ROLE OF BASOLATERAL EFFLUX TRANSPORTERS IN INTESTINAL ABSORPTION OF DRUGS AND PRODRUGS

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ABSTRACT

XIN MING: Role of Basolateral Efflux Transporters in Intestinal Absorption of Drugs and Prodrugs
(Under the direction of Dhiren R. Thakker, Ph.D.)

Polarized expression and cooperation of drug transporters in epithelial cells are critical in governing systemic exposure and thereafter drug actions. However, mechanisms for the basolateral efflux of hydrophilic compounds from intestine remain elusive. Studies described in this dissertation provide novel insights about the role of basolateral transporters in intestinal drug absorption: they are critical for the transcellular transport of hydrophilic drugs when apical transporters mediate uptake efficiently, or when prodrugs are metabolized into hydrophilic drugs intracellularly after diffusion through the apical membrane.

Investigation of the absorptive transport of fexofenadine in Caco-2 cells provided evidence for a vectorial transport system in its intestinal absorption, consisting of organic anion transporting polypeptide 2B1 for apical uptake and multidrug-resistance associated protein 3 (MRP3) for basolateral egress. The MRP inhibitor decreased the absorptive transport of fexofenadine moderately; further, this decrease was more pronounced when P-glycoprotein was also inhibited. It appears that apical efflux by P-glycoprotein may be the rate-limiting step in intestinal absorption of fexofenadine; however, MRP3-mediated basolateral efflux may control its absorption where P-glycoprotein expression/effect is low.

Adefovir dipivoxil, an ester prodrug of adefovir with improved bioavailability, diffuses across cell membranes readily and is rapidly hydrolyzed to adefovir intracellularly.

In MRP4-knockdown Caco-2 cells, basolateral availability of adefovir upon dosing the apical compartment with [^{3}H]adefovir dipivoxil was reduced at the level comparable to the chemical inhibition of MRPs in wild-type cells. Results showed that MRP4 is localized in the basolateral membrane of Caco-2 cells and mediates the basolateral efflux of adefovir generated intracellularly.

The diamidine drug, furamidine, was identified as a substrate for organic cation transporter 1 (OCT1), an intestinal basolateral transporter. It mediates efflux of furamidine at much lower rate than its uptake. A diamidoxime prodrug of furamidine diffuses into OCT1 transfected MDCKII cells, and is metabolized intracellularly to furamidine, which egresses across the basolateral membrane by OCT1-mediated transport. However, its significance is still questionable under physiological conditions due to the inefficient efflux and the low expression *in vivo*.

Overall, this work has provided new insights about carrier-mediated basolateral efflux mechanisms and defined when and how these basolateral transporters affect intestinal drug absorption.

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ABC	ATP binding cassette
ANOVA	Analysis of Variance
AUC	Area under the curve
CHO	Chinese Hamster Ovary
E1S	Estrone 3-sulfate
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	High Pressure Liquid Chromatography
J	Flux
J_{max}	Maximal Flux
K_m	Michaelis-Menten Constant
MDCKII	Madin Darby Canine Kidney II
MPP^+	1-methyl-4-phenylpyridinium
MRP	Multidrug resistance-associated protein
NEAA	Non-Essential Amino Acids
OAT	Organic anion transporters
OATP	Organic anion transporting polypeptides
OCT	Organic cation transporter
OCTN	Novel organic cation transporter
P_{app}	Apparent Permeability

PBS	Phosphate-buffered saline
P-gp	P-glycoprotein
RT-PCR	Reverse-transcription Polymerase Chain Reaction
S.D.	Standard Deviation
SLC	Solute Carrier Family
TEA	Tetraethylammonium
TEER	Transepithelial Electrical Resistance

CHAPTER 1

INTRODUCTION

A. INTRODUCTION

Oral administration is the primary drug delivery route and accounts for over 70% of all medications. In order to achieve pharmacological actions, oral drugs must dissolve in the stomach and then cross intestinal epithelium to arrive in the bloodstream. The villi of the intestinal epithelium and the presence of microvilli in each epithelial cell vastly increase the surface area for maximal absorption (Figure 1.1). The epithelial cell monolayer in the intestine constitutes a selective barrier to nutrients and xenobiotics (Figure 1.1). The intercellular spaces located between the enterocytes are around 10 Å in size and occupy less than 0.1% of the total surface area of the intestinal epithelium (Fordtran et al., 1968; Schultz et al., 1974). These spaces are further sealed by the presence of tight junctions, which effectively limit intestinal absorption through the paracellular spaces. Tight junctions also restrict the movements of lipids and proteins along the exoplasmic leaflet of the plasma membrane and separate the apical and basolateral membranes in the polarized enterocytes. Tight junctions and two membranes are the permeation barrier for intestinal drug absorption. In addition, abundant soluble and membrane bound enzymes, such as esterases and cytochrome P450 enzymes, are expressed inside the enterocytes to metabolize the drugs before they arrive in the blood circulation and these enzymes are the enzymatic barrier for drug absorption.

Bioavailable drugs overcome the barrier in the intestine in different ways (Figure 1.2). Lipophilic compounds traverse the intestinal epithelium via transcellular passive diffusion and are generally well-absorbed; in contrast, small hydrophilic compounds, which pass through the narrow paracellular pores, are generally poorly absorbed. However, nutrients and some hydrophilic drugs utilize carrier mediated transport systems and achieve

higher bioavailability than that expected from their physicochemical properties. Recently, drug vectorial transport systems have been established in elimination of drugs in the liver and kidney, in which uptake transporters in the basolateral membrane and efflux transporters in the apical membrane share similar substrate specificity, and the synergy of these transporters in polarized epithelial cells facilitates the unidirectional transport of a compound into the bile or urine (Ito et al., 2005). Accordingly, vectorial transport systems for intestinal drug absorption involve uptake transporters in the apical membrane and efflux transporters in the basolateral membrane of the intestine (Figure 1.2). Depending on their relative efficiency and capacity, apical uptake transporters or basolateral efflux transporters may control the transepithelial movement when the corresponding transport step becomes rate-limiting (Cheeseman, 1992). Knocking out the basolateral transporter organic solute transporter αβ (Ostαβ) in mice almost abolished transileal transport of taurocholate (Rao et al., 2008), which indicted that basolateral transporters are likely to be as important as apical transporters in efficient transcellular transport of hydrophilic compounds in the intestine.

Conceivably, intestinal basolateral transporters may also mediate efflux of hydrophilic compounds generated in the enterocytes, being either the active drugs from their prodrugs or the metabolites of the parent drugs (Figure 1.2). Due to the high lipophilicity of the parent compounds, they cross the apical membrane by passive diffusion and apical uptake transporters may not contribute to this process. However, they may be metabolized into more hydrophilic and/or charged metabolites by highly expressed esterases and CYP450 enzymes in the intestine, when the efflux transporters in the apical or basolateral membrane are needed to pump them out of the cells. The roles of the efflux transporters, in the canalicular (apical) and sinusoidal (basolateral) membranes, in elimination of the metabolites formed in the

hepatocytes has been well established (Zamek-Gliszczynski et al., 2006). However, the roles of the same transporters, such as MRP2 and MRP3, in the intestine have been unclear. In addition to mediating efflux of substrates out of the enterocytes, intestinal basolateral transporters in the solute carrier superfamily (SLC) may also play a role in intestinal secretion of xenobiotics via uptake of substrates from the blood circulation into the enterocytes (Arimori and Nakano, 1998). Mouse organic cation transporter 1 (mOct1) is functionally expressed in the intestinal basolateral membrane and facilitates the secretion of its substrates from the circulating blood into the intestinal lumen by mediating the basolateral uptake process (Jonker et al., 2001; Wang et al., 2002).

In this review, current advances of the research on intestinal basolateral transporters are summarized and the relevant apical transporters are also included in order to better describe the functions of the basolateral transporters. The following overview highlights members of both the SLC and ATP-binding cassette (ABC) superfamily of drug transporters with respect to intestinal expression, localization, substrate specificity, and contribution to intestinal drug absorption.

B. AMINO ACID TRANSPORTERS

A large number of amino acid transporters exist in both the apical and basolateral membranes of the intestinal epithelium and primarily mediate absorption of amino acids obtained from the diet (Figure 1.3, Table 1.1). Amino acid transporters represent the biggest transporter class and span eight of the total 46 SLC families. Many of these transporters have narrow substrate specificities transporting amino acids with molecular weight below 100, and, thus, are not significantly involved in absorption of drugs with typical molecular weight over 150. Only the transporters for large and neutral amino acids have been reported to contribute to the absorption of amino acid-like drugs, such as L-dopa and gabapentin, and will be discussed here.

B.1. Apical Transporters

Multiple transporters in the intestinal apical membrane are involved in uptake of large neutral amino acids, including $ATB^{0,+}$ (SLC6A14), B^0AT1 (SLC6A19), and $b^{0,+}AT$ (SLC7A8). $ATB^{0,+}$ is a Na^+ and Cl^- dependent amino acid transporter for hydrophobic neutral and cationic amino acids including amino acid-like drugs (Sloan and Mager, 1999). Valacyclovir, the valine prodrug of acyclovir, is substrate for $ATB^{0,+}$, suggesting that this transporter can be applied for improved oral absorption of amino acid prodrugs (Hatanaka et al., 2004; Umapathy et al., 2004). The regional expression of $ATB^{0,+}$ in the colon may be beneficial for colonic drug delivery. $b^{0,+}AT$ forms a heterodimer with its heavy subunit binding partner rBAT and translocates cationic and neutral amino acids as well as L-cysteine in an exchange fashion (Chillaron et al., 1996). Recently, it was reported that uptake of dipeptides via oligopeptide transporter 1 (PepT1) enhanced the uptake of gabapentin via trans-stimulation of $b^{0,+}AT$ exchange in rat intestine, indicating that apical uptake of

gabapentin is mediated by $b^{0,+}AT$ (Nguyen et al., 2007). However, whether gabapentin is a substrate for this transporter needs to be confirmed. B^0AT1 is localized in the apical membrane of the intestine and renal proximal tubule and transports neutral amino acids in a Na^+ dependent manner (Broer et al., 2004). Mutations in this transporter gene cause Hartnup disorder (Broer et al., 2004; Kleta et al., 2004; Seow et al., 2004). However, it has not been established whether it is involved in intestinal absorption of amino acid-like drugs.

B.2. Basolateral Transporters

B.2.a LAT1 (SLC7A5) and LAT2 (SLC7A8)

LAT1 and LAT2 are hetero(di)meric amino acid transporters composed of a catalytic subunit (LAT1 or LAT2, light chain) associated covalently with a glycoprotein 4F2hc/CD98 (heavy chain), transporting large branched and aromatic neutral amino acids (Verrey, 2003). LAT1 and LAT2 are obligatory amino acid exchangers with 1:1 stoichiometry and do not mediate net uptake of large and neutral amino acids (Meier et al., 2002). The net directional transport of amino acids depends on the parallel expression of a unidirectional transporter with overlapping selectivity, which recycles amino acids and drives the exchange function of LAT1 and LAT2 (Meier et al., 2002; Bauch et al., 2003). Both LAT1 and LAT2 transport L-dopa (Verrey et al., 2004), but only LAT1 transports gabapentin (Cundy et al., 2004). LAT1 is mainly expressed in growing cells. The presence of LAT1 in both luminal and abluminal membranes of microvessel endothelial cells in brain (Duelli et al., 2000) suggested that LAT1 plays an important role in large and neutral amino acids transfer across the blood-brain barrier, making LAT1 a good target to deliver amino acid-like drugs, such as L-dopa and gabapentin, to the brain (Pardridge, 2007). In rat intestine, LAT1 protein is most abundant in the colon but low in the small intestine, in contrast to the relatively homogeneous presence of

LAT2 throughout the digestive tract (Fraga et al., 2005). LAT2 is highly expressed in the basolateral membrane in mouse small intestine (Dave et al., 2004), indicating that it is mainly involved in the basolateral efflux step in transepithelial transport of amino acids. Both transporters are present in intestinal cell lines Caco-2 and IEC-6 cells at mRNA and protein levels (Fraga et al., 2005) and function as exchangers of neutral amino acids (Fraga et al., 2002).

B.2.b LAT3 (SLC43A1) and LAT4 (SLC43A2)

LAT3 and LAT4 belong to a new gene family of SLC43; they transport large and neutral amino acids in Na^+-, Cl^-- and pH-independent fashion (Babu et al., 2003; Bodoy et al., 2005). Northern blot analysis showed that *LAT3* mRNA was expressed at high level in pancreas, liver and skeletal muscle (Babu et al., 2003). A recent study using GeneChip arrays showed that *LAT3* mRNA is also highly expressed in human duodenum (Kim et al., 2007). Subcellular localization of LAT3 in polarized cells has not been attempted. *LAT4* mRNA is restricted to the epithelial cells of the distal tubule and the collecting duct in the kidney. However, it is mainly present in the cells of the crypt in the intestine (Bodoy et al., 2005). This transporter is expressed in the basolateral membrane of PCT kidney cells (Bodoy et al., 2005). LAT3 and LAT4 mediate net uptake of large and neutral amino acids (Babu et al., 2003; Bodoy et al., 2005), and may play a more important role than LAT1 and LAT2 in intestinal absorption of amino acids and amino acid-like drugs. However, substrate activity of amino acid-like drugs, such as L-dopa and gabapentin, towards these two novel transporters has not been studied.

B.2.c y^+LAT1 (SLC7A7) and y^+LAT2 (SLC7A6)

y^+LAT1 and y^+LAT2 were first identified on the basis of homology to LAT1 and were shown to function as obligatory amino acid exchangers of neutral and cationic amino acids. Transport of neutral amino acids by these two transporters is coupled to Na^+ (Torrents et al., 1998; Pfeiffer et al., 1999). y^+LAT1 was localized in the basolateral membrane in kidney proximal tubule (Bauch et al., 2003) and small intestine (Dave et al., 2004). In contrast to y^+LAT1, y^+LAT2 is widely expressed in non-epithelial and epithelial tissues (Verrey et al., 2004). The role of these transporters in intestinal drug absorption is not clear at present.

B.2.d TAT1 (SLC16A10)

T-type amino-acid transporter-1 (TAT1, SLC16A10) is highly expressed in the basolateral membrane of rat and mouse intestinal cells (Kim et al., 2001; Ramadan et al., 2006). TAT1 does not transport monocarboxylates, such as lactate and pyruvate, although most of members in the SLC16 family are monocarboxylate cotransporters (Kim et al., 2001). TAT1 mediates both uptake and efflux of aromatic amino acids, including L-dopa, in a Na^+- and pH-independent facilitative manner (Kim et al., 2001; Ramadan et al., 2006). TAT1 is able to mediate a net efflux of aromatic amino acids into the blood (Ramadan et al., 2006). Sequentially, these aromatic amino acids may drive obligatory exchange of other neutral amino acids into the blood via other basolateral transporters such as y^+LAT1 and LAT2 (Ramadan et al., 2006).

C. PEPTIDE TRANSPORTERS

C.1. Apical Peptide Transporters

Apical peptide transporters, mainly in the SLC15 family, are secondary active transporters that utilize the proton-motive force for uphill transport of short chain peptides and peptidomimetics into a variety of cells (Liang et al., 1995). Oligopeptide transporter 1 (PepT1, SLC15A1), the first identified peptide transporter (Fei et al., 1994), is mainly expressed in intestine with the highest expression in duodenum (Herrera-Ruiz et al., 2001). PepT1 expression has also been detected in Caco-2 cells, the most widely used *in vitro* model of intestinal epithelium (Brandsch et al., 1994). PepT1 transports a large variety of di- and tripeptides in a stereoselective manner (Terada and Inui, 2004), and also interacts with many peptide-like drugs, including β-lactam antibiotics cephalexin (Tamai et al., 1997), the ACE inhibitor enalapril (Zhu et al., 2000; Shu et al., 2001), and the anti-viral prodrug valacyclovir (Balimane et al., 1998; Ganapathy et al., 1998; Sugawara et al., 2000). The finding that intestinal PepT1 transports L-valine prodrugs such as valacyclovir provided a major advance toward the development of novel intestinal delivery systems via uptake transporters (Brodin et al., 2002). However, a recent study found that no correlation was observed between absorption of valacyclovir and PepT1 expression in human duodenum (Landowski et al., 2003). Interestingly, this study showed a significant positive correlation between valacyclovir absorption and the expression levels of human peptide transporter 1 (HPT1, also called Cadherin 17), which is also able to transport valacyclovir (Landowski et al., 2003). These results strongly supported the major contribution of HPT1 in intestinal absorption of peptides and peptide-like drugs, which was supported by a recent study showing that *HPT1* mRNA

level is the highest among 36 drug transporters in human jejunum (Hilgendorf et al., 2007). The knowledge about the apical transporters is summarized in Figure 1.3 and Table 1.1.

C.2. Basolateral Peptide Transporter

The basolateral peptide transporter, mediating the efflux of small peptides and peptide-like drugs from enterocytes to the blood, has not been cloned, although its functional activity was observed in the basolateral membrane in Caco-2 cells many years ago (Saito and Inui, 1993; Matsumoto et al., 1994). The proposed peptide transport model consists of active uptake by PepT1 and/or HPT1 driven by proton gradient followed by a downhill egress by the basolateral transporter to the mesenteric circulation (Terada and Inui, 2004). Functional activity of the basolateral peptide transporter has been studied in Caco-2 monolayers and the basolateral peptide transporter demonstrated the properties distinguished from the apical PepT1. Firstly, the basolateral uptake into Caco-2 cells led to the intracellular concentration of the substrates equal to the extracellular concentration and was shown to be less sensitive to the pH, suggesting that the basolateral peptide transporter in Caco-2 cells is a proton-independent facilitative transporter (Terada et al., 1999; Irie et al., 2001). This transporter can transport di- and tripeptides, peptide-like drugs and non-peptide PepT1 substrates (Terada et al., 2000). Consistent with the bi-directional property of facilitative transporters, the basolateral peptide transporter mediates both uptake and efflux of its substrates (Irie et al., 2004). The substrates showed much lower affinity for the basolateral peptide transporter at the intracellular side (Km = 30 mM) compared to PepT1 (Km = 1.6 mM) (Terada et al., 2000; Irie et al., 2004). This property may be adaptive to the higher intracellular concentration in the intestinal lumen, achieved by active uptake by PepT1. Low affinity of the basolateral peptide transporter prevents saturation of this carrier in order to retain its activity. This

kinetic property of peptide transporters is similar to that of the glucose transport system in the intestine, which consists of a secondary active transporter in the apical membrane, Na^+-glucose cotransporter (SGLT1, *Km* = 0.8 mM), and low affinity basolateral facilitate glucose transporter (GLUT2, *Km* = 15-20 mM) (Hediger and Rhoads, 1994).

A candidate protein of the basolateral peptide transporter was identified in rat jejunum by photoaffinity labeling (Shepherd et al., 2002). A single protein with the molecular weight of 112 kDa and *pI* of 6.5 was isolated and identified as a novel protein based on dissimilarity to PepT1 in protein sequence (Shepherd et al., 2002). However, no further studies on molecular cloning and kinetic characterization of this novel protein have been reported since this initial study. Therefore, the basolateral efflux transporter is still the major missing link in vectorial transport of small peptides and peptide-based drugs in the intestine.

D. NUCLEOSIDE TRANSPORTERS

D.1. Concentrative Nucleoside Transporters

Concentrative nucleoside transporters (CNTs, SLC28 family) mediate active, Na^+-dependent uptake of naturally occurring nucleosides and nucleoside-based drugs, such as zidovudine and 2'-2'-Didioxyinosine (Balimane and Sinko, 1999; Gray et al., 2004). The three subtypes differ in their substrate specificities: CNT1 transports pyrimidine-nucleoside, CNT2 prefers purine-nucleoside, and CNT3 transports both nucleosides (Gray et al., 2004). CNTs determine response to a variety of anticancer and antiviral nucleoside analogs, as they mediate the entry of these drugs into target cells (Cass et al., 1999). All three CNTs' mRNAs have been detected in the intestine (Wang et al., 1997; Ngo et al., 2001; Ritzel et al., 2001) but CNT2 showed the highest expression (Meier et al., 2007). CNT1 or CNT2 mRNAs were not found in Caco-2 cells suggesting that this model may not be appropriate to study nucleoside absorption (Ward and Tse, 1999). A recent study confirmed the expression of CNT1 and CNT2 mRNAs in the enterocytes in human duodenum using an *in situ* hybridization technique and localized their proteins predominantly in the apical membrane using immunohistochemical staining (Govindarajan et al., 2007). The broad substrate specificity of CNTs and intestinal expression suggest that these transporters play critical roles in intestinal nucleoside salvage and the absorption of nucleoside-based drugs.

D.2. Equilibrative Nucleoside Transporters

Equilibrative nucleoside transporters (ENTs, SLC29 family) transport naturally occurring nucleosides and nucleoside-based drugs in a Na^+-independent, bi-directional fashion. ENT1, ENT2 and ENT3 possess similar broad substrate specificities for purine and pyrimidine nucleosides, the latter two possessing additional activity to transport nucleobases

(Baldwin et al., 2004). ENT1 and ENT2 transport many anti-viral and anti-cancer drugs containing nucleoside moieties (Balimane and Sinko, 1999; Cass et al., 1999; Baldwin et al., 2004). All subtypes are widely distributed in mammalian tissues including human intestine, but at lower levels than CNT2 (Meier et al., 2007). In polarized cells, ENT1 is expressed in the basolateral membrane (Lai et al., 2002). However, a recent study showed that ENT1 and ENT2 mRNAs are present in the crypt cells, but not the absorptive enterocytes in the tip of villi in human duodenum (Govindarajan et al., 2007). Although their proteins are localized in the lateral membrane in the crypt cells, they are not co-localized with CNT1 and CNT2 in the same cells (Govindarajan et al., 2007), indicating they may not play a role in intestinal absorption of nucleoside-based drugs.

ENT4 was originally identified by genome database analysis and assigned to the SLC29 family based on the gene sequence homology (Acimovic and Coe, 2002). More recent studies showed that this transporter transports organic cations, such as TEA, MPP^+ and metformin, in a Na^+-independent and membrane potential-sensitive manner (Engel and Wang, 2005; Zhou et al., 2007). Thereafter, it was given a new name, plasma membrane monoamine transporter (PMAT), recognizing its function as a polyspecific organic cation transporter. PMAT protein is expressed in human small intestine and localized into the apical membrane of the enterocytes (Zhou et al., 2007), indicating that it may mediate apical uptake of cationic drugs such as metformin in the intestine.

E. BILE ACID TRANSPORTERS

E.1. Apical Sodium-Dependent Bile Salt Transporter (ASBT, SLC10A2)

It has been clearly established that bile acids are taken up at the ileal brush border membrane through a carrier-mediated, Na^+-dependent process in the intestine (Gallagher et al., 1976) and Caco-2 cells (Hidalgo and Borchardt, 1990). The molecular mechanism of ileal transport of bile acids was further clarified after molecular cloning of apical sodium-dependent bile salt transporter (ASBT, SLC10A2) from mouse (Saeki et al., 1999), rat (Shneider et al., 1995), and human (Wong et al., 1995) ileum. Transport of endogenous bile salts, including taurocholate, cholate, glycodeoxycholate, glycochenodeoxycholate, and glycoursodeoxy-cholate, by ASBT is electrogenic and Na^+-dependent (Weinman et al., 1998). ASBT plays an essential role in the (re-)absorption of bile acids that are secreted from the liver into bile and subsequently passed into the intestine, which has been proved by the studies showing that deficiency of the *ASBT* gene was associated with intestinal bile acid mal-absorption in humans (Oelkers et al., 1997), and targeted deletion of the *ASBT* gene eliminates enterohepatic cycling of bile acids in mice (Dawson et al., 2003).

ASBT has been utilized as a prodrug target to improve oral bioavailability (Balakrishnan and Polli, 2006). One bile acid conjugate of acyclovir achieved 16-fold greater acyclovir accumulation within hASBT transfected cells and a 2-fold increase in the bioavailability of acyclovir (Tolle-Sander et al., 2004). Further efforts have been expended in finding bile acid analogs with better substrate activity for hASBT (Balakrishnan et al., 2006a; Balakrishnan et al., 2006b). However, these efforts have resulted in limited success.

E.2. Organic Solute Transporter αβ (OSTαβ)

Similar to other intestinal transport systems, the identification of the basolateral efflux transporter of bile acids lagged behind the discovery of the apical transporter. The heteromeric transporter Ostαβ, was identified first in the liver of marine vertebrates using the expression cloning approach (Wang et al., 2001), and then in humans and mice (Seward et al., 2003). In contrast to other organic anion transporters identified to date, transport activity of this transporter requires the coexpression of two distinct gene products: a 340-amino acid protein with a predicted seven-transmembrane domain (OSTα) and a putative 128-amino acid ancillary polypeptide with a single-transmembrane domain (OSTβ) (Wang et al., 2001; Seward et al., 2003). This transporter was first regarded as a steroid transporter capable of transporting endogenous and xenogenous steroid compounds, including estrone 3-sulfate, digoxin, taurocholate and prostaglandin E2 (Wang et al., 2001; Seward et al., 2003; Ballatori, 2005). A role for Ostαβ in intestinal basolateral egress of bile acids was suggested after it was found that Ostαβ mRNAs are highly expressed in ileum and the protein is largely restricted to the basolateral membrane of ileal enterocytes (Ballatori et al., 2005; Dawson et al., 2005). In addition, expression levels of Ostαβ mRNAs are positively regulated via the bile acid-activated nuclear receptor farnesoid X receptor (FXR) (Boyer et al., 2006; Landrier et al., 2006; Lee et al., 2006). In a recent study, transileal transport of taurocholate was reduced by >80% in Ostα knockout mice compared to wild-type mice; the residual taurocholate transport was further reduced to background levels in gut sacs prepared from Ostα and Mrp3 double knockout mice (Rao et al., 2008). This result finally established the predominant role of Ostαβ in intestinal basolateral efflux of bile acids with MRP3 playing a minor role, which represents one of the most important advances in the area of intestinal

basolateral transporters in the past 5 years and proves that basolateral transporters are as important as apical transporters in efficient transcellular transport. Transport mediated by OSTαβ is facilitated diffusion (Ballatori et al., 2005); thus, OSTαβ may also play a role as the basolateral uptake transporter in intestinal secretion of steroid compounds such as digoxin.

F. ORGANIC CATION TRANSPORTERS

F.1. OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3)

Organic cation transporters (OCTs) in the SLC22A family, which are mainly expressed in the elimination organs such as the kidney (basolateral membrane of tubular cells) and liver (sinusoidal membrane of hepatocytes), have been most extensively studied in regards to hepatic and renal transport of relatively small organic cations (Koepsell et al., 2007). There are three distinct OCT transporters, namely OCT1, OCT2, and OCT3, which mainly mediate the entry of organic cations into cells (Koepsell et al., 2007). In humans, hOCT1 is the predominant OCT transporter expressed in the liver while hOCT2 is expressed predominantly in the kidney (Koepsell et al., 2007). The majority of OCT substrates are monovalent, and comparatively small, cations, the so called type I organic cations (Meijer et al., 1999; Wright, 2005) as exemplified by the prototypical substrates TEA and the neurotoxin, MPP^+ (Koepsell et al., 2007). In addition, OCTs interact with many drugs, including the H2 antagonist ranitidine (Bourdet et al., 2005), the antidiabetic drug metformin (Wang et al., 2002) and the anticancer drug oxaliplatin (Yokoo et al., 2007). Transport via OCTs is facilitative and driven by the negative membrane potential inside the cell. Uptake of positively charged molecules is thermodynamically favorable, which can produce over 10-fold higher intracellular concentrations compared with the dose concentration (Koepsell et al., 2007).

In contrast to the established role of OCTs in hepatic and renal disposition of organic cations, the roles of these transporters in intestinal absorption and secretion of cationic compounds have yet to be firmly established, although evidence for their expression in the intestinal tissues of humans and preclinical species have been reported (Grundemann et al.,

1994; Kekuda et al., 1998). Recently, quantification of mRNAs for OCTs by real-time RT-PCR showed that OCT1 and OCT3 are expressed in human intestine but at lower levels than in the elimination organs liver and kidney, and the expression of OCT2 was very low or undetectable (Terada et al., 2005; Hilgendorf et al., 2007). Immunostaining of OCT1 in human small intestine showed lateral localization (Muller et al., 2005). In Oct1-null mice, accumulation of TEA (Jonker et al., 2001) and metformin (Wang et al., 2002) was lower than wild type after intravenous administration, indicating that Oct1 is expressed in the basolateral membrane of mouse intestine and facilitates the elimination of its substrates from the circulating blood into the intestinal lumen. OCT1 is also capable of mediating efflux of cations, such as TEA and MPP^+, out of cells (Zhang et al., 1999; Keller et al., 2005). However, whether OCT1 plays a role in absorptive transport of cationic drugs by mediating basolateral egress has not been clear. Conceivably, efflux of cations by OCT1 may not be as efficient as the uptake process, because OCT1-mediated transport is driven by the membrane potential of positive outside and negative inside, which favors, thermodynamically, the uptake of positively charged molecules but resists the efflux process (Koepsell et al., 2007). Surprisingly, immunostaining of OCT1 (Ng, 2003) and OCT3 (Muller et al., 2005) has shown that both transporters are localized in the apical membrane of Caco-2 cells, which is consistent with the carrier mediated mechanism of uptake of ranitidine and metformin, OCT1 substrates, across the apical membrane of Caco-2 cells (Bourdet and Thakker, 2006; Proctor et al., 2008b). These observations from an *in vitro* transport model need to be validated in transport studies using *in vivo* models such as *in situ* intestinal perfusion.

F.2. OCTN1 (SLC22A4) and OCTN2 (SLC22A5)

Novel organic cation transporter 1 (OCTN1) and 2 (OCTN2) are expressed in human intestine (Terada et al., 2005; Hilgendorf et al., 2007; Meier et al., 2007). Indeed, they can be considered as zwitterion transporters because they transport zwitterions such as ergothioneine (OCTN1) and L-carnitine (OCTN2) much more efficiently than the prototypical OCT substrate TEA (Grundemann et al., 2005). OCTN1 is expressed in kidney, skeletal muscle, placenta, prostate, and heart with strong expression in erythrocytes and monocytes (Grundemann et al., 2005), which may be relevant to the protective role of ergothioneine, an intracellular antioxidant. Decreased proliferation and erythroid differentiation was observed in K562 cells depressed of *OCTN1* gene expression by siRNA knockdown, indicating this transporter may play a protective role in humans (Nakamura et al., 2007). OCTN1 also mediates renal excretion of an amino acid-type drug gabapentin, which is also a zwitterion, and genetic mutations of this transporter decreased renal clearance of gabapentin in humans (Urban et al., 2007). The subcellular localization of OCTN1 in the intestine has not been shown; therefore, the definite function in intestinal transport is unknown.

OCTN2 (SLC22A5) is a high-affinity, sodium-dependent transporter that plays a key role in the cellular uptake of L-carnitine (Koepsell et al., 2007). An important physiological role for L-carnitine involves transporting long-chain fatty acids across the inner mitochondrial membrane for oxidation. *OCTN2* mRNA have been detected in kidney, heart, skeletal muscle, intestine and placenta by Northern blotting (Tamai et al., 1998). In the Octn2 deficient mice, reduced oral bioavailability of L-carnitine was observed compared with wild-type mice, suggesting the possible involvement of Octn2 in the intestinal absorption of L-carnitine (Yokogawa et al., 1999). OCTN2 transports L-carnitine in a Na^+-dependent fashion

and small organic cations such as TEA in a Na^+-independent fashion (Wagner et al., 2000; Ohashi et al., 2001). OCTN2 also transports many drugs including β-lactam antibiotics cephaloridine (Ganapathy et al., 2000) and calcium channel blocker verapamil (Grube et al., 2006). In the Caco-2 model, hOCTN2 has been localized to the apical membrane using immunohistochemical technique, suggesting a potential role in mediating absorption of L-carnitine and cationic drug substrates (Elimrani et al., 2003). Despite the demonstrated expression and localization of OCTN2 in the intestine, their role in mediating drug absorption has not been fully established.

G. Organic Anion Transporting Polypeptides (OATP/SLCO Superfamily)

Organic anion transporting polypeptides (OATPs, SLCO superfamily) mediate bidirectional, Na^+-independent, pH-dependent transport of anions (Hagenbuch and Meier, 2004). Known substrates cover a wide range of chemical structure, including bile salts, steroid hormones and conjugates, thyroid hormones, and various drugs such as atorvastatin, fexofenadine, bencylpenicillin and enalapril, which are generally anionic amphipathic molecules with a molecular mass greater than 450 Daltons (Hagenbuch and Meier, 2004). Despite widespread study of OATPs expression and function in liver and other tissues, their role in the intestine remains unclear and controversial. Although multiple OATP isoforms have been suggested to be expressed in the intestine, only three of them have shown functional activity, however, none of them are localized in the basolateral membrane of the intestine.

G.1. Oatp1a5 (Slco1a5)

The first identified OATP isoform in the intestine is Oatp1a5 (Slco1a5, previously rOatp3), which was cloned from rat small intestine and localized to the apical membrane (Walters et al., 2000). It was suggested that Oatp1a5 plays a role as an influx transporter in the intestinal absorption of fexofenadine in rats (Kikuchi et al., 2006). However, the human homolog of this transporter has not been cloned (Hagenbuch and Meier, 2004), therefore, extrapolation of the results involving Oatp1a5 from rats to humans is questionable.

G.2. OATP1A2 (SLCO1A2)

OATP1A2 (SLCO1A2, previously OATP-A) is the first discovered human OATP transporter, which was originally cloned from a human liver cDNA library, but showed its strongest expression in human brain (Kullak-Ublick et al., 1995). Beside transporting

endogenous substances such as bile acids and steroid conjugates, OATP1A2 also transports some important pharmaceutical drugs such as the antihistamine fexofenadine (Cvetkovic et al., 1999), the HIV protease inhibitor saquinavir (Su et al., 2004), the anticancer agent methotrexate (Badagnani et al., 2006) and the antibacterial agent levofloxacin (Maeda et al., 2007). The oral bioavailability of metabolically stable fexofenadine in humans was significantly reduced when administered in the presence of fruit juices (Dresser et al., 2002; Dresser et al., 2005). *In vitro* results have demonstrated that grapefruit, orange, and apple juices inhibit fexofenadine transport by OATP1A2 suggesting that the observed decrease in oral bioavailability may result from inhibition of OATP1A2-mediated absorption (Dresser et al., 2002). More recently, it has been shown that OATP1A2 is expressed in the apical domain of intestinal enterocytes (Glaeser et al., 2007). However, the studies from other groups did not find the presence of *OATP1A2* mRNA in human intestines, casting doubt upon the role OATP1A2 in intestinal drug absorption (Tamai et al., 2000; Hilgendorf et al., 2007; Meier et al., 2007). In addition, the Michaels-Menten constant (K_m) of fexofenadine uptake by OATP1A2 is 6.4 μM (Cvetkovic et al., 1999). Following oral administration of a fexofenadine tablet, a concentration of greater than 1 mM in the intestinal lumen is achieved, indicating that OATP1A2 could be saturated completely and fail to play an important role even assuming its presence in human intestine.

G.3. OATP2B1 (SLCO2B1)

Initial investigation of human OATP isoform expression in the intestine has revealed the presence of mRNAs for OATP2B1 (previously OATP-B), OATP3A1 (previously OATP-D), and OATP4A1 (previously OATP-E) (Tamai et al., 2000). However, recent studies using real-time PCR showed that the expression of OATP2B1 is much higher than the other two

subtypes (Sai et al., 2006; Hilgendorf et al., 2007). OATP2B1 protein is localized at the brush-border (apical) surface of both human small intestine (Kobayashi et al., 2003) and Caco-2 cells (Sai et al., 2006). In contrast, it is localized to the basolateral membrane in the liver, showing an organ-specific membrane localization pattern. Functional studies demonstrated that OATP2B1 transports bile acids and endogenous sulfate conjugates such as estrone-3-sulfate (Nozawa et al., 2004), and the greater activity at lower pH broadens its specificity to include some clinically used drugs such as the HMG-CoA reductase inhibitor pravastatin, the antihistamine fexofenadine and the antidiabetic glibenclamide (Kobayashi et al., 2003). These studies clearly suggest a potential role for OATP2B1 in the intestinal absorption of anionic or zwitterionic drugs such as pravastatin and fexofenadine. Citrus juices and constituents are also able to inhibit OATP2B1 activity, identifying OATP2B1 as a potential site for diet-drug interactions (Satoh et al., 2005). The physiological and pharmacological roles of OATP2B1 in intestinal absorption may also vary between individuals as a single nucleotide polymorphism of this transporter in humans leads to over 50% reduction of its activity (Nozawa et al., 2002).

