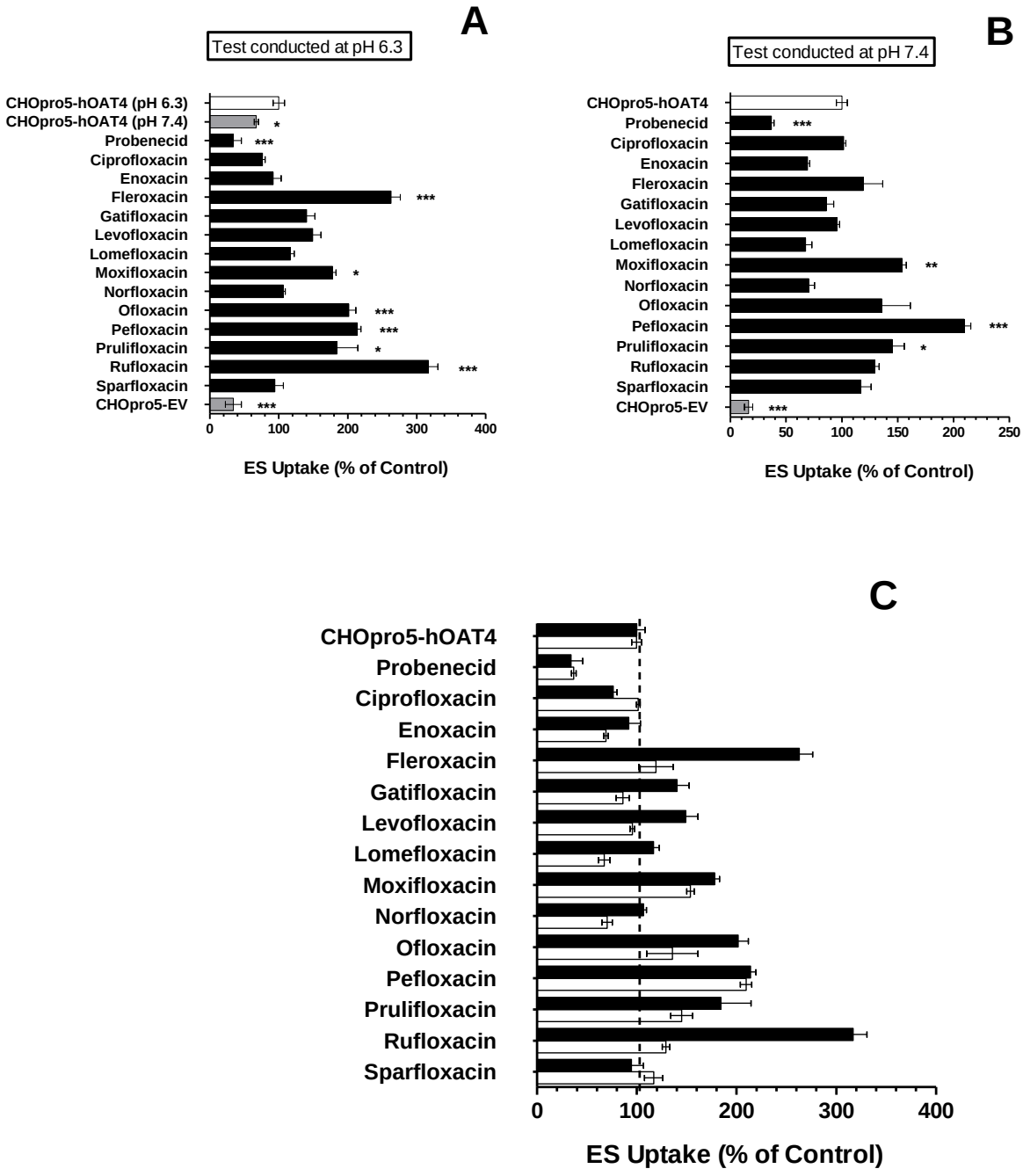

Figure 5.7. Influence of pH on the interaction of fluoroquinolones with human OAT4 - mediated transport

The uptake of 1 µM [^{3}H]ES was measured at room temperature and pH 6.3 (panel A), and pH 7.4 (Panel B) for 15 min using CHOpro5 cells stably expressing hOAT4 in presence of 1 mM unlabeled FQs (black bars); Panel A: 1 µM [^{3}H]ES solution at pH 7.4 was used as a control to test the influence of pH change on hOAT4-mediated ES uptake in absence of any interacting

compounds (dark grey bar); (Panel A, B): Probenecid (1 mM), a prototypical inhibitor of OATs, was used for the experiments as negative control (black bar). The empty-vector transfected CHOpro5-EV cells served as a reference for nonspecific background substrate accumulation (grey bar). Panel C: Comparison of the effect on FQ interactions with hOAT4 with change in pH conditions from 7.4 (open bars) to 6.3 (black bars).

Uptake on the X-axis is expressed as a percentage of the positive control (open bar). Values are expressed as means ± S.E.M. and the significant differences are analyzed between the positive control treatment (with no inhibitor) and treatments with FQs/probenecid/ES (at pH 7.4), using one-way ANOVA statistical analysis followed by Dunnet's *post hoc* test in GraphPad Prism® version 5.04 (*P<0.05, **P<0.01, ***P<0.001). All the experiments are conducted at least 3 times performed in triplicate and the graphs are single representative experiments;

5. D. DISCUSSION:

Recently, due to the toxicities associated with FQs, there has been an increasing interest in understanding the mechanisms governing their *in vivo* disposition kinetics. Consequently, the FQs have been studied for interactions with different transporter families expressed in the body, which could potentially mediate their *in vivo* absorption, distribution, and elimination, and even be responsible for clinically significant drug interactions (116). However, additional studies would need to be conducted to further elucidate such interactions of FQs with the SLC transporters (116). We studied the FQ – OCT interactions in Chapter 4, where none of the FQs demonstrated significant interactions with hOCT2; however, gatifloxacin, moxifloxacin, prulifloxacin and sparfloxacin were found to be moderate inhibitors of hOCT1, and moxifloxacin was the only FQ found to inhibit hOCT3. This study was designed to evaluate the role of OATs in the kinetic disposition of the same selected subset of FQs (n=13), using stably transfected cell lines expressing mouse and/or human OATs.

The renally expressed basolateral transporters, hOAT1 and hOAT3 investigated in this study are known to function as organic anion/dicarboxylate exchangers using the outwardly directed endogenous α-ketoglutarate gradient to drive uptake of anionic subtrates from systemic circulation into the RPTC (115, 166, 169, 171). Hence, these OATs might represent a rate-limiting step for uptake of FQs from the blood circulation (116). Previous work has already characterized mOat3 interactions with ciprofloxacin, gatifloxacin, norfloxacin and ofloxacin, as well as identified ciprofloxacin and gatifloxacin interactions with hOAT3 (187). This study thus tested a larger dataset of FQs, identified from our earlier systemic review with the mouse as well as human orthologs of OAT1 as well as hOAT4 (116). The kinetic characterization of the FQs as inhibitors for mOat3, allowed an assessment of their inhibition potency for this transporter.

Preliminary interaction studies conducted on mOat3 demonstrated significant inhibitory interactions of all the tested FQs (Figure 5.1). The kinetically characterized FQs (demonstrating ≥ 50% inhibition in preliminary studies, Figure 5.1) were found to competitively inhibit mOat3, with sparfloxacin showing the strongest inhibition, followed by prulifloxacin, enoxacin, levofloxacin, lomefloxacin, fleroxacin and finally moxifloxacin (Figure 5.2 and Table 5.1). A similar preliminary interaction study for hOAT3 using the same dataset of FQs, demonstrated a substantial difference in the inhibition pattern as compared to mOat3, suggesting possible species differences in FQ specificity (Figure 5.3). Ciprofloxacin was found to inhibit mOat3 (~54%) as well as hOAT3 (~40%) – mediated ES uptake, which concurred with an earlier observation (Figure 5.1 and 5.3; 187). However, gatifloxacin which previously demonstrated a moderate hOAT3 interaction, showed no significant interaction in this study (Figure 5.3; 187). Such species differences in FQ specificity for the OATs were also evident from the FQ interaction studies with mOat1 and hOAT1 (Figure 5.4 and 5.5). For example, fleroxacin and pefloxacin were found to be weak inhibitors of hOAT1 with IC_{50} values of 2252 ± 135 µM and 2228 ± 84.3 µM, respectively, while they did not interact with mOat1 (Figure 5.4, 5.5 and 5.6, Table 5.2). The preliminary inhibition study for hOAT1-FQ interactions demonstrated lomefloxacin to inhibit hOAT1-mediated PAH uptake by ~50% (Figure 5.6). This suggested that hOAT1 could be the OAT mediating *in vivo* interactions of the loop diuretic, furosemide (also transported by hOAT1) with lomefloxacin (mentioned above), causing decreased CL_{ren} and subsequent increased AUC_{∞} for this FQ, with no changes observed in furosemide PK (34, 59, 162).

