

INTERPLAY BETWEEN METFORMIN AND SEROTONIN TRANSPORT
IN THE GASTROINTESTINAL TRACT: A NOVEL MECHANISM FOR THE
INTESTINAL ABSORPTION AND ADVERSE EFFECTS OF METFORMIN

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ABSTRACT

**TIANXIANG HAN: Interplay between Metformin and Serotonin Transport
in the Gastrointestinal Tract: A Novel Mechanism for the Intestinal
Absorption and Adverse Effects of Metformin
(Under the direction of Dhiren R. Thakker, Ph.D.)**

Metformin is a widely prescribed drug for Type II diabetes mellitus. Previous studies have shown that this highly hydrophilic and charged compound traverses predominantly paracellularly across the Caco-2 cell monolayer, a well-established model for human intestinal epithelium. However, oral bioavailability of metformin is significantly higher than that of the paracellular probe, mannitol (~60% vs ~16%). Based on these observations, the Thakker laboratory proposed a “sponge” hypothesis (Proctor et al., 2008) which states that the functional synergy between apical (AP) transporters and paracellular transport enhances the intestinal absorption of metformin. This dissertation work aims to identify AP uptake transporters of metformin, determine their polarized localization, and elucidate their roles in the intestinal absorption and adverse effects of metformin.

Chemical inhibition and transporter-knockdown studies revealed that four transporters, namely, organic cation transporter 1 (OCT1), plasma membrane monoamine transporter (PMAT), serotonin reuptake transporter (SERT) and choline high-affinity transporter (CHT) contribute to AP uptake of metformin in Caco-2 cells. Although AP intestinal localization of PMAT, SERT and CHT has been established, the intestinal membrane localization of OCT1 is ambiguous in the literature. In this dissertation work,

functional and immunostaining studies showed that OCT1 is localized in the AP membrane of Caco-2 cells and human and mouse intestinal epithelia.

Since this dissertation data showed for the first time that metformin is a substrate of SERT, the *in vivo* role of this transporter in mediating metformin oral absorption was investigated. The SERT-selective chemical inhibitor, paroxetine, decreased metformin systemic AUC_{0-6h} by 9% ($p < 0.05$), and in mSert^{-/-} mice, metformin systemic AUC_{0-6h} was lowered by 6% ($p < 0.05$).

Because SERT mediates the reuptake of serotonin which modulates gastrointestinal (GI) functions, its role in metformin-mediated GI adverse effects, such as diarrhea, was investigated in a mouse model. Results showed that metformin induced a 4-fold increase ($p < 0.001$) in intestinal motility, and a 50% increase ($p < 0.05$) in water content of the large intestine. These effects were diminished by serotonin receptor 4 antagonist GR113808, suggesting that metformin could cause GI adverse effects, such as diarrhea, by inhibiting SERT-mediated serotonin re-uptake and subsequent serotonin-induced increased GI motility and water-retention.

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DEDICATION

To my wise parents, for giving me life and inspiring me in science and medicine,

To my loving wife, for giving me the reason to continue and prosper.

May this work make a difference in people's lives.

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LIST OF ABBREVIATIONS

5HT ₄	Serotonin Receptor 4
AMPK	5'-Adenosine Monophosphate -Activated Protein Kinase
ANOVA	Analysis of Variance
AP	Apical
ATP	5'-Adenosine Triphosphate
AUC	Area Under the Curve
BCA	Bicinchoninic acid
BL	Basolateral
CHO	Chinese Hamster Ovary
CHT	Choline High-affinity Transporters
CTL	Choline Transporter-Like Protein
DAPI	4',6-diamidino-2-phenylindole
DDI	Drug-Drug Interaction
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase

K_m	Michaelis-Menten Constant
MATE	Material and Toxin Extrusion
MPP ⁺	1-methyl-4-phenylpyridinium
NEAA	Non-Essential Amino Acids
OCT	Organic Cation Transporter
OCTN	Novel Organic Cation Transporter
Papp	Apparent Permeability
PAGE	Polyacrylamide Gel Electrophoresis
P-gp	P-glycoprotein
PMAT	Plasma Membrane Monoamine Transporter
qRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium-Dodecyl Sulfate
SERT	Serotonin Re-uptake Transporter
SLC	Solute Carrier
TEA	Tetraethylammonium
TEER	Transepithelial Electrical Resistance
TRIS	Tris(hydroxymethyl)aminomethane
V_{\max}	Maximal Velocity

CHAPTER 1

INTRODUCTION

1.A. INTRODUCTION

For orally administered compounds, such as drugs and nutrients, traversing the intestine is the first and pivotal step to entering the blood stream, a process that plays a significant role in the absorption, and thus biological functions of these compounds. The intestinal epithelial monolayer acts as the major barrier to intestinal absorption (Figure 1.1). This monolayer is polarized into an apical (AP) side facing the intestinal lumen, and a basolateral (BL) side facing the blood, which are separated by a protein complex called the tight junctions. In order to be absorbed into the blood system, compounds can pass through the intestinal epithelium either by the paracellular or transcellular pathways (Figure 1.2). Paracellular transport is regulated by the tight junctions, which allows certain small and hydrophilic compounds to pass through and be absorbed. Transcellular transport of compounds can be achieved by two processes: passive diffusion and an active process mediated by transporter proteins. Lipophilic compounds can readily diffuse through the cell membrane, whereas for hydrophilic compounds, transporters are required for facilitating their transcellular transport. Conversely, some transporters such as P-glycoprotein (P-gp), can prevent the absorption of certain compounds by effluxing them back into the intestinal lumen.