H. Multiple-Resistance Associated Proteins (MRPs)

One of the largest sub-families of the ATP-binding cassette (ABC) transporters affecting drug disposition is the ABCC family (multidrug resistance-associated protein, MRP). MRPs can transport a large range of organic anions, including anionic drugs and drugs conjugated to glutathione (GSH), sulfate, or glucuronate, in contrast to P-gp transporting lipophilic cationic compounds (Reid et al., 2003b). MRPs 1-5 are expressed in the intestine with differential expression profiles in human intestinal segments (Zimmermann et al., 2005), however, only MRP2, MRP3 and MRP4 showed evidence of protein expression and functional activities in the intestine and are discussed here.

H.1. MRP2 (ABCC2)

MRP2 (ABCC2) is localized exclusively to the apical membrane of polarized cells, such as hepatocytes, renal proximal tubule epithelia and intestinal epithelia (Buchler et al., 1996). This localization supports the function of MRP2 in the terminal excretion and detoxification of endogenous and xenobiotic organic anions, particularly the substances conjugated with glutathione, glucuronate, or sulfate (Zamek-Gliszczynski et al., 2006). Compared to the extensively studied and established role of MRP2 in detoxification of drugs in the liver, its role in the intestine has been less well studied. MRP2 is highly expressed in human jejunum (Hilgendorf et al., 2007). Studies utilizing wild-type and Mrp2 deficient rats have clearly demonstrated a significantly reduced intestinal excretion of MRP2 substrate DNP-SG in the deficient rat after intravenous administration of the unconjugated parent (Gotoh et al., 2000). However, MRP2 may be less important than P-gp in limiting the absorption of drugs, because an 8.5-fold increase in oral bioavailability of paclitaxel in Mdr1a^{-}/b^{-} mice was observed compared to the wild-type mice, whereas change was not

detected in Mrp2$^{-/-}$ mice, although Mrp2 plays an important role in biliary excretion of paclitaxel (Lagas et al., 2006).

H.2. MRP3 (ABCC3)

MRP3 transports organic compounds conjugated to glutathione, sulfate, or glucuronate, also bile salts and unconjugated drugs such as methotrexate (Borst et al., 2007). Studies in knockout mice have shown that Mrp3 contributes to the transport of morphine-3-glucuronide (Zelcer et al., 2005) and fexofenadine (Matsushima et al., 2008a; Tian et al., 2008a) from the liver into blood. *MRP3* mRNA is highly expressed in human jejunum with a similar level as P-gp (Hilgendorf et al., 2007), and increases to even higher levels in ileum and colon (Zimmermann et al., 2005). Mrp3 protein is localized in the basolateral membrane in rat small intestine and colon (Rost et al., 2002). Mrp3 plays a minor role (<20%) in the basolateral efflux of bile acids (Zelcer et al., 2006; Rao et al., 2008). Studies in rat intestinal basolateral membrane vesicles (BLMV) demonstrated uptake of MRP3 substrates estradiol-17-β-glucoronide and taurocholate is ATP-dependent and correlated with the expression levels of Mrp3 in the BLMV prepared from rat jejunum, ileum, and colon (Shoji et al., 2004), which showed the functional activity of Mrp3 in the basolateral membrane of rat intestine. Clearly, further studies will be necessary for a more complete understanding of the physiological and pharmacological roles of MRP3 in intestinal absorption.

H.3. MRP4 (ABCC4)

MRP4, the fourth member of the ABCC family, was initially identified as a homolog of MRP1 (ABCC1) by screening databases of human expressed sequence tags (Kool et al., 1997). MRP4 was the first identified MRP isoform without a third (N-terminal) membrane spanning domain (Kool et al., 1997). The “short” members of the family, MRP4, MRP5,

MRP8 and MRP9, are distinguished by their ability to confer resistance to nucleoside based agents and to transport cyclic nucleotides (Kruh et al., 2007). The substrate specificity of MRP4 is particularly broad and it transports cAMP, cGMP, p-aminohippurate, urate, dehydroepiandrosterone sulfate, methotrexate, and estradiol-17β-D-glucuronide as well as adefovir (Schuetz et al., 1999; van Aubel et al., 2002; Zelcer et al., 2003; van Aubel et al., 2005). MRP4 is the only transporter in ABCC family that shows tissue specific localization: it assumes basolateral localization in most organs such as liver (Rius et al., 2003), whereas in kidney proximal tubules it is located at the apical surfaces (van Aubel et al., 2002). A recent study clearly showed the protein expression of Mrp4 in mouse intestine (Belinsky et al., 2007). $Mrp4^{-/-}$ mice were significantly more sensitive to adefovir and histopathological analysis indicated that small intestine and colon suffered increased toxicity in the knockout mice as compared to wild-type mice (Belinsky et al., 2007). These results supported the notion that MRP4 reduces the accumulation of adefovir entering into the enterocytes from the blood side, and protects the enterocytes from adefovir cytotoxicity. The results also provide circumstantial evidence that MRP4 is present in the basolateral membrane of the enterocytes. However, Mrp4 was localized primarily to the basal cytoplasmic region, but not distinctly localized to the basolateral membrane of enterocytes in rats (Johnson et al., 2006).

I. CONCLUSION

Understanding the roles of transport proteins in the basolateral membrane of the intestine represents a major missing link in transporter mediated drug absorption. The majority of drug transporters in the intestine have been identified in the apical membrane with very few identified in the basolateral membrane. In addition, the functional roles of some known basolateral transporters in intestinal absorption are still not clear. For example, MRP3 is highly expressed in the basolateral membrane of the intestine and capable of transporting bile acids, however, its role in bile acids absorption was proved to be neglectable by the studies using Mrp3 knockout mice (Zelcer et al., 2006; Rao et al., 2008). Without significant progress in understanding the roles and functions of intestinal basolateral transporters, a complete understanding of vectorial transport in drug absorption will remain lacking.

Compared to the slow progress in the research on the mechanisms of basolateral efflux in drug absorption, several vectorial transport systems for nutrient absorption have been established and lessons can be learned from the basolateral efflux mechanisms in nutrient absorption. The first example of a vectorial transport system was identified in intestinal absorption of D-glucose (Hediger and Rhoads, 1994), which was described as a classic model of transcellular transport (Lodish et al., 2000). In this model, Na^+-K^+ pump in the basolateral membrane transports Na^+ out of the cells to generate an inward Na^+ gradient, which is utilized by the apical sodium/glucose cotransporters 1 (SGLT1) for uphill transport of glucose into the enterocytes. Facilitative glucose transporter (GLUT2), which possesses similar substrate specificity to SGLT1 but is expressed in the basolateral membrane, mediates the egress of glucose into the blood circulation. The features of this model include:

1. Apical uptake transporters are secondary active transporters that mediate uphill transport into cells by utilizing ion gradients;

2. Basolateral efflux transports are facilitative transporters that mediate downhill transport of substrates, and their affinity is lower than apical transporters in order not to be saturated by the high intracellular concentration.

This model of vectorial transport can be applied to the absorption systems for other nutrients. For example, the neutral amino acids transport system consists of $ATB^{0,+}$ (Na^+ and Cl^- dependent) plus B^0AT1 (Na^+ dependent) in the apical membrane and TAT1 (facilitative) plus LAT2 (exchanger) in the basolateral membrane; small peptide transport system is the apical PepT1 and/or HPT1 (H^+ dependent) coupled to the basolateral peptide transporter (facilitative); bile acids transport system includes the apical ASBT (Na^+ dependent) and the basolateral OSTαβ (facilitative). Identification of OSTαβ as the major basolateral efflux transporter of bile acids represents one of the most important steps forward in this area in the past 5 years. Knocking out Ostα in mice almost abolished transileal transport of taurocholate (Rao et al., 2008), which indicted that basolateral transporters are as important as apical transporters in efficient transcellular transport. Many nutrient transporters also transport drugs containing the chemical moiety that can be recognized by the transporters. Therefore, it is desired to continue studying these vectorial transport systems of nutrients and explore their pharmacological functions in drug absorption. According to this vectorial model, OATP2B1, which can utilize H^+ gradient in small intestine, may represent another vectorial transport system for drugs when coupled with the basolateral transporters with similar substrate selectivity such as MRPs since they have similar substrate specificity in transporting anionic and zwitterionic compounds. Apical and basolateral OCTs also have similar substrate

specificity; however, efflux by OCTs is thermodynamically unfavorable and thus inefficient, which may not play a role in intestinal absorption of cationic compounds under physiological conditions.

The vectorial transport system in the intestine is fundamentally different from those in the liver and the kidney, which consist of a facilitative transporter, for the basolateral uptake of drugs into the hepatocytes or renal tubular cells, and a primary active transporter in ABC superfamily for the apical efflux of drugs into the bile or urine. For example, hepatic excretion of pravastatin involves the basolateral uptake via OATP1B1 and the apical efflux by MRP2 (Matsushima et al., 2005), whereas renal excretion of adefovir involves the basolateral uptake via organic anion transporter 1 (OAT1) (Cihlar et al., 1999) followed by efflux into the urine via the apical MRP4 (Imaoka et al., 2007). Clearly, intestinal drug transport lags behind the current understanding of hepatic and renal drug transport. The strategies that are successful in studying the hepatic and renal vectorial systems should be applied to identify those systems for drug absorption in the intestine. Those technological advances include transcellular transport studies in double transfected cells with uptake and efflux transporters in two membranes (Ito et al., 2005) and organ perfusion techniques in transporter knockout mice (Tian et al., 2008a). Renal tubular cell lines LLC-PK1 and MDCKII cells are routinely used to construct double transfected transporter systems. Differences in localization of the transporters in the enterocytes and renal cells should be noticed. For example, OATP2B1 is an apical transporter in the intestine (Kobayashi et al., 2003). However, it is expressed in the basolateral membrane upon transfection into MDCKII cells (Kopplow et al., 2005), therefore, it will not form a vectorial transport system with a basolateral transporter to study intestinal drug transport involving OATP2B1. Transport of

bile acids using everted gut sac of Ostα$^{-/-}$ and Ostα$^{-/-}$Mrp3$^{-/-}$ mice (Rao et al., 2008) represents a breakthrough in this area because it was the first study of the *in vivo* functions of intestinal basolateral transporters. This technique will be applied to study other intestinal basolateral transporters in the near future.

To fully establish the function of intestinal basolateral transporters, information from clinical studies is needed. Recently, understanding the impacts of genetic variations in drug transporters on drug disposition is beginning to emerge. Clearly, a loss or reduction of transporter activities due to genetic variation in intestinal basolateral transporters could potentially limit intestinal absorption of their substrates. Therefore, clinical studies on the effect of genetic variation in intestinal basolateral transporters on drug absorption will be an important area of future research. The oral bioavailability is determined by both the fraction absorbed in the intestine and the presystemic elimination, mainly in the liver and the intestine. Because many transporters are expressed in both intestine and liver, clarification of mechanisms of altered drug bioavailability in the intestine or liver will be difficult. A human *in vivo* jejunal perfusion technique enables direct determination of the effective jejunal permeability of drugs (Tannergren et al., 2003b) and will help understand mechanisms about intestinal transporters from clinical data in the future.

In conclusion, the research on intestinal basolateral transporters will lead to a more complete understanding of carrier-mediated intestinal drug absorption and help design and develop successful oral drugs and delivery systems. Further, understanding of species differences in intestinal transporter expression and function may lead to choosing the right animal models to predict absorption in humans. Strategies to utilize intestinal vectorial transport systems may aid in improving absorption of poorly absorbed drugs. Knowledge of

drug-drug interactions via transporters and their genetic variations may reduce the potential of therapeutic failure and toxicity when co-administration of oral drugs is needed.

J. RATIONALE AND OVERVIEW OF PROPOSED RESEARCH

Polarized expression and functional synergy of drug transporters in the apical and basolateral membranes of epithelial cells are critical in determining the net transcellular transport and, ultimately, in governing drug disposition in the body. Compared to the abundant information about apical transporters in the intestine, understanding of the roles of intestinal basolateral transporters has remained missing in carrier mediated drug absorption. Indeed, very few transporters are identified in the basolateral membrane and function of known basolateral transporters in intestinal drug absorption is often unclear. However, several vectorial transport systems in the absorption of nutrients, such as glucose and bile acids, have been elucidated, clearly showing that the basolateral transporters are as important as the apical transporters in an efficient transcellular transport of the substrates in the intestine. The overall objective of this project is to understand the roles of the basolateral transporters in intestinal absorption of drugs and prodrugs. *The central hypothesis of this project is that basolateral transporters can determine the transcellular transport rate when (i) apical transporters mediate uptake efficiently, or (ii) prodrugs diffuse through the apical membrane and are metabolized efficiently within the enterocytes into hydrophilic drugs.* Transport of fexofenadine and adefovir dipivoxil was investigated in Caco-2 cell monolayers to test the two aspects of this hypothesis.

Fexofenadine, a zwitterionic anti-allergy medicine, represents a large class of drugs that contain both acidic and basic groups and possess molecular weight over 300 (Table 1.3). The charged nature of fexofenadine at the pH values encountered in the gastrointestinal lumen makes it hard to permeate the cell membranes of enterocytes. In addition, fexofenadine is an excellent substrate for P-glycoprotein (P-gp) (Cvetkovic et al., 1999),

which would make its intestinal absorption even less effective. However, fexofenadine achieves greater bioavailability than that expected from its physicochemical properties and efflux by P-gp. Therefore, vectorial transport systems, including apical uptake and basolateral efflux transporters, have been suggested to explain this dichotomy. Literature knowledge of intestinal transporters for fexofenadine is limited to the apical transporters. OATP1A2 was reported to mediate apical uptake of fexofenadine in the intestine (Cvetkovic et al., 1999), however, whether this transporter is expressed in the intestine is still controversial.

Adefovir is a nucleoside drug for treatment of hepatitis B (Table 1.3). Mrp4 knockout mice showed severe toxicity in the intestine compared to wild-type mice, which indicated that Mrp4 prevents entry of adefovir into enterocytes from the mesenteric circulation, and thus protects the intestine. The prodrug of adefovir, adefovir dipivoxil (Table 1.3), is completely metabolized into active adefovir in the enterocytes during absorptive transport. Therefore, MRP4 may be a good candidate as a basolateral transporter that mediates egress of adefovir formed from its prodrug during oral absorption.

Besides the main body of the studies, this dissertation project will continue the research in the Thakker laboratory on the role of OCT transporters in drug absorption by testing whether OCT1 can mediate the basolateral efflux of cations in the intestine. Theoretically, efflux by OCT1 is thermodynamically unfavorable. However, OCT1 is the only cation selective transporter expressed in the basolateral membrane of the intestine, therefore to evaluate the efflux function of OCT1 is of interest. Disposition of diamidine compounds like pentamidine (Table 1.3) in the body suggests that the diamidine drug, furamidine (Table 1.3) may be a substrate for OCT1. Furamidine diamidoxime (Table 1.3) is

metabolized to active furamidine in the enterocytes, and OCT1 might mediate the egress of the active drug from enterocytes to the blood circulation.

The major goals of this research plan are:

Specific Aim 1: To elucidate vectorial transport of fexofenadine.

1a) Determine substrate activity toward apical transporters in singly transfected cells.

1b) Evaluate the role of MRP3 and OSTαβ in the basolateral egress of fexofenadine in Caco-2 cells.

Specific Aim 2: Evaluate the role of MRP4 in intestinal transport of adefovir dipivoxil.

2a) Determine cellular localization of MRP4 in Caco-2 cells.

2b) Evaluate the role of MRP4 by knocking it down in Caco-2 cells using MRP4-specific siRNA.

Specific Aim 3: Evaluate the efflux function of hOCT1 in the basolateral membrane.

3a) Determine uptake and efflux of furamidine by hOCT1 in hOCT1 transfected cells.

3b) Evaluate the role of hOCT1 in transport of furamidine diamidoxime in hOCT1 transfected MDCKII cells.

Several *in vitro* cell models including transfected cell models and Caco-2 cells that retain intestinal transport mechanisms are utilized to identify apical uptake transporters and basolateral efflux transporters and determine their relative roles in intestinal drug absorption. This project will generate new knowledge about: 1. novel substrate activity of test compounds towards intestinal transporters; 2. expression and localization of candidate

transporters in Caco-2 cells; 3. the roles and importance of the basolateral transporters in intestinal absorption of drugs and prodrugs; 4. the factors influencing the importance of the intestinal basolateral transporters in drug absorption. This information will provide a theoretical foundation in understanding drug-drug and food-drug interactions, predicting individual variability in drug absorption, designing drugs that will be effective when administered orally, and developing strategies to optimize oral absorption.

Table 1.1. Major Human Intestinal Nutrient Transporters

Protein Name	*Gene Symbol*	*Localization*[a]	*Transport type*[b] */Coupling ions*	*Substrates*
$ATB^{0,+}$	SLC6A14	AP	C/Na^+, Cl^-	Neutral and cationic amino acids
B^0AT1	SLC36A1	AP	C/Na^+	Neutral amino acids
$b^{0,+}AT$	SLC7A9	AP	E	Cationic amino acids, large neutral amino acids
LAT1	SLC7A5	BL	E	Large neutral amino acids
LAT2	SLC7A8	BL	E	Large neutral amino acids
LAT3	SLC43A1	Unknown	F	Large neutral amino acids
LAT4	SLC43A2	BL	F	Large neutral amino acids
y^+LAT1	SLC7A7	BL	E	Cationic and neutral amino acids
y^+LAT2	SLC7A6	BL	E	Cationic and neutral amino acids
TAT1	SLC16A10	BL	F	Aromatic amino acids
PepT1	SLC15A1	AP	C/H^+	Di- and tripeptides
HPT1	CDH17	AP	C/H^+	Di- and tripeptides
CNT1	SLC28A1	AP	C/Na^+	Pyrimidine nucleosides, adenosine
CNT2	SLC28A2	AP	C/Na^+	Purine nucleosides, uridine
CNT3	SLC28A3	AP	C/Na^+	Broadly selective for pyrimidines and purines
ENT1	SLC29A1	BL	F	Purine and pyrimidine nucleosides
ENT2	SLC29A2	BL	F	Purine and pyrimidine nucleosides and nucleobases
ENT4	SLC29A4	AP	F	Adenosine, organic cations
ASBT	SLC10A2	AP	C/Na^+	Bile acids
OST$\alpha\beta$		BL	F	Bile acids, steroids

[a] AP: apical; BL: basolateral.

[b] C: cotransporters; E: exchangers; F; facilitative transporters.

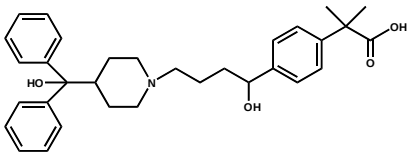
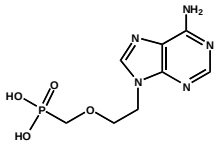
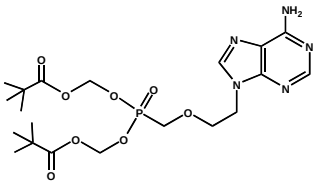
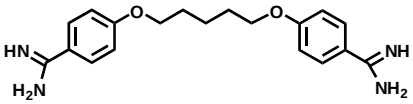
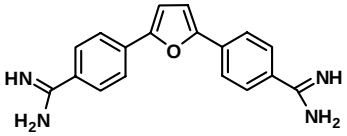
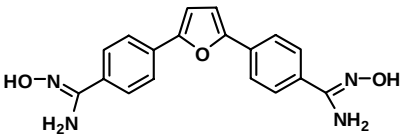
Table 1.2 Major Human Intestinal Transporters for Xenobiotics

Protein Name	Gene Symbol	Localization[a]	Transport type[b] /Coupling ions	Substrates
OCT1	SLC22A1	BL	F	Organic cations
OCT3	SLC22A3	AP	F	Organic cations
OCTN1	SLC22A4	Unknown	F or E/H^+	Ergothioneine, organic cations
OCTN2	SLC22A5	AP	C/Na^+ (L-carnitine) or F (organic cations)	L-carnitine; organic cations
OATP2B1	SLCO2B1	AP	F	Bulky organic anions
OATP1A2	SLCO1A2	AP	F	Bulky organic anions
MDR1/P-gp	ABCB1	AP	ABC	Broad substrate selectivity, preferring cationic compounds
BCRP	ABCG2	AP	ABC	Broad substrate selectivity
MRP2	ABCC2	AP	ABC	Organic anions, conjugates
MRP3	ABCC3	BL	ABC	Organic anions, conjugates and bile salts
MRP4	ABCC4	BL	ABC	Organic anions, conjugates, bile salts and nucleotides

[a] AP: apical; BL: basolateral.

[b] C: cotransporters; E: exchangers; F; facilitative transporters; ABC: ABC transporters.

Table 1.3. Structures and Physicochemical Properties of Test Compounds

Name	Structure	Log P	pK_a(s)	Molecular Weight
Fexofenadine		4.8	4.3, 9.5	501.66
Adefovir		-2.1	2.0, 6.8	273.19
Adefovir dipivoxil		2.4		501.47
Pentamidine		1.7	11.6	340.42
Furamidine		2.7	10.4, 11.8	304.35
Furamidine diamidoxime		3.1	4.4, 5.2	336.34

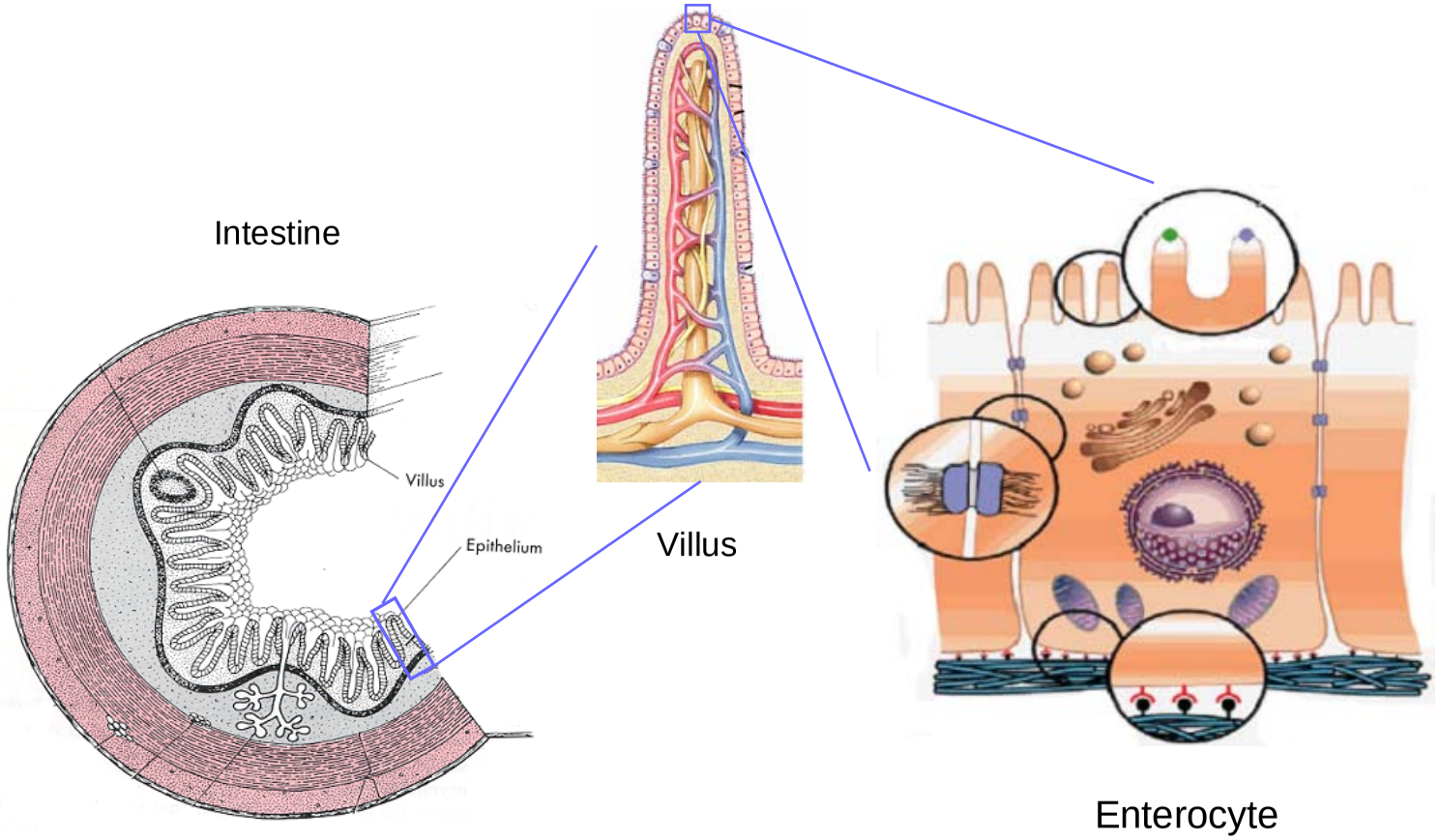


Figure 1.1. Barrier properties of intestinal epithelia. Epithelial cell monolayer constitutes a selective barrier for absorption. Tight junctions and two membranes are the permeation barrier for intestinal absorption. Abundant soluble and membrane bound enzymes in the enterocytes are the enzymatic barrier for intestinal absorption. (Sources: http://img.tfd.com/ and http://www.uic.edu)

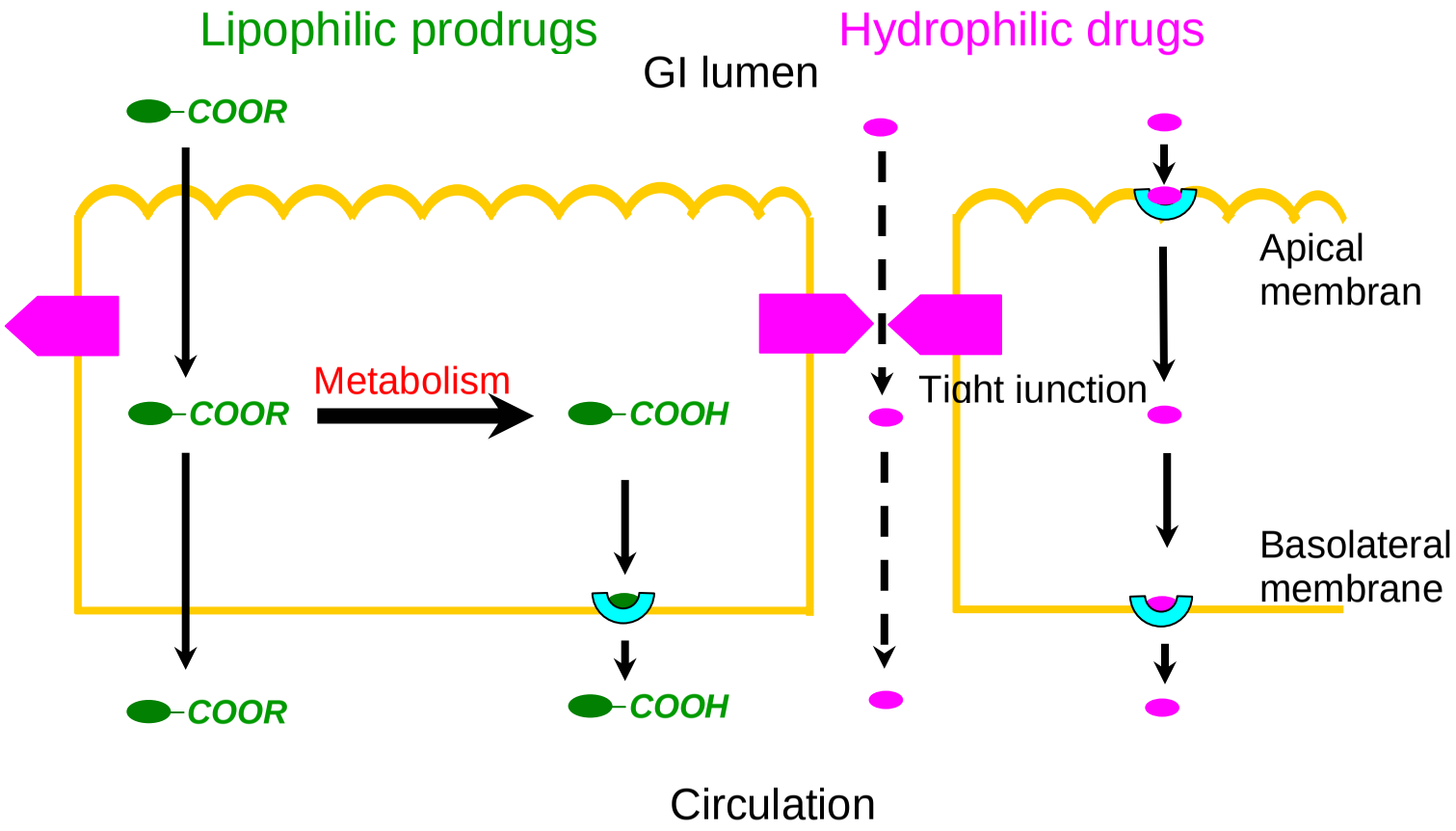


Figure 1.2. Roles of basolateral transporters in intestinal absorption of drugs and prodrugs. For hydrophilic drugs, basolateral transporters are as important as apical transporters in efficient transcellular transport of drugs in the intestine. For some prodrugs, carrier-mediated basolateral efflux may control the transcellular transport of the prodrugs.

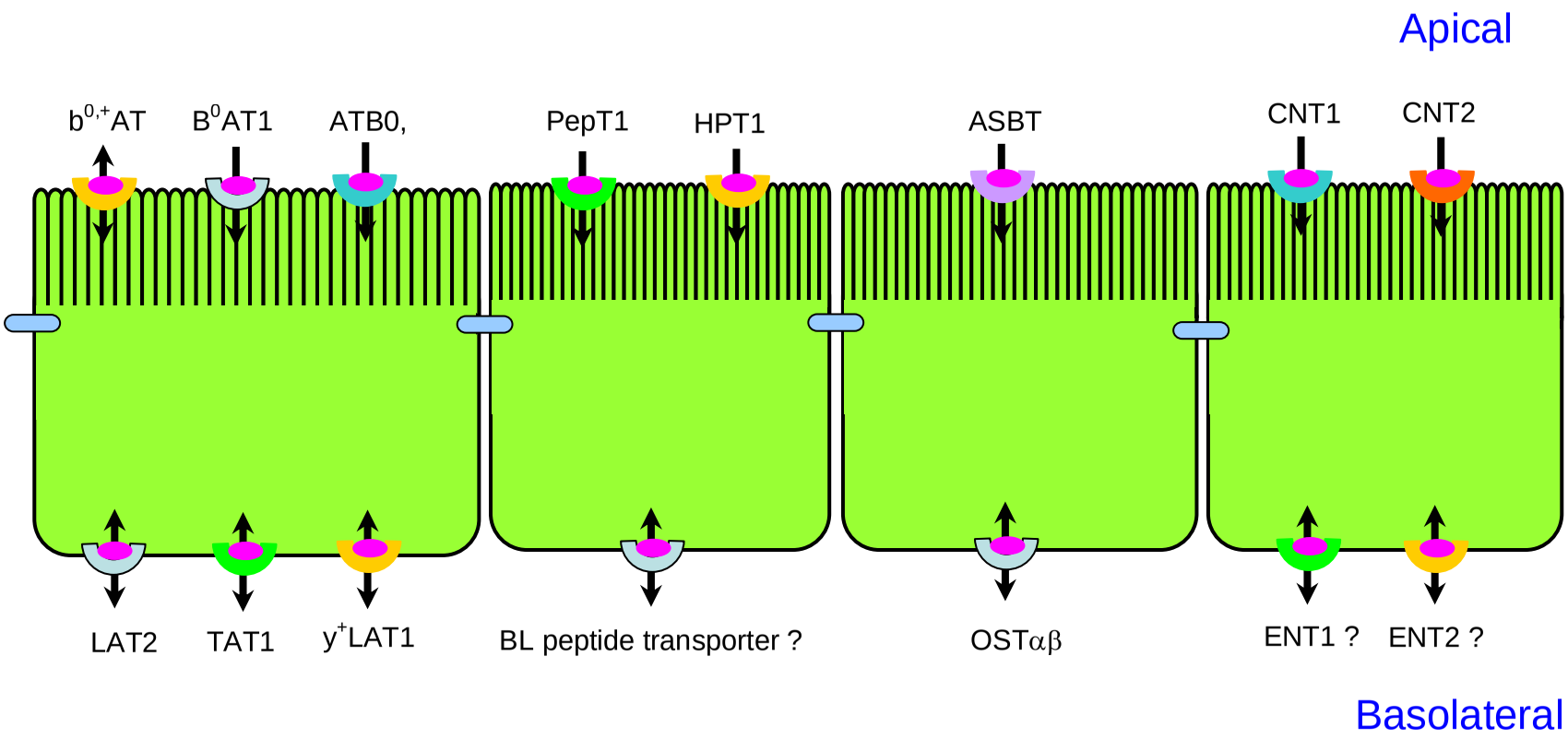


Figure 1.3. Major human intestinal nutrient transporters involved in drug absorption. Arrows denote transport direction. Unidentified or proposed transporters also are indicated (?).

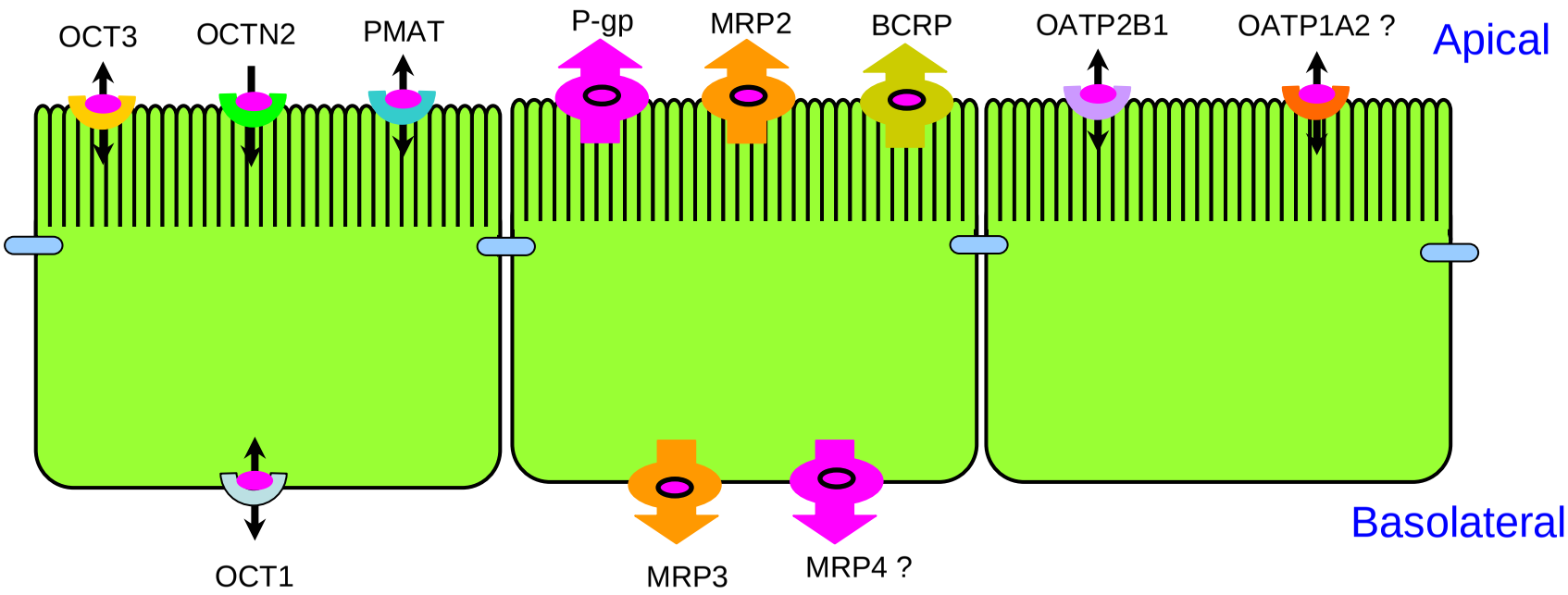


Figure 1.4. Major human intestinal transporters transporting xenobiotics. Arrows denote transport direction. Unidentified or proposed transporters also are indicated (?). Localization of OCT1 indicates the localization in human intestine, but not Caco-2 cells.

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CHAPTER 2

VECTORIAL TRANSPORT OF FEXOFENADINE ACROSS CACO-2 CELLS: INVOLVEMENT OF APICAL UPTAKE AND BASOLATERAL EFFLUX TRANSPORTERS

This chapter will be submitted to the *Journal of Pharmacology and Experimental Therapeutics* and is presented in the style of that journal.