In conjunction with the inhibition of OAT-mediated transport, stimulatory effects by some FQs on hOAT3, mOat1 and hOAT1-mediated substrate uptake were also observed (Figure 5.3, 5.4 and 5.5). Such stimulatory effects were found to be sporadic among the tested FQs and were

transporter-specific, e.g., mOat3 demonstrated no stimulation of ES uptake in presence of 1 mM FQs; while at the same concentrations, enoxacin, levofloxacin, moxifloxacin and prulifloxacin stimulated hOAT3-mediated ES uptake (Figure 5.1 and 5.3). The stimulatory effect of ciprofloxacin on m/hOAT1-mediated PAH uptake demonstrated in Figure 5.4 and 5.5, was also observed in an earlier study (187). Moreover, when these FQs were tested with hOCTs in our previous study (Chapter 4), the stimulatory/inhibitory behavior of the FQs also varied between the individual hOCTs, exhibiting no consistent pattern (Appendix III). In addition a previous study demonstrated sparfloxacin to be a 'borderline stimulator' for MRP2 (ABC transporter) mediated transport (130). Such *in vitro* stimulation also has been observed for different drug classes such as steroids, anticancer chemotherapeutics and non-steroidal anti-inflammatory drugs, with transporters like OATP1B1 and 1B3 (SLC superfamily) and MRP2 (ABC superfamily) (87, 130, 187). These studies have postulated the existence of transporter-specific allosteric binding sites for such drug molecules which could stimulate the transporter-mediated substrate (another drug or endogenous substrate) uptake, without the drug molecules being transported themselves (Chapter 4; 87, 130). However, some FQs were confirmed to be competitive inhibitors for mOat3 (Figure 5.2, Table 5.1) (187). Also, OATs exhibit considerable amino-acid sequence identity between species (~78% between mOat3 and hOAT3, ~80% between mOat1 and hOAT1) and paralogs (~48% between mOat3 and mOat1, ~49% between hOAT3 and hOAT1) (3). Hence such an allosteric binding site (if existing), would be very unique for each OAT, and may demonstrate a narrow specificity across the class FQs (as only some FQs showed stimulation with m/hOAT1, hOAT3). Overall, the preliminary OAT-FQ interaction studies demonstrated that among the tested FQs, only ciprofloxacin exhibited similarities in interaction with mouse and human species of OATs, i.e., significant stimulation

effects of mOat1 and hOAT1-mediated PAH uptake, and a significant inhibition of mOat3 and hOAT3-mediated ES uptake. No consistent pattern of stimulation or inhibition was observed for the other FQs (See Appendix III). In general, these studies suggest that in the mouse/human RPTC, OAT/Oat1 and 3 may be involved in the basolateral uptake of some FQs.

In addition to exploring the FQ interactions with the basolateral OATs, i.e., OAT1 and 3, additional analysis was performed to study potential FQ interactions with apically localized hOAT4. Though studies have confirmed the apical localization of hOAT4 in human RPTC (absent in rodents), its mechanism of action is unclear due to conflicting results indicating its function as a facilitated diffusion carrier and an exchanger (15, 32, 57, 186). Thus it is still unknown whether hOAT4 mediated exchange mechanism would cause the efflux of drugs from the RPTCs into the urinary space, or whether it would result in the tubular reabsorption of compounds (57, 186). More recent studies have demonstrated the pH-dependent increase in substrate (ES) uptake by hOAT4, and have postulated one of the mechanisms to be facilitating reabsorption of compounds by hydroxyl ion exchange (18, 57). Based on this postulated mechanism of transport by hOAT4, and our previous systematic review indicating potential renal tubular reabsorption of some FQs, we studied whether this transporter could mediate apical reabsorption of the FQs. Due to its apical localization in the RPTC, hOAT4 is exposed to a lower urinary pH of ~6.3. Furthermore, literature has suggested that FQ ionic species are sensitive to pH change (116). Thus, in order to identify potential interactions under simulated physiological pH conditions, the experiments were conducted at pH ~6.3. Additional experiments were conducted at pH = 7.4 to compare the pH-sensitivity of these FQ-hOAT4 interactions. The hOAT4-mediated ES uptake in absence of FQs at pH 6.3 (positive control) demonstrated stimulation as compared to that at pH = 7.4 (Figure 5.7, Panel B), which was consistent with

earlier findings (18, 57). If hOAT4 is considered to transport the FQs by a reabsorptive mechanism, then at pH 6.3, the FQ uptake from the extracellular medium would consequently decrease the intracellular [H^3]ES accumulation. On the contrary, our study results demonstrated stimulation of hOAT4-mediated ES uptake at this pH (on comparison with the positive control treatment at pH =6.3) (Figure 5.7, Panel B and C), with the exception of ciprofloxacin, enoxacin, pefloxacin and sparfloxacin, which demonstrated no significant change in ES uptake as a function of pH (Figure 5.7, Panel C). These pH-sensitive stimulatory effects shown by hOAT4 in presence of FQs could, yet again, be attributed to the potential allosteric binding mechanisms of specific FQs with a unique site on hOAT4, similar to that observed with the basolateral OATs (explained above).

In addition to the OATs 1, 3 and 4 which have been identified in our study, other OAT paralogs could potentially mediate FQ disposition in the body. For example, OAT2 is known to be expressed in humans on the basolateral membrane in the RPTC and on the sinusoidal membrane (assumption in literature based on animal immunolocalization studies) in hepatocytes (16, 90, 186). Hence, OAT2 could be important for the basolateral uptake of FQs in the RPTC. hURAT1, an OAT known to mediate active reabsorption of urate from the urinary space, is also known to be localized to the apical membrane in the RPTC (186). This renal OAT could be potentially involved in the tubular reabsorption of some FQs, thus affecting their overall CL_{ren} and $t_{1/2}$s (Chapter 3). Along with OAT2 mentioned above, hOAT7, a human-specific OAT, is also known to be specifically localized in the liver (sinusoidal membrane in hepatocytes) (186). As some FQs undergo considerable hepatic metabolism along with renal elimination (e.g., sparfloxacin, moxifloxacin), hepatically expressed OATs (i.e., hOAT2 and/or hOAT7) could potentially mediate their hepatic uptake and subsequent metabolism. Also, potentially due to

higher ‘pre-systemic concentrations’ (before hepatic first-pass effect) attained by FQs following gastrointestinal uptake, OAT2 or OAT7 - mediated hepatic DDIs could potentially occur with concomitantly administered drugs/endogenous molecules which are substrates for these transporters. However, it is essential to consider that the overall flux governing FQ disposition is a ‘net’ process which is also dependent on individual contributions of other SLC transporter members like OCTs, MATEs, OATPs, as well as the ABC transporters (4, 116). Thus any OAT-FQ interactions would contribute as one of the components driving the overall FQ flux for each organ (e.g., renal excretion or potential hepatic metabolism).

Following the identification and *in vitro* characterization of FQ interactions with OATs, a quantitative assessment was conducted to determine whether these OATs could potentially mediate any clinically relevant FQ DDI on concomitant administration of other drugs which are OAT substrates/inhibitors, for example, probenecid and furosemide (39, 42, 59, 75, 95, 149, 157, 187, 197). Based on the recent DDI guidance drafted by the FDA (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf), the ratio of unbound maximum concentration (unbound C_{max}) to K_i or IC_{50} values of the drugs (DDI index) (See Chapter 4; 73) were calculated for hOAT1 and mOat3. A DDI ratio ≥ 0.1 is suggested to indicate that the transporter-mediated DDI would achieve clinical relevance and additional *in vivo* DDI studies will have to be conducted with the co-administered drugs before obtaining FDA approval (73). Thus, unbound C_{max} values were calculated by compiling the human (for hOAT1) and mouse (for mOat3) *in vivo* pharmacokinetic and protein binding studies for each FQ as shown in Tables 5.1 and 5.2. Further, the DDI indices were calculated for FQ interactions with hOAT1 and mOat3 (187). As can be seen from Table 5.1 and 5.2, all the DDI indices were < 0.1, indicating that interactions of these FQs with OATs may not

result in clinically relevant DDIs. Nevertheless, this study has indeed identified an important component for the pathway of renal elimination of this dataset of FQs. Such *in vitro* transport studies would need to be conducted for the newer marketed FQs, as well as for those under development, in order to design safer antimicrobials and reduce the occurrence of any new DDIs.