A. ABSTRACT

Fexofenadine (Allegra®) is a non-sedative antihistamine that exhibits good oral bioavailability despite its zwitterionic chemical structure and efflux by P-gp. Evidence exists that multiple uptake and efflux transporters play a role in hepatic disposition of fexofenadine. However, the roles of specific transporters and their inter-relationship in intestinal absorption of this drug are not yet clear. The purpose of this study was to elucidate vectorial absorptive transport of fexofenadine across Caco-2 cells involving specific apical uptake and basolateral efflux transporters. Studies with cellular models (e.g. MDCKII and CHO cells) expressing single transporters showed that OATP2B1 stimulated uptake of fexofenadine at pH 6.0. Apical uptake of [^{3}H]fexofenadine into Caco-2 cells was decreased by 45% with 200 μM estrone 3-sulfate (E1S), an OATP inhibitor, at pH 6.0, but not at pH 7.4, indicating that OATP2B1 mediates apical uptake of fexofenadine into these cells. Examination of fexofenadine efflux from pre-loaded Caco-2 cells in the presence and absence of the MRP inhibitor MK-571 and the P-gp inhibitor GW918 showed that P-gp and MRP both play a role in the apical efflux, whereas MRP plays a role in the basolateral efflux. These results also showed that while OSTαβ is functionally active in the basolateral membrane of Caco-2 cells, it does not play a role in the transport of fexofenadine as evidenced digoxin and E1S, substrates for OSTαβ, not having any effect on its transport. MK-571 decreased the absorptive transport of fexofenadine by 17%. However, the decrease in absorptive transport by MK-571 was 42% when P-gp was inhibited by GW918. The results showed apical entry of fexofenadine into Caco-2 cells is predominantly attenuated by P-gp, with a small contribution by MRP2; whereas basolateral efflux is predominantly mediated by MRP3. It appears that apical efflux by P-gp may be the rate-limiting step in the absorptive transport of

fexofenadine across intestinal epithelium; however, basolateral efflux by MRP3 may control absorption rate of fexofenadine where P-gp expression/effect is low.

B. Introduction

Fexofenadine, a zwitterionic anti-allergy medicine, has been used as a probe to investigate carrier mediated transport processes due to its low passive permeability and relatively low extent of metabolism (Molimard et al., 2004). Fexofenadine is charged at physiological pH because it is a zwitterion with two pK_a's of 4.25 and 9.53 (Yasui-Furukori et al., 2005). Fexofenadine is an excellent substrate for P-glycoprotein (P-gp) and its limited brain penetration has been suggested to arise from P-gp mediated efflux and low intrinsic membrane permeability (Cvetkovic et al., 1999; Chen et al., 2003; Polli et al., 2003; Petri et al., 2004). Despite its zwitterionic chemical structure and efflux by P-gp, fexofenadine has 33% bioavailability in humans (Dresser et al., 2005). Both hepatic and renal excretion contribute to elimination of fexofenadine in the body (Tahara et al., 2006) because it is a substrate for renal organic anion transporter 3 (OAT3) (Tahara et al., 2006) and hepatic organic anion transporting polypeptides 1B3 (OATP1B3) and 1B1 (OATP1B1) (Shimizu et al., 2005; Matsushima et al., 2008b). Hepatic disposition of fexofenadine has been studied extensively and multiple transporters have been found to be involved. After uptake by OATP1B3 and OATP1B1 into hepatocytes, fexofenadine is excreted into the bile by multiple efflux transporters in the canalicular membrane including multidrug resistance-associated protein 2 (Mrp2) (Tian et al., 2008b) and bile salt export pump (BSEP) (Matsushima et al., 2008a). Surprisingly, P-gp does not seem to contribute to the biliary excretion of fexofenadine, at least in mice, although it plays a major role in the efflux of fexofenadine in the intestine and blood-brain barrier (Tahara et al., 2005b). In addition, efflux by Mrp3 in the sinusoidal membrane attenuates hepatic excretion of fexofenadine (Matsushima et al., 2008a; Tian et al., 2008a).

Fexofenadine was initially used as an *in vivo* P-gp probe (Cvetkovic et al., 1999; Yasui-Furukori et al., 2005; Shimizu et al., 2006). Interestingly, using a human *in vivo* jejunal perfusion technique, concurrent administration of verapamil or ketoconazole, P-gp inhibitors, failed to increase the effective jejunal permeability of fexofenadine (Tannergren et al., 2003a; Tannergren et al., 2003b). This observation may be explained when one considers that verapamil and ketoconazole not only inhibit P-gp, but also other transporters that facilitate absorptive transport of fexofenadine in the intestine, for example, OATPs. This notion was supported by a clinical study, which showed that fruit juices decreased oral absorption of fexofenadine by inhibiting the uptake transporter in apical membrane of enterocytes (Dresser et al., 2002). Fruit juices inhibit multiple OATP isoforms including OATP1A2 (SLCO1A2, formerly OATP-A) (Dresser et al., 2002) and OATP2B1 (SLCO2B1, formerly OATP-B) (Satoh et al., 2005). Fexofenadine is a substrate for OATP1A2 (Cvetkovic et al., 1999); however, conflicting results have been reported in the literature about the substrate activity of fexofenadine towards OATP2B1 (Nozawa et al., 2004; Shimizu et al., 2005). *OATP1A2* gene does not appear to be expressed in human jejunum (Tamai et al., 2000; Hilgendorf et al., 2007; Meier et al., 2007), the major absorption site of fexofenadine (Petri et al., 2006), whereas OATP2B1 is highly expressed in this region (Hilgendorf et al., 2007; Meier et al., 2007) and has been localized in the apical membrane of human small intestine (Kobayashi et al., 2003). In addition, human organic cation transporter 3 (hOCT3, SLC22A3) (Muller et al., 2005) and human organic cation/carnitine transporter (hOCTN2, SLC22A5) (Elimrani et al., 2003) are also present in the apical membrane of enterocytes. They transport cations and zwitterions with 1-methyl-4-phenylpyridinium (MPP^+) and L-carnitine as their protypical substrates, respectively (Koepsell et al., 2007). It

is conceivable that these transporters, particularly hOCTN2, may play a role in the absorptive transport of fexofenadine because of its zwitterionic functionality.

Intestinal absorptive transport involves entry into the enterocytes across the apical membrane from gastrointestinal lumen followed by intracellular translocation and exit from the cells across the basolateral membrane. Depending on their relative efficiency and capacity, an uptake transporter in the apical membrane or an efflux transporter in the basolateral membrane can become rate-limiting for transepithelial movement (Cheeseman, 1992). However, it is still unclear whether basolateral transporters play an important role in intestinal transport of fexofenadine, and if so, which one. Limited information is available about transporters that are expressed in the basolateral membrane in intestine. Among the known basolateral transporters in the intestine, two classes of transporters may contribute to basolateral efflux of fexofenadine: heteromeric organic solute transporter αβ (OSTαβ) and multidrug resistance-associated protein 3 (MRP3).

Ostαβ is highly expressed in ileum as evidenced by the presence of mRNA levels in this tissue; the expression appears to be largely restricted to the lateral and basal membranes of ileal enterocytes (Ballatori et al., 2005; Dawson et al., 2005). It was recently established that Ostαβ plays the predominant role in egress of bile acids through the ileal basolateral membrane - transileal transport of taurocholate was reduced by >80% in Ostα knockout mice compared to wild-type mice (Rao et al., 2008). Transport activity of Ostαβ requires the coexpression of two distinct gene products, a predicted seven-transmembrane domain protein (OSTα) and a putative single-transmembrane domain ancillary polypeptide (OSTβ) (Wang et al., 2001; Seward et al., 2003). Transport mediated by OSTαβ is facilitated diffusion, indicating this transporter plays dual roles of basolateral efflux in intestinal absorption and

basolateral uptake in intestinal secretion (Ballatori et al., 2005). Besides bile acids, Ostαβ is capable of transporting other endogenous and xenogenous steroid compounds including E1S, digoxin, and prostaglandin E2 (Wang et al., 2001; Seward et al., 2003; Ballatori, 2005). Interestingly, these compounds are also substrates for OATPs.

Multidrug resistance-associated proteins (MRPs) belong to the subfamily C of the ABC gene superfamily (ABCC). The members of MRPs predominantly transport anionic substances, which contrasts with P-gp transporting lipophilic cationic compounds (Reid et al., 2003b). MRPs 1-5 are expressed with differential expression profiles in human intestinal segments; MRP3 is the most abundantly expressed isoform in the human intestine, followed by MRP2 (Zimmermann et al., 2005). In Caco-2 cells, MRP2 is the most highly expressed MRP isoform, followed by MRP3 and MRP4 (Prime-Chapman et al., 2004). MRP2 was localized to apical membranes in rat small intestine (Mottino et al., 2000). Basolateral localization was assumed for other MRPs (Deeley et al., 2006); however, only Mrp3 showed clear basolateral localization in rat small intestine and colon (Rost et al., 2002). MRP4 shows tissue-specific localization, which is expressed in apical membrane of kidney tubules (van Aubel et al., 2002) and in basolateral membrane of hepatocytes (Rius et al., 2003). However, its localization in enterocytes is unclear.

In this study, vectorial transport of fexofenadine involving specific apical uptake and basolateral efflux transporters was studied in Caco-2 cells. The results showed that OATP2B1 mediates apical uptake of fexofenadine and that P-gp, and to a lesser extent MRP2, attenuates the uptake across this membrane. The results also show that MRP3 mediates its efflux across the basolateral membrane, and may play a critical role in the intestinal absorption of fexofenadine under certain circumstances.

C. MATERIALS AND METHODS

Materials

MDCKII, CHO and Caco-2 cells were obtained from the American Tissue Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), F-12 Nutrient Mixture and Eagle's minimum essential medium (EMEM) with Earle's salts and L-glutamate, nonessential amino acids (NEAA, 100x), N-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES, 1M) and penicillin-streptomycin-amphotericin B solution (100x) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) and trypsin-EDTA solution (1x) were obtained from Sigma Chemical Co. (St. Louis, MO). Hank's balanced salt solution (HBSS) was obtained from Mediatech, Inc. (Herndon, VA). Geneticin and hygromycin B were obtained from Invitrogen Co. (Carlsbad, CA). [^{3}H]E1S (77 Ci/mmol), [^{3}H]L-carnitine and [^{3}H]MPP$^+$ (85 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]fexofenadine (53 Ci/mmol) was obtained as a gift from GlaxoSmithKline (Research Triangle Park, NC) and originally custom synthesized by Amersham Life Sciences (Piscataway, NJ). GW918 was obtained as a gift from GlaxoSmithKline (Research Triangle Park, NC). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

OCTN2 and OATP2B1 cDNAs in pcDNA3.1/hygro vector were provided by Professor Heyo K. Kroemer (Ernst Moritz Arndt University, Germany) and Professor Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany), respectively. hOCT3 cDNA in pSPORT1 vector was provided by Dr. Vadivel Ganapathy (Medical

College of Georgia, Augusta, GA) and was subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection

MDCKII cells were transfected with pcDNA3.1 empty vector or the vectors containing the full-length hOCTN2 and OATP2B1 cDNAs using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol. Transfectants were selected with 0.2 mg/ml hygromycin B for 10 days. A clone with the highest uptake activity with respect to the probe substrate, [^{3}H]L-carnitine and [^{3}H]E1S for hOCTN2 and OATP2B1, respectively, was chosen as a stably transfected cell line for further studies. The stably transfected MDCKII cells were cultured in DMEM with 10% FBS, 10% NEAA, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B plus 0.2 mg/ml hygromycin B. CHO cells, stably transfected with hOCT3, and the mock cells were constructed with a similar method. The transfectants were selected with 0.5 mg/ml geneticin, and further screened for the highest uptake activity of model substrate, [^{3}H]MPP$^+$. The stably transfected CHO cells were cultured in F-12 Nutrient Mixture with 10% FBS, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B plus 0.5 mg/ml geneticin. Caco-2 cells were cultured in EMEM, supplemented with 10% FBS, 1% NEAA, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Caco-2 cells were seeded at a density of 60,000 cells/cm^2 on Transwell^{TM} filters. Medium was changed the day after seeding, and every other day thereafter. The cells were cultured for 21-25 days before use. All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

RT-PCR

Total RNA was isolated from Caco-2 cells using TRIzol® reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed with 1 μg total RNA and random hexamer primers. Normalized first-strand cDNA from human ileum was a part of the Human Digestive System MTC™ panel (BD Clontech, Palo Alto, CA). Gene-specific primers were designed based on the published gene sequences (Seward et al., 2003). Multiplex amplification reactions (target gene and β-actin) were performed on a Robocycler 96 PCR system (Stratagene, La Jolla, CA) for 25 cycles. Reactions contained 5 ng of cDNA from tissue mRNA or 150 ng of cDNA from Caco-2 total RNA. The PCR products were analyzed by electrophoresis in agarose gel containing ethidium bromide.

Uptake Studies

Stably transfected MDCKII or CHO cells were grown in monolayers in 24-well plates. Medium was changed every other day. The cells were used 5-7 days post-seeding. Cells were pre-incubated for 30 minutes at 37ºC in transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES pH 7.4). Experiments were initiated by replacement of the transport buffer with 400 μl ml of dose solution. Uptake was determined within the linear uptake region after which the dose solution was aspirated and cells were washed three times with 4ºC transport buffer. Cells were dissolved in 500 μl 0.1 N NaOH/0.1% SDS for 4 hours with shaking. Radioactivity was determined by scintillation counting. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Uptake studies were also conducted in Caco-2 cells grown on Transwell™ filters. Cell monolayers were pre-incubated for 30 min at 37ºC in transport buffer. Experiments were initiated by replacement of the apical transport buffer with 400 µl of transport buffer containing test compounds in the absence or presence of inhibitors. Uptake was determined within the linear uptake region, after which the monolayers were washed three times and dissolved in 500 µl 0.1 N NaOH/0.1% SDS for 4 hours with shaking. Radioactivity was determined with the same method as the uptake study in the transfected cells.

Efflux Studies

Caco-2 cell monolayers were incubated for 1 hour with 100 µM fexofenadine in both apical and basolateral sides in the absence or presence of inhibitors. After washing both sides 3 times with cold transport buffer, efflux was determined over 2 minutes in the absence or presence of inhibitors. Then monolayers were dissolved in 300 µl 1% Triton X-100 for 4 hours with shaking and the solution was extracted with 1 ml ethyl acetate. The organic phase was evaporated to dryness under nitrogen gas, and the residue was reconstituted with 200 µl 25% methanol. The effluxed and cellular samples were analyzed by LC-MS/MS with cetirizine as an internal standard. The effluxed and cellular amounts together added up to the total loaded amount in the cells, and efflux clearance was calculated based efflux rates and cellular concentration after loading.

Transport Studies

Caco-2 cell monolayers were incubated for 30 min with the transport buffer with or without inhibitors. Transport studies were initiated by replacing donor chambers with 10 µM

fexofenadine in the absence or presence of inhibitors. Receptor chambers were sampled at selected times and analytes were quantified by LC-MS/MS.

LC-MS/MS Analysis of Fexofenadine

LC-MS/MS was performed using API-4000 triple-quadropole mass spectrometer (Applied Biosystems, Foster City, CA). Mobile phases of 0.1% formic acid in water and 0.1% formic acid in methanol were used with a 5-95% methanol linear gradient. The column used was a Phenomenex Synergy Polar RP, 30 x 2 mm (Phenomenex, Torrance, CA). A flow rate of 0.8 ml/min and injection volume of 15 µl were utilized. Samples were ionized using APCI and ions were monitored at the following transitions: 503/466 for fexofenadine and 389/201 for the internal standard, cetirizine.

Immunofluorescent Staining

Caco-2 cells were grown on 6.5-mm diameter Transwell™ inserts for the purpose of immunostaining. Inserts were washed three times in phosphate-buffered saline (PBS); similar washes were included between each of the following stages. Cells were fixed in 1% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific binding sites were blocked by incubation for 30 min with 5% normal sheep serum. Inserts were incubated in primary antibody M3II-9 (Covance, Berkeley, CA) (diluted to 5 µg/ml with PBS) for 60 min. Primary antibody was detected by incubation with Alexa Flour 488 goat anti-mouse antibody for 60 min. Inserts were washed and mounted in Permount* Mounting Medium (Fisher Scientific, Pittsburgh, PA). Staining was viewed using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY).

Data Analysis

Data are expressed as mean ± S.D. from three measurements unless otherwise noted. Statistical significance was evaluated using unpaired *t* tests or ANOVA followed by Dunnet's test for multiple comparisons as appropriate. The data were analyzed with SigmaStat 2.0 (Systat Software, Inc., San Jose, CA). Uptake clearance (CL_{up}) was determined using Equation (1):

$$CL_{up} = \frac{dX/dt}{C_o} \quad (1)$$

where *dX/dt* is the flux determined from the amount taken up (*X*) over time (*t*) during the experiment, and C_o is the initial concentration in the donor side. Kinetic constants (J_{max}, K_m *and* K_d) were obtained by fitting a model incorporating saturable and nonsaturable components to the uptake data at 37ºC. The following model was utilized:

$$J = \frac{J_{\max} * C}{K_m + C} + K_d * C \quad (2)$$

where J_{max} is the maximal uptake rate, K_m is the kinetic constant for saturable uptake, K_d is the kinetic constant for nonsaturable uptake, and *C* is the fexofenadine concentration. Apparent permeability (P_{app}) was determined using Equation (3):

$$P_{app} = \frac{dQ/dt}{A \cdot C_o} \quad (3)$$

where *dQ/dt* is the flux determined from the amount transported (*Q*) over time (*t*) during the experiment, *A* is the surface area of the porous membrane, and C_o is the initial concentration in the donor side. Efflux clearance (CL_{eff}) was calculated using Equation (4):

$$CL_{eff} = \frac{dX/dt}{C_o} \quad (4)$$

where *dX/dt* is the amount effluxed (*X*) over time (*t*) determined in the linear region of efflux and C_o is the initial concentration of fexofenadine loaded in the cells. Initial intracellular concentrations were calculated using the amount loaded at $t = 0$ and Caco-2 cellular volume of 3.66 µl/mg protein (Bourdet and Thakker, 2006).

D. RESULTS

Uptake of Fexofenadine into MDCKII Cells that are Stably Transfected with OATP2B1, hOCT3, and hOCTN2

As shown in Figure 2.1A, uptake of 10 nM [^{3}H]E1S at pH 6.0 into the OATP2B1-expressing MDCKII was 8.7-fold ($p < 0.001$) greater than that in the mock cells and was abolished by 200 μM unlabeled E1S. Uptake of [^{3}H]fexofenadine at pH 6.0 into MDCKII-OATP2B1 cells was 2.3-fold ($p < 0.01$) greater than that into the mock cells, and was also abolished by 200 μM unlabeled E1S (Figure 2.1B). Uptake of [^{3}H]MPP$^+$ (model substrate for hOCT3) and [^{3}H]L-carnitine (model substrate for hOCTN2) into CHO-hOCT3 or MDCK-hOCTN2 cells was 7.7-fold or 89-fold greater, respectively, than those into the mock cells, and was abolished by high concentrations of the unlabeled model substrates (Figures 2.2A and 2.3A). However, uptake of [^{3}H]fexofenadine into CHO-hOCT3 and MDCK-hOCTN2 cells was equivalent to the control and was not affected by the unlabeled model substrates (Figures 2.2B and 2.3B).

pH-dependent Apical Uptake of Fexofenadine into Caco-2 Monolayers

As shown in Figure 2.4A, uptake of 10 nM [^{3}H]E1S at pH 6.0 was 1.7-fold ($p < 0.05$) greater than that at pH 7.4 into the apical membrane of Caco-2 cells and was reduced by 200 μM unlabeled E1S ($p < 0.01$). As shown in Figure 2.4B, uptake of 100 nM [^{3}H]fexofenadine at pH 6.0 was 1.9-fold ($p < 0.01$) greater than that at pH 7.4 into the apical membrane of Caco-2 cells, and unlabeled E1S (200 μM) reduced the uptake at pH 6.0 by 40% ($p < 0.05$), but not at pH 7.4.

Basolateral Uptake of Fexofenadine into Caco-2 Monolayers

As shown in Figure 2.5, RT-PCR of RNA isolated from Caco-2 cells and human ileum gave the bands at 526 and 254 base pairs for OSTα and OSTβ, respectively, and demonstrated the presence of mRNAs of these two subunits in Caco-2 cells. Basolateral uptake of [^{3}H]E1S into Caco-2 cells showed a combination of saturable and passive diffusion mechanism (K_m = 589 μM, V_{max} = 36 pmol/mg/min, and K_d = 0.012 μl/mg/min) (Figure 2.6A). The K_m value was comparable with that in oocytes expressing hOSTαβ (320 μM) (Seward et al., 2003). Digoxin (500 μM), another hOSTαβ substrate/inhibitor (Seward et al., 2003), inhibited basolateral uptake of [^{3}H]E1S (10 nM) by 45% ($p < 0.01$), whereas 500 μM fexofenadine did not reduce it in Caco-2 cells (Figure 2.6B). In addition, both E1S and digoxin at 500 μM did not reduce the basolateral uptake of 100 nM [^{3}H]fexofenadine in Caco-2 cells, neither did 500 μM unlabeled fexofenadine (Figure 2.6C).

Apical and Basolateral Efflux of Fexofenadine in Caco-2 Monolayers

The cellular localization of MRP3 in Caco-2 was examined by confocal laser scanning microscopy. As shown in Figure 2.7, MRP3 was localized in the basolateral membrane of Caco-2 cells, as expected based on its localization in rat intestine (Rost et al., 2002). As shown in Figure 2.8A, GW918 (1 μM) and MK-571 (25 μM) stimulated the accumulation of fexofenadine into Caco-2 cells by 4-fold ($p < 0.01$) and 2-fold ($p < 0.05$), respectively, when cells were exposed from both sides. After preloading, fexofenadine was preferentially transported out of the cells across the apical membrane (2-fold, $p < 0.01$), whereas efflux to the basolateral side was preferred by 1.5-fold ($p < 0.01$) when P-gp was inhibited (Figures 2.7B and 2.7C). MK-571 inhibited the efflux of cellular fexofenadine

across the apical and basolateral membranes by 41% ($p < 0.05$) and 57% ($p < 0.05$), respectively (Figures 2.8B and 2.8C). GW918 nearly completely inhibited apical efflux ($p < 0.01$) and also inhibited basolateral efflux of fexofenadine ($p < 0.05$) (Figure 2.8B).

Absorptive and Secretory Transport of Fexofenadine across Caco-2 Monolayers

As shown in Figure 2.9A, MK-571 (25 μM) decreased absorptive transport by 17% whereas GW918 (1 μM) increased the transport by 2.3-fold ($p < 0.01$). When P-gp was inhibited with GW918, further treatment with MK-571 decreased the transport rate by 42% ($p < 0.01$). MK-571 alone increased secretory transport of fexofenadine by 32% ($p < 0.05$) (Figure 2.9B).

E. DISCUSSION

Fexofenadine is a zwitterionic compound that must depend on one or more intestinal transporter(s) for oral absorption. The observation that fruit juices decreased oral absorption of fexofenadine in humans, possibly by inhibiting intestinal uptake transporter, supported the notion that transporters contribute to intestinal absorption of fexofenadine (Dresser et al., 2002). However, the identity of the specific uptake transporter involved in the intestinal absorption of fexofenadine remains unclear. OATP1A2 was first suggested to mediate the apical uptake of fexofenadine into enterocytes (Dresser et al., 2002; Glaeser et al., 2007). However, the studies from several other groups did not show the presence of *OATP1A2* mRNA in human intestine (Tamai et al., 2000; Hilgendorf et al., 2007; Meier et al., 2007) or Caco-2 cells (Hilgendorf et al., 2007), casting doubt on the role of OATP1A2 in the intestinal absorption of fexofenadine. OATP2B1 is highly expressed in human jejunum (Hilgendorf et al., 2007; Meier et al., 2007) and Caco-2 cells (Hilgendorf et al., 2007), and has been localized in the apical membrane of human small intestine (Kobayashi et al., 2003). The results of this study showed that among the apical intestinal transporters tested, OATP2B1, but not OCT3 or OCTN2, stimulated uptake of [^{3}H]fexofenadine into transfected cells. In addition, apical uptake of [^{3}H]fexofenadine in Caco-2 cells was decreased by 45% with 200 μM E1S at pH 6.0, but not at pH 7.4, which is consistent with a greater activity of OATP2B1 at lower pH (Nozawa et al., 2004; Shimizu et al., 2005). This result supported the proposition that OATP2B1 mediates apical uptake of fexofenadine into Caco-2 cells at acidic pH because OATP2B1 is the only OATP isoform showing pH dependent activity. OATP2B1-mediated uptake of fexofenadine may be the major transport mechanism *in vivo* because of the acidic microclimate in small intestine (Daniel et al., 1985). These results also explain why

OATP2B1 would not play a significant role in hepatic uptake of fexofenadine because of the minimal activity of this transporter in fexofenadine uptake at neutral pH of the blood.

This study also showed that basolateral efflux of fexofenadine is mediated by a MK-571-sensitive transporter,3 but not OSTαβ in Caco-2 cells. OSTαβ is a bidirectional facilitative transporter mediating both uptake and efflux processes. In this report, we first showed the presence of OSTαβ mRNAs (Figure 2.5) and the functional activity of E1S transport in the basolateral side of Caco-2 cells (Figure 2.6A). However, fexofenadine did not inhibit the basolateral uptake of E1S in Caco-2 cells, although the OSTαβ substrate, digoxin, inhibited E1S uptake by this transporter (Figure 2.6B). In additioin, OSTαβ substrates/inhibitors did not decrease uptake of [^{3}H]fexofenadine across the basolateral membrane of Caco-2 cells (Figure 2.6C). Furthermore, the basolateral uptake of fexofenadine, up to 500 μM, did not show a saturable mechanism (Figure 2.6C). Therefore, we concluded that the basolateral transport of fexofenadine does not involve OSTαβ. However involvement of other facilitative transporters cannot be ruled out.

In contrast, the MRP inhibitor, MK-571, reduced the basolateral efflux of fexofenadine from pre-loaded Caco-2 cells, suggesting that the basolateral MRPs mediate transport of fexofenadine out of Caco-2 cells. The leukotriene LTD4 receptor antagonist MK-571 specifically modulates MRP associated multidrug resistance, but not the drug resistance of P-gp (Gekeler et al., 1995). MK-571 is an effective inhibitor of MRP2 (Chen et al., 1999), MRP3 (Zeng et al., 2001) and MRP4 (Reid et al., 2003a), and showed the highest potency ($IC_{50} \approx 5$ μM), inhibiting MRP3-mediated methotrexate transport among 17 inhibitors tested in that study (Zeng et al., 2001). MK-571 also inhibits hepatic transporter OATP1B3 (Letschert et al., 2005). However, this transporter is not expressed in human

intestine and Caco-2 cells (Hilgendorf et al., 2007). Furthermore, MK-571 at 25 μM did not inhibit apical and basolateral uptake of fexofenadine into Caco-2 cells. Also, at this concentration MK-571 did not inhibit uptake of [^{3}H]E1S in OATP2B1 transfected MDCKII cells; only at the highest concentration tested (500 μM), it inhibited [^{3}H]E1S uptake by 30%. These results indicated that the modulation of fexofenadine transport by MK-571 in Caco-2 cells is not due to inhibition of OATPs. While this article was in preparation, Matsushima et al. reported that fexofenadine was a substrate for MRP3, but not for MRP4 (Matsushima et al., 2008a), and that Mrp3 played an important role in sinusoidal efflux of this drug in mice (Matsushima et al., 2008a; Tian et al., 2008a). Immunofluorescence studies using laser scanning confocal microscopy showed that MRP3 was localized to the basolateral membrane of Caco-2 cells. Combination of the results from the literature and this study led to the conclusion that basolateral MRP3 in Caco-2 cells mediates the efflux of fexofenadine out of the cells. The results also showed that MK-571 reduced the apical efflux of fexofenadine from preloaded Caco-2 cells. MRP2 was localized to the apical membrane of enterocytes (Mottino et al., 2000), indicating that MRP2 may be another transporter mediating apical efflux of fexofenadine besides P-gp. A recent study on hepatic disposition of fexofenadine supported this observation, showing that Mrp2 is primarily responsible for the biliary excretion of fexofenadine in mice (Tian et al., 2008b).

Inhibition of MRPs reduced the absorptive transport moderately, whereas inhibition of P-gp increased the transport over 2-fold. The results showed that when P-gp is present, modulation of MRPs does not affect the overall transport rate dramatically, indicating that apical entry, predominantly influenced by P-gp-mediated efflux is rate-limiting in absorptive transport of fexofenadine in Caco-2 cells. However, when P-gp is inhibited, MRP inhibition

can decrease the overall rate by over 40%, clearly providing evidence for the role of an MRP transporter, presumably MRP3, in the basolateral efflux and vectorial absorptive transport of fexofenadine in Caco-2 cell monolayers. The role of MRP3 in mediating basolateral efflux was likely underestimated because apical MRP2 is also inhibited with MK-571, and this inhibition leads to an increase in the absorptive transport rate, counteracting the effect of inhibition of basolateral MRP3. Further work is needed to design effective siRNA sequence to suppress MRP2 or MRP3 specifically in Caco-2 cells in order to fully elucidate the roles of apical MRP2 and basolateral MRP3 in the intestinal absorption of fexofenadine. It is conceivable that basolateral efflux by MRP3 may control absorption rate of fexofenadine in human small intestine when P-gp is not fully effective in attenuating the cellular entry of this drug across the apical cell membrane.

Studies described in this report provide a novel insight about the roles of basolateral transporters in intestinal drug absorption. In order for a drug with poor membrane permeability to cross the intestinal epithelia efficiently, the basolateral transporter is required for egress of the drug into the blood side after apical transporters mediate uptake into enterocytes. This notion has been established in a study of bile acid absorption, showing that knocking out the basolateral transporter Ostαβ in mice almost abolished transileal transport of taurocholate (Rao et al., 2008). Altered drug absorption of fexofenadine due to modulation of MRP3 activity may have important clinical implications. Concomitant administration of the P-gp inhibitor verapamil (1 mM in the jejunal perfusate) failed to increase the effective jejunal permeability of fexofenadine (Tannergren et al., 2003b). Verapamil at 1 mM inhibits MRP3 activity by 70% (Zeng et al., 2001). Therefore, reduction of MRP3 mediated basolateral efflux by verapamil simultaneously may partially explain the failure of

modulation of fexofenadine permeability by this P-gp inhibitor. On the other hand, in the patients with diminished P-gp activity due to genetic variations or coadministration with a P-gp inhibitor, inhibition of intestinal MRP3 may reduce fexofenadine bioavailability dramatically. Furthmore, hepatic MRP3 attenuates hepatic elimination of fexofenadine (Matsushima et al., 2008b; Tian et al., 2008a) and, thus, increases the systemic exposure of this drug, which is synergistic with the function of intestinal MRP3 defined in this study. Therefore, the synergistic action of MRP3 in the intestine and liver will produce more dramatic effects on pharmacokinetics of fexofenadine, and, thereafter, the magnitude and duration of fexofenadine actions. Pharmacokinetic studies of fexofenadine in Mrp3 knockout mice, and further studies in humans are required to define the *in vivo* function of MRP3 in oral absorption of drugs. Collectively, the vectorial transport system consisting of apical OATP2B1 and basolateral MRP3 (and/or other basolateral MRPs) may be an important pathway to deliver hydrophilic anionic and zwitterionic drugs such as pravastatin and fexofenadine into human bodies.

F. ACKNOWLEDGEMENTS

We gratefully acknowledge Professor Heyo K. Kroemer (Ernst Moritz Arndt University, Germany) for providing the plasmid of pcDNA3.1/hygro/hOCTN2, Professor Dietrich Keppler (German Cancer Research Center, Germany) for providing the plasmid of pcDNA3.1/hygro /OATP2B1 and Dr. Vadivel Ganapathy (Medical College of Georgia, USA) for providing the plasmid of pSPORT1/hOCT3. Xin Ming was supported by Eli Lilly Pre-Doctoral Fellowship.

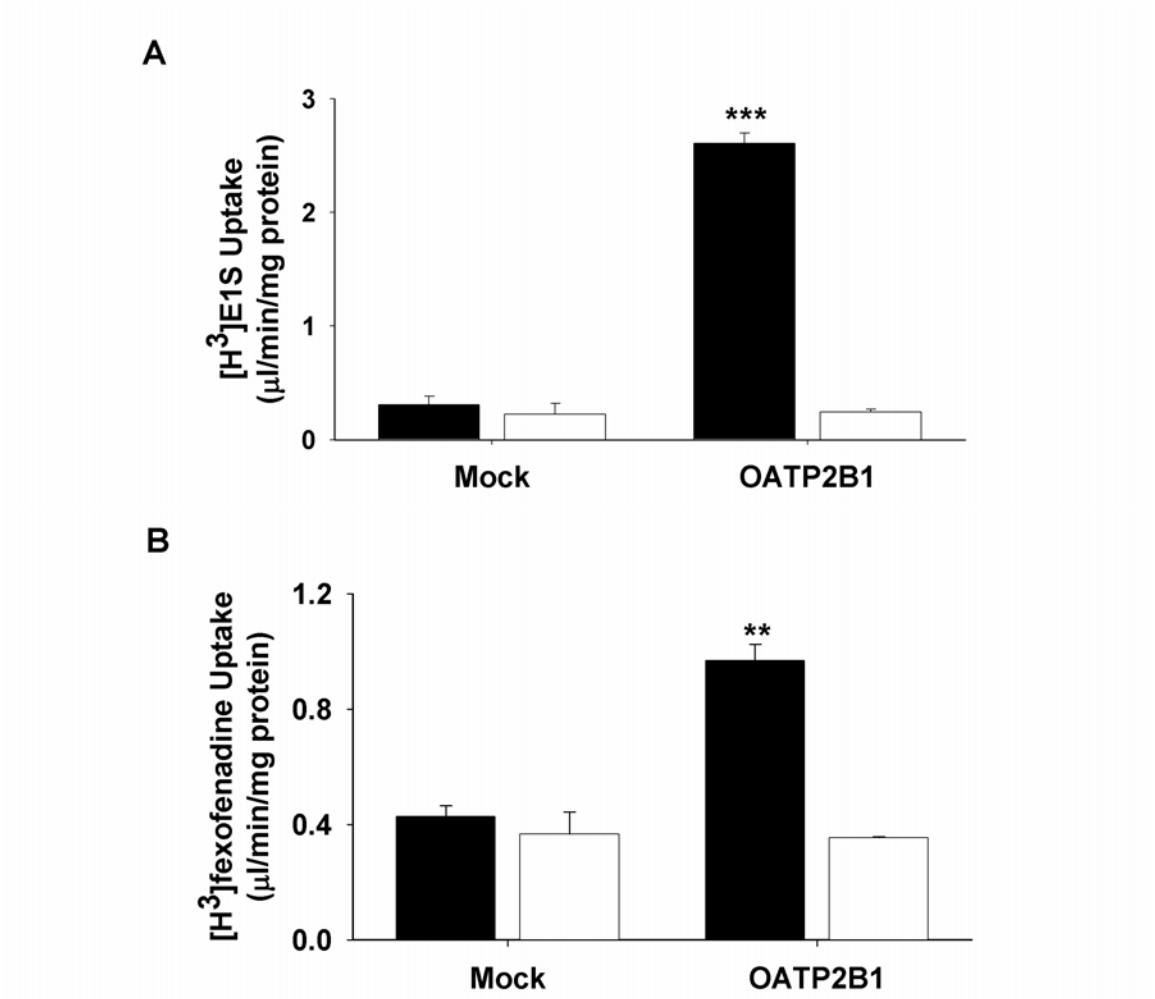

Figure 2.1. Uptake clearance of [^{3}H]E1S (A) and [^{3}H]fexofenadine (B) in MDCKII cells stably transfected with OATP2B1. MDCKII cells stably transfected with empty plasmid (Mock) or OATP2B1 cDNA (OATP2B1) were incubated with 10 nM [^{3}H]E1S (A) or 100 nM [^{3}H]fexofenadine (B) in the absence (solid bar) or presence (blank bar) of 200 µM unlabelled E1S for 3 minutes and uptake clearance of test compounds in these cells was determined. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

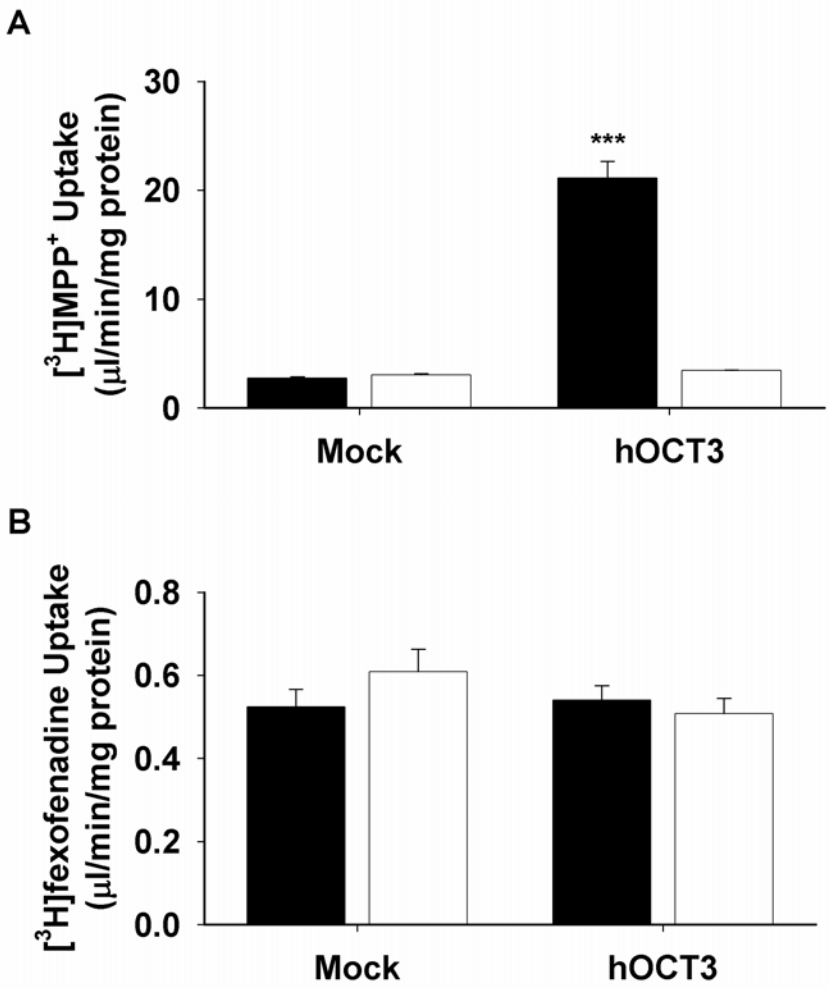

Figure 2.2. Uptake clearance of [^{3}H]MPP$^+$ (A) and [^{3}H]fexofenadine (B) in CHO cells stably transfected with hOCT3. CHO cells stably transfected with empty plasmid (Mock) or hOCT3 cDNA (hOCT3) were incubated with 1 µM [^{3}H]MPP$^+$ (A) or 100 nM [^{3}H]fexofenadine (B) in the absence (solid bar) or presence (blank bar) of 500 µM unlabelled MPP$^+$ for 3 minutes and uptake clearance of test compounds in these cells was determined. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

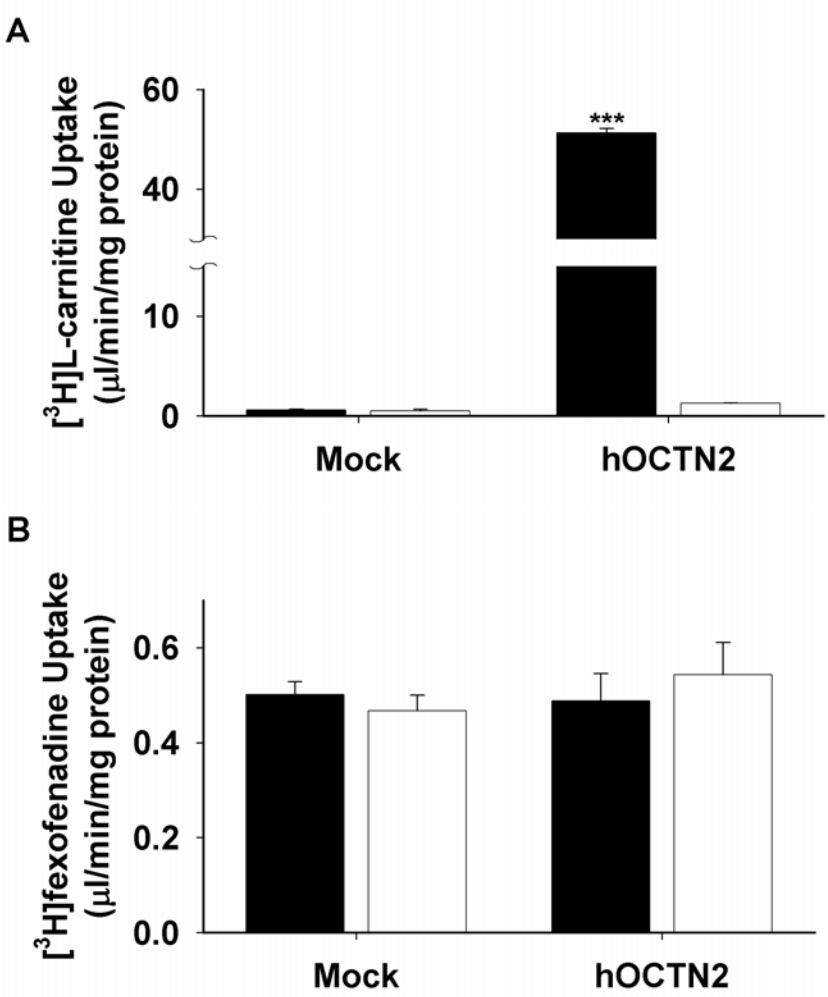

Figure 2.3. Uptake clearance of [^{3}H]L-carnitine (A) and [^{3}H]fexofenadine (B) in MDCKII cells stably transfected with hOCTN2. MDCKII cells stably transfected with empty plasmid (Mock) or hOCTN2 cDNA (hOCTN2) were incubated with 10 nM [^{3}H]L-carnitine (A) or 100 nM [^{3}H]fexofenadine (B) in the absence (solid bar) or presence (blank bar) of 500 µM unlabelled L-carnitine for 3 minutes and uptake clearance of test compounds in these cells was determined. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

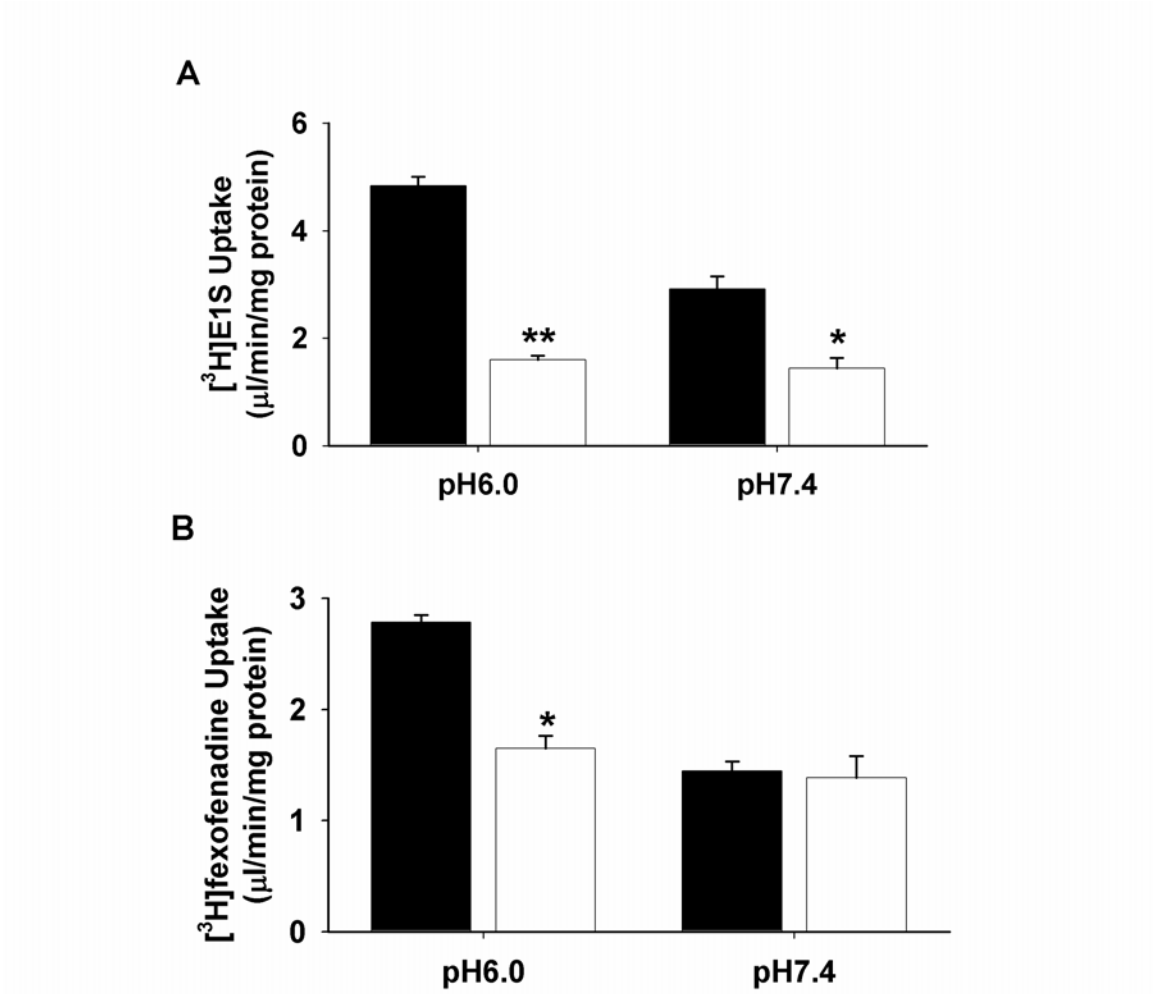

Figure 2.4. pH-specific apical uptake clearance of [^{3}H]E1S (A) and [^{3}H]fexofenadine (B) in Caco-2 cells. Caco-2 cells were incubated at pH 6.0 and pH 7.4 from the apical side with 10 nM [^{3}H]E1S (A) or 100 nM [^{3}H]fexofenadine (B) in the absence (solid bar) or presence (blank bar) of 200 µM unlabelled E1S for 3 minutes and uptake clearance of test compounds in these cells was determined. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

Figure 2.5. Detection of *hOSTαβ* mRNA in Caco-2 cells by RT-PCR. Lane 1, 100 bp DNA ladder; Lane 2, hOSTα in Caco-2 cells; Lane 3, hOSTα in human ileum; Lane 4, hOSTα in water; Lane 5, hOSTβ in Caco-2 cells; Lane 6, hOSTβ in human ileum; Lane 7, hOSTβ in water.

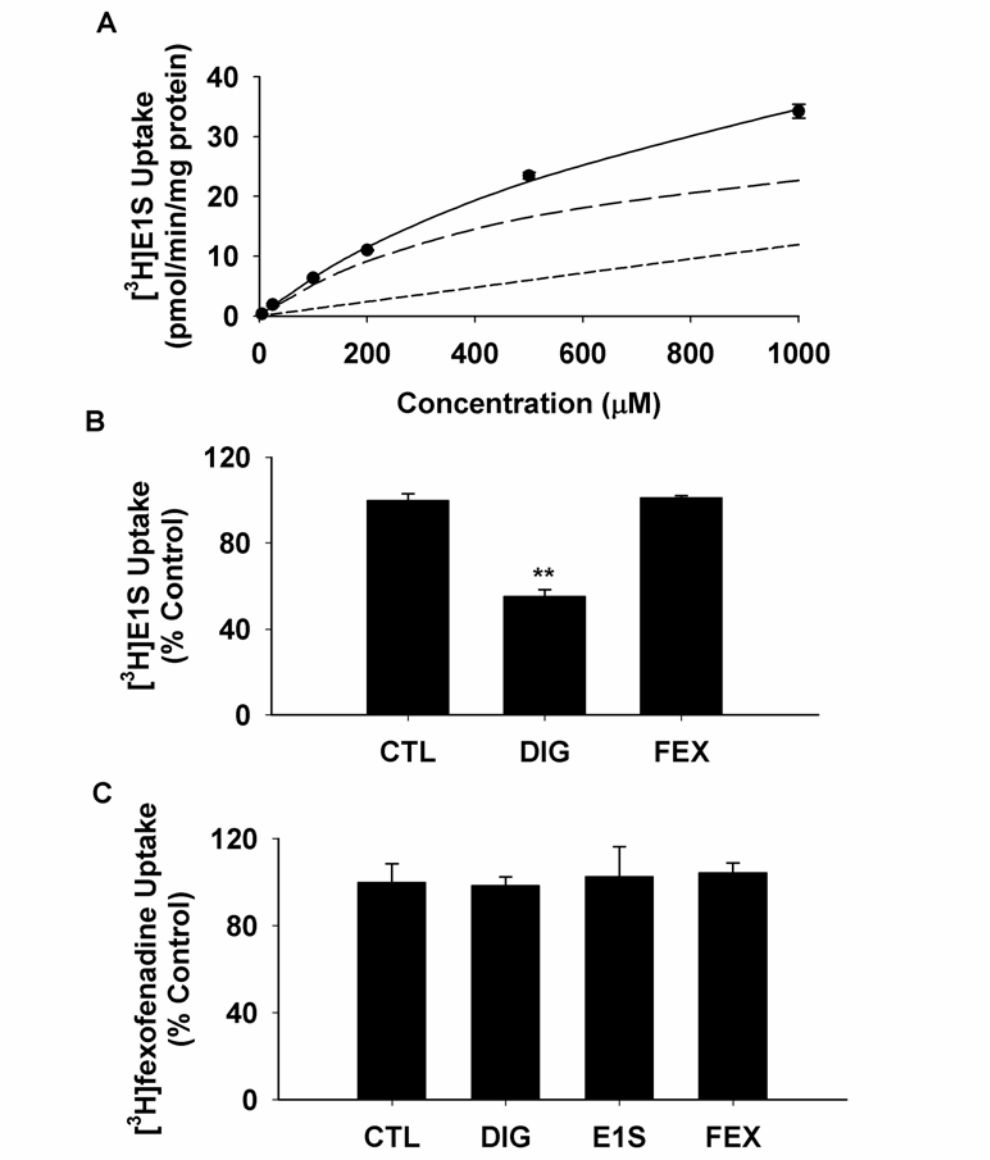

Figure 2.6. Basolateral uptake of [^{3}H]E1S (A, B) and [^{3}H]fexofenadine (C) in Caco-2 cells. (A). Basolateral uptake of [^{3}H]E1S in Caco-2 cells was determined over 3 minutes at the indicated concentrations. The solid, dashed and dotted lines represent the best fit to the [^{3}H]E1S uptake data, the saturable and passive components of the uptake, respectively. (B). Basolateral uptake of [^{3}H]E1S in Caco-2 cells was determined over 3 minutes in the absence (CTL) or presence of 500 μM unlabelled digoxin (DIG) or 500 μM fexofenadine (FEX). (C). Basolateral uptake of [^{3}H]fexofenadine in Caco-2 cells was determined over 3 minutes in the

absence (CTL) or presence of 500 μM unlabelled digoxin (DIG), 500 μM E1S (E1S) or 500 μM fexofenadine (FEX). Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

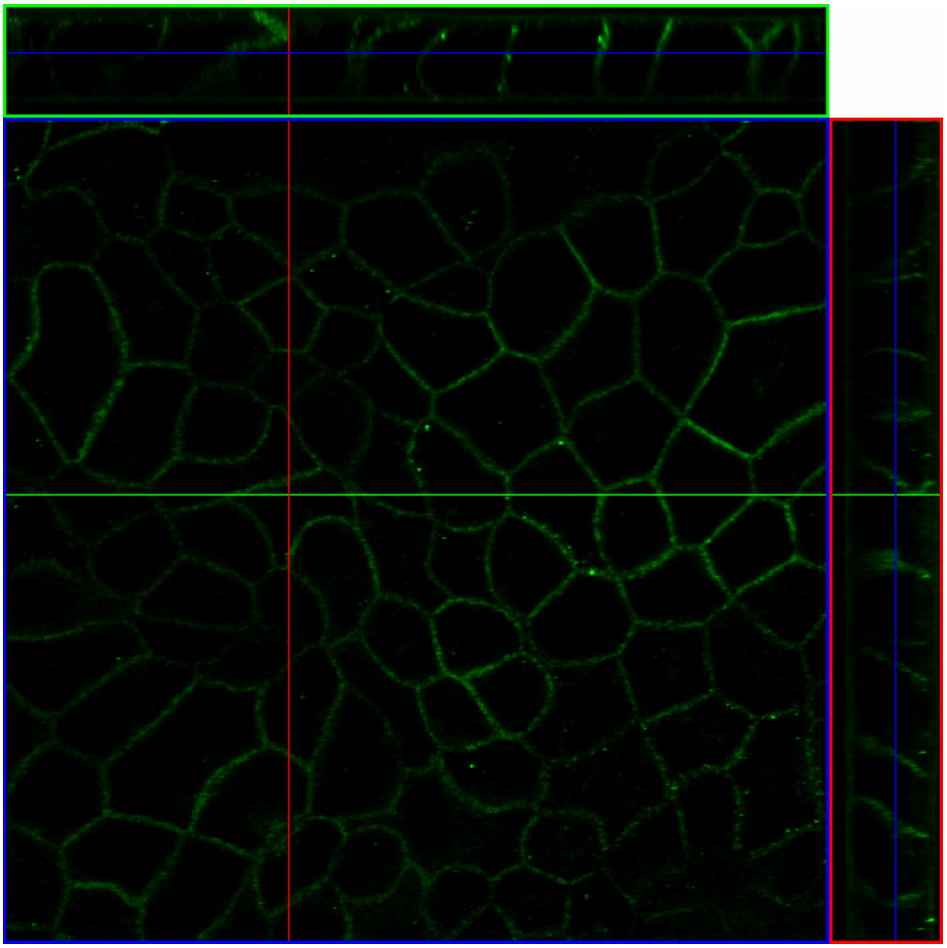

Figure 2.7. Immunostaining for MRP3 in Caco-2 cell monolayers. Indirect immunofluorescent staining was performed and viewed via confocal laser scanning microscopy. The X-Y image (center) is shown as projections through the whole plane of the cell monolayer viewed en face, and perpendicular X-Z (upper) and Y-Z (right) images are shown.

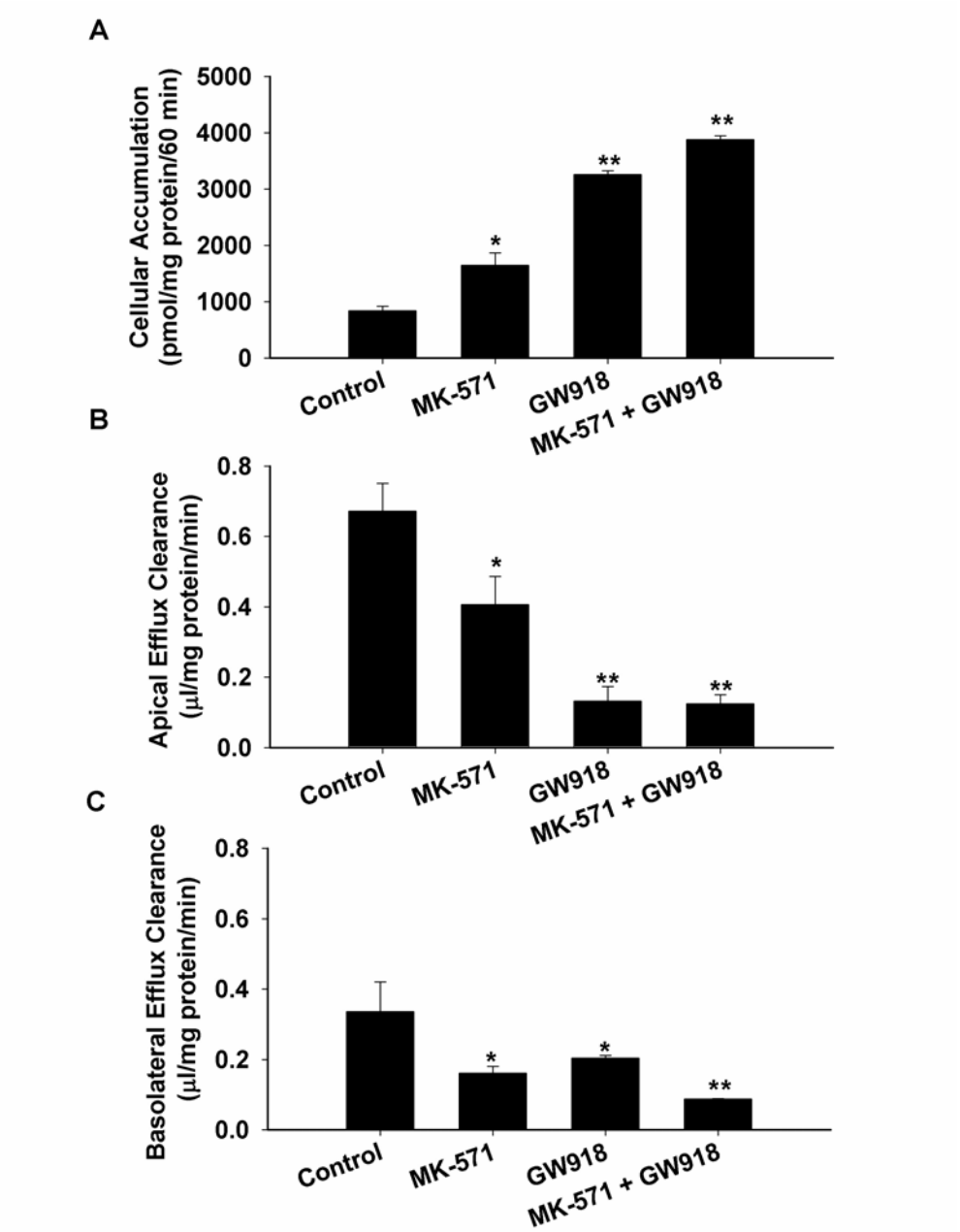

Figure 2.8. Accumulation (A) of fexofenadine in Caco-2 cells and its efflux (B, C) from the pre-loaded cells. Accumulation of fexofenadine from apical and basolateral sides of Caco-2 monolayers was determined over 1 hour in the absence (CTL) or presence of 25 μM MK-571, 1 μM GW918 or both. (B). Apical efflux clearances of fexofenadine from Caco-2 monolayers was determined over 3 minutes after 1-hour preloading in the absence (CTL) or presence of 25 μM MK-571, 1 μM GW918 or both. (C). Basolateral efflux clearances of

fexofenadine from Caco-2 monolayers was determined over 3 minutes after 1-hour preloading in the absence (CTL) or presence of 25 μM MK-571, 1 μM GW918 or both. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

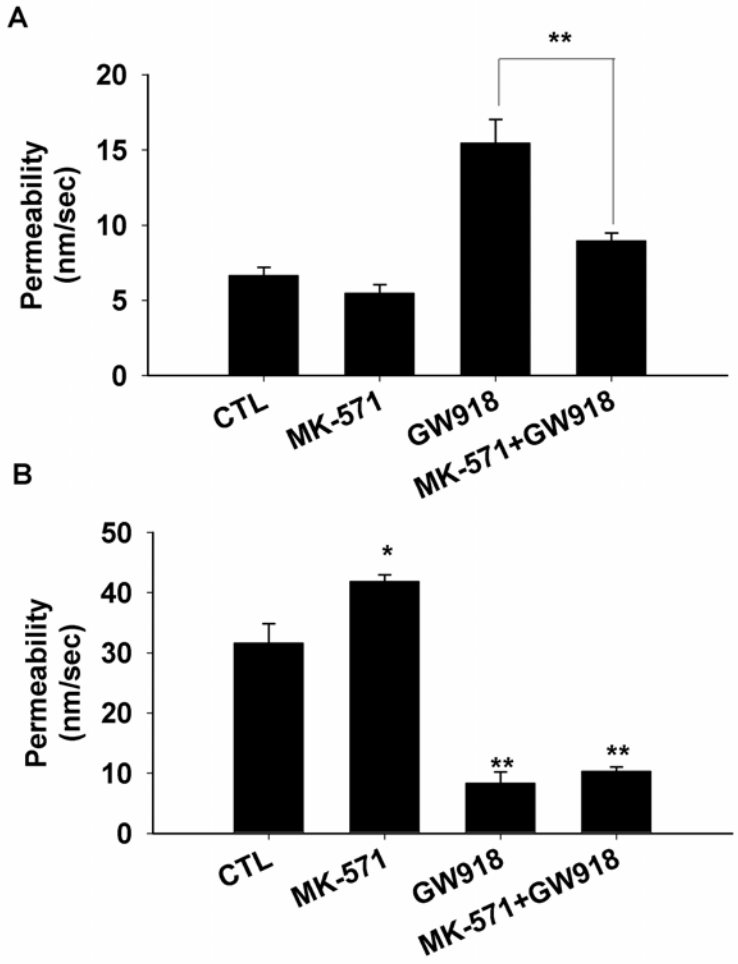

Figure 2.9. Absorptive and secretory transport of fexofenadine across Caco-2 cells. (A). Absorptive transport of fexofenadine across Caco-2 monolayers was determined in the absence (CTL) or presence of 25 μM MK-571, 1 μM GW918 or both. (B). Secretory transport of fexofenadine across Caco-2 monolayers was determined in the absence (CTL) or presence of 25 μM MK-571, 1 μM GW918 or both. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

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CHAPTER 3

MRP4 MEDIATES BASOLATERAL EFFLUX OF ADEFOVIR FORMED IN CACO-2 CELLS FROM ITS PRODRUG - ADEFOVIR DIPIVOXIL

This chapter will be submitted to the *Journal of Pharmacology and Experimental Therapeutics* and is presented in the style of that journal.

A. ABSTRACT.

Adefovir dipivoxil, a diester prodrug of the antiviral drug adefovir, shows higher oral bioavailability than the active drug (32-45% compared to <12%). The prodrug is known to be metabolized completely into the active drug in the enterocytes. However, it is not known how the highly charged and hydrophilic active drug crosses the basolateral membrane in the intestine. The purpose of this study is to determine the role of specific basolateral transporter(s) in the egress of adefovir using Caco-2 cell monolayers as a model of intestinal epithelium. Results showed that multidrug resistance-associated protein 4 (MRP4), a known adefovir transporter, plays a role in its basolateral efflux when generated from adefovir dipivoxil in Caco-2 cells. Western blotting of Caco-2 cell lysates showed the presence of MRP4 protein in this cell line. Images of confocal microscopy showed that MRP4 is localized in the basolateral membrane of Caco-2 cells. This localization was further confirmed by Western blotting of the basolateral membrane fractions isolated by a novel method involving biotinylation of membrane proteins followed by affinity enrichment. MRP4-knockdown Caco-2 cells, constructed using a pRNATin-H1.2 siRNA expression vector, showed reduced MRP4 protein expression and 39% reduction in the availability of adefovir on the basolateral side when dosed with [^{3}H]adefovir dipivoxil on the apical side. These results established that MRP4 plays a significant role in the egress of adefovir across the basolateral membrane of Caco-2 cells. A comparable reduction in the availability of adefovir on the basolateral side (48%) by the general MRP inhibitor indomethacin (30 μM) further established that among the MRP transporters, MRP4 plays a predominant role in the basolateral egress of adefovir in Caco-2 cells. The results highlight the importance of the basolateral MRP4 in oral absorption of adefovir dipivoxil, and suggest that the oral

bioavailability of adefovir can be significantly affected if an MRP4 inhibitor is co-administered with the prodrug, adefovir dipivoxil.

B. INTRODUCTION.

Acyclic nucleotide phosphonate adefovir is a reverse transcriptase inhibitor with the activities against a wide range of viruses such as retroviruses, immuno-deficiency virus type 1 and 2 (HIV1 and HIV2), herpes viruses, and hepadnaviruses (De Clercq, 2003). However, the permeability of adefovir across biological membranes is very low due to the presence of highly negatively charged phosphonate group (successive *pKa*'s = 2.0 and 6.8), which results in low oral bioavailability of adefovir in humans (less than 12%) (Cundy et al., 1995). Therefore, esters of adefovir were synthesized in order to mask the negative charges and improve its membrane permeability. Adefovir dipivoxil is one of those prodrugs. Bioavailability of adefovir after oral administration of adefovir dipivoxil in humans is approximately 32-45% (Barditch-Crovo et al., 1997). The active phosphonate form of adefovir was the only component that was detected in plasma after oral administration of adefovir dipivoxil in humans (Noble and Goa, 1999), indicating that adefovir dipivoxil is converted to the active drug by presystemic metabolism. Further study showed that adefovir dipivoxil was not detected in the mesenteric vein in rat intestinal perfusion experiments (Annaert et al., 2000), which proved that adefovir dipivoxil is metabolized completely into its active phosphonate form in the intestine. The study using Caco-2 cell model confirmed this mechanism by showing that adefovir was the major component that was measured from the basolateral chamber when adefovir dipivoxil was dosed into the apical chamber of Caco-2 monolayers (Annaert et al., 1997). Accordingly, intestinal absorptive transport of adefovir dipivoxil involves the diffusion of the prodrug across the apical membrane, followed by cellular metabolism into adefovir, and subsequent basolateral efflux of this active drug into the blood (Figure 3.6). It remains unclear as to how the highly charged and hydrophilic active

drug crosses the basolateral membrane of the enterocytes. A previous study showed that the basolateral efflux of adefovir in Caco-2 cells may involve a carrier-mediated transport process, and that the efflux was not mediated by P-gp (Annaert et al., 1998). Whether multidrug resistance-associated proteins (MRPs, ABCC subfamily) were involved in the intestinal basolateral efflux of adefovir was not known.

MRP4, the fourth member of the ABCC family, was initially identified as a homolog of MRP1 (ABCC1) by screening databases of human sequence tags (Kool et al., 1997). MRP4 was the first MRP isoform identified that does not have a third (N-terminal) membrane spanning domain (Kool et al., 1997). The "short" members of this family, MRP4, MRP5, MRP8 and MRP9, are distinguished by their ability to transport cyclic nucleotides and nucleoside-based agents (Kruh et al., 2007). MRP4 is up-regulated in adefovir-resistant cells, which indicated that the enhanced efflux of adefovir by MRP4 leads to this resistance (Schuetz et al., 1999). The substrate specificity of MRP4 is particularly broad; it transports cAMP, cGMP, *p*-aminohippurate, urate, dehydroepiandrosterone sulfate, methotrexate, and estradiol-17β-D-glucuronide as well as adefovir (Schuetz et al., 1999; van Aubel et al., 2002; Zelcer et al., 2003; van Aubel et al., 2005). In human kidney, MRP4 is abundantly expressed (Hilgendorf et al., 2007) and localized in the apical membrane of the proximal tubules (van Aubel et al., 2002). Adefovir is actively secreted in the urine, and most of the administered dose is recovered in the urine as intact drug (Cundy, 1999). It has been established that the active renal secretion of adefovir involves uptake into tubular cells by the basolateral organic anion transporter 1 (OAT1, *SLC22A6*) (Cihlar et al., 1999), and the apical efflux into urine by MRP4 (Imaoka et al., 2007).

Recently, studies with Mrp4 knockout ($Mrp4^{-/-}$) mice suggested that Mrp4 is expressed in mouse intestine (Belinsky et al., 2007). $Mrp4^{-/-}$ mice were significantly more sensitive to adefovir, as indicated by a clear separation of the dose-response curves for knockout and wild-type mice, and the intestine in the knockout mice suffered increased toxicity as compared to the wild-type mice (Belinsky et al., 2007). This result supported the notion that Mrp4 prevents the entry of adefovir into the enterocytes from the circulating blood in order to protect the enterocytes from the adefovir cytotoxicity; it further suggested that Mrp4 is present in the basolateral membrane of the enterocytes. Therefore, it is proposed that MRP4 may mediate the basolateral efflux of adefovir formed in the enterocytes during oral absorption of adefovir dipivoxil. In the present study, the expression and localization of MRP4 was investigated in Caco-2 cells and its possible role in the basolateral efflux of adefovir was examined during absorptive transport of adefovir dipivoxil in wild-type and MRP4-knockdown Caco-2 cells.

C. MATERIALS AND METHODS.

Materials

Caco-2 cells were obtained from the American Tissue Culture Collection (Manassas, VA). Eagle's minimum essential medium (EMEM) with Earle's salts and L-glutamate, nonessential amino acids (NEAA, 100x), N-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES, 1M) and penicillin-streptomycin-amphotericin B solution (100x) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) and trypsin-EDTA solution (1x) were obtained from Sigma Chemical Co. (St. Louis, MO). Hank's balanced salt solution (HBSS) was obtained from Mediatech, Inc. (Herndon, VA). Geneticin was obtained from Invitrogen Co. (Carlsbad, CA). [^{3}H]adefovir dipivoxil (11 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

Caco-2 cells were cultured in EMEM, supplemented with 10% FBS, 1% NEAA, 1% penicillin-streptomycin-amphotericin B solution at 37°C in a humidified atmosphere with 5% CO2. The cells were passaged every 4 days using trypsin-EDTA, and plated at densities of 1:5 in 75 cm^2 T-flasks. Caco-2 cells were seeded at a density of 60,000 cells/cm^2 on Transwell^{TM} filters. Medium was changed the day after seeding, and every other day thereafter. The cells were cultured for 21-25 days before use. Transepithelial electrical resistance (TEER) was measured to ensure cell monolayer integrity. Measurements were obtained using an EVOM Epithelial Tissue Voltammeter and an Endohm-12 electrode

(World Precision Instruments, Sarasota, FL). Cell monolayers with TEER values greater than 300 Ω•cm2 were used in transport experiments.

Immunoblot Analysis

Caco-2 cells were lysed in a solution containing 1% SDS, 1 mM EDTA, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The protein concentration of the clear cell lysate was determined with the BCA protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Ten or fifty µg of total protein per lane were resolved by electrophoresis on NuPAGE 4 to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and were transferred onto polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membrane was blocked and incubated with the M4I-10 antibody (0.3 µg/ml) and then with an anti-rat IgG, horseradish peroxidase-linked antibody (Pierce, Rockford, IL). Signals were developed using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

Immunofluorescent Staining

Caco-2 cells were grown on 6.5-mm diameter Transwell^{TM} inserts (Corning Inc., Lowell, MA) for the purpose of immunostaining. Inserts were washed three times in phosphate-buffered saline (PBS); similar washes were included between each of the following stages. Cells were fixed in 1% paraformaldehyde for 15 minutes and then permeabilized with 0.1% Triton X-100 for 10 minutes. Nonspecific binding sites were blocked by incubation for 30 minutes with 5% normal sheep serum. Inserts were incubated with primary antibody M4I-10 (Axxora, San Diego, CA) diluted to 5 µg/ml with PBS, for 60 minutes. Primary antibody was detected by incubation with Alexa Flour 568 goat anti-rat

antibody for 60 minutes. Cell nuclei were stained with SYTO® 13 or DAPI (Invitrogen, Carlsbad, CA). Inserts were washed and mounted in Permount* Mounting Medium (Andwin Scientific, Addison, IL). Staining was viewed using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY). To obain X-Z image in immunostaining Caco-2 cells, inserts were frozen in Tissue-Tek Cryo-OCT Compound (Fisher Scientific, Pittsburgh, PA) and sectioned in longitudinal direction.

Immunoblot Analysis of Apical and Basolateral Membrane Fractions of Caco-2 Cells

Caco-2 cells were grown on six-well Transwell™ filters with the method described above. Biotinylation experiments were conducted 21 days post-seeding using the Cell Surface Protein Isolation Kit (Pierce, Rockford, IL) according to the manufacturer's protocol with modifications. After washing with ice-cold PBS, sulfo-*N*-hydroxysuccinimide-SS-biotin (1.5 mg/ml in PBS) was added to the apical chambers of three Transwell™ filters or the basolateral chambers of the other three Transwell™ filters and incubated for 30 minutes at 4°C. Cells were washed three times with PBS, containing 100 mM glycine, and incubated for 20 minutes at 4°C with the same buffer. Cells were washed with PBS, scraped into 1 ml of PBS, and pelleted by centrifugation at 800*g* for 2 minutes. The pellet was treated with 700 µl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 7.4, and protease inhibitors) for 1 h at 4°C with shaking. Cell lysates were spun at 16,000*g* for 10 minutes and the supernatant was added to the column with NeutrAvidin™ Gel. The column was incubated for 60 minutes at room temperature with end-over-end mixing and then was washed with the washing buffer 4 times. The biotinylated proteins were released by incubation of the gel with 2 x Laemmli buffer for 30 minutes at room temperature. Samples of the biotinylated fractions (25 µl) were subjected to Western analysis

for detection of MRP4 with M4I-10 antibody (Axxora, San Diego, CA) after they were diluted to 0.3 μg/ml with PBS, and for detection of the basolateral protein Na^+-K^+ ATPase with the antibody (Abcam, Cambridge, MA) after dilution to 0.4 μg/ml.

siRNA Knockdown Experiments

An siRNA sequence was designed to silence the *MRP4* gene expression based on a published report (Reid et al., 2003b). Chemically synthesized oligonucleotide encoding the siRNA sense strand, 5'-gatcccaatcctgcacatgcaccatcttgatatccggatggtgcatgtgcaggatttttttttccaaa-3', was annealed with the complementary DNA oligonucleotide, 5'-agcttttggaaaaaaaatcctgca-catgcaccatccggatatcaagatggtgcatgtgcaggattgg-3'. The resulting double-stranded DNA was inserted into the BamH I and Hind III sites of the pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ). Caco-2 cells at 60% confluence were transfected with the MRP4 siRNA plasmid, using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol specific to Caco-2 cells. Transfectants were selected with 0.2 mg/ml hygromycin B for 3 weeks and screened by Western blot for MRP4 expression. A clone with the lowest MRP4 expression was chosen as a stably transfected cell line for further functional study.