Although only some FQs demonstrated significant inhibition interactions with the human OAT-mediated substrate transport, further consideration is needed for the unanticipated stimulatory effects demonstrated by the other FQs (Figures 5.3, 5.4, 5.5 and 5.7). If such observations are truly a result of allosteric binding mechanisms, then in such a scenario, concomitant use of FQs with OAT substrates (victim drugs) could potentially cause increased renal elimination (in case of hOAT1 and 3) or reabsorption (for hOAT4) of the interacting substrates (victim drug), with no significant effect on the 'OAT-mediated elimination' of FQs. This could subsequently affect the systemic concentrations of the 'victim' drugs, further increasing/decreasing their efficacies (depending on the site of action) and/or causing potential toxicities. For example, consider a drug like the loop diuretic – furosemide, known to be transported by hOAT1 (IC_{50} = 18 ± 1.1 µM), hOAT3 (IC_{50} = 7.3 ± 0.81 µM), as well as hOAT4 (IC_{50} = 44.5 ± 2.53 µM) (34, 59, 184). In such a case, if a FQ causing stimulation of hOAT1, e.g., ciprofloxacin (FQs with considerable renal elimination), is co-administered with furosemide, it may result in increased renal basolateral uptake of furosemide due to ciprofloxacin-mediated hOAT1 stimulation (See Figure 5.5). Similarly effect may be seen when a FQ found to stimulate hOAT3 (e.g., levofloxacin) (Figure 5.3) is co-administered with furosemide. Assuming that the apical efflux transporters like MRP4 and BCRP (known to transport furosemide) (60) are uninhibited by the FQs in such a DDI scenario, this may result in increased urinary concentrations of furosemide, further causing an enhanced natriuretic effect by

this diuretic (34, 184). On the contrary, co-administration of a FQ like ofloxacin (stimulated hOAT4, Figure 5.7, Panel B) with furosemide may increase the hOAT4-mediated reabsorption of furosemide, further decreasing its natriuretic effect due to lower concentrations in the urine (34, 59, 184). However, as the *in vivo* scenario includes multiple transporters (uptake and efflux) mediating renal transport of furosemide as well as these FQs, such effects may be counterbalanced during their transcellular flux, producing no "net" significant impact on furosemide PK and resultant pharmacodynamic response. Although the clinical implications of such stimulatory effects of FQs have not been assessed, these may have an important role especially in multi-drug regimen interactions (poly-pharmacy) where due to inhibition of multiple transporters by different drugs, such stimulation of substrate transport may exhibit enhanced activity/toxic effects.

CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Due to their wide spectrum of antimicrobial activity, longer durations of action and general availability, FQs rank among the most highly prescribed medications for the past thirty years (9). As a class, FQs exhibit intestinal, hepatic, and renal elimination; with renal excretion representing a major component in determining the systemic and urinary concentrations of many of these agents. In the course of FQ development, there has been accumulating evidence establishing a clear role for active transport mechanisms in their systemic disposition. However, until recently, work aimed at identifying the specific transport mechanisms/transporters involved in FQ kinetics has been limited. It now appears that multiple members from the ABC and SLC transporter superfamilies play an active role in FQ disposition, not only in renal elimination, but also in the overall flux of these zwitterionic molecules in the body. This information, in turn, provides potential explanations, at the molecular level, for clinically observed drug-drug interactions, organ-specific adverse effects, and inter-patient variability in FQ pharmacokinetics and pharmacodynamics. As indicated by the results of our systematic review (discussed in Chapter 3), for some FQs the renal handling appears to be the driving force behind the differences in their duration of action and clinical dose frequency. This dissertation thus intended to study the role of SLC22 transporter family members in the ‘net’ renal tubular

secretion/reabsorption (i.e., $CL_{ren,tub}$) of a selected dataset of FQs (identified from the systematic review discussed in Chapters 2 and 3). Further, the *in vitro* studies (Chapter 4 and 5) characterizing the interactions between FQs and the OCTs and OATs (Figure 1.2), allowed an assessment of the *in vivo* contribution of these transport proteins to the net renal elimination of these antimicrobials, as well as identified transporters potentially influencing their overall absorption, distribution, metabolism and elimination. These *in vitro* studies examining FQ-OCT interactions (discussed in Chapter 4), demonstrated that hOCT1 and hOCT3 are likely to mediate the renal basolateral uptake of some FQs. Based on its localization, hOCT3 may be involved in the intestinal and hepatic uptake of moxifloxacin (Figures 4.4 and 4.5, Table 4.1). Due to its abundant expression in the liver (basolateral membrane), hOCT1 could mediate the hepatic uptake of gatifloxacin, moxifloxacin, prulifloxacin and sparfloxacin, amongst others, for which a preliminary interaction was detected (Figures 4.1, 4.2 and 4.6, Table 4.1). Also, as hOCT1 is expressed on the basolateral membrane in the enterocytes (Figure 4.6), this transporter could also potentially mediate the intestinal uptake of FQs from the 'presystemic' blood circulation (prior to first-pass metabolism), further facilitating GI efflux of these FQs by the apically localized ABC transporters and reducing their bioavailability (4, 14, 23, 40, 52, 96, 148, 205). Moreover, hOCT1 could potentially be a rate-limiting transporter for FQ prodrugs like prulifloxacin, wherein its metabolic conversion (to active metabolite, ulifloxacin) would be an essential step to elicit an *in vivo* therapeutic action (discussed in Chapter 4).

In the *in vitro* studies with renally expressed human and mouse orthologs of OATs (Chapter 5), some of the FQs moderately inhibited OAT-mediated transport activity. The studies demonstrated that due to its abundant renal expression (basolateral membrane), hOAT1 may be involved in the uptake of FQs like fleroxacin and pefloxacin from the systemic circulation

(Figure 5.6, Table 5.2). However, in mice, renally expressed mOat3 was more likely to be involved in the basolateral uptake of FQs like enoxacin, fleroxacin, levofloxacin, lomefloxacin, moxifloxacin, prulifloxacin and sparfloxacin; with varying affinities (Figure 5.2, Table 5.1). Transporters such as hOAT3 and mOat1 demonstrated only moderate interactions with the FQs. Some moderate OAT-mediated interactions (e.g., ciprofloxacin with hOAT3, lomefloxacin with hOAT1) also further allowed identification of the transport pathways likely to mediate the clinically observed DDIs (e.g., lomefloxacin with furosemide; ciprofloxacin with probenecid), and thus indicated their involvement in the renal elimination of FQs (75, 95, 162). Additionally, the studies with human and mouse orthologs of OATs demonstrated the existence of species differences in selectivity and relative affinities of the FQs for OAT1 and OAT3 - mediated transport (hOAT1 versus mOat3, hOAT1 versus mOat1).

In addition to the OATs encompassed by this dissertation, such FQs interactions have yet to be studied for renally expressed hURAT1 (SLC22A12), which is known to be localized to the apical membrane in the RPTC, and is involved in active tubular reabsorption of urate from the urinary space (186). This transporter could potentially mediate the renal tubular reabsorption of FQs, thus explaining the prolonged $t_{1/2}$s for some FQs. In addition, hOAT2 known to be expressed in RPTC (basolateral membrane) and hepatocytes (sinusoidal membrane), as well as hOAT7 (human-specific, hepatically expressed on sinusoidal membrane) could potentially mediate the hepatic uptake of FQs, further affecting their metabolism and/or renal uptake.

Following identification of the significant inhibitory interactions of selected FQs with the OCTs and OATs, DDI indices were calculated for hOCT1, hOCT3, hOAT1, and mOat3. This DDI index analysis enabled a quantitative assessment of the potential of these SLC22 transporters to mediate clinically relevant DDIs for FQs, according to the FDA DDI draft

guidance:

(http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf) (discussed in Chapters 4 and 5, Table 4.1, 5.1 and 5.2). However, the DDI indices suggested that these SLC22 transporters are not likely to be involved in causing clinically relevant DDIs on co-administration of the tested FQs with other OAT/OCT substrates or inhibitors. In addition, the impact of mOat3 on the renal elimination of some FQs (enoxacin, norfloxacin, moxifloxacin, sparfloxacin) was assessed, by analyzing the relationship between CL_{ren}^{u} of FQs in mice (obtained from preclinical data) and their respective K_i values for mOat3. The results demonstrated no significant relationship (See Appendix IV) (187). These observations suggested that although the SLC22 transporters are potentially involved in the systemic disposition of FQs, they may not be the rate-limiting step. This might be attributed to the physiological interplay of different transporter gene families (ABC as well as SLC transporters) that mediate the pathways governing overall FQ kinetics, a result of which, being no single transporter represents the rate-limiting step in their renal/hepatic elimination. Assessments of clinical DDI indices lead to similar conclusion. For example, the clinical DDI studies with concomitant administration of FQs and probenecid demonstrated only ~25-60% decrease in CL_{ren} (42, 75, 95, 149). Probenecid has exhibited *in vitro* inhibition of OATs, OATPs (rat Oatp1 and 2), MDR1 (weak inhibition), and MRPs (MRP2, 4 and 5); and has been shown to completely shut down *in vitro* OAT – mediated transport (~100% inhibition: Figure 5.1, 5.3) (25, 45, 77, 133, 163). These results may indicate that on concomitant administration of probenecid and FQs, despite the inhibition anion transporters, FQ kinetics could be mediated by uninhibited cation/zwitterion transporters such as OCTs, OCTNs and MATEs, known to interact with these antimicrobials (116). Similar results were found with the cation/zwitterion SLC transporters

upon FQs combination with cationic drugs like cimetidine (26, 42, 111, 146, 150). Under these conditions, FQ kinetics might largely be mediated by the uninhibited anion and ABC efflux transporters (116). These data indicate that the resultant PK endpoints obtained in our systematic review (discussed in Chapter 3) might represent the 'combined function' of multiple transporters, from a variety of gene families, expressed in several tissues. Such a 'combined functioning' of transporters establishing the ultimate *in vivo* distribution profile of a given substrate, could be tested by determining the effects of probenecid and cimetidine co-administration on the overall kinetics of FQ disposition. However, clinical studies may not investigate such interactions, considering the safety and toxicities associated with the FQs.