Accumulation Study

MRP4-knockdown and wild-type Caco-2 cells were seeded at 5×10^5 cells per well in six-well plates and grown for 48 hours. Cells were incubated with 10 μM [^{3}H]adefovir dipivoxil in the absence or presence of 30 μM indomethacin for 2 hour. Then the dose solution was aspirated and cells were washed three times with 4ºC transport buffer. Cells were dissolved in 500 μl 0.1 N NaOH/0.1% SDS for 4 hours with shaking. Radioactivity was

determined by scintillation counting. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Transport Studies

Caco-2 cell monolayers were incubated for 30 minutes with transport buffer in the absence or presence of 30 μM indomethacin. Transport studies were initiated by replacing the donor chamber solution with 10 μM [^{3}H]adefovir dipivoxil with or without 30 μM indomethacin. The receptor chamber was sampled at selected times and radioactivity was determined by scintillation counting.

Data Analysis

Data are expressed as mean ± SD from three measurements unless otherwise noted. Statistical significance was evaluated using *t*-test or ANOVA followed by Dunnet's test for multiple comparisons. The data were analyzed with SigmaStat 2.0 (Systat Software, Inc., San Jose, CA).

Basolateral availability (F_b) was defined as a new parameter to represent the availability of adefovir in the basolateral (receptor) compartment after dosing adefovir dipivoxil in the apical (donor) compartment, and was determined using Equation (1):

$$F_b = \frac{dQ/dt}{C_o} \qquad (1)$$

where *dQ/dt* is determined from the amount of adefovir appearing in the basolateral compartment (Q) over time (t) during the experiment, and C_o is the initial concentration of adefovir dipivoxil in the donor side.

D. RESULTS.

Expression and Localization of MRP4 Protein in Caco-2 cells

Western blot analysis was performed to measure the expression of MRP4 protein in Caco-2 cells. The M4I-10 antibody for human MRP4 labeled a protein in the lysates of Caco-2 cells and MCF-7/ADR cells (positive control) with an apparent molecular weight around 200 kDa (Figure 3.1). These results suggest that human MRP4 protein is expressed in Caco-2.

Laser scanning confocal images from immunostaining Caco-2 cells by MRP4 antibody (X-Y sections) showed that the MRP4 stain was at the borders, surrounding nuclear staining, indicating the lateral localization (Figure 3.2). X-Z images of MRP4 staining showed distinct basal and lateral membrane localization and the stain was not co-localized with nuclear staining (Figure 3.2), supporting the basolateral localization of MRP4.

As shown in Figure 3.3, immunoblot analysis of apical and basolateral membrane fractions of Caco-2 cells showed the separation of two membrane fractions. Western blotting with MRP4 and Na^+-K^+ ATPase antibodies showed that the same membrane fraction was enriched with MRP4 that was also enriched with Na^+-K^+ ATPase. Since Na^+-K^+ ATPase is a basolateral membrane marker, it confirmed that MRP4 is mainly expressed in the basolateral membrane of Caco-2 cells.

Construction of Caco-2 Cells that are Stably Expressing MRP4-specific siRNA

Caco-2 cells were transfected with the MRP4 siRNA plasmid. After selection by hygromycin B, thirty-two clones were obtained and evaluated by Western blotting. As shown in Figure 3.4A, MRP4 expression in Caco-2 clones #5, 16 and 21 was suppressed compared

to the wild-type Caco-2 cells. The MRP4 function of those constructed cell lines was examined by measuring accumulation of [^{3}H]adefovir dipivoxil with or without the MRP inhibitor indomethacin. Consistent with the reduced MRP4 expression, the ratios of the accumulation in the absence vs. presence of indomethacin decreased from 3.5 for wild-type Caco-2 cells to 1.4 for Clone #16 (Figure 3.4B). Cell Clone #16 showed the lowest MRP4 protein expression and functional activity of MRP4, and was chosen to be used in the transport study.

Transport of Adefovir Dipivoxil in MRP4-Knockdown and Wild-type Caco-2 Cells

When absorptive transport of adefovir dipivoxil across Caco-2 cell monolayers was examined, only adefovir was detected in the basolateral compartment, indicating that (i) the dipivoxil prodrug was hydrolyzed completely in the cells and (ii) adefovir appeared in the basolateral compartment by egress across the basolateral membrane. Figure 3.5 depicts the basolateral availability (F_b) of adefovir in wild-type Caco-2 cells and those that are stably expressing MRP4-specific siRNA (Clone #16). The basolateral availability of adefovir was lower in the MRP4-knockdown Caco-2 cells than in the wild-type cells by 39%. For comparison, indomethacin (30 μM) treatment of the wild-type Caco-2 cells reduced the basolateral availability of adefovir by 48% with respect to that in the wild-type Caco-2 cells.

E. DISCUSSION.

Our understanding about the role of transporters that either facilitate or attenuate intestinal absorption of drugs has grown rapidly over the past few years (Kunta and Sinko, 2004; Thwaites and Anderson, 2007; Dobson and Kell, 2008). However, most of the reports in this area have focused on the apical transporters involved in trafficking of compounds between intestinal lumen and the cellular compartment of enterocytes. Our understanding of the transport of hydrophilic and/or charged compounds are transported from the enterocytes into the mesenteric blood circulation is relatively poor. In a previous study (Chapter 2), it was discovered that MRP3 in the basolateral membrane of Caco-2 cells transports a zwitterionic drug, fexofenadine, out of the Caco-2 cell across the basolateral membrane. These results suggested that MRP3 likely plays a role in the intestinal transport of hydrophilic zwitterionic compounds that are able to enter the cells via one or more apical transporters. Another member of the ABCC family, MRP4, appears to be localized to the basolateral membrane of enterocytes as evident from the observations that (i) its mRNA was detected in human jejunum and Caco-2 cells (Hilgendorf et al., 2007), and the Mrp4 protein was detected in mouse intestine (Belinsky et al., 2007); (ii) intravenous dosing of adefovir caused increased toxicity to the intestine in the knockout mice as compared to the wild-type mice (Belinsky et al., 2007), suggesting that MRP4 may be functionally active in the basolateral membrane of the enterocytes to protect them from the cytotoxicity of adefovir in the blood circulation.

In the present study, it was hypothesized that MRP4 plays an important role in the egress of anionic hydrophilic compounds like adefovir across the basolateral membrane of Caco-2 cells, used as a model for human intestinal epithelium. Immunostaining followed by

laser scanning confocal microscopy provided evidence that MRP4 is localized on the lateral and basal membranes of Caco-2 cells grown as monolayers. The localization of MRP4 was further investigated by isolation of apical and basolateral cell membrane and subjecting the membrane fractions to Western blot analysis. Isolation of the apical and the basolateral membranes was accomplished by a novel approach in which the surface proteins on each membrane were biotinylated in two separate experiments by reacting the cell monolayers with a biotin-linked electrophile on the apical or the basolateral side. Subsequent affinity enrichment allowed the isolation of apical and basolateral membrane fragments. Western blot analysis of the apical and basolateral membrane fractions revealed that the basolateral membrane was enriched in MRP4. Since Caco-2 cells are a good model for enterocytes in small intestine in terms of the qualitative transporter expression profile (Taipalensuu et al., 2001; Hilgendorf et al., 2007), one can extrapolate these results to human intestinal epithelium and suggest that MRP4 protein is expressed in the basolateral membrane of the intestine. However, this needs to be confirmed considering at least one other study, which reported that Mrp4 was localized primarily to the basal cytoplasmic region, but not distinctly localized to the basolateral membrane of enterocytes in rats (Johnson et al., 2006).

To determine the role played by MRP4 in the egress of hydrophilic anionic compound adefovir across the basolateral membrane of Caco-2 cells, the following experimental model was developed: [^{3}H]adefovir dipivoxil, a prodrug of adefovir in which two phosphate anionic sites were masked by esterification, was placed in the apical compartment in a transport experiment; the basolateral compartment was sampled for [^{3}H]adefovir as a function of time. It has been shown previously that adefovir dipivoxil can enter Caco-2 cells across the apical membrane, and is completely hydrolyzed in the cytosol

so that only adefovir is detected in the basolateral compartment (Annaert et al., 1997). The above experiment was performed in the wild-type Caco-2 cells and in MRP4-knockdown Caco-2 cells, which were created by stably expressing MRP4-specific siRNA (Figure 3.4). The fraction of the adefovir dipivoxil dose appearing on the basolateral side as adefovir was reduced by 39% when MRP4-knockdown Caco-2 cells were used instead of the wild-type Caco-2 cells. These results provided evidence that MRP4 plays an important role in the egress of adefovir formed in Caco-2 cells from adefovir dipivoxil. When wild-type Caco-2 cells were treated with indomethacin, a general MRP inhibitor, the fraction of the adefovir dipivoxil dose appearing on the basolateral side as adefovir was reduced by 48%. Comparing these results with those obtained in the MRP4-knockdown cells, it can be concluded that MRP4 is the major player among ABCC subfamily in facilitating basolateral egress of adefovir in Caco-2 cells.

Besides MRP4, other "short" members of the ABCC family, MRP5, MRP8 and MRP9, are also capable of transporting nucleoside based agents such as adefovir (Deeley et al., 2006; Kruh et al., 2007). Among these, MRP5 is minimally expressed in Caco-2 cells (Prime-Chapman et al., 2004). MRP8 is expressed in the apical membrane in polarized cells, whereas MRP9 shows basolateral localization (Kruh et al., 2007). The expression levels of MRP8 and MRP9 in the enterocytes or Caco-2 cells have not been determined. Therefore, their possible involvement in adefovir transport across the basolateral membrane of Caco-2 cells is unknown. MRP8, if present in the apical membrane of the Caco-2 cells, could decrease adefovir egress across the basolateral membrane by pumping it across the apical membrane. Another apical transporter of adefovir is breast cancer resistance protein (BCRP,

ABCG2) (Takenaka et al., 2007), however, its role was not examined in this study due to its minimal expression level in Caco-2 cells (Taipalensuu et al., 2001; Hilgendorf et al., 2007).

The physiological functions of MRPs in ABCC subfamily in the intestine have been unknown despite of their relatively high expression levels. Detoxifying the enterocytes by pumping out drugs and metabolites of the intestine was proposed (Kruh et al., 2007). However, definitive evidence is still missing. The present study suggests that the basolateral MRP4 may play an important role in the oral absorption of adefovir dipivoxil, an ester prodrug of the anionic drug adefovir, by mediating the egress of the anionic drug formed inside the enterocytes. The study thus provides a new insight about the role of MRPs in the intestinal absorption of hydrophilic anionic drugs. Design of ester prodrugs is a major strategy to improve the permeability of drugs that are higher charged and/or hydrophilic (Beaumont et al., 2003). However, due to the extensive presence of esterases in the enterocytes, many prodrugs are hydrolyzed in the intestine. The advantage of presystemic activation is that only the active drug enters systemic circulation, minimizing or eliminating the exposure of most organs and tissues to the prodrug or intermediate metabolites. Therefore, design of prodrugs for the drug, which can be transported by intestinal basolateral transporters, will yield not only good oral bioavailability but also minimal systemic exposure to additional chemical entities other than the active drug as depicted for adefovir and its dipivoxil prodrug (Figure 3.6). However, this study also shows that transporter-based drug-drug interactions can occur during uptake into enterocytes but also during egress across the basolateral membrane.

F. ACKNOWLEDGEMENTS.

We gratefully acknowledge Wendy Salmon (Michael Hooker Microscopy Facility, University of North Carolina at Chapel Hill, Chapel Hill, NC) for her kind assistance with laser scanning confocal microscopy experiment. Xin Ming was supported by Eli Lilly Pre-Doctoral Fellowship.

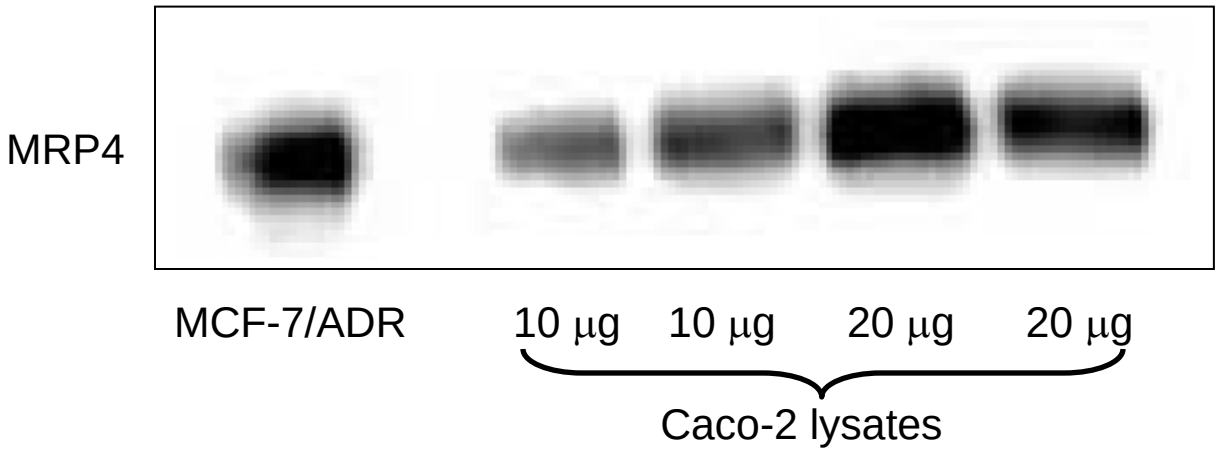

Figure 3.1. Immunoblot analysis of MRP4 in Caco-2 cells. Caco-2 cells after 21-day culture on the transwell were lysed and total protein was subjected to Western blot analysis. The M4I-10 antibody (Axxora, San Diego, CA) was used to stain the membrane for detection of MRP4, and MCF-7/ADR cells were used as a positive control.

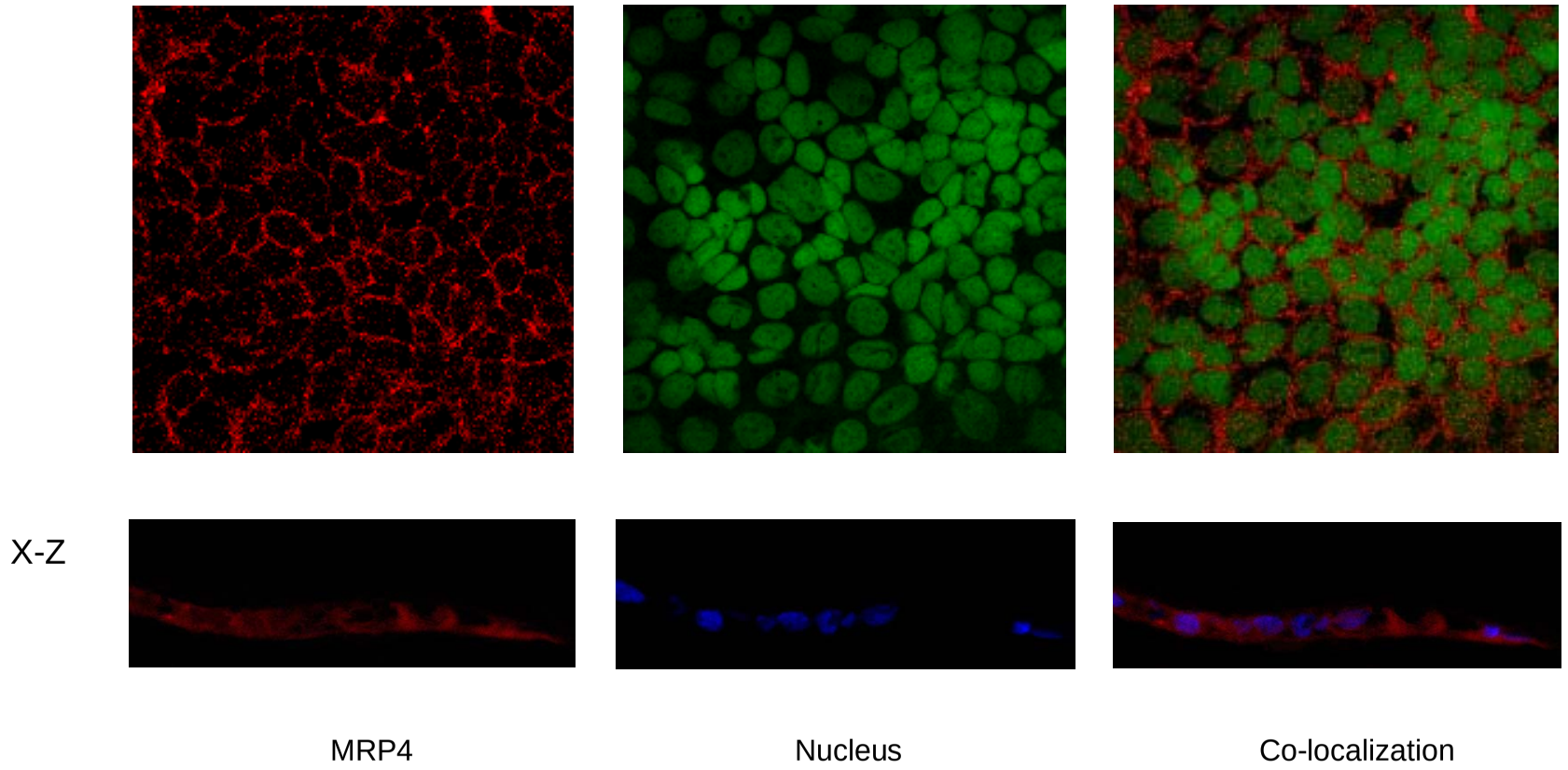

Figure 3.2. Immunolocalization of MRP4 in Caco-2 cells. Indirect immunofluorescent staining was performed and viewed via focal laser scanning microscopy. MRP4 staining is shown in red and nucleus in green (X-Y) or blue (X-Z). The X-Y image (upper) is shown as projections through the whole plane of the cell monolayer viewed en face, and perpendicular X-Z (lower) images are also shown.

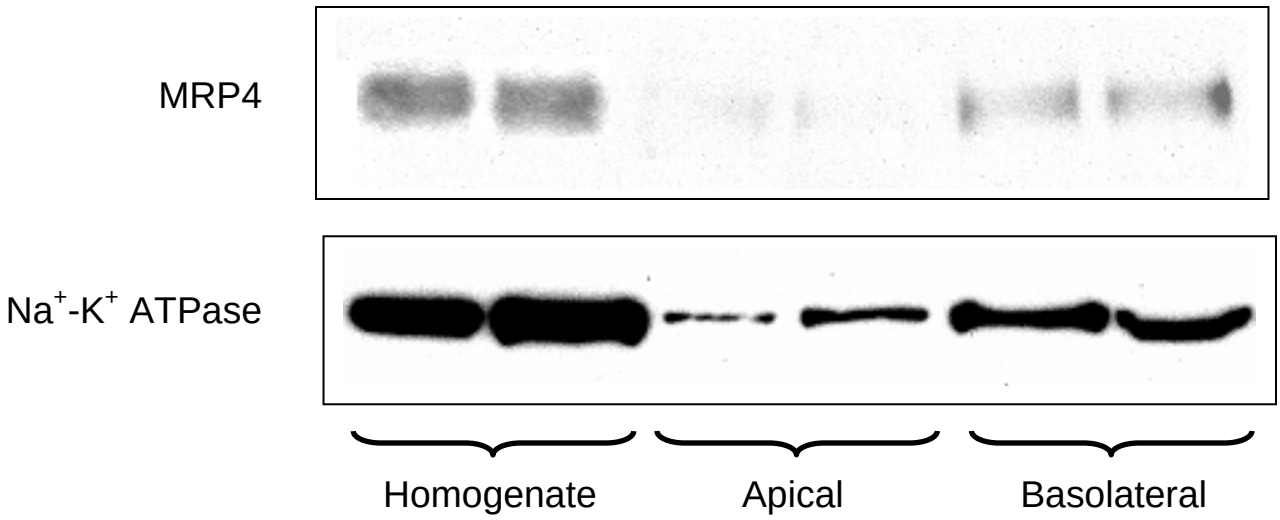

Figure 3.3. Immunoblot analysis of MRP4 in apical and basolateral membrane fractions of Caco-2 cells. Apical and basolateral membrane fractions of Caco-2 cells were isolated by biotinylation of plasma membrane proteins followed by affinity enrichment of the membrane fractions as described in Materials and Methods. Samples were analyzed by Western blot for MRP4 expression. Na^+-K^+ ATPase protein served as a basolateral marker.

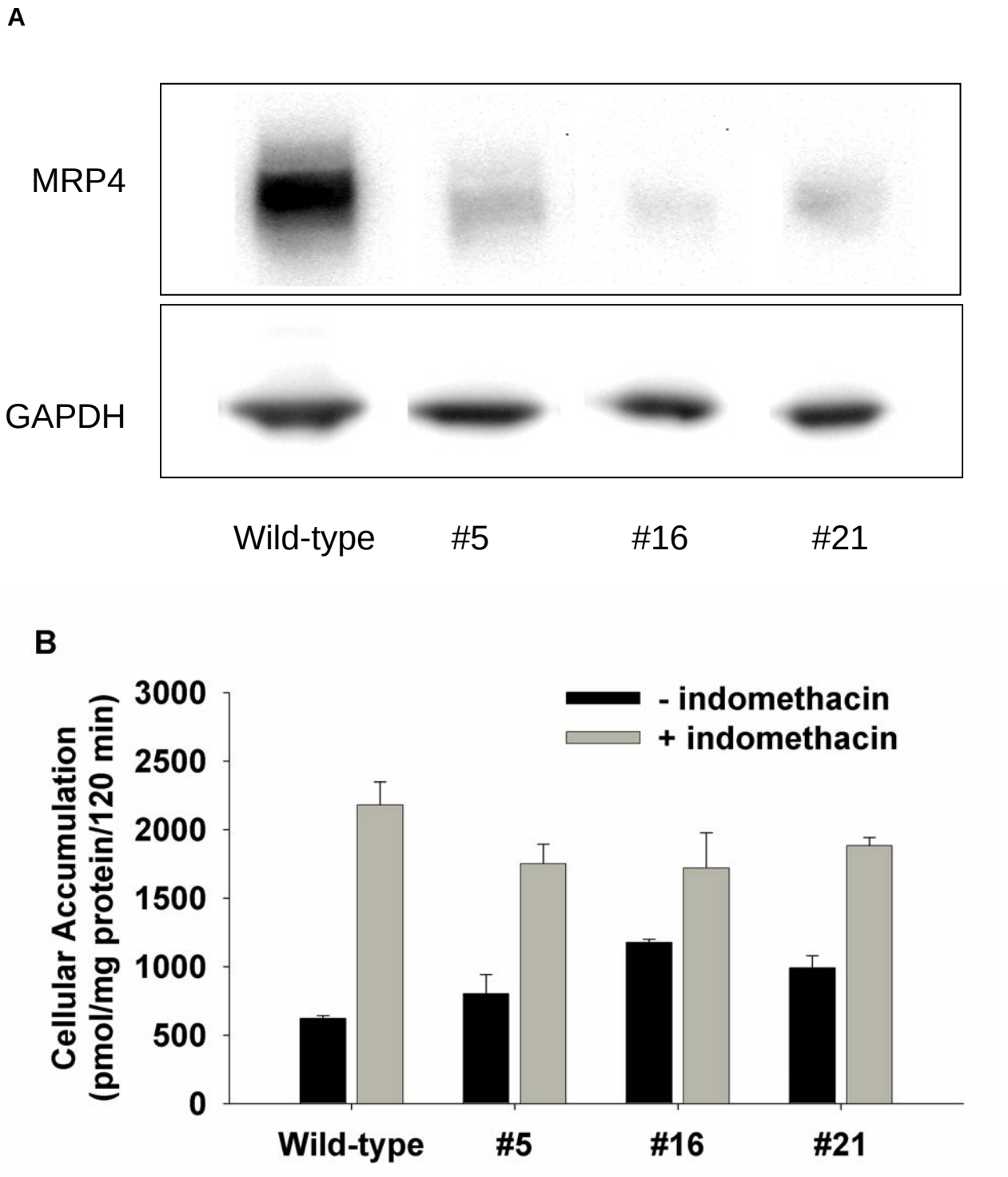

Figure 3.4. Effects of siRNA on MRP4 expression and [^{3}H]adefovir dipivoxil accumulation in Caco-2 cells. (A) The level of MRP4 protein in Caco-2 cells, stably transfected with MRP4-specific siRNA plasmid. The MRP4-specific siRNA plasmid was transfected into

Caco-2 cells using the Nucleofector System (Amaxa, Gaithersburg, MD) and transfectants were selected with 0.2 mg/ml hygromycin B for 3 weeks. Total proteins were analyzed by Western blot for MRP4 expression. GAPDH protein served as an internal control. (B) Accumulation of [^{3}H]adefovir dipivoxil after treatment of wild-type Caco-2 cells and MRP4-knockdown clones in the presence and absence of indomethacin (30 μM). After culturing for 4 days, the amount of [^{3}H]adefovir dipivoxil (10 μM), with or without indomethacin in MRP4-knockdown clones of Caco-2 cells was examined. Data represent mean ± S.D. of a representative experiment in triplicate.

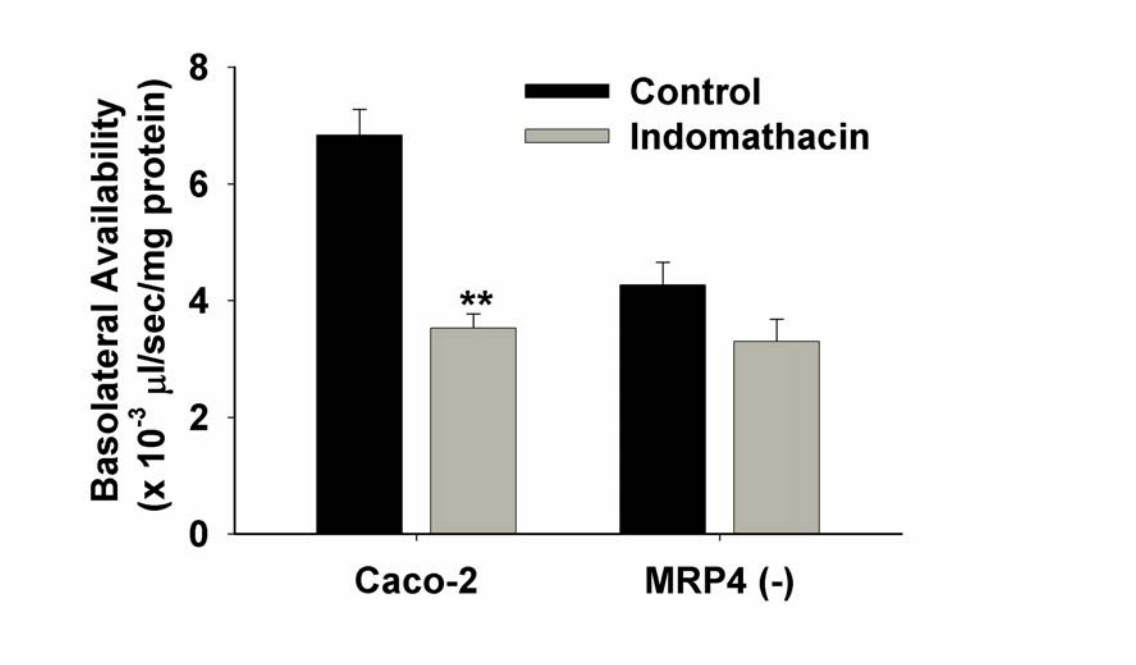

Figure 3.5. Basolateral availability of adefovir in wild-type or MRP4-knockdown Caco-2 cells. Transport of 10 μM [^{3}H]adefovir dipivoxil in the absence or presence of 30 μM indomethacin across MRP4 knockdown or wild-type Caco-2 cell monolayers were performed. Adefovir (radioactivity) was measured in the basolateral compartment. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

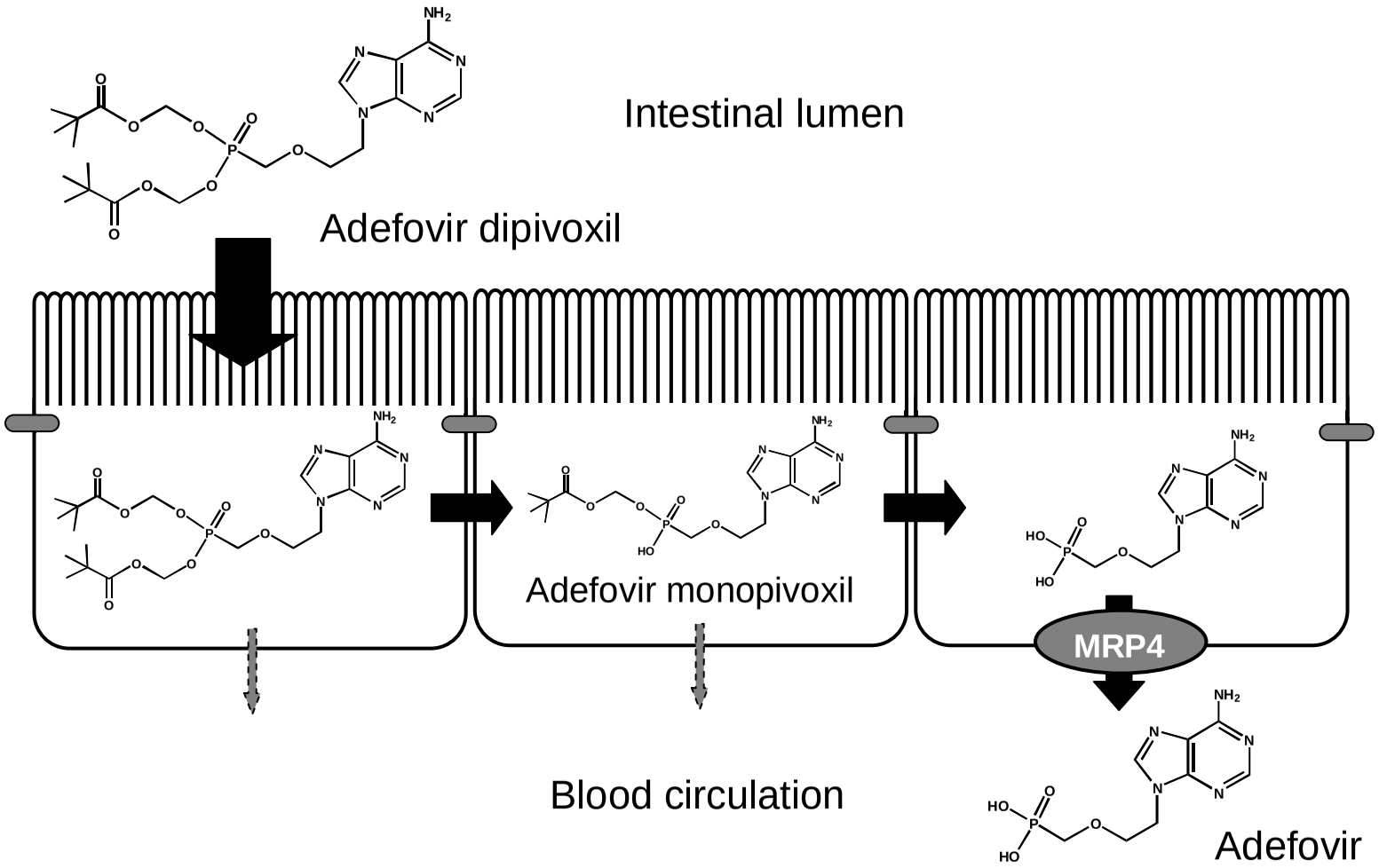

Figure 3.6. Model for transport of adefovir dipivoxil across intestinal epithelia. Adefovir dipivoxil diffuses through the apical membrane very rapidly and is metabolized by intracellular esterases to monoester first, and then adefovir. Basolateral MRP4 mediates the efflux of adefovir into the circulating blood.

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CHAPTER 4

OCT1 MEDIATES BASOLATERAL EFFLUX OF FURAMIDINE FROM ITS DIAMIDOXIME PRODRUG

This chapter will be submitted to the *Journal of Pharmacology and Experimental Therapeutics* and is presented in the style of that journal.

A. ABSTRACT.

Oral activity of antiparasitic aromatic diamidine drugs is poor due to low membrane permeability across intestinal epithelia. Prodrug strategies to mask positively charged amidine groups have been applied for improving their oral bioavailability. Diamidoxime prodrugs of diamidines have shown oral activity in rats, however, furamidine diamidoxime failed to show enhanced membrane permeability in Caco-2 and MDCKII cells, compared to furamidine itself. The goal of this study is to investigate whether human organic cation transporter 1 (hOCT1) in intestinal basolateral membrane enhances transcellular transport of furamidine diamidoxime by facilitating basolateral efflux of furamidine formed in enterocytes. Efflux of [^{14}C]furamidine was inhibited by quinidine but *trans*-stimulated by TEA in CHO cells stably transfected with hOCT1. The results showed that OCT1 interacts with furamidine on the intracellular side and mediates efflux of this diamidine. Then, polarized expression of hOCT1 in Caco-2 cells and hOCT1 stably transfected MDCKII cells was examined by uptake of [^{14}C]furamidine from apical and basolateral sides. The results showed that apical uptake of [^{14}C]furamidine was 2.4-fold higher than basolateral uptake in Caco-2 cells, however, uptake was 2.0-fold higher from basolateral side compared to apical side in MDCKII-hOCT1 cells, indicating that hOCT1 is expressed in apical membrane of Caco-2 cells but mainly in basolateral membrane of MDCKII-hOCT1 cells. Finally, transport studies for furamidine diamidoxime in MDCKII-hOCT1 cells showed that prodrug was converted to furamidine in the cells and the rate of furamidine basolateral appearance was 1.5-fold higher than that in mock cells and was *trans*-stimulated by TEA. These results indicated a possible role of OCT1 in the egress of cationic drugs in the intestine, although

this role is still questionable under physiological conditions since the electrical potential across the cell membrane does not favor efflux aided by the electrogenic transporter OCT1.

B. INTRODUCTION.

Pentamidine, an aromatic diamidine, has been used clinically since 1939 and continues to be an important agent to treat early-stage human African trypanosomiasis, *Pneumocystis carinii* pneumonia (PCP) in AIDS patients, and antimony-resistant leishmaniasis (Barrett et al., 2007). Furamidine is a diamidine analog of pentamidine in which the alkoxy chain linking the phenyl rings has been replaced with a furan ring (Figure 4.1). Furamidine has shown excellent *in vitro* and *in vivo* activities in mouse and monkey models of early-stage African trypanosomiasis (Barrett et al., 2007). Clinical use of aromatic diamidines has been restricted by their poor oral activity. At physiologic pH, the two positive charges associated with the diamidine groups (successive *pKa*'s = 10.4 and 11.8) make furamidine highly hydrophilic (Saulter, 2005), which leads to poor oral absorption and consequently poor oral activity. Strategies for improving oral bioavailability for diamidine drugs have focused on masking these positive charges. For example, pafuramidine, the O-methylamidoxime prodrug of furamidine, achieved over 100-fold higher permeability across Caco-2 monolayers compared to furamidine itself (Zhou et al., 2002a), and exhibits enhanced oral activity in animal models (Boykin et al., 1996) and humans (Yeates, 2003; Yeramian et al., 2005; Chen et al., 2007).

Pafuramidine is efficiently converted to furamidine via stepwise O-demethylation and N-dehydroxylation of both O-methylamidoxime groups in tandem (Zhou et al., 2002b). Cytochrome P450 4F (CYP4F) enzymes are the major enzymes responsible for O-demethylations (Wang et al., 2006) and N-dehydroxylation of amidoxime is mediated by cytochrome b5 and NADH cytochrome b5 reductase (Saulter et al., 2005). Multiple biotransformations may increase potential for interindividual variability due to drug-drug

interactions involving metabolic enzymes. CYP4F isoforms also metabolize a large number of endogenous substances and xenobiotics (Kalsotra and Strobel, 2006); therefore drug-drug and drug-food interactions involving these CYP enzymes may inhibit the conversion of the prodrug to active drug (Tirkkonen and Laine, 2004). Furamidine diamidoxime, formed by two O-demethylations of pafuramidine, is uncharged as both amidines are masked as amidoximes (Figure 4.1). Activation of furamidine diamidoxime to active furamidine only involves cytochrome b5 and NADH cytochrome b5 reductase (Figure 4.1) (Kurian et al., 2004; Saulter et al., 2005). Therefore, furamidine diamidoxime may be a better prodrug candidate with good membrane permeability and lower potential for interindividual variability and drug-drug interaction compared to pafuramidine. Previous studies have shown that diamidoxime prodrugs of pentamidine and its analogs possess oral activity against *Pneumocystis carinii* in rats (Hall et al., 1998), although less active than pafuramidine. However, furamidine diamidoxime did not achieve higher transcellular permeability than furamidine itself in Caco-2 and MDCKII cell monolayers, although the loss of the dose in donor compartment was comparable to pafuramidine (Saulter, 2005). The possible explanation was that furamidine diamidoxime is metabolized into furamidine by reductases quickly, but highly charged furamidine cannot cross basolateral membrane to achieve good permeability.

Recently, furamidine and pentamidine were proved to be substrates for human organic cation transporter 1 (hOCT1, SLC22A1) (Appendix), which is mainly expressed in the sinusoidal membrane of hepatocytes and mediates entry of organic cations into cells (Koepsell et al., 2007). hOCT1 interacts with many drugs, including the H2 antagonist ranitidine (Bourdet et al., 2005), the antidiabetic drug metformin (Wang et al., 2002), and the

anticancer drug oxaliplatin (Yokoo et al., 2007), and plays an important role in their disposition. OCT1 is also expressed in the basolateral membrane of intestine (Jonker et al., 2001; Wang et al., 2002; Muller et al., 2005). Immunostaining of OCT1 in human small intestine showed basolateral localization (Muller et al., 2005). In Oct1-null mice, accumulation of TEA (Jonker et al., 2001) and metformin (Wang et al., 2002) was lower than wild type after intravenous administration, indicating that Oct1 is expressed in the basolateral membrane of mouse intestine and mediates basolateral uptake of cations into enterocytes. OCT1 is also capable of transporting cations such as TEA and MPP^+ out of cells (Busch et al., 1996; Zhang et al., 1999). However, whether OCT1 plays a role in absorptive transport of cationic drugs has not been clear. Ranitidine, a hydrophilic cationic drug with good oral bioavailability, egresses across the basolateral membrane of Caco-2 cells via a non-saturable mechanism (Bourdet and Thakker, 2006). In contrast, metformin, a hydrophilic cationic drug with much higher *pKa* than ranitidine, does not cross the basolateral membrane readily after being transported into the Caco-2 cells via a cation-selective transporter in the apical membrane (Proctor et al., 2008). Immunostaining of hOCT1 showed that hOCT1 was localized in apical membrane of Caco-2 cells (Ng, 2003), whereas OCT1 is localized in basolateral membrane of the mouse and human intestinal epithelium (Jonker et al., 2001; Wang et al., 2002; Muller et al., 2005). Because of the disparity of localization of OCT1 in the intestine and in Caco-2 cells, it is conceivable that the poor membrane permeability of furamidine diamidoxime across Caco-2 cell monolayers may be due to the absence of OCT1 mediated basolateral efflux in these cells. The results in this study demonstrate that hOCT1 mediates efflux of furamidine, although at lower rate than the uptake process. Further,

expression of hOCT1 in the basolateral membrane of MDCKII can enhance the absorptive permeability of furamidine diamidoxime across the cell monolayers.

C. MATERIALS AND METHODS.

Materials

F-12 Nutrient Mixture, penicillin-streptomycin-amphotericin B solution (100x) and N-hydroxyethyl-piperazine-N'-2-ethanesulfonate (HEPES, 1M) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) and trypsin-EDTA solution (1x) were obtained from Sigma Chemical Co. (St. Louis, MO). Geneticin was obtained from Invitrogen Co. (Carlsbad, CA). Hank's balanced salt solution (HBSS) was obtained from Mediatech, Inc. (Herndon, VA). TEA chloride, MPP^+ and quinidine were purchased from Sigma (St. Louis, MO). Furamidine dihydrochloride salt (purity > 98%) was synthesized by Medichem (Chicago, IL) using previously described methods (Das and Boykin, 1977). Furamidine diamidoxime (purity > 98%) was a gift from Dr. David W. Boykin (Georgia State University, Atlanta, GA). [^{3}H]MPP^+ (85 Ci/mmol) and [^{14}C]TEA (55 mCi/mmol) were obtained from American Radiolabelled Chemicals (St. Louis, MO). [^{14}C]furamidine (55.3 mCi/mmol, purity 96%) was obtained from Huntingdon Life Sciences (Huntingdon, UK). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Chinese Hamster Ovary (CHO) cells and Madin-Darby Canine Kidney II (MDCKII) cells were obtained from the American Tissue Culture Collection (Manassas, VA). hOCT1 cDNA in pcDNA3.1 vector was provided by Professor Hermann Koepsell (Julius-Maximilians-University, Würzburg, Germany).

Cell Culture and Transfection

Construction of hOCT1 stably transfected CHO cells was described in Appendix. MDCKII cells were transfected with pcDNA3.1 empty vector or the vector containing the

full-length hOCT1 using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol specific for MDCKII cells. Transfectants were selected with 500 μg/ml geneticin for 10 days. A clone with the highest [^{3}H]MPP$^+$ uptake activity was chosen to create a stably transfected cell line for further studies. The stably transfected MDCKII cells were cultured in DMEM with 10% FBS, 10% NEAA, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B plus 0.5 mg/ml geneticin. The stably transfected CHO cells were cultured in F-12 Nutrient Mixture with 10% FBS, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B plus 0.5 mg/ml geneticin. Caco-2 cells were cultured in EMEM, supplemented with 10% FBS, 1% NEAA, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Caco-2 cells were seeded at a density of 60,000 cells/cm^2 on Transwell^{TM} filters. Medium was changed the day after seeding, and every other day thereafter. The cells were cultured for 21-25 days before use. All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Uptake Studies

In general, uptake studies were carried out 5-7 days post-seeding. Cells were pre-incubated for 30 minutes at 37ºC in transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES pH 7.2). Experiments were initiated by replacement of the transport buffer with 400 μl of radiolabelled dose solutions in transport buffer. Uptake was determined within the linear uptake region, after which the dose solution was aspirated and cells were washed three times with 4ºC transport buffer and lysed in 500 μl 0.1 N NaOH/0.1% SDS by shaking for 4 hours. Radioactivity was determined by scintillation counting. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

In *trans*-stimulation studies, each well of cells was preincubated with either 400 μl of transport buffer or 400 μl of transport buffer with 2 mM non-radiolabelled TEA, 200 μM quinidine or 100 μM furamidine at 37°C for 1 h. Cells were then rinsed twice with ice-cold transport buffer and once with 1 ml of the transport buffer before the uptake studies were performed as described above.

Uptake studies were also conducted in Caco-2 cells and MDCKII-hOCT1 cells grown on Transwell™ filters, and 1 μM [^{14}C]furamidine was added into the apical or basolateral chamber of monolayers and the uptake studies were performed as described above.

Efflux Studies

CHO-hOCT1 cells were incubated for 2 hours with 10 μM [^{14}C]TEA or [^{14}C]furamidine. After washing twice with ice-cold transport buffer and once with the transport buffer, efflux was determined within the linear efflux region in the absence or presence of 2 mM non-radiolabelled TEA and 200 μM quinidine. Then the dose solution was aspirated and cells were washed three times with 4ºC transport buffer and lysed in 500 μl 0.1 N NaOH/0.1% SDS by shaking for 4 hours. Radioactivity of the effluxed and cellular samples was measured by scintillation counting. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Transport Studies

Caco-2 cell monolayers were incubated for 30 min with the transport buffer. Transport studies were initiated by replacing the donor chamber with 25 μM furamidine diamidoxime in the absence or presence of 2 mM TEA added only to the receiver chamber.

The receiver chamber was sampled at selected times and furamidine and furamidine diamidoxime were quantified by fluorescence HPLC.

Analytical Methods

Analytical methods for furamidine and its diamidoxime prodrug were employed as described previously (Zhou et al., 2002a) with modification. Samples were analyzed by reverse-phase HPLC with a photodiode array detector and a fluorescence detector (Hewlett Packard model 1100, Palo Alto, CA, USA). The column used was a 150 × 2.1 mm Zorbax Bonus-RP (5 μm particle size) column (Agilent Technologies, Wilmington, DE). UV detection with wavelength at 359 nm and fluorescence detection with excitation at 359 nm and emission at 461 nm were chosen for routine detection, without omitting any observable peaks. The solvent system consisted of solvent A (15 mM ammonium formate-30 mM formic acid in 100% HPLC-grade water) and solvent B with identical components in 4:1 acetonitrile-HPLC-grade water. A gradient elution was used with a starting concentration of 95% solvent A and 5% solvent B. This gradient increased linearly to 80% solvent B over 12 min, followed by an 8-min reequilibration time to initial solvent conditions. The flow rate was 0.35 ml/min. Six-point standard curves of furamidine or furamidine diamidoxime were generated with concentration ranges covering the concentration in the samples.

Data Analysis

Data are expressed as mean ±SD from three measurements unless otherwise noted. Statistical significance was evaluated using T-test and ANOVA followed by Dunnett's test for multiple comparisons. The data were analyzed with SigmaStat 2.0 (Systat Software, Inc., San Jose, CA). Uptake clearance (CL_{up}) was determined using Equation. (1):

$$CL_{up} = \frac{dX/dt}{C_o} \quad (1)$$

where *dX/dt* is the flux determined from the amount taken up (*X*) over time (*t*) during the experiment, and C_o is the initial concentration in the donor side.

Basolateral availability (F_b) was defined as a parameter to represent the availability of furamidine in the basolateral (receptor) compartment after dosing furamidine diamidoxime in the apical (donor) compartment, and was determined using Equation (2):

$$F_b = \frac{dQ/dt}{C_o} \quad (2)$$

where *dQ/dt* is determined from the amount of furamidine appearing in the basolateral compartment (*Q*) over time (*t*) during the experiment, and C_o is the initial concentration of furamidine diamidoxime in the donor side.

D. RESULTS.

trans-Stimulation of TEA Uptake Studies

trans-Stimulation of [^{14}C]TEA uptake studies was used to investigate whether furamidine interacts with hOCT1 on the intracellular side. As shown in Figure 4.2, after pre-incubating CHO-hOCT1 cells with TEA (2 mM) for 1 h at 37°C, [^{14}C]TEA uptake was enhanced by 2.8-fold ($p < 0.01$). Pre-incubating cells with 200 μM quinidine or 100 μM furamidine resulted in a significant decrease in [^{14}C]TEA uptake (apparent *trans*-inhibition) to 16% ($p < 0.01$) and 13% ($p < 0.01$) of the control, respectively, indicating that furamidine binds to hOCT1 on the intracellular side, but its translocation rate is much slower than that of TEA. These *trans*-stimulation effects were not observed in mock cells.

Efflux of Furamidine from CHO-hOCT1 Cells

Efflux of [^{14}C]TEA after preloading 10 μM [^{14}C]TEA in CHO-hOCT1 cells for 1 hour was linear up to 20 minutes (data not shown). As shown in Figure 4.3A, efflux of [^{14}C]TEA from CHO-hOCT1 cells over 5 minutes was reduced by 74% ($p < 0.01$) by 200 μM quinidine, but enhanced 1.8-fold ($p < 0.01$) by 2 mM TEA indicating that hOCT1 can mediate efflux of cations. Efflux of [^{14}C]furamidine after preloading 10 μM [^{14}C]furamidine in CHO-hOCT1 cells for 2 hour was linear up to 10 minutes (data not shown). As shown in Figure 4.3B, the efflux of [^{14}C]furamidine over 5 minutes from CHO-hOCT1 cells was reduced by 21% ($p < 0.05$) by 200 μM quinidine, but enhanced 1.5-fold ($p < 0.05$) by 2 mM TEA, proving that hOCT1 also transports furamidine out of cells.

Cellular Localization of Functional hOCT1 in Caco-2 cells and MDCKII-hOCT1 Cells.

Uptake of 1 μM [^{14}C]furamidine from both sides of Caco-2 cell monolayers was linear up to 30 minutes (data not shown). Apical uptake of [^{14}C]furamidine was 2.4-fold ($p < 0.01$) higher than basolateral uptake in Caco-2 cells, and 1 mM ranitidine, an OCT1 substrate/inhibitor (Bourdet et al., 2005), reduced it by 83% ($p < 0.01$); in contrast, the basolateral uptake was not reduced by ranitidine (Figure 4.4). These results provided evidence for expression of functional hOCT1 mainly in the apical membrane of Caco-2 cells, and supported the previous report (Ng, 2003) which provided immunohistochemical evidence for the presence of hOCT1 on the apical membrane of Caco-2 cells. Uptake of 1 μM [^{14}C]furamidine from both sides of MDCKII-hOCT1 cell monolayers was linear up to 30 minutes (data not shown). In MDCKII-hOCT1 cells, the apical and basolateral uptake was 1.5-fold ($p < 0.05$) and 2.6-fold ($p < 0.01$) higher, respectively, than that in mock cells (Figure 4.5), providing evidence for expression of functional hOCT1 mainly in the basolateral membrane of stably transfected MDCKII-hOCT1 cells.

Transport of Furamidine Diamidoxime across MDCKII-hOCT1 Cells

Based on the basolateral localization of hOCT1 in MDCKII-hOCT1 cells, this cell line was chosen for further transport study. The experiment involved placing 25 μM furamidine diamidoxime in the apical compartment, and measuring the concentration of furamidine appearing in the basolateral compartment as a function of time. It was anticipated that the diamidoxime prodrug would be converted rapidly and quantitatively to furamidine in the MDCKII-hOCT1 cells, which would then appear in the basolateral compartment with the assistance of hOCT1 expressed in the basolateral membrane of MDCKII-hOCT1 cell monolayers. As shown in Figure 4.6, the basolateral availability of furamidine in MDCKII-

hOCT1 cell monolayers was 1.57-fold ($p < 0.05$) greater than that of mock cells. With 2 mM TEA in the receptor compartment (*trans*-stimulation), the basolateral availability of furamidine in MDCKII-hOCT1 cells was 2-fold ($p < 0.01$) greater than that across mock cells.

E. DISCUSSION.

Investigation of transporters in the basolateral membrane of intestinal epithelium that may play a role in the oral absorption of hydrophilic drugs with net charge have revealed the presence of MRP transporters as well as OSTαβ, both of which can transport zwitterionic and/or anionic compounds. The present study was undertaken to determine if there are transporters in the basolateral membrane of intestinal epithelium that can facilitate egress of hydrophilic cations. Evidence exists for the presence of OCT1 in the basolateral membrane of human as well as mouse intestinal epithelium (Jonker et al., 2001; Wang et al., 2002; Muller et al., 2005). Furamidine was shown to be a substrate for OCT1 (Appendix); furthermore, the diamidoxime prodrug of furamidine is available in which the positive charges are masked and which is able to enter Caco-2 cell monolayers, used as a model for intestinal epithelium, across apical membrane via passive diffusion, and rapidly converted to furamidine via facile reductive metabolism mediated by cytochrome *b5* and NADH cytochrome *b5* reductase. Hence furamidine and its diamidoxime prodrug provided an excellent pair of compounds to determine if OCT1 can assist egress of hydrophilic cations across the basolateral membrane of enterocytes.

Initial experiments with CHO-OCT1 cells could demonstrate that furamidine can egress out of cells via OCT1-mediated transport and that an organic cation outside the cell can accelerate this efflux (Figure 4.3). However, quinidine only decreased furamidine efflux by 21%, compared to 74% decrease for TEA efflux, indicating that hOCT1 mediated efflux of furamidine is not as efficient as that of TEA. Furthermore, activity of furamidine efflux by hOCT1 is lower than its uptake, which showed over 5-fold higher activity in CHO-hOCT1 cells than mock cells (Appendix). Transport via OCTs is driven by membrane potential, and

the uptake of positively charged molecules is thermodynamically favorable while the efflux is unfavorable (Koepsell et al., 2007). With two positive charges in each molecule, aromatic diamidines have more energy to drive uptake but also have to overcome higher energy barrier in the efflux process. In addition, furamidine binds to DNA with high capacity (Lansiaux et al., 2002), which therefore decreases the free furamidine concentration inside the cells and reduces the driving force for cellular efflux. The divalent positive charge and high DNA binding of furamidine may result in its inefficient efflux by hOCT1.

Functional studies further showed that OCT1 is expressed in the apical membrane of Caco-2 cells (Figure 4.4), which are routinely used as a model for intestinal epithelium. Hence Caco-2 cells were deemed inappropriate as a model for studies designed to assess the role of OCT1 in basolateral efflux of compounds. As a result, hOCT1 was stably expressed in MDCKII cells, and results in Figure 4.5 provided evidence that hOCT1 was localized in the basolateral membrane, thus making them a suitable cellular model. Results reported in Figure 4.6 showed that furamidine appeared in the basolateral compartment when diamidoxime prodrug of furamidine was placed in the apical compartment of MDCKII-hOCT1 cell monolayers. Further, these results showed that the appearance of furamidine in the basolateral compartment is 1.6-fold faster in the MDCKII-hOCT1 cells compared to mock cells. The rate of furamidine appearance was significantly increased when an organic cation, TEA was placed in the basolateral compartment due to *trans*-stimulation.

The overall permeability of furamidine diamidoxime cannot be calculated because the pure standard of the intermediate product, furamidine monoamidoxime, is not available. Based on the appearance of furamidine diamidoxime and furamidine in the donor compartment, the permeability of about 20 nm/sec is still much lower than that of

pafuramidine in MDCKII cells (355 nm/sec) (Saulter, 2005), though the increase of furamidine diamidoxime transport was shown in the presence of hOCT1 in the basolateral membrane of MDCKII cells. Furamidine diamidoxime is a neutral and lipophilic molecule with a ClogP of 3.1, which is close to that of pafuramidine (ClogP of 4.3) (Saulter, 2005). Thus, the transcellular permeability of furamidine diamidoxime is still lower than that expected based on its lipophilicity. Intestinal absorptive transport involves uptake across the apical membrane followed by cytosolic translocation and subsequent basolateral efflux into the blood. In the intestinal transport of furamidine diamidoxime, apical uptake of lipophilic prodrug is quick and not rate-limiting. After metabolism into furamidine by cellular reductases, the diamidine molecule binds to DNA extensively, leading to inefficient cytosolic translocation. Passive diffusion of the highly charged diamidine across the basolateral membrane is minimal and hOCT1 facilitates the efflux of furamidine, however, with lower efficiency compared to the uptake of furamidine by the same transporter. Therefore, cellular sequestration and basolateral efflux of furamidine are the rate limiting steps in transcellular transport of furamidine diamidoxime in the intestine. Binding of aromatic diamidines to DNA in kinetoplastid and mitochondrion of target trypanosomes has been considered as one of the main mechanisms for their antiparasitic action (Barrett et al., 2007). Therefore, it is not rational to design a diamidine compound without DNA binding in order to decrease the cellular sequestration. Design of diamidine prodrugs that are only metabolized by enzymes in the blood may be a better strategy to avoid the metabolism of prodrug followed by cellular sequestration in the intestine.

F. ACKNOWLEDGEMENTS.

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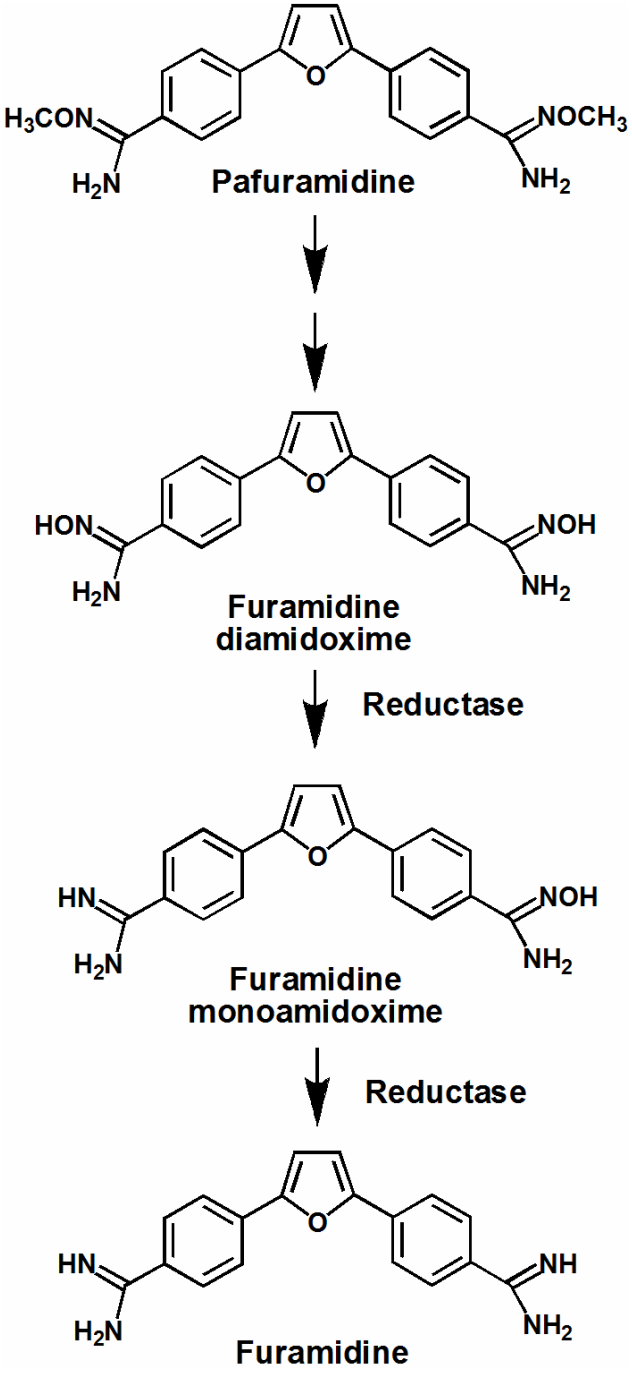

Figure 4.1. Metabolism of furamidine diamidoxime. The conversion of furamidine diamidoxime to furamidine monoamidoxime and the subsequent conversion to furamidine are both reductive N-dehydroxylation reactions. Such reactions have been shown to be catalyzed by the cytochrome *b5/b5* reductase system .

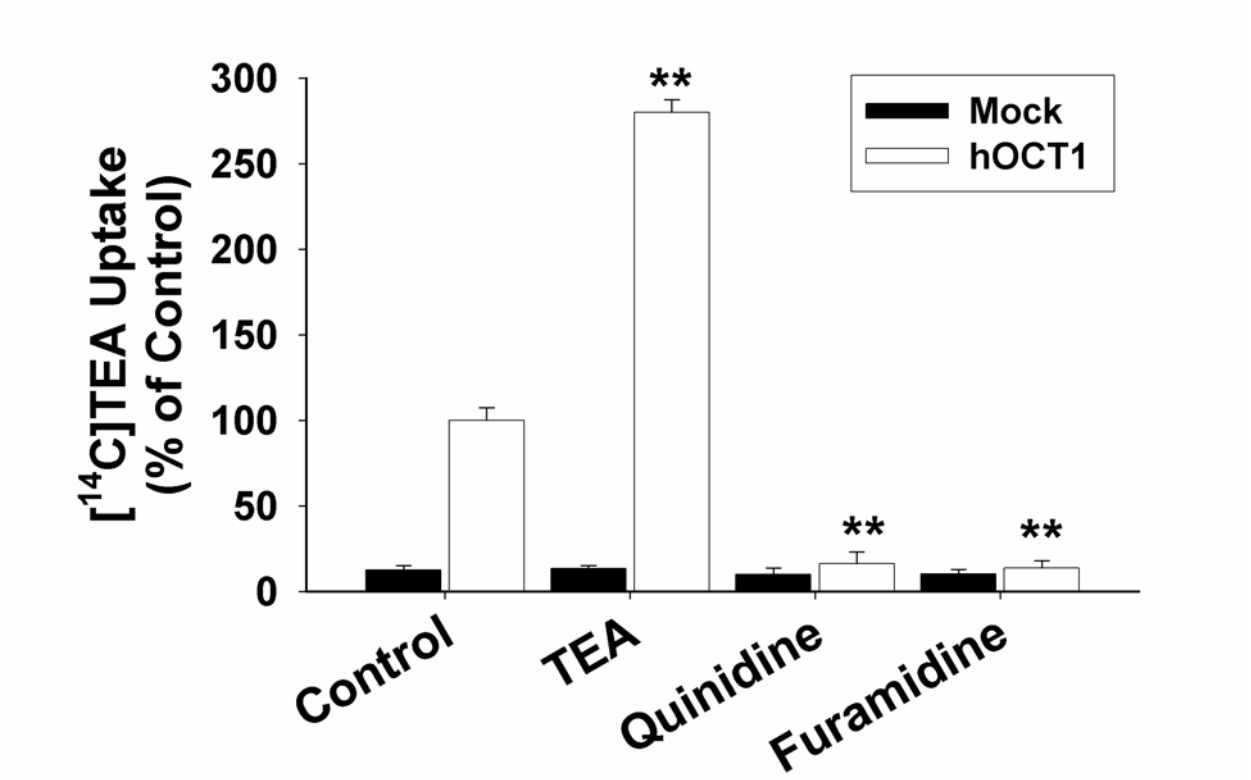

Figure 4.2. *trans*-stimulation of [^{14}C]TEA uptake in CHO-hOCT1 cells. CHO-hOCT1 (open bar) and mock cells (solid bar) were pre-incubated with either 0.4 ml of transport buffer (Control) or 0.4 ml of transport buffer with 2 mM non-radiolabelled TEA (TEA), 200 μM quinidine (Quinidine) or 100 μM furamidine (Furamidine) at 37°C for 1 h. Cells were then rinsed twice with ice-cold transport buffer and once with 1 ml of the buffer before [^{14}C]TEA uptake was performed. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

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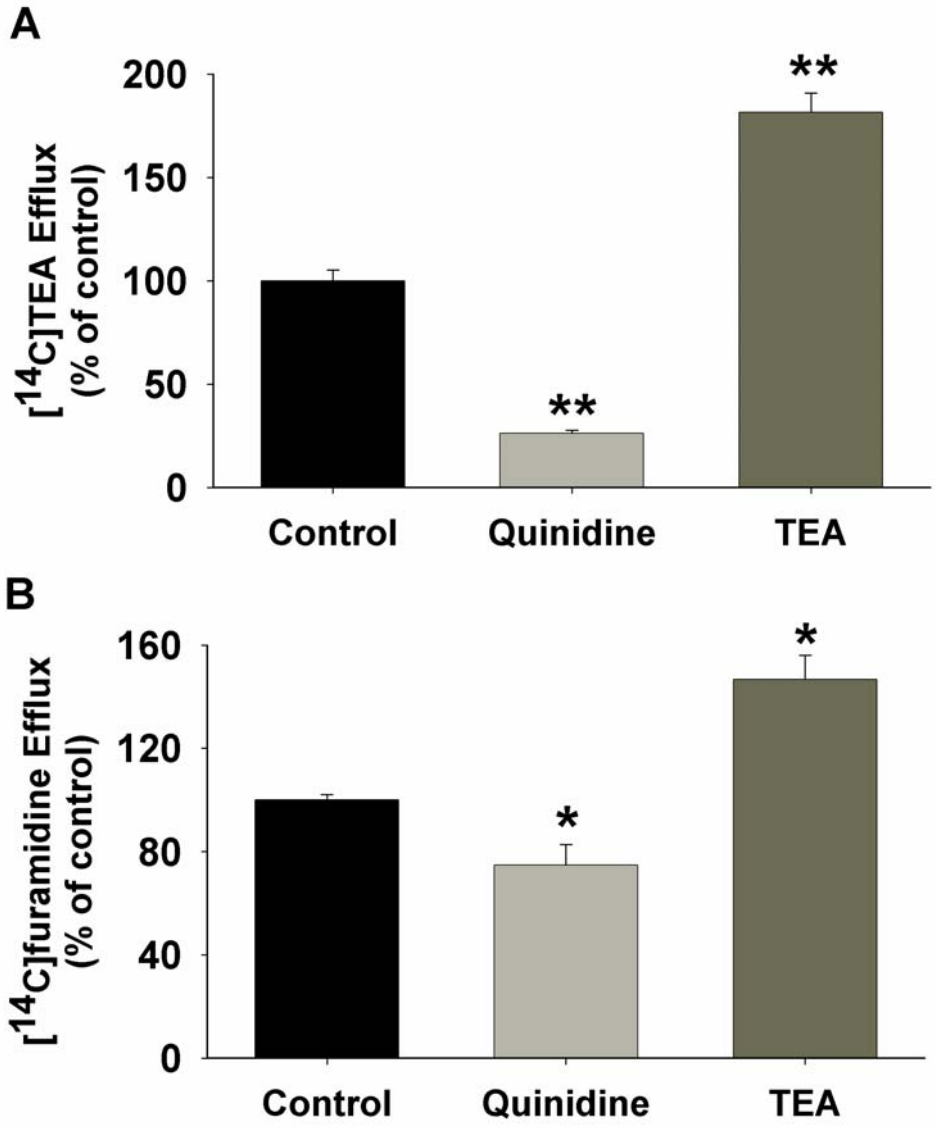

Figure 4.3. Efflux of [^{14}C]TEA (A) and [^{14}C]furamidine (B) from CHO-hOCT1 Cells. CHO-hOCT1 cells were incubated for 2 hour with 10 μM [^{14}C]TEA (A) or [^{14}C]furamidine (B). After washing, efflux was determined within the linear efflux region within in the absence (Control) or presence of 2 mM non-radiolabelled TEA (TEA) and 200 μM quinidine (Quinidine). Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

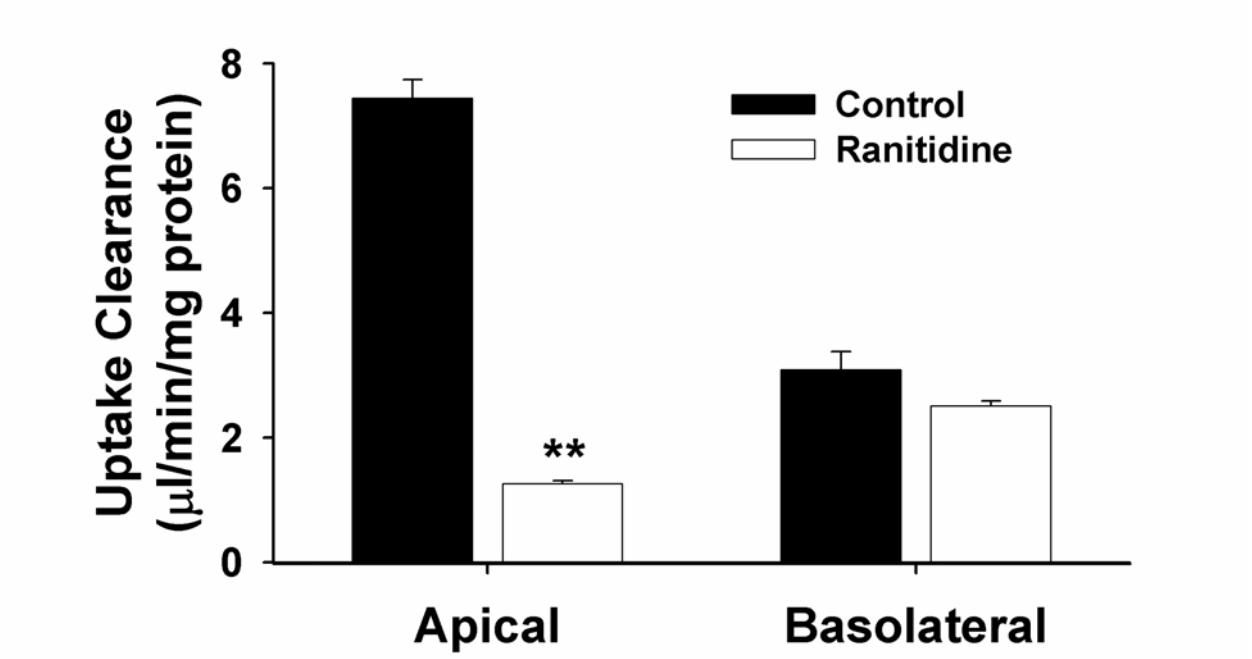

Figure 4.4. Uptake of [^{14}C]furamidine in Caco-2 Cells. After pre-incubation in transporter buffer for 30 minutes, 1 μM [^{14}C]furamidine in the absence (solid bar) or presence of 1 mM ranitidine (open bar) was added into the apical or basolateral chamber of Caco-2 cell monolayers and uptake studies were performed. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with control.

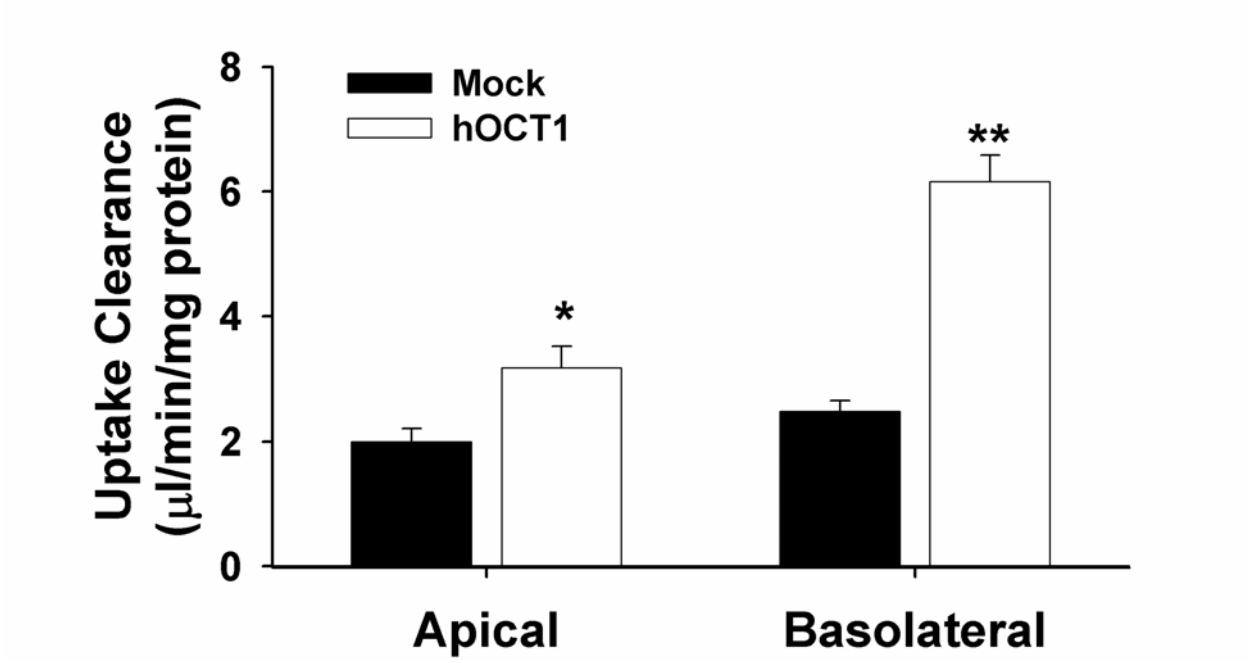

Figure 4.5. Uptake of [^{14}C]furamidine in MDCKII-hOCT1 Cells. MDCKII-hOCT1 (open bar) and mock cells (solid bar) were pre-incubated in transporter buffer for 30 minutes, 1 μM [^{14}C]furamidine was added into the apical or basolateral chamber of the cell monolayers and uptake studies were performed. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

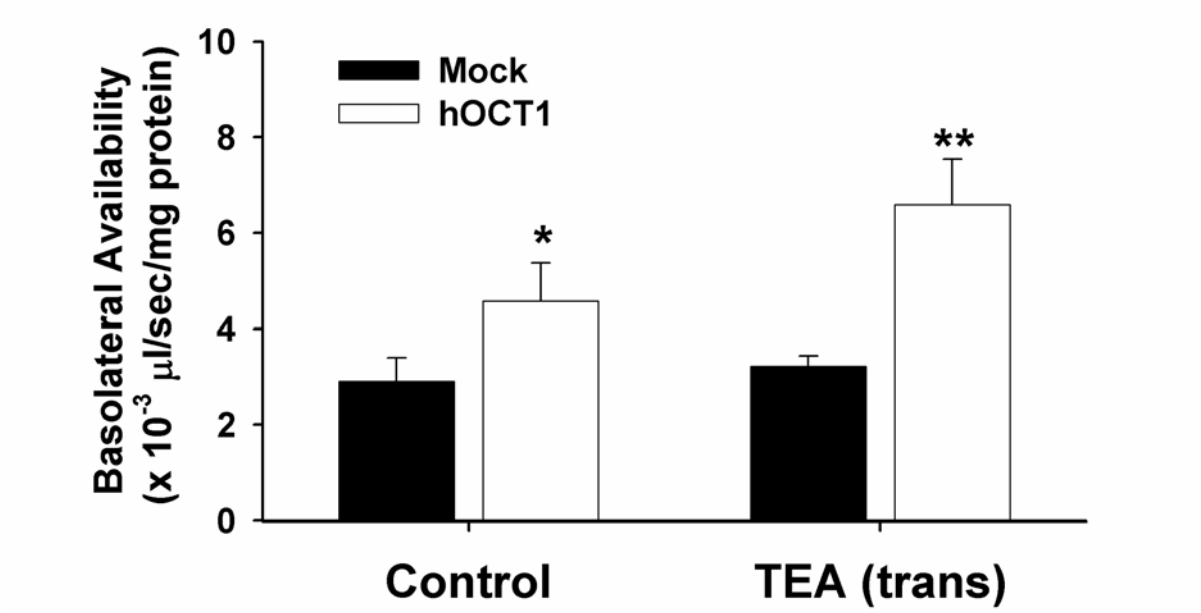

Figure 4.6. Basolateral availability of furamidine across MDCKII-hOCT1 cell monolayers. MDCKII-hOCT1 (open bar) and mock cells (solid bar) were pre-incubated in transporter buffer for 30 minutes, 25 μM furamidine diamidoxime was added into the apical chamber of the cell monolayers and transport studies were performed in the absence (Control) or presence of 2 mM non-radiolabelled TEA (TEA(trans)) in the basolateral chamber. Appearance of furamidine in the basolateral chamber was measured to obtain the basolateral availability of furamidine. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

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CHAPTER 5

CONCLUSIONS

Roles of Basolateral Transporters in Intestinal Drug Absorption

The studies undertaken for this dissertation project have broadly focused on elucidating the roles of the basolateral transporters in intestinal absorption of drugs and prodrugs. An understanding of the basolateral efflux mechanism in the intestine is of much interest because it is still a major missing link in transporter mediated drug absorption. In contrast, the presence of many transporters in the apical and basolateral membrane has been established in the liver and the kidney, and their roles in governing drug disposition in these organs have been elucidated. The importance of the basolateral transporters has been established for intestinal absorption of nutrients such as glucose and bile acids. Extrapolating from the known functions of basolateral transporters in the body, it was hypothesized that intestinal basolateral transporters may determine the transcellular transport of drugs when apical transporters mediate uptake efficiently, or prodrugs diffuse through the apical membrane efficiently and are metabolized into hydrophilic and/or charged drugs in the enterocytes.