An unexpected observation in the *in vitro* studies (Chapters 4 and 5) was the stimulation of OCT- and OAT`- mediated transport by some FQs. Some previous studies also demonstrated FQ stimulation of transport activity including sparfloxacin as a stimulator of MRP2 (ABC transporter), ciprofloxacin causing stimulation of mOat1- and hOAT1- mediated PAH uptake (130, 187). Such stimulatory mechanisms by FQs could have potentially significant clinical manifestations by increasing the clearance (i.e., decreasing the $t_{1/2}$) of the 'victim' (interacting) drugs (Chapter 4 and 5). This observation may be of particular importance in cases where multi-drug regimens are administered to patients. Future investigations could explore such stimulatory mechanisms in vivo, for example, determining if co-administration of FQs and PAH increases renal PAH clearance or co-administration of FQ and metformin (OCT substrate) accelerates metformin clearance, and to explore if these mechanisms could in fact have any significant clinical implications in multiple-drug interactions.

These *in vitro* studies have only explored FQs as inhibitors for the SLC22 transporters, leaving open the question of whether they are actual substrates. Thus, future studies should assess whether cellular accumulation can be confirmed.

It would also be of interest to study the molecular-level binding characteristics of FQs with the transport proteins. Quantitative structure-activity relationship (QSAR) and comparative molecular field analysis (CoMFA) studies could aid in identifying the key factors influencing the sporadic inhibition/stimulation mechanisms seen with the individual FQs, and allow the prediction of any allosteric binding mechanisms of these agents with these transporters (24, 79, 94). Finally, although this study has functionally characterized that FQs are likely be moderate inhibitors of the SLC22 transporters, the impact of various physiological (endogenous substrates and xenobiotics, protein binding, pH conditions) and genetic (SLC22 single nucleotide polymorphisms (SNPs)) factors could affect (increase/decrease) the 'apparent' affinities of these agents for the transporters (71, 83, 180). Nevertheless, the existence of such transporter SNPs as well as disease states in patient populations could potentially make such moderate FQ-transporter interactions more clinically relevant.

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APPENDIX I

SUMMARY OF HUMAN PHARMACOKINETIC STUDIES FOR FLUOROQUINOLONES

1. AMIFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Cook JA et. al.(21)	Healthy, n = 48 males. 18-46 yrs	62-102 kg	400, oral	0-12 hrs	0-24 hrs	HPLC with UV	0.10-5 µg/ml	0.5-100 µg/ml	Non-compart-mental	1.34	-	-	99 ± 14

2. ANTOFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min /kg)	CL_{ren} (ml/min/kg)
Wang J et. al. (194)	Healthy, n = 12 males. 20-28 yrs	52-70	400, IV Infusion over 120 min (1mg/ml)	0-96 hrs	0-96 hrs	HPLC with UV	37 µg/ml	40 µg/ml	Non-compart-mental	3.23	3.72	2.1	1.22

3. CIPROFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Lettieri JT et. al.(97)	Healthy, n = 12 males. 27.4 ± 4.3 yrs	73 ± 6.7	300 or 400 mg, IV Infusion over 60 min (200 ml volume)	0-24 hrs	0-24 hrs	HPLC	0.025-5.0 µg/ml	0.025-5.0 µg/ml	Non-compart-mental and two Compart-mental	0.52 (300 mg) 0.68 (400 mg)	2.44 (300 and 400 mg)	8.26 (300 mg) 8.18 (400 mg)	5.32 (300 mg) 4.9 (400 mg)
Wingender W et. al.(199)	Healthy, n = 6 males. 29 ± 9 yrs	75 ± 11	100 mg IV bolus (with infusion pump within 5 min)	0-48 hrs	0-48 hrs	Micro-biolog-ical assay and HPLC	0.01 µg/ml	0.05 µg/ml	Mammillary three compart-mental open model	0.1456	1.98 ± 0.4	9.62 ± 0.93	4.42 ± 0.43

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Höffken G et al. (64)	Healthy, n = 12 Males and Females, 22-34 yrs	51 - 80.5	50 or 100 mg IV infusion (with an infusion pump over 15 min)	0-105 min and 2-24 hrs	0-24 hrs	Micro-biolog-ical Assay and HPLC	0.008 µg/ml with K. *pneumo niae* and 0.07 µg/ml with B. *subtilis*	0.2 µg/ml (HPLC)	Open two and three compart-mental models	0.072 (50 mg) 0.18 (100 mg)	-	18 (50 mg) 13.89 (100 mg)	11.14 (50 mg) 8.78 (100 mg)
Davis R et. al. (27)	Healthy, n = 12 Males, 23-32 yrs	75.9 ± 8.5	200 mg IV infusion over 30 min	0-45 min and 1-24 hrs	0-48 hrs	HPLC	0.02 µg/ml	0.01 µg/ml	Non-compart-mental	0.383	2.25 ± 0.48	7.02	4.57

4. ENOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Misiak PM et. al. (111)	Healthy, n = 10, Male and female, 19 -52 yrs	56.4 – 98.4	400 mg, IV Infusion over 60 min	0-48 hrs	0-48 hrs	HPLC	0.1 µg/ml	3.0 µg/ml	Non-compart-mental	1.056	2.0	5.14	2.86

5. FLEROXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Stuck AE et. al. (161)	Healthy, n = 6, Male and female, 25-58 yrs	52–74	100 mg, IV Infusion over 20 min	0-60 min and 2-72 hrs	0-96 hrs	HPLC	20 ng/ml	20 ng/ml	Non-compart-mental	1.14	1.4 ± 0.34	1.41 ± 0.23	0.93 ± 0.3

6. GATIFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Gajjar DA et. al. (43)	Healthy, n = 40 (8 in each study group), Male and female, 18-45 yrs	61-96	400 mg, IV Infusion over 60 min	0-72 hrs	0-24 hrs	HPLC	Not mentioned		Non-compart-mental	1.72	1.5 ± 0.2	2.62	2.17

7. GEMIFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Allen A et. al. (2)	Healthy, n = 19 (n=4 received this dose), Male, 18-45 yrs	21-41	160 mg, oral	0-48 hrs	0-24 hrs	HPLC	0.01 µg/ml	1.0 µg/ml	Non-compart-mental	0.33	-	-	2.1
Allen A et. al. (1)	Healthy, n = 22, Male and female, 18-60 yrs	-	320 mg, oral	0-48 hrs	Pre-dose on Day 1 and day 5	HPLC	0.01 mg/ml	0.01 mg/ml	Non-compart-mental	0.312	-	-	4.53

8. GREPAFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Efthymiopoulos, C. et al. (31)	Healthy, male and female, n = 18, 21-37 yrs	70-89	200, 400, 600, 800, 1200 mg, oral	0-72 hrs	0-72 hrs	HPLC	0.00466 µg/ml	0.00466 µg/ml	Non-compart-mental	0.31 (200mg), 0.68 (400 mg), 1.18 (600 mg), 1.66 (800 mg), 2.7(1200 mg)	-	-	0.5 (200 mg), 0.66 (400, 600, 800 mg), 0.83 (1200 mg)

9. LEVOFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/ kg)	CL_{ren} (ml/min/ kg)
Chow AT. et al. (19)	Healthy, male and female, n = 18 (n=4 with normal renal functions assessed), 26-54 yrs	-	750 mg, IV Infu-sion over 1.5 hrs	0-72 hrs	Not deter-mined	HPLC	Not men-tioned (valida-tion range: 0.125-13.75 µg/ml)	Not deter-mined	Two Compart-mental using linear disposi-tion	4.04	1.51	2.66	-
Chien SC et. al. (17)	Healthy, male, n = 18 (10 for IV), 18-55 (20-44 for IV levofloxaci n treatment) yrs	94.4 ± 10.5	500 mg, IV Infu-sion with infu-sion pump over 60 min	0, 0.5, 1 hr (during IV inf.) and 0.5-60 hrs post-treatment	8hrs prior to dosing, 0-48 hrs post-dosing	HPLC	Not men-tioned (valida-tion range: 0.082-10.5 µg/ml)	Not men-tioned (valida-tion range: 2-1132 µg/ml)	Non-compart-mental	3.32	0.94	1.66	1.01

LOMEFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Gros I. et al. (53)	Healthy, female, n = 6, 26-49 yrs	50-67	400 mg, oral	0-24 hrs	0-24 hrs	HPLC	0.05 µg/ml	2 µg/ml	Open Two Compart-mental model	2.22	-	-	2.06
Stone JW et. al. (160)	Healthy, male, n = 6, 24-42 yrs	68-81	400 mg, oral	0-90 min and 2 –25 hrs post-dosing	0 —48 hrs	Antibiotic assay (plate diffusion method) and HPLC (serum samples from 2 volunteers)	0.25 µg/ml (plate diffusion assay), HPLC: < 0.12 µg/ml	0.25 µg/ml) (plate diffusion assay)	Open Two Compart-mental linear model	1.93	-	-	2.09

10. MOXIFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min /kg)	CL_{ren} (ml/min /kg)
Siefert HM et. al. (145)	Healthy, male adults	85 (aver age)	102 mg, IV infu-sion over 30 mins	0-48 hrs	-	HPLC and radio-active scintilla-tion	Not specified for human assay (0.005 µg/ml for animal plasma samples)	- (radio-active method used)	Non-compart-mental	0.54	2 ± 1.08	2.2	0.43
Stass H et. al. (158)	Healthyn = 12, male, 23-41 yrs	71-112	400 mg, IV infu-sion over 1 hr	0-1hr during infusion, and 1.5 – 96 hrs post-dosing	0 — 96 hrs	HPLC	2.5 µg/ml	2.5 µg/ml	Non-compart-mental	2.08	2.05 ± 1.15	2.27	0.51

11. NORFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Eandi M et. al. (30)	Healthy n = 12 (6 were healthy), male and female, 26-31 yrs	54-72	400 mg, oral	0-12 hrs	0-24 hrs	Liquid chromat-ography	Not mentioned (final range: 0.31 -5.0 µg/ml)	Not men-tioned (final range: 50 -600 µg/ml)	Non-compart-mental	0.27	-	-	7.47

12. OFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Lode H et. al. (99)	Healthy n = 18, male and female, 25-46 yrs	54-74	25, 50, 100, 200 mg, IV infused during 30 min	0-2 hrs and at 3-72 hrs post-dosing	0-72 hrs post-dosing	Liquid chromato-graphy	Detection limits: 20 µg/ml	Detection limits: 200 µg/ml	Oral two and three compart-mental model	0.09 (25 mg), 0.19 (50 mg), 0.44 (100 mg), 0.86 (200 mg)	-	4.3 (25 mg), 4.2 (50 mg), 3.5 (100, 200 mg)	3.7 (25 mg), 3.42 (50 mg), 2.8 (100 mg), 2.88 (200 mg)

13. PEFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Montay G et. al. (114)	Healthy n = 6, male and female, 19-29 yrs	54-75	400 mg, oral	0-24 hrs	0-72 hrs post-dosing	Biological, Fluorimetric assay and HPLC	0.1 µg/ml (Fluorimetric assay)	Not mentioned	Oral two and three compartmental model	2.89	-	-	0.19

14. RUFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min /kg)	CL_{ren} (ml/min/kg)
Kisicki JC et. al. (89)	Healthy n = 40, male, 19-29 yrs	54-75	400 and 600 mg, oral	0-12 hrs	0-24 hrs	HPLC	0.05 µg/ml	0.05 µg/ml	One compart-mental open model	10.56 (400 mg), 16.63 (600 mg)	-	-	0.27 (400 mg), 0.3 (600 mg)
Perry G et. al. (131)	Healthy n = 24, male and female, n=6 assessed with creatinine clearance > 80 ml/min/ 1.73 m^2, 40 ± 7 yrs	70 ± 4	400 mg, oral	0-96 hrs	0-96 hrs	HPLC	0.5 µg/ml	0.3 µg/ml	two compart-mental open model with first order input and elimination	9.24	-	-	0.24

15. SPARFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Montay G et. al. (113)	Healthy n = 12, male, 20-28 yrs	70 ± 6.8	200, 400, 600 and 800 mg, oral	0-120 hrs	0-120 hrs	HPLC	15 ng/ml	250 ng/ml	Non-compart-mental model	1.13 (200 mg), 1.96 (400 mg), 2.75 (600 mg), 3.45 (800 mg)	-	-	0.27 (200 mg), 0.28 (400, 600 mg), 0.29 (800 mg)
Fillastre JP et. Al. (36)	Healthy n = 20, male and female, n=6 assessed with creatinine clearance between 75-133 ml/min/ 1.73 m^2, 55 ± 10 yrs	70 ± 11	400 mg, oral	0-120 hrs	0-120 hrs	HPLC	15 ng/ml	250 ng/ml	Non-compart-mental model	1.89	-	-	0.3

16. TEMAFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min/ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Grannemann RG et. al. (51)	Healthy n = 30, male, 19-28 yrs	60-89	100, 200, 400, 600 and 800 mg, oral	0-60 hrs	0-60 hrs	HPLC	0.01 µg/ml	0.01 µg/ml	Non-compart-mental model and one-compart-mental open model	0.45 (100 mg), 0.90 (200 mg), 1.78 (400 mg), 2.97 (600 mg), 3.52 (800 mg)	-	-	1.76 (100 mg), 1.82 (200 mg), 1.75 (400, 1.33 (600 mg), 1.75 (800 mg)

17. TROVAFLOXACIN:

Study	Population	BW (kg)	Dose and Route (mg)	Sampling Schedule		Assay	LOQ		PK Analysis	PK Endpoints			
				Plasma	Urine		Plasma	Urine		AUC (mg*min /ml)	Vd_{ss} (L/kg)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)
Vincent J et. al. (192)	Healthy n = 16, male, 18-42 yrs	64-90.7	30, 100, 200, 300 mg, IV infu-sion over 1 hr	0-73 hrs	0-72 hrs	HPLC	0.1 µg/ml	0.1 µg/ml	Non-compart-mental model	ND (30 mg), 0.98 (100 mg), 1.87 (200 mg), 2.6 (300 mg)	ND (30 mg), 1.21 ± 0.1 (100 mg), 1.3 ± 0.23 (200 mg), 1.4 ± 0.23 (300 mg)	ND (30 mg), 1.42 (100 mg), 1.28 (200 mg), 1.62 (300 mg)	0.19 (30 mg), 0.16 (100 mg), 0.12 (200 mg), 0.19 (300 mg)

APPENDIX II

COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF FLUOROQUINOLONES ACROSS THE GROUPS SHOWING NET TUBULAR SECRETION (GROUP 3), REABSORPTION (GROUP 1) AND NO NET TRANSPORT (GROUP 2)

FQs discussed in Chapter 3 were distributed into three Groups based on 'net' renal tubular clearance ($CL_{ren,tub}$):

1. **Group 1 FQs:** Pefloxacin, Rufloxacin, Sparfloxacin

   (Net tubular reabsorption, $CL_{ren,tub} < -1$)

2. **Group 2 FQs:** Antofloxacin*(excluded in the analysis as physicochemical properties could not be obtained by the ACD software), Fleroxacin, Grepafloxacin, Levofloxacin, Lomefloxacin, Moxifloxacin, Temafloxacin, Trovafloxacin

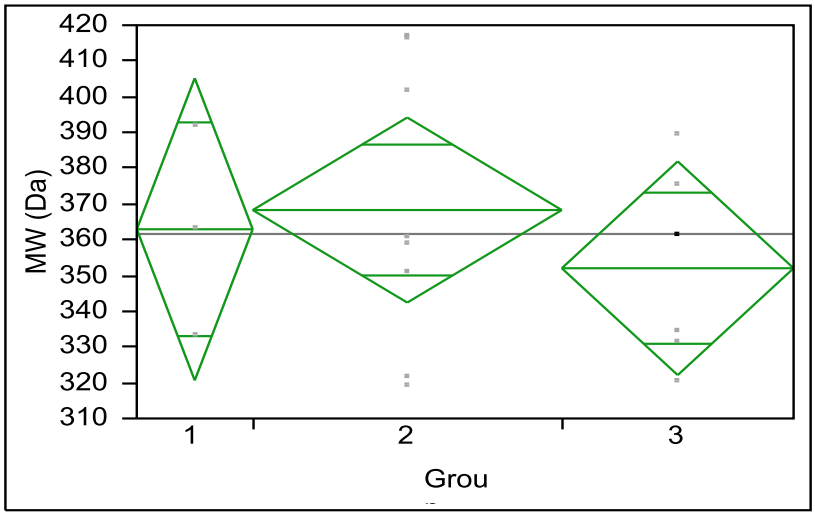
   (Little/No net transport, $-1 < CL_{ren,tub} < 1$)

3. **Group 3 FQs:** Amifloxacin, Ciprofloxacin, Enoxacin, Gatifloxacin, Gemifloxacin, Norfloxacin, Ofloxacin

   (Net tubular secretion, $CL_{ren,tub} > 1$)

Molecular Properties compared among the Groups of FQs: Molecular weight, Log D (Logarithmic distribution coefficient) at pH 7.4, hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), number of rotatable bonds (nRot), molar volume. Software: ACD Labs PhysChem Suite, Version 12. Statistical Analysis was conducted using JMP statistical software, Version 9.0.2.

1. **Molecular Weight (MW):**



One-way ANOVA:

Summary of Fit:

Rsquare	0.053
Adj Rsquare	-0.082
Root Mean Square Error	34.139
Mean of Response	361.608
Observations (FQs)	17

Analysis of Variance:

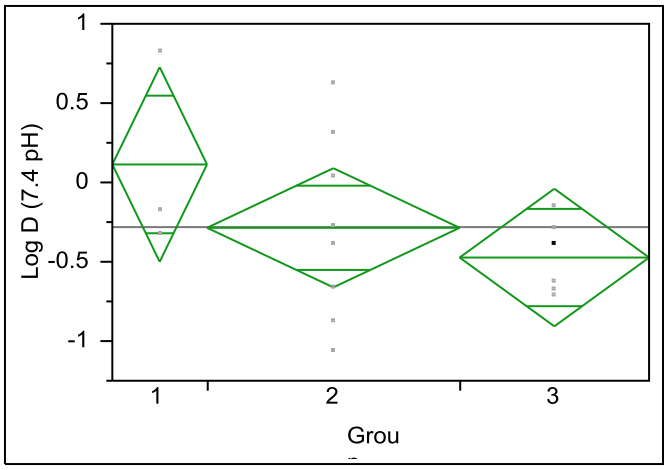
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Group	2	914.86	457.43	0.39	0.6826
Error	14	16316.15	1165.44		
C. Total	16	17231.01			

ANOVA indicates no significant difference among Group 1, 2 and 3 for MW.

Summary Statistics for MW of the 3 Groups:

Group	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	362.9	19.71	320.7	405.2
2	8	368.3	12.07	342.4	394.2
3	6	352.0	13.94	322.1	381.9

2. Log D at pH 7.4:



One-way ANOVA:

Summary of Fit:

Rsquare	0.167
Adj Rsquare	0.0480
Root Mean Square Error	0.495
Mean of Response	-0.282
Observations (FQs)	17

Analysis of Variance:

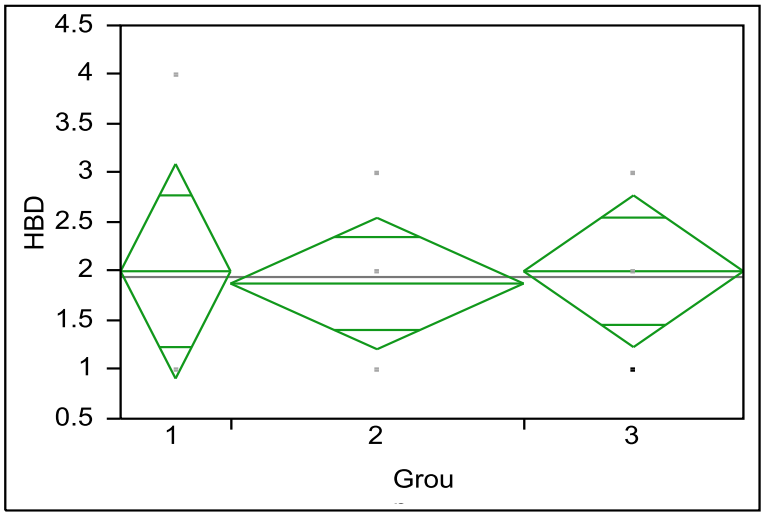
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Group	2	0.69	0.34	1.40	0.2782
Error	14	3.43	0.25		
C. Total	16	4.12			

ANOVA indicates no significant difference among Group 1, 2 and 3 for Log D at pH 7.4.

Summary Statistics for LogD of the 3 Groups:

Group	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	0.1	0.29	-0.5	0.7
2	8	-0.3	0.18	-0.7	0.1
3	6	-0.5	0.20	-0.9	-0.04

3. Hydrogen Bond Donor (HBD):



One-way ANOVA:

Summary of Fit:

Rsquare	0.0061
Adj Rsquare	-0.135
Root Mean Square Error	0.881
Mean of Response	1.941
Observations (or Sum Wgts)	17

Analysis of Variance:

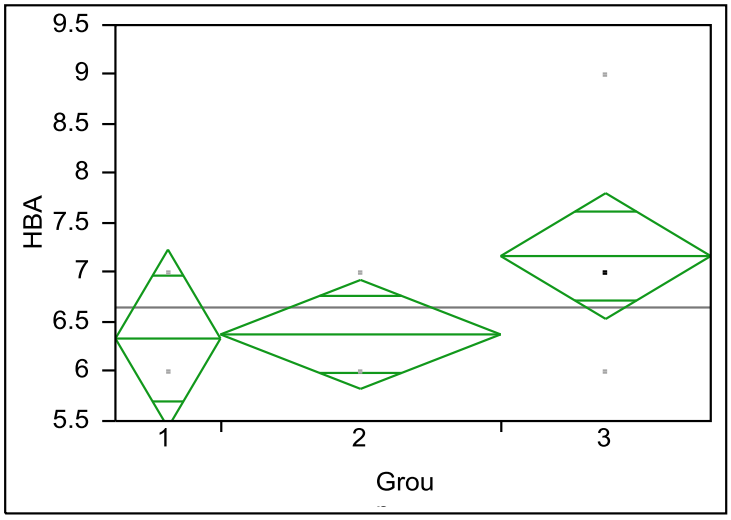
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Group	2	0.07	0.033	0.043	0.9584
Error	14	10.88	0.78		
C. Total	16	10.94			

ANOVA indicates no significant difference among Group 1, 2 and 3 for HBD.

Summary Statistics for HBD of the 3 Groups:

Group	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	2.0	0.51	0.9	3.1
2	8	1.9	0.31	1.2	2.5
3	6	2.0	0.36	1.2	2.8

4. Hyrdogen Bond Acceptors (HBA):



One-way ANOVA:

Summary of Fit:

Rsquare	0.254
Adj Rsquare	0.147
Root Mean Square Error	0.726
Mean of Response	6.647
Observations (FQs)	17

Analysis of Variance:

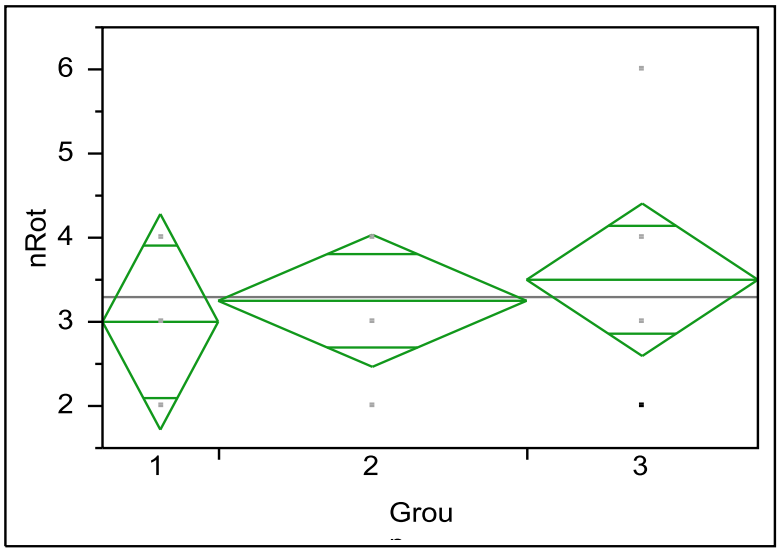
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Group	2	2.507	1.254	2.380	0.1289
Error	14	7.375	0.527		
C. Total	16	9.882			

ANOVA indicates no significant difference among Group 1, 2 and 3 for HBA.