Fexofenadine, a zwitterionic anti-allergy medicine, represents a large class of drugs that contain both acidic and basic groups and possess molecular weight over 300. The charged nature of these compounds in the physiological pH makes it hard for them to permeate biological membranes. In addition, many of them are substrates for P-gp, which would make their intestinal absorption even less effective. However, these bulky zwitterionic drugs achieve greater bioavailability than that expected from their physicochemical properties and efflux by P-gp. Therefore, this work was undertaken to test the hypothesis that vectorial transport systems, including apical uptake and basolateral efflux transporters, play a pivotal role in the intestinal absorption of zwitterionic drugs such as fexofenadine. In Chapter

2 of this dissertation, the apical uptake transporter of fexofenadine was re-examined first. The results showed that OATP2B1, but not OCT3 or OCTN2, mediates transport of [^{3}H]fexofenadine into transfected cells at pH 6.0. Apical uptake of fexofenadine decreased by half with an OATP inhibitor at pH 6.0, but not at pH 7.4. This result supported that OATP2B1 mediates apical uptake of fexofenadine into Caco-2 cells at acidic pH because OATP2B1 is the only OATP isoform showing pH dependent activity. OATP2B1-mediated uptake of fexofenadine may be the major mechanism *in vivo* because of the acidic microclimate in small intestine. The basolateral efflux of fexofenadine in Caco-2 cells was examined, and the studies focused on the facilitative transporter OSTαβ and the ABC transporter MRP3. These two transporters showed either protein localization (MRP3) or functional activity (OSTαβ) in the basolateral membrane of Caco-2 cells. However, studies showed that OSTαβ did not contribute to the basolateral transport of fexofenadine; this conclusion was based on the observations that its substrates/inhibitors did not interact with fexofenadine transport in the basolateral side of Caco-2 cells. MRP inhibitor reduced the basolateral efflux of cellular fexofenadine by 57%. This result indicated that basolateral efflux of fexofenadine is mediated by MRP but not by OSTαβ in Caco-2 cells. Combining the findings from this work with the literature evidence showing that fexofenadine is a substrate for MRP3 but not for MRP4 (Matsushima et al., 2008a), it was concluded that MRP3 plays a role in the basolateral efflux of fexofenadine in Caco-2 cell monolayers. The MRP inhibitor MK-571 decreased the absorptive transport of fexofenadine moderately. However, MK571 decreased more dramatically when P-gp was also inhibited. The results showed apical entry dominated by P-gp mediated efflux is rate limiting in absorptive transport of fexofenadine in Caco-2 cells. However, basolateral efflux by MRP3 may control

the absorption rate of fexofenadine in human small intestine when P-gp's influence in the intestinal absorption is diminished due to the inhibition by drugs and diets, and genetic variation of *MDR1* gene.

Another situation in which basolateral efflux transporters could be important is when lipophilic prodrugs diffuse through the apical membrane and are metabolized into the hydrophilic drugs in the enterocytes efficiently. Adefovir dipivoxil is known to be metabolized completely into adefovir in the enterocytes (Annaert et al., 1997; Annaert et al., 2000). The studies in Chapter 3 examined whether MRP4, a known adefovir transporter (Schuetz et al., 1999), in Caco-2 cells plays a role in the basolateral efflux of adefovir generated from adefovir dipivoxil inside the cells. The presence of MRP4 protein in Caco-2 cell was established by Western blotting and its basolateral localization was confirmed by immunofluorescence microscopy and Western blotting of membrane fractions isolated using a method involving biotinylation of surface membrane proteins followed by affinity enrichment. MRP4-knockdown Caco-2 cells were constructed in which the MRP4 protein level and activity were reduced; the availability of [^{3}H]adefovir in the basolateral compartment after apical dosing the cell monolayers with [^{3}H]adefovir dipivoxil was reduced by 39% in the MRP4-defficient Caco-2 cells. In a similar experiment, chemical inhibition of MRP4 in wild type cells reduced the availability of [^{3}H]adefovir in the basolateral compartment by 48%. These results demonstrated that MRP4 in the basolateral membrane of Caco-2 cells contributes predominantly to the efflux of adefovir generated intracellularly from its prodrug, thus highlighting the importance of the basolateral MRP4 in oral absorption of adefovir dipivoxil.

The first two parts of this project focused on the roles of the ABC transporters, transporting anionic compounds, in the basolateral membrane of the intestine. Besides the ABC transporters, many SLC transporters are also expressed in the basolateral membrane of the intestine, including OCT1 that mainly transports cationic compounds (Muller et al., 2005; Koepsell et al., 2007). In the previous studies in the Thakker laboratory, it was found that this transporter is expressed in the apical membrane of Caco-2 cells (Ng, 2003). Further, evidence did not support an OCT1-mediated efflux of OCT1 substrates, ranitidine and metformin, across the basolateral membrane of Caco-2 cells; rather, both ranitidine and metformin appeared to be transported across the apical membrane via an OCT-like transporter (Bourdet and Thakker, 2006; Proctor et al., 2008a). In Chapter 4 of this dissertation project, hOCT1 was expressed in the basolateral membrane of MDCKII cells. The possible efflux function of this transporter was examined using dicationic drugs pentamidine and furamidine. Both of these compounds were found to be substrates for hOCT1, which represents the first report showing that dicationic drugs are substrates for an OCT transporter. It is conceivable that OCT1 may mediate hepatic elimination and toxicity of these aromatic diamidine drugs (Appendix). Whether hOCT1 in the intestinal basolateral membrane enhances the transcellular transport of the diamidoxime prodrug of furamidine, by facilitating basolateral efflux of furamidine formed in the enterocytes, was tested in Chapter 4. The results showed that efflux of [^{14}C]furamidine from hOCT1 stably transfected CHO cells was inhibited by quinidine but *trans*-stimulated by TEA, indicating that hOCT1 can mediate efflux of this diamidine. Then, basolateral expression of hOCT1 in MDCKII-hOCT1 cells was confirmed by the result of 2.6-fold higher basolateral uptake of [^{14}C]furamidine in the transfected cells compared to mock cells. Finally, transport of furamidine diamidoxime in MDCKII-hOCT1

cells showed that availability of furamidine on the basolateral side, after apical dosing of the diamidoxime prodrug, was 1.5-fold higher than that in mock cells and was *trans*-stimulated by TEA in the basolateral compartment. This study showed that hOCT1 facilitates the basolateral efflux of furamidine after metabolism from its diamidoxime prodrug in the cells. However, the efflux of furamidine by OCT1 is not as efficient as the uptake process, because OCT1-mediated transport is driven by the outside-positive membrane potential, which resists thermodynamically the efflux process of positively charged molecules (Koepsell et al., 2007). In addition, intracellular metabolism and consequently cellular sequestration may play an important role in limiting the transport of furamidine diamidoxime across the intestinal epithelium.

In conclusion, intestinal basolateral transporters may determine the transcellular transport rate when apical transporters mediate uptake efficiently; or prodrugs diffuse through the apical membrane efficiently and are metabolized into hydrophilic drugs in the enterocytes. Although not tested in this dissertation project, intestinal basolateral transporters in ABC superfamily may also protect the enterocytes preventing entry of drugs or their metabolites from blood; for example, Mrp4 prevented the entry and toxicity of adefovir into the enterocytes from the blood circulation (Belinsky et al., 2007). In addition, the basolateral transporters in the SLC family may play a role in intestinal secretion of xenobiotics via uptake of substrates from blood circulation into the enterocytes (Arimori and Nakano, 1998), for example, the basolateral Oct1 facilitates the secretion of its substrates from the circulating blood into the intestinal lumen by mediating the basolateral uptake process (Jonker et al., 2001; Wang et al., 2002).

Factors Influencing the Importance of Intestinal Basolateral Transporters

Being a drug substrate for an intestinal basolateral transporter does not necessarily mean that this transporter must play an important role in intestinal absorption of the drug. In addition to the substrate activity of the drug toward the basolateral transporter, the importance of this transporter in overall absorptive transport is also influenced by other factors, including the paralleled mechanisms in the basolateral efflux step and the other steps in the transcellular transport process.

The transport across the basolateral membrane is a combination of carrier-mediated transport and passive diffusion as described in Equation 1. If the clearance for passive diffusion $K_d >> J_{max}/K_m$ (intrinsic clearance of the transporter), modulation of the basolateral transporter activity, which is represented as $J_{max}*C/(K_m+C)$, will not affect the overall transport rate J dramatically.

$$J = \frac{J_{\max} * C}{K_m + C} + K_d * C \qquad (1)$$

The basolateral transporter activity is represented as $J_{max}*C/(K_m+C)$. The membrane expression level of the transporter determines J_{max}. The basolateral transporter only functions when the substrate does not saturate it (when $C << K_m$), so the activity is also related to the intracellular concentration of the compound, which is controlled by multiple factors including apical entry, cellular binding and basolateral efflux. The basolateral transporters have typical high K_m values to ensure not being saturated. *In vitro* measurement of the K_m value of a specific substrate for a specific transporter is still important to assess whether this transporter is functional in the basolateral egress of the test compound *in vivo*.

Barrier epithelia are known to have more permeable basolateral membranes compared to apical membranes (Zeidel, 1996), due to higher fluidity of the exofacial leaflet of the basolateral membranes (Negrete et al., 1996). The higher membrane permeability of digoxin across the basolateral membrane has been reported in the transcellular transport across MDCKII cells (Ito et al., 1999). Therefore, changes in basolateral transporter activity will not affect the transport rate across of the basolateral membrane if a drug is reasonably lipophilic. In addition, if a drug crosses apical membrane by passive diffusion efficiently, then it transports through the basolateral membrane will be even faster via the same mechanism. Therefore, if an apical uptake transporter is not involved in intestinal transport of a lipophilic drug, then the possibility is even lower that a carrier-mediated process is important in the basolateral egress, unless the lipophilic drug is metabolized into hydrophilic metabolites in the enterocytes.

Another factor that affects passive permeability is the ionization of the compounds. In small intestine, intestinal lumen has acidic microclimate with pH of 6.0-6.5 (Daniel et al., 1985), and intracellular cytosol of villus tips and circulating blood are at neutral pH (around 7.4) (Hayashi and Suzuki, 1998) (Figure 5.1). The different pH values across the intestinal epithelium can lead to differential ionization of a drug in these three compartments (apical, intracellular, and basolateral) and therefore influence the permeability of acidic and basic compounds differently across the apical and basolateral membranes of the enterocytes. For an acidic compound, the passive permeability across the apical membrane is likely to be greater than that across the basolateral membrane because the acidic compound is less ionized in intestinal lumen than intracellular cytosol. However, similar pH values between the cytosolic compartment and the blood make the permeability across the basolateral

membrane lower than that across the apical membrane. In contrast, the differential pH values lead to higher permeability across the basolateral membrane than the apical one for basic drugs. Because of the lower passive diffusion across the basolateral membrane, acidic compounds may have greater component of carrier-mediated transport compared to basic compounds. In addition, small cationic (basic) compounds tend to transport across the intestinal epithelium in paracellular pathway due to the favorable interaction with negative charges in the paracellular space including tight junctions (Lee and Thakker, 1999; Anderson, 2001; Bourdet and Thakker, 2006; Proctor et al., 2008a), thus carrier-mediated basolateral egress would be less important for small cationic drugs. The pH gradient across the intestinal epithelium and its influence on the differential permeability of acidic and basic drugs across the apical and basolateral membranes may explain why only anion-specific MRPs, and none of the cation-specific ABC transporters, are present on the basolateral membrane of intestinal epithelium

The importance of basolateral transporters in absorptive transport of a compound is also influenced by other steps in the transcellular transport process. Intestinal drug absorption involves at least three steps: the apical entry, cytosolic translocation and basolateral egress. According to Equation 2,

$$\frac{1}{Pt} = \frac{1}{Pa} + \frac{1}{Pc} + \frac{1}{Pb} \qquad (2)$$

(where *Pt, Pa, Pc* and *Pb* represent the permeability of a compound across the intestinal epithelium, apical membrane, cytosol and basolateral membrane, respectively), the overall transcellular transport is limited by the slowest step, so called the rate-limiting step. Accordingly, if the basolateral transport is not rate limiting, the overall rate will not be

sensitive to the modulation of the basolateral transporter, even if a carrier-mediated transport mechanism contributes to the basolateral egress of a compound. For example, inhibition of MRPs in Caco-2 cells only decreased the absorptive transport of fexofenadine moderately, but when P-gp was inhibited, inhibition of MRPs decreased the absorptive transport more dramatically. The results indicate that apical entry, dominated by P-gp mediated efflux, is rate limiting in the absorptive transport of fexofenadine in Caco-2 cells. When the rate limiting step is shifted by inhibition of P-gp, the overall transport is more sensitive to the modulation of the basolateral MRP3.

Predicting the Importance of Intestinal Basolateral Transporters

It is difficult to predict the importance of intestinal basolateral transporters in drug absorption because many factors may influence *in vivo* functions of these transporters besides their activity for transporting drugs. Biopharmaceutics Classification System (BCS) has been applied to categorize drugs into four classes according to their solubility and permeability in order to predict *in vivo* pharmacokinetics of drug products, mainly oral bioavailability, from *in vitro* measurements of permeability and solubility (Figure 5.2) (Amidon et al., 1995). Recently, BCS was applied to predict drug disposition *in vivo*, including effects of efflux and absorptive transporters on drug absorption (Wu and Benet, 2005). This system is used here as a guideline to predict the importance of intestinal basolateral transporters in drug absorption.

Effects of intestinal basolateral transporter will be important for a few Class 1 compounds. The high permeability and solubility of the class 1 compounds leads to high concentrations in the intestinal lumen that saturate any transporter. Therefore, Class 1 compounds may be substrates for transporters using *in vitro* cellular systems, but transporter effects may not be important clinically. Furthermore, most of class 1 compounds are

lipophilic and able to cross both membranes by passive diffusion. However, some class 1 compounds achieve higher permeability because they act as substrates for absorptive transporters; for example, L-dopa is classified into Class 1 (Wu and Benet, 2005) because it is absorbed via high capacity amino acid transport systems in the intestine (Lennernas et al., 1993). In this case, the intestinal basolateral transporters such as TAT1 (Ramadan et al., 2006) and LAT2 (Soares-da-Silva et al., 2004) are as important as the apical transporters in intestinal absorption of L-dopa. Therefore, effects of intestinal basolateral transporter will be important for the class 1 compounds that achieve higher permeability by absorptive transporters, including L-dopa and valacyclovir.

Effects of intestinal basolateral transporter will be minimal for Class 2 compounds, but may be important for disposition of their metabolites.

The class 2 compounds are typically bulky and lipophilic and are able to cross both membranes by passive diffusion. The low solubility leads to low concentrations in intestinal lumen. Many of the class 2 compounds are substrates for apical efflux transporters such as P-gp, and the rate-limiting step of intestinal transport is the apical entry controlled by the efflux by P-gp. Therefore, the effects of the basolateral transporters will be minimal because of the high passive permeability and efflux by apical transporters. However, some of class 2 compounds are prodrugs that are metabolized in the intestinal epithelium, for example, adefovir dipivoxil; or they are the active drugs but are metabolized to active metabolites in the enterocytes, for example, terfenadine is metabolized to fexofenadine, which possesses even higher antihistamine activity, in the intestine (Grant et al., 1999). For these class 2 compounds that are prodrugs or generate active metabolites in the enterocytes, the intestinal basolateral transporters may be important for their oral activity.

Intestinal basolateral transporter effects will predominate for Class 3 and 4 compounds, and are more important for anionic compounds. For the Class 3 and 4 compounds, absorptive transport systems, including apical uptake and basolateral efflux transporters, will be necessary to overcome the poor passive permeability characteristics of these compounds. Many class 3 and 4 compounds are ionizable, and are either positively or negatively charged at physiological pH in the intestine. For the hydrophilic anionic drugs such as pravastatin and methotrexate, the passive permeability across apical membrane is higher than that across the basolateral membrane; therefore, the basolateral transporters may have a more significant influence on their oral absorption. On the contrary, for the hydrophilic cationic drugs such as ranitidine, a basolateral transporter may have less influence on oral absorption because of their likely relatively high permeability across the basolateral membrane. In addition, the preferred paracellular transport of hydrophilic cationic drugs also decreases the importance of the basolateral transporters. The basolateral transporter effects in BCS were summarized in Figure 5.2.

Future Direction

The obstacles to understanding the role of the basolateral transporters in intestinal drug absorption are the small number of these transporters identified in the intestine and somewhat unclear *in vivo* functions of the known basolateral transporters. A combination of mechanistic studies with *in vitro* systems and functional research in animals and humans is needed to overcome these obstacles, which is the future direction in this field.

Molecular cloning of novel transporters is needed for identification of the key basolateral transporters in the intestine; for example, the peptide basolateral transporter, which is expressed in the basolateral membrane of Caco-2 cells (Terada and Inui, 2004), has

not yet been identified in the intestine. Localization of "orphan" transporters, for example, MRP5, MRP8 and MRP9, in the enterocytes and characterization of their functional activity may be an important near-term objective. In addition, for the known basolateral transporters such as MRP3 and OSTαβ, their substrate specificity should be explored by measuring substrate activity in the transporter transfected models because few drugs have proven to be substrates for these transporters.

In vivo functional characterization of intestinal basolateral transporters in drug absorption is more demanding compared to the *in vitro* studies. Pharmacokinetic studies in transporter knockout mice have been a successfully used method in exploring the roles of transporters in hepatic and renal disposition of drugs and their metabolites. Recently, transport of bile acids using everted gut sac and absorption of bile acids were studied in Ostα$^{-/-}$ and Ostα$^{-/-}$Mrp3$^{-/-}$ mice (Rao et al., 2008). This is the first study to investigate the *in vivo* function of intestinal basolateral transporters, and represents a significant breakthrough in this area. This kind of studies in transporter knockout animals will be applied to study other intestinal basolateral transporters in the near future.

The most convincing evidence of transporter effects is from clinical studies. Co-administration of a test drug as a transporter substrate with a transporter inhibitor is a typical design in these clinical studies. However, limited by the availability of an appropriate substrate and/or an inhibitor that is suitable for human use and the broad distribution of transporters, these studies have generated ambiguous results. Understanding of the impact of genetic variations of drug transporters on drug disposition continues to emerge. Knowing the effects of mutations on transporter activity using *in vitro* systems along with pharmacokinetic analysis of clinical data tend to generate more meaningful conclusions on transporter effects.

Clearly, the loss or reduction of transporter activities in the basolateral membrane due to genetic variation in these transporters could potentially limit intestinal absorption of their substrates. Therefore, clinical studies on the effect of genetic variation in these transporters on drug absorption will be an important area of future research. To avoid being confused by the effects of liver transporters, a human *in vivo* jejunal perfusion technique, which enables direct determination of the effective jejunal permeability of drugs (Tannergren et al., 2003b), may help understand mechanisms about intestinal transporters from clinical data.

Research on intestinal basolateral transporters will lead to more complete understanding of carrier-mediated intestinal drug absorption, for example, the failure of modulation of fexofenadine permeability by P-gp inhibitor verapamil may be partly explained by the reduction of MRP3 mediated basolateral efflux by this inhibitor simultaneously (Zeng et al., 2001; Tannergren et al., 2003b). Current research has focused on the drug-diet interaction mediated by intestinal apical transporters; however, plant phenols in vegetables, fruits and many food sources inhibit activity of multiple MRP transporters in intestinal basolateral membrane (Wu et al., 2005), which may lead to alterations to drug absorption mediated by these transporters. Although modulation of intestinal basolateral transporters does not always change pharmacokinetics of drugs dramatically, their effects definitely increase the inter-individual variability of drugs due to drug-drug interactions and genetic variations of the basolateral transporters. On the other hand, if a drug is a substrate for intestinal basolateral transporters, but not for the apical transporters, then design of their prodrugs, which are labile to intestinal enzymes, can also lead to great oral availability. In conclusion, knowledge generated from the research on intestinal basolateral transporters will provide the theoretical foundation in understanding drug-drug and food-drug interactions,

predicting individual variability in drug absorption, designing and developing drugs that will be effective when administered orally.

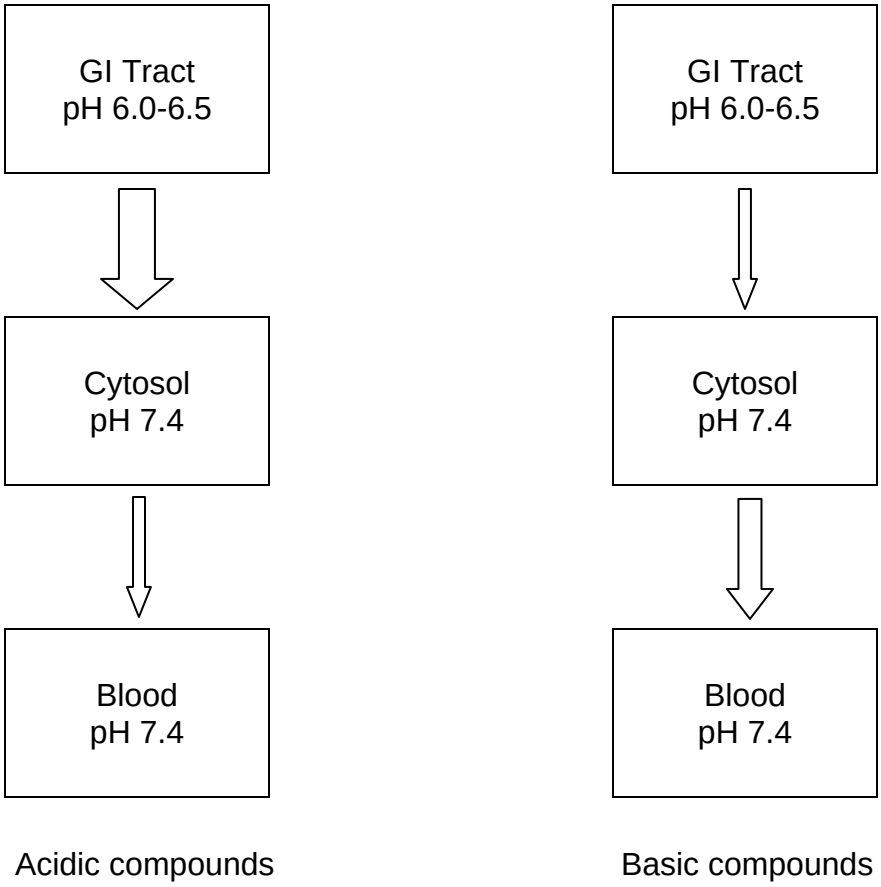

Figure 5.1. pH dependent passive diffusion of acidic or basic compounds across apical and basolateral membranes of the enterocytes. Based on the pH partition theory, acidic compounds cross apical membrane with higher permeability than that of basolateral membrane, whereas basic compounds cross basolateral membrane with higher permeability.

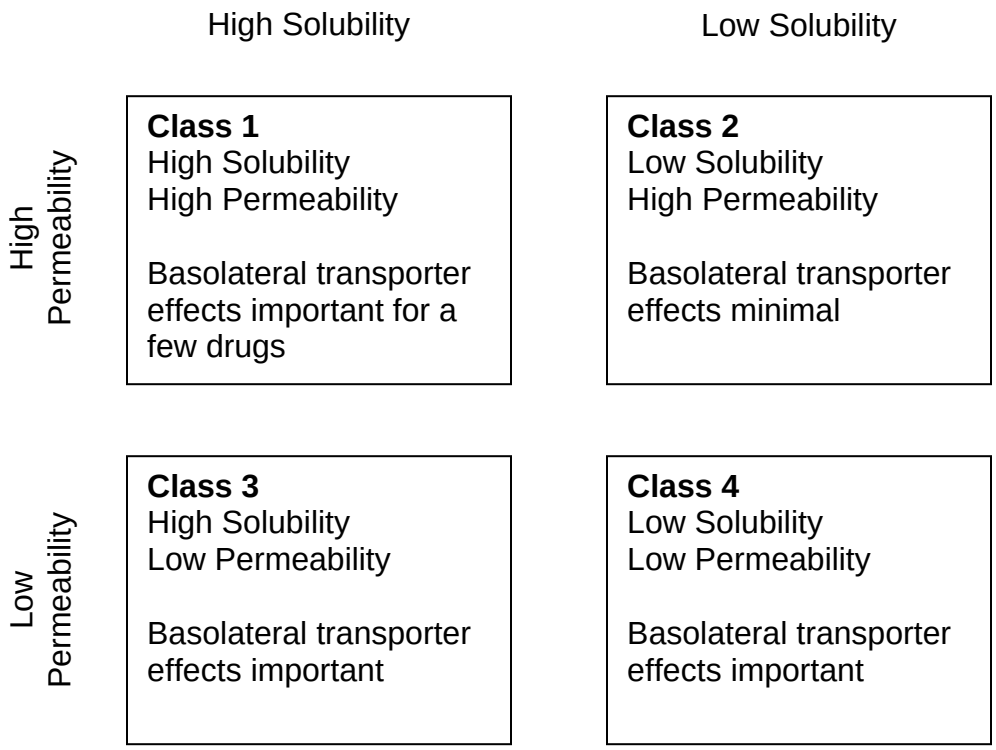

Figure 5.2. Intestinal basolateral transporter effects on drug absorption by BCS class.

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APPENDIX

TRANSPORT OF DICATIONIC DRUGS PENTAMIDINE AND FURAMIDINE BY HUMAN ORGANIC CATION TRANSPORTERS

This appendix was submitted to the *Journal of Pharmacology and Experimental Therapeutics* and was presented in the style of that journal.

A. ABSTRACT.

Transport by membrane transporters in pathogenic protozoa is critical for the antiparasitic activity of aromatic diamidine drugs, pentamidine and furamidine. However, no such diamidine transporter has been identified in mammalian cells. Aromatic diamidines are primarily eliminated in the bile, but cause toxicity in both liver and kidney, suggesting a role of transporters in trafficking these molecules in both elimination organs. The goal of this study is to investigate whether human organic cation transporters (hOCTs, SLC22A1-3) mediate the elimination and cytotoxicity of aromatic diamidines in humans. Inhibitory and substrate activities of pentamidine and furamidine were studied in stably transfected Chinese Hamster Ovary (CHO) cells. The results of [^{3}H]1-methyl-4-phenylpyridinium (MPP^+) uptake inhibition study showed that pentamidine is a potent inhibitor for all three OCT isoforms (IC_{50} < 20 μM), whereas furamidine is a potent inhibitor for hOCT1 and hOCT3 ($IC_{50} \leq 20$ μM), but less potent inhibitor for hOCT2 (IC_{50} = 189.2 μM). Both diamidines are good substrates for hOCT1 (K_m = 36.4 and 6.1 μM, respectively), and weak substrates for hOCT2, but neither is a substrate for hOCT3. hOCT1 enhanced the cytotoxicity of pentamidine and furamidine by 4.4-fold and 9.3-fold, respectively, in CHO-hOCT1 cells compared to mock cells. Ranitidine, an OCT1 inhibitor, reversed the OCT1-mediated potentiation of toxicity. This is the first finding that aromatic diamidines are substrates for mammalian transporters. These transporters may play important roles in the disposition of aromatic diamidines in humans, and resultant drug-drug interactions as well as toxicity involving diamidine drugs.

B. INTRODUCTION.

Pentamidine, an aromatic diamidine, has been used in the clinic for over 60 years against early-stage human African trypanosomiasis, to treat *Pneumocystis carinii* pneumonia (PCP) in AIDS patients, and to treat antimony-resistant leishmaniasis (Barrett et al., 2007). Furamidine is a diamidine analog of pentamidine in which the alkoxy chain linking the phenyl rings has been replaced with a furan ring (Figure A.1). Furamidine has shown excellent *in vitro* and *in vivo* activity in mouse and monkey models of early-stage African trypanosomiasis (Barrett et al., 2007). The O-methyl amidoxime prodrug of furamidine, pafuramidine, is in phase III clinical trials to orally treat human African trypanosomiasis (Barrett et al., 2007).

Pentamidine is eliminated slowly in humans with a plasma half-life of over four days after intravenous administration (Vohringer and Arasteh, 1993). Hepatic metabolism and excretion is the major elimination pathway for pentamidine, and renal excretion only accounts for a small fraction (<10%) of the administered dose in humans (Conte, 1991; Bronner et al., 1995). Furamidine is metabolically stable, and hepatic excretion is the major elimination pathway in rats (unpublished data). The elimination pathway of the diamidines is unusual because renal excretion is preferred over hepatic elimination for small cationic molecules (Meijer et al., 1999), partly due to the presence of multiple cation transport systems in kidney. Adverse drug reaction is the primary limitation for parenteral pentamidine therapy; nephrotoxicity, hepatotoxicity, gastrointestinal discomfort and pancreatitis accounted for the majority of toxicity in humans (Balslev and Nielsen, 1992; O'Brien et al., 1997). Furamidine was also associated with severe nephrotoxicity and hepatotoxicity in mice (Werbovetz, 2006).

Carrier mediated uptake and accumulation in target parasites is crucial for the toxicity of aromatic diamidines to parasites (de Koning et al., 2005; Barrett et al., 2007). The intracellular concentration of pentamidine achieved was over 1 mM in trypanosomes when exposed to 1 μM of the drug (Carter et al., 1995). This process is mediated by multiple transport systems, including the P2 transporter, the high-affinity pentamidine transporter 1, and the low-affinity pentamidine transporter 1 (Barrett et al., 2007). Mutations of these transporters in target parasites cause resistance of the target parasites to pentamidine and other aromatic diamidines (Barrett et al., 2007). However, no such diamidine transporter has been identified in mammalian cells, although carrier mediated transport in mammalian cells has been speculated because furamidine was rapidly taken up by B16 melanoma cells (Lansiaux et al., 2002). Renal clearance of pentamidine showed saturation at high doses, indicating possible involvement of an active secretion mechanism in kidney (Lidman et al., 1994). Poola et al. (2003) studied the renal excretion of pentamidine in the isolated perfused rat kidney and showed that tetraethylammonium (TEA), a protypical substrate for organic cation transporters (OCTs), reduced renal excretion of pentamidine, indicating the possible role of OCTs in its renal excretion. However, the definite role of OCTs in pharmacokinetics and toxicity of pentamidine in humans has not been established.

In this study, aromatic diamidines, pentamidine and furamidine, were first tested as substrates and inhibitors for OCTs in the SLC22A family, which are mainly expressed in the major elimination organs, kidney (the basolateral membrane of tubular cells) and liver (the sinusoidal membrane of hepatocytes) (Koepsell et al., 2007). There are three distinct OCT transporters, namely OCT1, OCT2, and OCT3, which mediate the entry of organic cations into cells (Koepsell et al., 2007). In humans, hOCT1 is the predominant OCT transporter

expressed in the liver while hOCT2 is expressed predominantly in the kidney (Koepsell et al., 2007). The majority of OCT substrates are monovalent, and comparatively small, cations, the so called type I organic cations (Meijer et al., 1999; Wright, 2005) as exemplified by the protypical substrates TEA and the neurotoxin, MPP^+ (Koepsell et al., 2007). In addition, OCTs interact with many drugs, including the H2 antagonist ranitidine (Bourdet et al., 2005), the antidiabetic drug metformin (Wang et al., 2002) and the anticancer drug oxaliplatin (Yokoo et al., 2007). The results in this study clearly demonstrate that the aromatic diamidines, pentamidine and furamidine, are potent inhibitors for hOCT1, hOCT2, and hOCT3, and are substrates for hOCT1, and to a lesser extent for hOCT2. The results further demonstrate the ability of hOCT1 to potentiate the cellular toxicity of the diamidines.

C. MATERIALS AND METHODS.

Materials

F-12 Nutrient Mixture, penicillin-streptomycin-amphotericin B solution (100x) and N-hydroxyethyl-piperazine-N'-2-ethanesulfonate (HEPES, 1M) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) and trypsin-EDTA solution (1x) were obtained from Sigma Chemical Co. (St. Louis, MO). Geneticin was obtained from Invitrogen Co. (Carlsbad, CA). Hank's balanced salt solution (HBSS) was obtained from Mediatech, Inc. (Herndon, VA). Pentamidine isethionate salt (purity > 98%), TEA chloride, MPP^+ and quinidine were purchased from Sigma (St. Louis, MO). Furamidine dihydrochloride salt (purity > 98%) was synthesized by Medichem (Chicago, IL) using previously described methods (Das and Boykin, 1977). [^{3}H]MPP^+ (85 Ci/mmol) was obtained from American Radiolabelled Chemicals (St. Louis, MO). [^{3}H]pentamidine (4.6 Ci/mmol, purity 97.4%) was obtained from Moravek Biochemicals (Brea, CA). [^{14}C]furamidine (55.3 mCi/mmol, purity 96%) was obtained from Huntingdon Life Sciences (Huntingdon, UK). Chinese hamster ovary (CHO) cells were obtained from the American Tissue Culture Collection (Manassas, VA).

cDNA

hOCT1 cDNA in pcDNA3.1 vector and hOCT2 cDNA in pCMV vector were provided by Professor Hermann Koepsell (Julius-Maximilians-University, Würzburg, Germany). hOCT3 cDNA in pSPORT1 vector was provided by Dr. Vadivel Ganapathy (Medical College of Georgia, Augusta, GA, USA) and was subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection

CHO cells were transfected with pcDNA3.1 empty vector or the vectors containing the full-length hOCT1, hOCT2 and hOCT3 using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol specific for CHO cells. Transfectants were selected with 500 μg/ml geneticin for 10 days. A clone with the highest [^{3}H]MPP$^+$ uptake activity was chosen as a stably transfected cell line for further studies. The stably transfected CHO cells were cultured in F-12 Nutrient Mixture with 10% FBS, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B plus 500 μg/ml geneticin. All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Cellular Transport

Stably transfected CHO cells were grown as monolayers in 24-well plates. Medium was changed every other day. The cells were used 5-7 days post-seeding. Cells were pre-incubated for 30 minutes at 37ºC in transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES pH 7.2). Experiments were initiated by replacement of the transport buffer with 0.4 ml of radiolabelled dose solutions in transport buffer. Uptake was determined within the linear uptake region after which the dose solution was aspirated and cells were washed three times with 4ºC transport buffer and dissolved in 500 μl 0.1 N NaOH/0.1% SDS for 4 hours with shaking. Radioactivity was determined by scintillation counting. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

For kinetic studies, varying amounts of unlabeled diamidines were added to the uptake solutions to give increasing total (radiolabeled plus unlabeled) substrate

concentrations. Nonspecific cell-associated radioactivity was determined by measuring substrate uptake in mock cells at each substrate concentration, and these values were then subtracted from the results in transfected cells to obtain uptake values and the final kinetic data. The K_m and V_{max} values were obtained by fitting the Michaelis-Menten equation $V = V_{max} \times [S]/(K_m + [S])$ to the data using WinNonlin (Pharsight, Mountain View, CA), where V refers to the rate of substrate transport, V_{max} refers to the maximum rate of substrate transport, $[S]$ refers to the concentration of substrate, and K_m is defined as the concentration of substrate at the half-maximal transport rate.