Summary Statistics for HBD of the 3 Groups:

Group	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	6.3	0.42	5.4	7.2
2	8	6.4	0.26	5.8	6.9
3	6	7.2	0.30	6.5	7.8

5. Number of Rotatable Bonds (nRot):



One-way ANOVA:

Summary of Fit:

Rsquare	0.034
Adj Rsquare	-0.104
Root Mean Square Error	1.035
Mean of Response	3.294
Observations (or Sum Wgts)	17

Analysis of Variance:

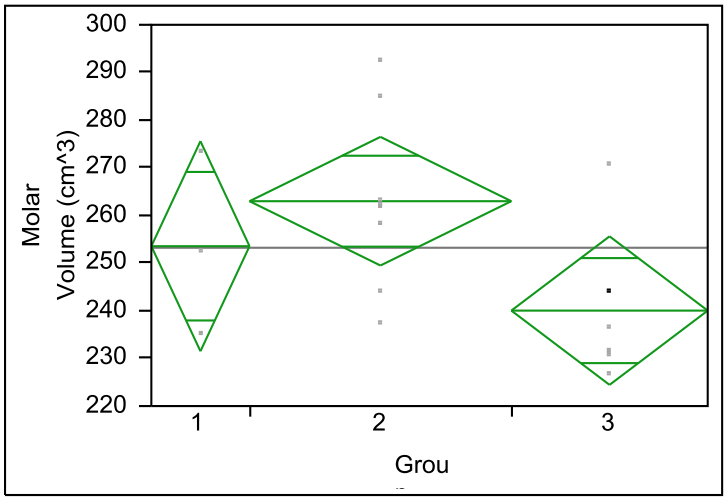
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Group	2	0.529	0.265	0.247	0.7844
Error	14	15.000	1.071		
C. Total	16	15.529			

ANOVA indicates no significant difference among Group 1, 2 and 3 for nRot.

Summary Statistics for nRot of the 3 Groups:

Group	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	3.0	0.60	1.7	4.3
2	8	3.3	0.36	2.5	4.0
3	6	3.5	0.42	2.6	4.4

6. Molar Volume:



One-way ANOVA:

Summary of Fit:

Rsquare	0.290
Adj Rsquare	0.188
Root Mean Square Error	17.795
Mean of Response	253.218
Observations (FQs)	17

Analysis of Variance:

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Group	2	1807.402	903.701	2.854	0.0913
Error	14	4433.398	316.671		
C. Total	16	6240.801			

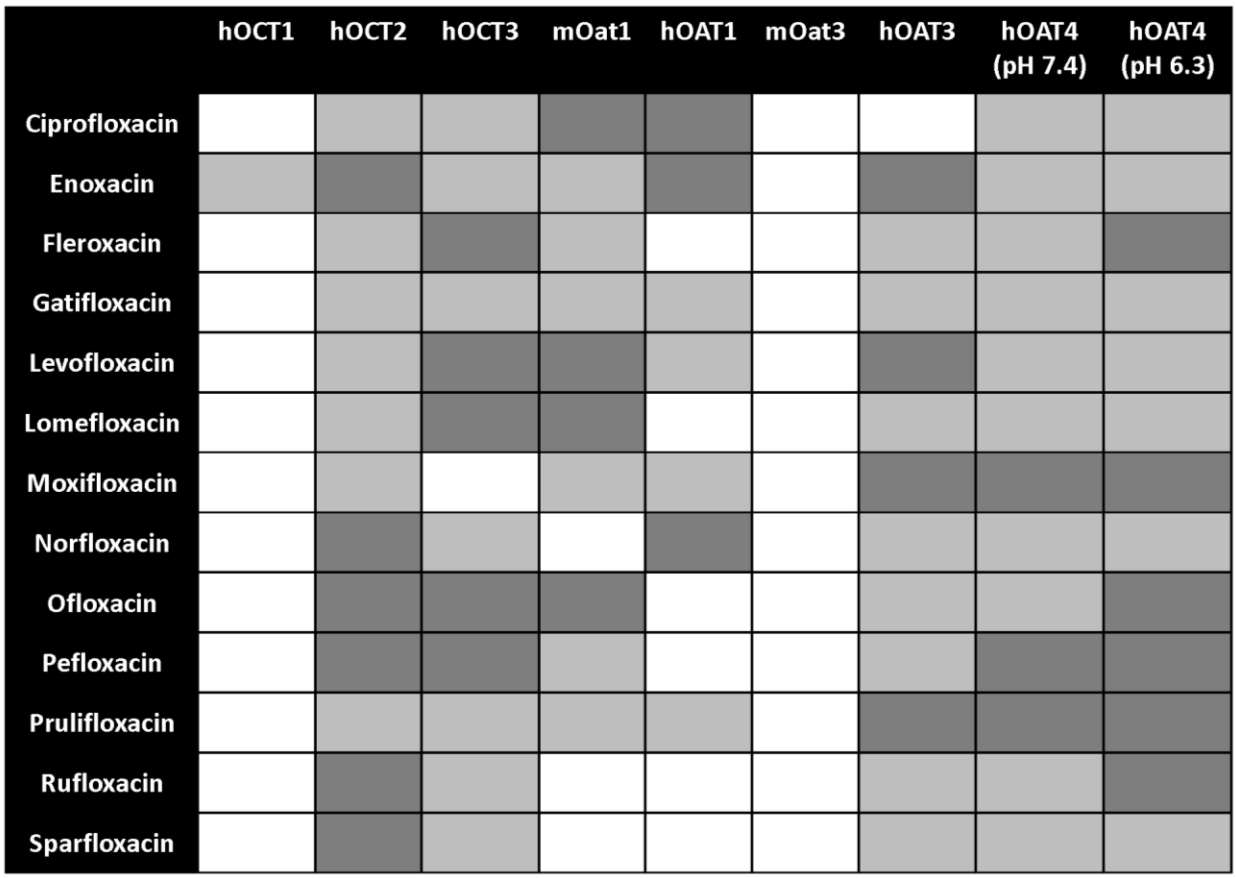
ANOVA indicates no significant difference among Group 1, 2 and 3 for Molar Volume.

Summary Statistics for Molar Volume of the 3 Groups:

Group	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	253.543	10.274	231.51	275.58
2	8	262.988	6.292	249.49	276.48
3	6	240.030	7.265	224.45	255.61

APPENDIX III

INTERACTIONS OF THE FLUOROQUINOLONES IN THE PRELMINARY INTERACTION EXPERIMENTS WITH THE SOLUTE CARRIER 22 TRANSPORTERS: INHIBITION, STIMULATION, OR NO INTERACTION



Inhibition ; Stimulation ; No Interaction

No consistent pattern of interaction was observed for any particular FQ, when tested with: hOCT1, hOCT2, hOCT3, mOat1, hOAT1, mOat3, hOAT3, hOAT4 (treatment at pH 7.4 and 6.3). Individual interactions are represented in the Figure above.

APPENDIX IV

ASSESSMENT OF THE CLINICAL RELEVANCE OF MOUSE ORGANIC ANION TRANPORTER 3 IN THE RENAL ELIMINATION OF FLUOROQUINOLONES

Inhibition constant (K_i values) were obtained for mOat3 with a selected subset of FQs (discussed in Chapter 5 and (187)). The following FQs were studied further to examine whether mOat3 could potentially be a rate-limiting step in their in vivo elimination, suggesting possible species difference in renal handling of FQs (as human OAT3 did not demonstrate significant in vitro interactions with FQs in preliminary studies).

Pharmacokinetic preclinical studies of FQs conducted in mice:

1. ENOXACIN:

FQ: Study	Species, BW	Dose and Route (mg)	Sampling Schedule		Assay	LOQ	PK Analysis	PK Endpoints					K_i in mOat3 (µM)
			Plasma	Urine		Plasma and/or Urine		AUC (mg*min/ml)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)	f_u (%)	CL_{ren}^u (ml/min/kg)	
Shinichi N et. al. (117)	Std-DDY mice, n=40 (plasma tests), 9 (urine tests), 26-32 g	50 mg/kg (1.45 mg, chosen on average)	0-8 hrs	0-24 hrs	Bioassay: thin layer cup-method with E.coli; Protein binding study by rapid ultrafiltration	0.04 µg/ml	One-compart-mental model	0.45	-	65.6	72.4	90.6	396 ± 15 (Chapter 5)

2. NORFLOXACIN:

FQ: Study	Species, BW	Dose and Route (mg)	Sampling Schedule		Assay	LOQ	PK Analysis	PK Endpoints					K_i in mOat3 (µM)
			Plasma	Urine		Plasma and/or Urine		AUC (mg*min/ml)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)	f_u (%)	CL_{ren}^u (ml/min/kg)	
Norfloxacin: Shinichi N et. al. (117)	Std-DDY mice, n=40 (plasma tests), 9 (urine tests), 26-32 g	50 mg/kg (1.45 mg, chosen on average)	0-4 hrs	0-24 hrs	Bioassay: thin layer cup-method with E.coli; Protein binding study by rapid ultrafiltration	0.04 µg/ml	One-compartmental model	0.065	-	34.8	60.2	57.8	558 ± 75 (187)

3. MOXIFLOXACIN:

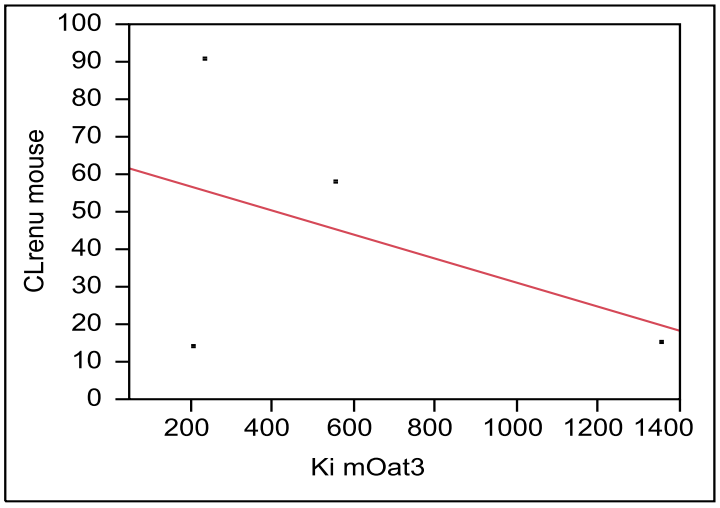
FQ: Study	Species, BW	Dose and Route (mg)	Sampling Schedule		Assay	LOQ	PK Analysis	PK Endpoints					K_i in mOat3 (µM)
			Plasma	Urine		Plasma and/or Urine		AUC (mg*min/ ml)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/ kg)	f_u (%)	CL_{ren}^u (ml/min/ kg)	
Siefert HM et. al. (145)	Male NMRI mice, n= 3 per time point, 26-43 g, 6 weeks old	9.2 mg/kg, IV dose	0-4 hrs	-	HPLC, Urine (and plasma) were assay-ed by scintill-ation count-ing; Protein binding assay by ultrafiltr ation	5.0 µg/ml for plasma	Non-compart-mental	0.13	70.2	10.3	69	14.9	1356 ± 114 (Chapter 5)

4. SPARFLOXACIN:

FQ: Study	Species, BW	Dose and Route (mg)	Sampling Schedule		Assay	LOQ	PK Analysis	PK Endpoints					K_i in mOat3 (µM)
			Plasma	Urine		Plasma and/or Urine		AUC (mg*min /ml)	CL_{tot} (ml/min/kg)	CL_{ren} (ml/min/kg)	f_u (%)	CL_{ren}^u (ml/min/ kg)	
Siefert HM et. al. (145)	Male Std-ddY mice, n= 5 per time point, 22-38 g, 6 weeks old	5 mg/kg, IV dose	0.25 - 8 hrs	0-48 hrs poole d for group of 5 mice	Agar well diffu-sion metho d with E. *coli* Kp; Protein bind-ing consid ered from a prev-ious analy-sis	0.01 µg/ml	One or two-compart-ment open model	0.044	-	7.6	56	13.5	205 ± 12 (Chapter 5)

From these mice preclinical studies, and the K_i values determined from an earlier study in Chapter 5, it was further studied if the increase/decrease in K_i values (i.e., binding affinity) of FQ inhibitors for mOat3 could potentially affect their CL_{ren}^{u} (further affecting $CL_{ren,tub}$ in mice). Statistical Analysis was conducted using JMP statistical software, Version 9.0.2.

FQ	**~K_i values for mOat3 (µM)**	**CL_{ren}^{u} from the studies in mice (ml/min/kg)**
Enoxacin	396	90.6
Norfloxacin	558	57.8
Moxifloxacin	1356	14.9
Sparfloxacin	205	13.5



Linear Fit Equation: CL_{ren}^{u} mouse = 63.168 - 0.0321*K_i mOat3 (UM)

Summary of Fit:

RSquare	0.215
RSquare Adj	-0.178
Root Mean Square Error	40.267
Mean of Response	44.275
Observations (FQs)	4

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	886.49	886.49	0.5467
Error	2	3242.88	1621.44	**Prob > F**
C. Total	3	4129.37		0.5367

When this univariate relationship was assessed by the linear regression analysis, it demonstrated that there was no significant linear relationship between the CL_{ren}^{u} in mice and the K_i values of the FQs for mOat3. This demonstrated that mOat3 may not be the rate-limiting transporter for the overall renal clearance of these FQs. However, a larger dataset of FQ preclinical (mice) studies and mOat3 K_i values would be essential to build stronger conclusions.

VITA

Aditi Mulgaonkar was born on April 12, 1986 in Mumbai, India and is an Indian citizen.
She graduated from MET's Institute of Pharmacy, University of Mumbai, India with a Bachelors in Pharmaceutical Sciences in 2007. She worked as a Research Analyst in the knowledge services division at WNS Global Services Inc., Mumbai, India for one year before joining the PK-PD-Drug Transporter Research Group at Department of Pharmaceutics, Virginia Commonwealth University (VCU) in 2008.

During her PhD graduate education, Aditi has published 8 abstracts and 2 manuscripts. She has presented her research at Annual Meetings of the American Association of Pharmaceutical Scientists (AAPS, 2010, 2011), Experimental Biology Conference (EB, 2012) and Office of Clinical Pharmacology (FDA) Science Day (2009) in addition to the poster presentations within the School of Pharmacy.
She received an AAPS-Pharmacokinetics, Pharmacodynamics and Drug Metabolism (PPDM) Section Travelship Award in 2009 and 2010. In addition to this, she has also received VCU School of Pharmacy Jyotsna and Mavji Thacker Award for academic excellence in Department of Pharmaceutics in 2009, and Joseph Schwarz Award for recognition of achieving the greatest distinction in areas of scholarship, research and service in 2011, from the VCU School of Pharmacy.
Aditi served as the GSA President in Department of Pharmaceutics from 2010-2011. She also served as AAPS Student Chapter at VCU Chair-elect (2010-2011) and AAPS Student Chapter at VCU Chair (2011-2012). In addition, she also serves as a student representative in the executive council of the PPDM Section in the AAPS organization since 2010.

PUBLICATIONS:

1 Gloriane W. Schnabolk, Bhawna Gupta, Aditi Mulgaonkar, Mrugaya Kulkarni and Douglas H. Sweet. Organic anion transporter 6 (SLC22a20) specificity and sertoli cell-specific expression provide new insight on potential endogenous roles. *J. Pharmacol. Exp. Ther.* September 2010; **334** (3): 927-935.

2 Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Fluoroquinolone disposition: Identification of the contribution of renal secretory and reabsorptive drug transporters. *Exp. Opin. Drug Metab. Toxicol.* May 2012 (doi:10.1517/17425255.2012.674512).

ABSTRACTS:

Extra-mural:

1. Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Role of Drug Transporters in the Systemic Disposition of Fluoroquinolones (FQ). Experimental Biology Conference, April 2012, San Diego, CA.
2. Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Interaction of Organic Anion Transporters 1 and 3 with Fluoroquinolones Exhibiting Net Tubular Clearance in Humans. AAPS Annual Meeting and Exposition, Washington DC, October 2011.
3. Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Role of Drug Transporters (DT) in the Systemic Pharmacokinetics (PK) of Fluoroquinolones (FQ). FIP PSWC/AAPS Annual Meeting and Exposition, New Orleans, LA, November 2010.
4. Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Role of Drug Transporters (DT) in the Systemic Pharmacokinetics (PK) of Fluoroquinolones (FQ). 15th Annual Office of Clinical Pharmacology (OCP) Science Day, Baltimore, Maryland, October 2010.

Intra-mural:

1. Elizaveta Budko, Aditi Mulgaonkar, Douglas H. Sweet. Role of Human Organic Cation Transporters (hOCTs) in renal aminoglycoside toxicity. VCU School of Pharmacy Research and Career Day, Richmond, VA, October 2011.
2. Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Interaction of Organic Anion Transporters 1 and 3 with Fluoroquinolones Exhibiting Net Tubular Clearance in Humans. VCU School of Pharmacy Research and Career Day, Richmond, VA, October 2011.
3. Aditi Mulgaonkar, Jürgen Venitz, Douglas H. Sweet. Role of Drug Transporters (DT) in the Systemic Pharmacokinetics (PK) of Fluoroquinolones (FQ). VCU School of Pharmacy Research and Career Day, Richmond, VA, October 2010.
4. Aditi Mulgaonkar and Douglas H. Sweet. Characterizations of Organic anion transporter mOat6 expression in the mouse testis using laser capture micro-dissection (LCM) technique. VCU School of Pharmacy Research and Career Day, Richmond, VA, October 2009.