For inhibition studies, different concentrations of unlabeled inhibitors were added to uptake solution containing 1 μM [^{3}H]MPP$^+$. Nonspecific cell-associated radioactivity was determined by measuring [^{3}H]MPP$^+$ uptake in mock cells at each inhibitor concentration, and these values were then subtracted from the values in hOCT1-transfected cells to give corrected data that were used for generation of the inhibition curves. The IC_{50} value was estimated by fitting the data to the equation $V = V_o/[1 + (I/IC_{50})^n]$ using WinNonlin (Pharsight, Mountain View, CA), where V is the uptake rate of [^{3}H]MPP$^+$ in the presence of inhibitor, V_o is the uptake rate of [^{3}H]MPP$^+$ in the absence of inhibitor, I is the concentration of inhibitor, and n is the Hill coefficient.

Cytotoxicity Assay

The cytotoxicity of diamidines was measured by the Alamar Blue assay (Schoonen et al., 2005). In brief, cells were seeded in 96-well plates at 3000 cells/well. After 24 hours, cells were exposed to different concentrations of diamidines in the presence or absence of 1 mM ranitidine, an OCT1 inhibitor (Bourdet et al., 2005), for 24 hours. Drug-containing medium was replaced with fresh medium, and cells were incubated for another 24 hours.

Alamar Blue reagent was added and incubated for 4 hour. The samples were read in a microplate reader set at 544 nm excitation wave length and 584 nm emission wave length. The IC_{50} values were obtained by fitting F, the percentage of the maximal cell growth at different drug concentrations, to the equation $F = 100/[1 + (C/IC_{50})^n]$ using WinNonlin (Pharsight, Mountain View, CA); the maximal cell growth was the cell growth in the medium without diamidine; C is the concentration of diamidine, and n is the slope factor.

Statistical Analysis

Data are expressed as mean ± SD from three measurements unless otherwise noted. Statistical significance was evaluated using ANOVA followed by Dunnet's test for multiple comparisons. The data were analyzed with SigmaStat 2.0 (Systat Software, Inc., San Jose, CA).

D. RESULTS.

Expression of Functional hOCT1, hOCT2, and hOCT3 in Stably Transfected CHO Cells.

Uptake of 1 μM [^{3}H]MPP$^+$, a model substrate for OCTs, as a function of time was determined in CHO cells transfected with hOCT1 (CHO-hOCT1), hOCT2 (CHO-hOCT2), and hOCT3 (CHO-hOCT3), and compared with its uptake in mock cells. Uptake of [^{3}H]MPP$^+$ in each transfected cell line was linear up to 5 minutes, and was several-fold greater than in the mock cells (Figure A.2), providing evidence for expression of functional hOCT transporters in stably transfected CHO cells.

Inhibition of hOCT1, hOCT2, and hOCT3 by Aromatic Diamidines in CHO Cells

As expected, uptake of [^{3}H]MPP$^+$ into CHO-hOCT1, CHO-hOCT2, and CHO-hOCT3 cells was inhibited in a concentration-dependent manner by TEA and quinidine (Figure A.3), with quinidine showing over an order of magnitude greater potency than TEA (Table A.1). The two aromatic diamidine compounds tested, pentamidine and furamidine (Figure A.1), proved to be potent inhibitors of all three hOCTs. The IC_{50} values in Table A.1 show that pentamidine inhibits all three hOCTs with similar potency; it inhibits hOCT1 and hOCT2 with a potency (IC_{50} of 16.4 ± 1.7 and 10.6 ± 2.4 μM, respectively) similar to that of quinidine and is a more potent hOCT3 inhibitor than quinidine (IC_{50} of 14.8 ± 3.4 μM). Furamidine, a structural analog of pentamidine, also is a potent inhibitor of hOCT1 and hOCT3 (IC_{50} of 7.4 ± 0.9 and 20.4 ± 2.8 μM, respectively), but a weak inhibitor of hOCT2 with the IC_{50} value of 182.0 ± 30.3 μM, which is close to that of TEA (189.2 ± 11.2 μM).

Uptake of Pentamidine and Furamidine into hOCT1-, hOCT2-, and hOCT3-expressing CHO Cells

As shown in Figure A.4A, the uptake of 0.1 μM [^{3}H]pentamidine into CHO-hOCT1 cells was 5.1-fold ($p < 0.001$), greater than that into mock cells. The [^{3}H]pentamidine uptake into CHO-hOCT2 and CHO-hOCT3 cells was only marginally greater (1.2-fold, $p < 0.05$) than the control and equivalent to the control, respectively. The uptake of 1 μM [^{14}C]furamidine was 4.6-fold ($p < 0.001$) and 1.6-fold ($p < 0.01$) greater into CHO-hOCT1 and CHO-hOCT2 cells, respectively, compared with mock cells, whereas the uptake into CHO-OCT3 cells was not above the control value (Figure A.5A). These data suggest that pentamidine and furamidine are good substrates of hOCT1 and weak substrates of hOCT2, but neither of them is a substrate of hOCT3. In CHO-hOCT1 cells, accumulation of pentamidine (Figure A.4B) and furamidine (Figure A.5B) was linear over 10 minutes. The uptake, measured as a function of concentration, was saturable (Figures A.4C and A.5C) with K_m values of 36.4 ± 8.3 μM and 6.1 ± 1.1 μM, respectively, and V_{max} values of 156.0 ± 3.7 pmol/mg/min and 27.2 ± 1.3 pmol/mg/min, respectively. The uptake of two diamidine drugs in mock cells also showed concentration-dependent mechanism (data not shown), indicating endogenous transporters in CHO cells may transport aromatic diamidines.

Cytotoxicity of Diamidines in CHO-hOCT1 Cells

The question that was addressed next was whether the hOCT1-mediated cellular uptake of the diamidines could increase their cytotoxicity. The toxicity of pentamidine toward CHO-hOCT1 cells was 4.4-fold greater than toward mock cells (IC_{50} of 46.2 ± 5.3 μM and 202 ± 16.4 μM, respectively), indicating that hOCT1 enhances pentamidine-induced cytotoxicity (Figure A.6A, Table A.2). The IC_{50} values of furamidine in CHO-hOCT1 cells

and mock cells were 1.1 ± 0.05 and 10.2 ± 0.7 μM, respectively, indicating that expression of hOCT1 greatly enhances furamidine-induced cytotoxicity (Figure A.6B, Table A.2). The cytotoxicity of pentamidine and furamidine was also determined in the presence or absence of 1 mM ranitidine, an OCT1 substrate and inhibitor (Bourdet et al., 2005). At this concentration, ranitidine itself showed no cytotoxicity toward CHO or CHO-hOCT1 cells. Ranitidine treatment almost completely eliminated the hOCT1-mediated enhancement of the diamidine cytotoxicity (Figures A.6A and A.6B). The IC_{50} values of pentamidine and furamidine in CHO-hOCT2 and CHO-hOCT3 cells were not significantly different than those in CHO-Mock cells (data not shown), consistent with the results reported here that the diamidines are not good substrates of hOCT2 or hOCT3.

E. DISCUSSION.

Pentamidine, an aromatic diamidine, has been used to treat protozoal infections (Barrett et al., 2007). Both pentamidine (Conte, 1991; Bronner et al., 1995) and furamidine (unpublished data) are mainly eliminated in the liver in contrast to small cationic molecules, such as metformin (Pentikainen et al., 1979) and cimetidine (Weiner and Roth, 1981), which are mainly excreted via the kidney. Renal cation transport systems appear to contribute to the preferred renal elimination of these compounds since both metformin (Kimura et al., 2005) and cimetidine (Tahara et al., 2005) are superior substrates for hOCT2, predominantly expressed in the kidney, rather than hOCT1 that is predominantly expressed in the liver (Koepsell et al., 2007). In the present study, we examined the substrate activity of pentamidine and furamidine towards hOCTs in order to determine the molecular mechanisms responsible for disposition of aromatic diamidines in the liver and the kidney, the major elimination organs. The results showed that pentamidine and furamidine are indeed good substrates for hOCT1; in contrast, these diamidines are poor substrates for hOCT2, and do not exhibit any substrate activity toward hOCT3. These results are consistent with previous reports that pentamidine (Conte, 1991; Bronner et al., 1995) and furamidine (unpublished data) are eliminated predominantly via hepatic clearance mechanisms. These results also provide the first evidence that aromatic diamidines are substrates for mammalian transporters.

OCTs had previously been found to interact mainly with monovalent organic cations (Type I cations) (Wright, 2005; Koepsell et al., 2007). Recently, one divalent organic cation, paraquat, has been identified to be substrate for OCT2 (Chen et al., 2007). Our finding of aromatic diamidines, divalent organic cations, as substrates of hOCT1 suggests that this transporter, like hOCT2, also can transport dications. This is the first report in which a drug

with divalent cation functionality is shown to be an OCT substrate. The three OCT isoforms seem to have differential inhibition profiles and substrate activities for dicationic compounds. As shown in the present study, aromatic diamidines are better substrates for hOCT1 compared to hOCT2. In contrast, paraquat has been reported as a better substrate for hOCT2 (Chen et al., 2007). Although aromatic diamidines are potent inhibitors of hOCT3, the aromatic diamidines are not transported by it. Irrespective of the mechanism, the data suggest that hOCT1 contributes to and may be the predominant mechanism for the uptake of aromatic diamidines by the hepatocytes.

Parenteral pentamidine therapy caused adverse drug reactions including nephrotoxicity, hepatotoxicity, gastrointestinal discomfort and pancreatitis (Balslev and Nielsen, 1992; O'Brien et al., 1997). Pentamidine and furamidine are DNA minor groove binders (Barrett et al., 2007) and furamidine selectively accumulates in nuclei of tumor cells (Lansiaux et al., 2002). The rapid entry of these charged compounds into cells could not be accounted for by a simple diffusion process (Lansiaux et al., 2002). Specific transporters, analogous to those for the entry of aromatic diamidines into parasites (Barrett et al., 2007), were speculated to promote intracellular accumulation. In this study, we observed that overexpression of hOCT1 not only increased the uptake of pentamidine and furamidine, but also enhanced the cytotoxicity of these agents. OCT-mediated transport has been reported to enhance the cytotoxicity of cisplatin (Ciarimboli et al., 2005), oxaliplatin (Zhang et al., 2006) and paraquat (Chen et al., 2007). Transport via OCTs is driven by membrane potential, and uptake of positively charged molecules is thermodynamically favorable, which can produce over 10-fold higher intracellular concentrations compared with the dose concentration (Koepsell et al., 2007). With two positive charges in each molecule, aromatic diamidines can

achieve even higher intracellular concentrations, driven by membrane potential. The results in this study suggest that hOCT1 promotes the accumulation of aromatic diamidines in mammalian cells, and may play an important role in diamidine-induced cytotoxicity in the organs expressing hOCT1, such as liver.

In human kidney, hOCT2 is highly expressed, but not hOCT1 (Koepsell et al., 2007). Aromatic diamidines are poor substrates for hOCT2; therefore, transport by renal OCTs may not account for the severe toxicity of pentamidine in human kidney (Balslev and Nielsen, 1992; O'Brien et al., 1997). In a recent study, the expression of *hOCT1* mRNA was shown to be only a few-fold lower than that of hOCT2 in the kidney (Hilgendorf et al., 2007), indicating that hOCT1 may also contribute to basolateral uptake of cations, including diamidines, in the kidney. Therefore, whether OCTs modulate nephrotoxicity of diamidines needs further investigation. In rats, the highest Oct1 expression was observed in the kidney (Grundemann et al., 1994), which may explain why TEA reduced the renal excretion of pentamidine in this species (Poola et al., 2003). These results highlight the possibility that differential expression of transporters across different species may contribute to different profile of organ toxicities for molecules like diamidines whose toxicity appears to be dependent on OCT1-dependent cellular uptake.

Drug-drug interaction via OCTs has been well documented (Ho and Kim, 2005; Li et al., 2006). For example, cimetidine is a potent hOCT2 inhibitor and coadministration with cimetidine reduced renal clearance of other cationic drugs (Ayrton and Morgan, 2001). Pentamidine is a potent inhibitor of hOCT2 with an IC_{50} value below that of quinidine and is one the most potent hOCT2 inhibitors among the marketed drugs. Therefore, pentamidine may affect the renal clearance of other cations by inhibiting hOCT2. Further, pentamidine

and furamidine are potent inhibitors to hOCT1 and may be able to reduce the hepatic uptake of other hOCT1 substrates, such as metformin, and thus change their pharmacokinetics and pharmacodynamics (Shu et al., 2007; Shu et al., 2008).

F. ACKNOWLEDGEMENTS.

We gratefully acknowledge Professor Hermann Koepsell (Justus-Liebig-University, Germany) for providing the plasmids of pcDNA3.1/hOCT1 and pCMV/hOCT2 and Dr. Vadivel Ganapathy (Medical College of Georgia, USA) for providing the plasmid of pSPORT1/hOCT3. This study was supported by a grant from the Bill and Melinda Gates Foundation. Xin Ming was supported by Eli Lilly Pre-Doctoral Fellowship.

Table A.1.

Inhibition of hOCT1-, hOCT2-, or hOCT3-mediated [^{3}H]MPP$^+$ Uptake by Pentamidine and Furamidine in CHO Cells.

Inhibitor	*hOCT1 IC_{50} (μM)*	*hOCT2 IC_{50} (μM)*	*hOCT3 IC_{50} (μM)*
Quinidine	5.7 ± 0.9	13.3 ± 0.7	22.7 ± 1.9
Pentamidine	16.4 ± 1.7	10.6 ± 2.4	14.8 ± 3.4
Furamidine	7.4 ± 0.9	182.0 ± 30.3	20.4 ± 2.8
TEA	469.7 ± 12.7	189.2 ± 11.2	1476.6 ± 160.1

Table A.2.

Cytotoxicity of Pentamidine and Furamidine in hOCT1 Transfected and Mock CHO Cells.

Drug	*hOCT1 IC_{50} (μM)*	*Mock IC_{50} (μM)*	*Fold change* [a]
Pentamidine	46.2 ± 5.3	202.2 ± 16.4	4.4
+ ranitidine	109.9 ± 12.4	170.2 ± 25.6	1.5
Furamidine	1.1 ± 0.05	10.2 ± 0.7	9.3
+ ranitidine	7.3 ± 0.4	10.3 ± 1.3	1.4

[a] Fold increase in the sensitivity of CHO-hOCT1 cells to pentamidine and furamidine compared with mock cells incubated under identical conditions.

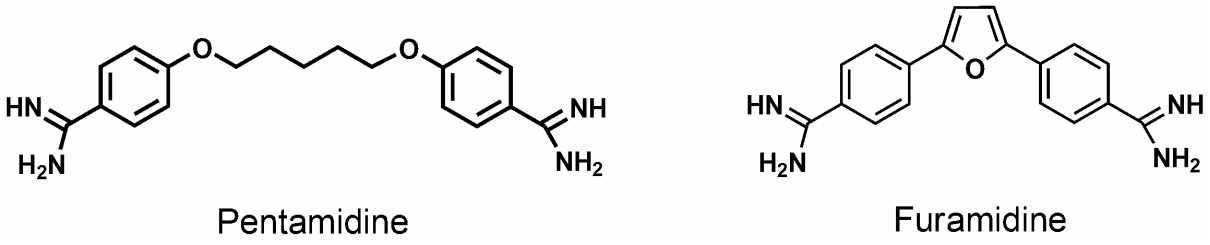

Figure A.1. Chemical structures of pentamidine and furamidine.

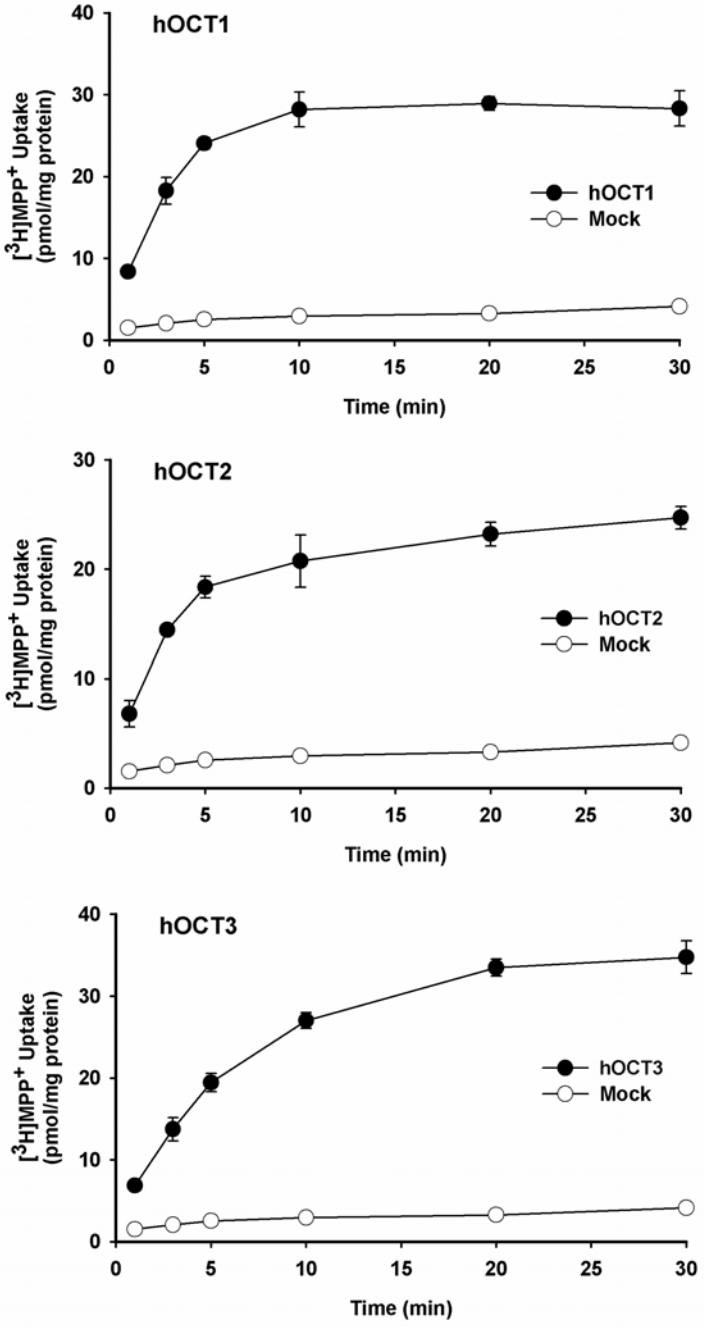

Figure A.2. Functional expression of hOCT1, hOCT2, and hOCT3 in stably transfected CHO cells. CHO cells, stably transfected with hOCT1, hOCT2, hOCT3 cDNA (●) or empty vector (Mock, ○), were incubated with [^{3}H]MPP$^+$ (1 μM) at 37ºC for the indicated time. Data represent mean ± S.D. from a representative experiment.

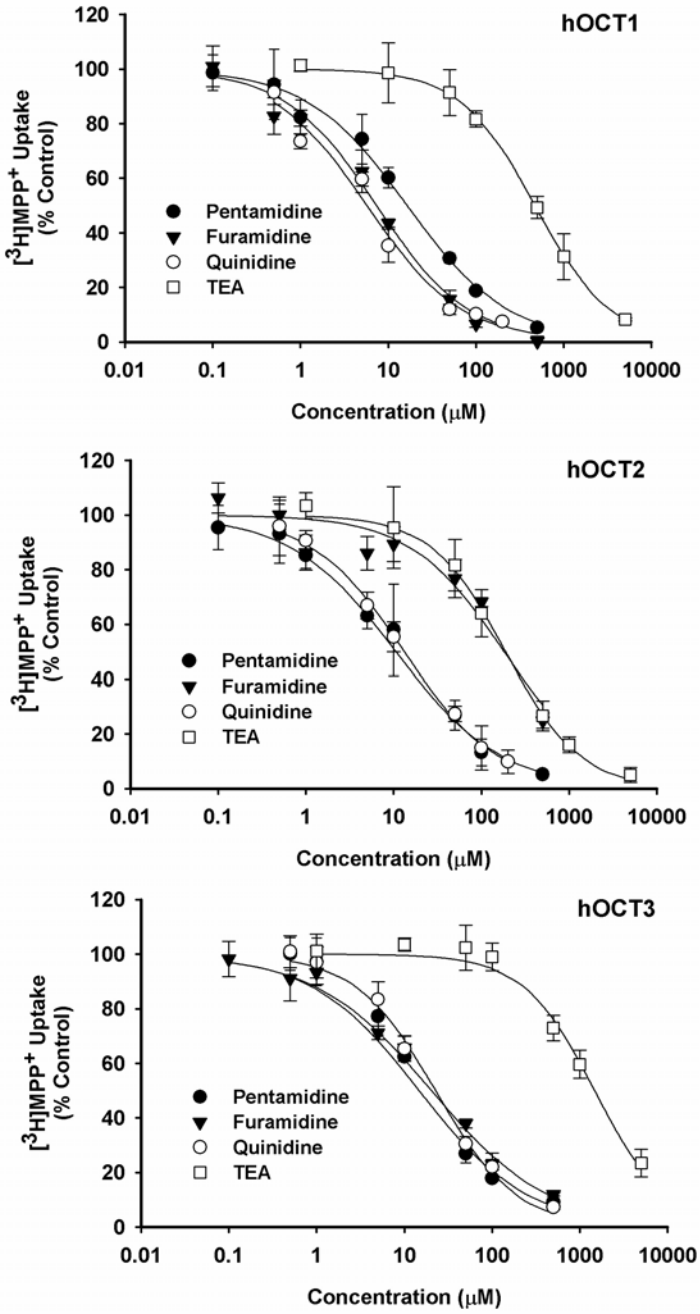

Figure A.3**.** Concentration-dependent inhibition hOCT1, hOCT2, and hOCT3 in CHO cells by organic cations. Uptake of [^{3}H]MPP$^+$ in CHO-hOCT1, CHO-hOCT2, CHO-hOCT3 and mock cells was determined in the absence or presence of increasing concentrations of pentamidine (●), furamidine (▼), quinidine (○) and TEA (□) for 4 minutes. Nonspecific cell-

associated radioactivity was determined by measuring substrate uptake in mock cells at each inhibitor concentration, and these values were then subtracted from the values in hOCT1-transfected cells to give corrected data that were used for generation of the inhibition curves. Thus, the data represent inhibition of the transporter-mediated portion of $[^3H]MPP^+$ uptake. Data are expressed as mean ± S.D. of experiments in triplicate.

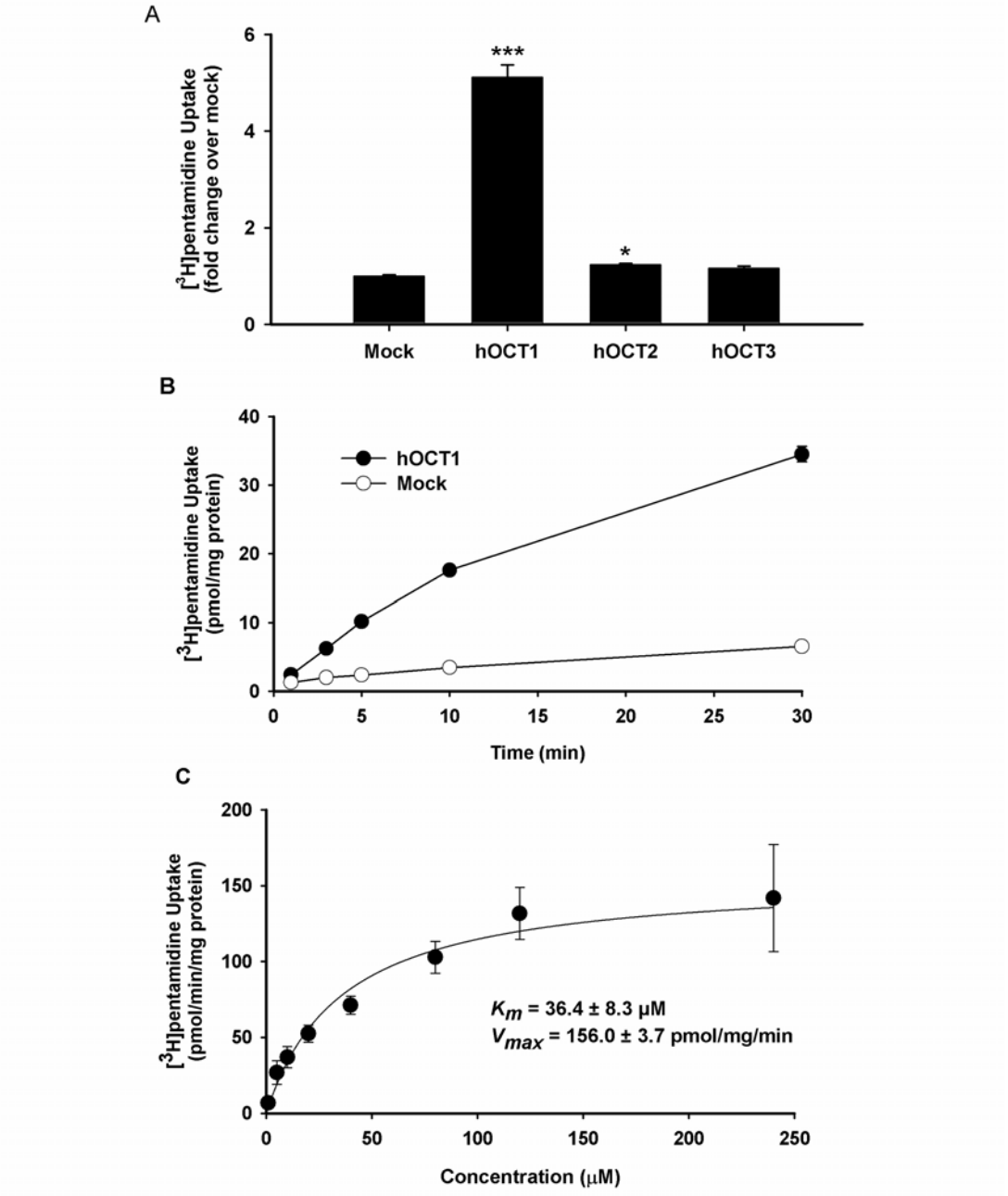

Figure A.4. Uptake of [^{3}H]pentamidine by CHO-hOCT1, CHO-hOCT2, and CHO-hOCT3 cells. A, uptake of [^{3}H]pentamidine (0.1 μM) was determined in CHO-hOCT1, CHO-hOCT2, and CHO-hOCT3 and mock cells over 10 minutes. B, uptake of [^{3}H]pentamidine (0.1 μM) was determined in mock or CHO-hOCT1 cells for the indicated time periods. C, uptake of [^{3}H]pentamidine (indicated concentration) was determined in mock or CHO-

hOCT1 cells for 10 minutes. Nonspecific cell-associated radioactivity was determined by measuring the compound uptake in mock cells at each substrate concentration, and these values were then subtracted from the values in hOCT1-transfected cells to obtain the data used to generate final kinetic curves. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

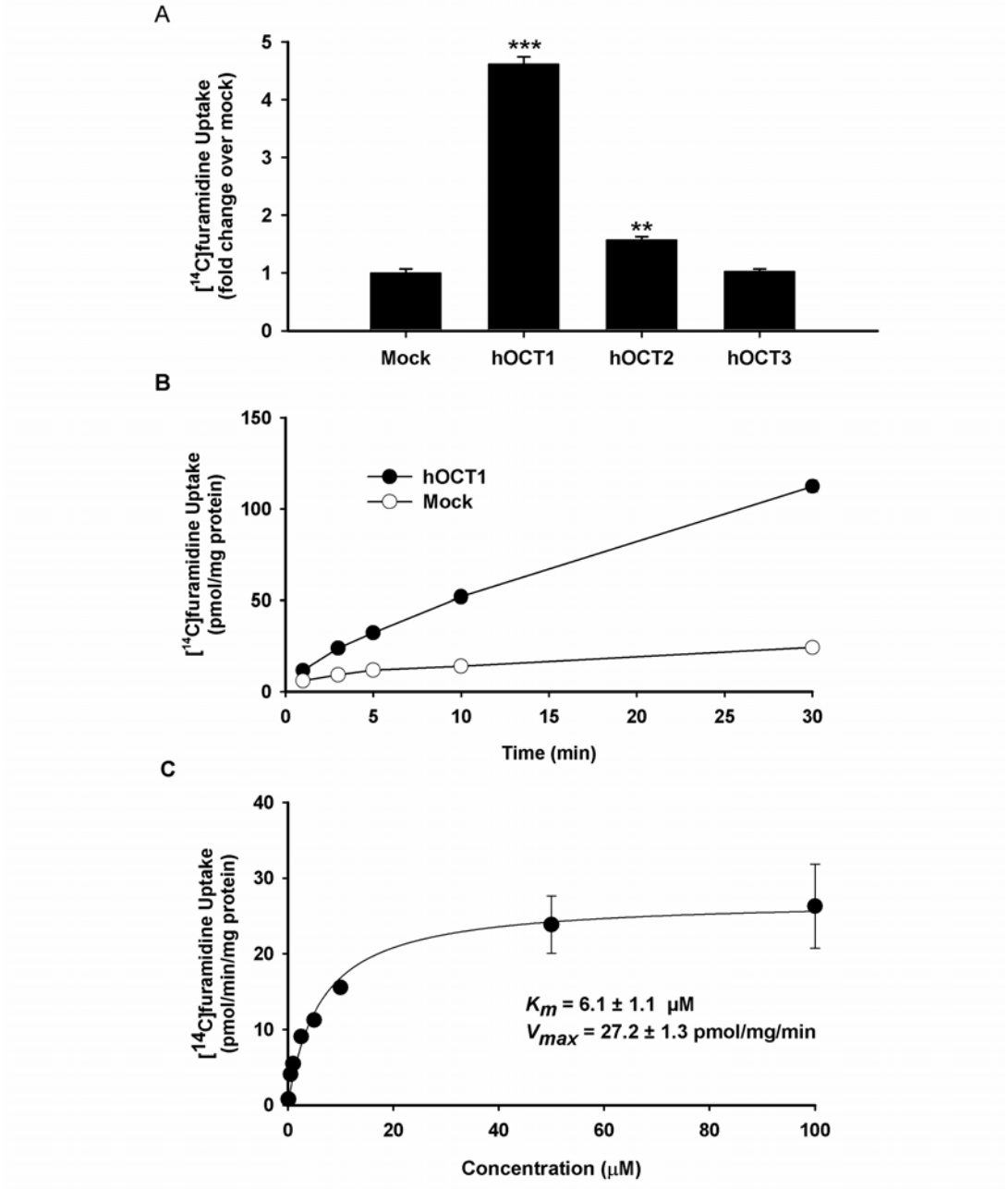

Figure A.5. Uptake of [^{14}C]furamidine by CHO-hOCT1, CHO-hOCT2, and CHO-hOCT3 cells. A, uptake of [^{14}C]furamidine (1 μM) was determined in CHO-hOCT1, CHO-hOCT2, and CHO-hOCT3 and mock cells over 10 minutes. B, uptake of [^{14}C]furamidine (1 μM) was determined in mock or CHO-hOCT1 cells for the indicated time periods. C, uptake of [^{14}C]furamidine (indicated concentration) was determined in mock or CHO-hOCT1 cells for

10 minutes. Nonspecific cell-associated radioactivity was determined by measuring the compound uptake in mock cells at each substrate concentration, and these values were then subtracted from the values in hOCT1-transfected cells to obtain the final kinetic curves. Data represent mean ± S.D. of a representative experiment in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with uptake in mock cells.

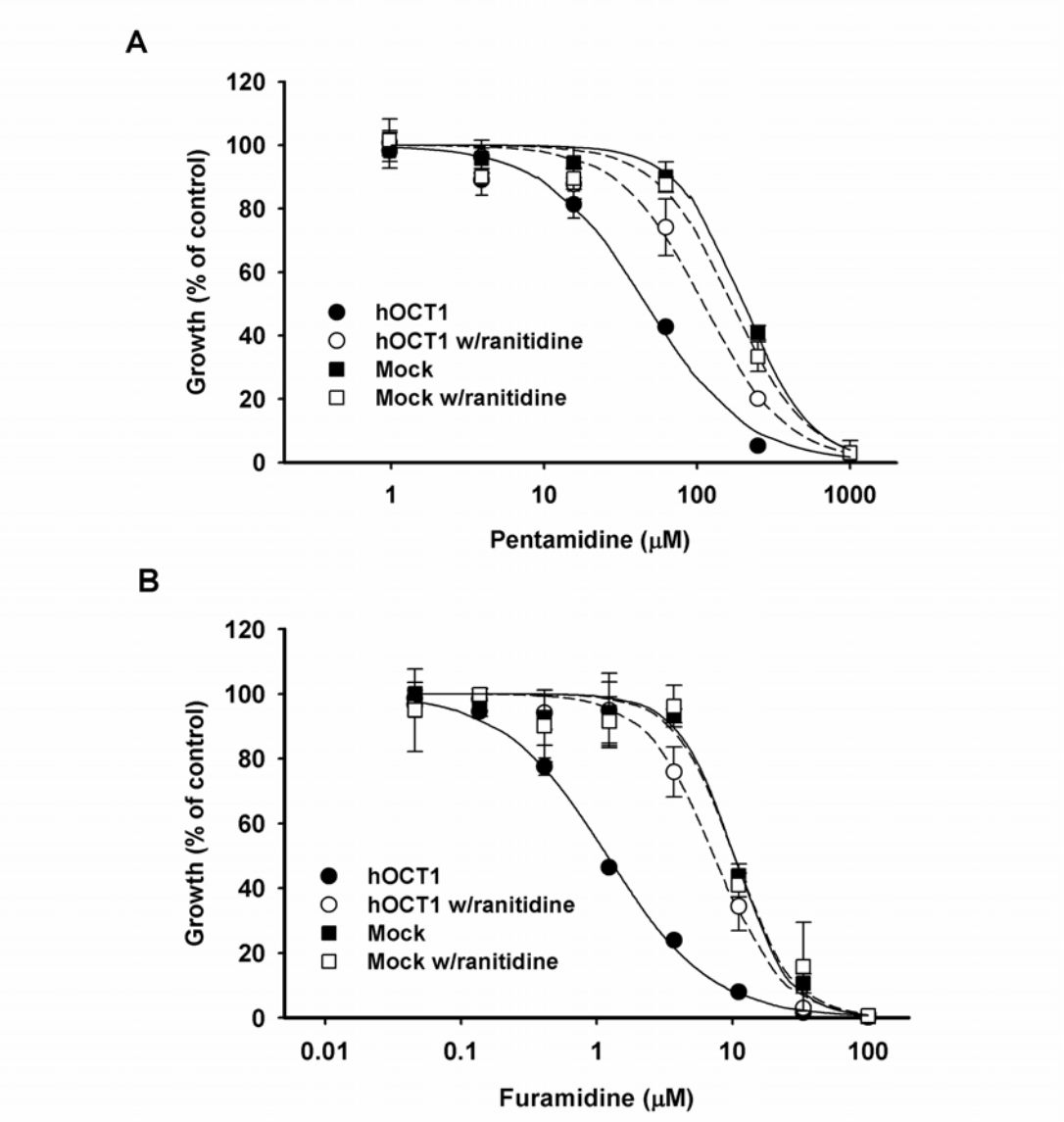

Figure A.6. Effect of hOCT1 on pentamidine and furamidine cytotoxicity. After 1-day culture in 96-well plates, CHO-hOCT1 (circle) or mock cells (square) were incubated with different concentrations of pentamidine (A) or furamidine (B) in the presence (solid) or absence (empty) of 1 mM ranitidine, an OCT1 inhibitor, for 24 hours followed by another 24-hour culture in fresh media. Alamar blue assays were performed afterwards and the IC_{50} values of cell growth inhition were calculated. Studies were performed in triplicate in each individual experiment.

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