In addition to the absorption processes through the intestinal epithelium, the distribution and elimination of drugs are also critical processes. The highly permeable lipophilic compounds can be readily distributed into different tissues, while hydrophilic compounds require transporters for their distribution. Furthermore, transporters play a significant role in the elimination of drugs via the liver and kidney. In the BL membrane of hepatocytes and kidney proximal tubules, transporters mediate the uptake of their substrates from the blood into cells, while efflux transporters in the AP membranes of hepatocytes and proximal tubules eliminate these compounds into bile and urine, respectively. More than 400 membrane transporters in two major superfamilies, namely ATP-binding cassette (ABC) (Schinkel and Jonker, 2003), and solute carrier (SLC) (Hediger et al., 2004) (Koepsell et al., 2007), have been annotated in humans, and play significant roles in the absorption, distribution, and elimination of various compounds.

Since a large portion (~40%) of orally administered drugs are organic cations or weak bases at physiological pH (Neuhoff et al., 2003), and numerous endogenous compounds of important biological functions are cations (such as monoamine neurotransmitters and hormones), extensive attention have been focused on cation-selective transporters in the recent literature (Koepsell et al., 2007). Figure 1.3 summarizes the major cation-selective transporters expressed in the intestine, liver and kidney (adapted from (Koepsell et al., 2007)). This chapter reviews the roles of cationic transporters in the absorption, distribution, and elimination, as well as physiological and pharmacological functions of cationic compounds, with an emphasis on their intestinal adverse effects and toxicity, and sets the stage for the dissertation work discussed in subsequent chapters.

1.B. CATION-SELECTIVE TRANSPORTERS

1.B.1. Organic Cation Transporters (OCTs)

OCTs, which belong to the SLC22A family, have been most extensively studied in the absorption, distribution and elimination of small organic cations (Koepsell et al., 2007). There are three isoforms of OCTs, namely OCT1, OCT2, and OCT3 (SLC22A1-3), which exhibit broad expression in different organs. For example, it is well established that OCT1 and 3 are localized in the sinusoidal (BL) membrane of human hepatocytes, and are responsible for the uptake of cations from the blood into the hepatocytes (Chen et al., 2010; Faber et al., 2003; Nies et al., 2009); similarly in the kidney, OCT2 is localized in the BL membrane of human renal proximal tubules and is responsible for the uptake of cations from the blood (Karbach et al., 2000; Motohashi et al., 2002; Okuda et al., 1996). In the intestine, OCT3 is expressed in the AP membrane of enterocytes, and mediates the uptake of cations from the lumen into the enterocytes (Koepsell et al., 2007; Muller et al., 2005). The localization of OCTs ensures their function as uptake transporters.

The majority of OCT substrates are monovalent, and comparatively small cations, the so-called type I organic cations (Meijer et al., 1999; Wright, 2005), exemplified by the prototypical substrates tetraethylammonium (TEA) and the neurotoxin, 1-methyl-4-phenylpyridinium (MPP⁺) (Koepsell et al., 2007). In addition, OCTs interact with several drugs, including the H₂-receptor antagonist ranitidine (Bourdet et al., 2005), the antidiabetic drug metformin (Wang et al., 2002) and the anticancer drug oxaliplatin (Yokoo et al., 2007). Recently, the divalent organic cations pentamidine and paraquat, have been identified as substrates for OCT1 (Ming et al., 2009b) and OCT2 (Chen et al., 2007), respectively. Transport via OCTs is facilitative and driven by a negative

membrane potential inside the cell. Uptake of positively charged molecules is thermodynamically favorable, and can produce over 10-fold higher intracellular concentrations compared to the dose concentration (Koepsell et al., 2007).

1.B.2. Novel Organic Cation Transporters (OCTNs)

OCTN1 and OCTN2, belong to the SLC22A4 and SLC22A5 families, respectively, and transport organic cations. OCTN1 is also considered as an ergothioneine transporter because it transports ergothioneine much more efficiently than TEA (Grundemann et al., 2005), while OCTN2 is a high-affinity, sodium-dependent L-carnitine transporter that plays a key role in the cellular uptake of L-carnitine (Koepsell et al., 2007). Both transporters play important physiological roles by transporting ergothioneine and L-carnitine into cells. OCTN1 is expressed in kidney, skeletal muscle, placenta, prostate, and heart with strong expression in erythrocytes and monocytes (Grundemann et al., 2005), and OCTN2 has been detected in kidney, heart, skeletal muscle, intestine and placenta (Tamai et al., 1998). Both transporters are localized in the AP membrane of proximal tubular epithelial cells in the kidney (Tamai et al., 2001; Tamai et al., 2004). OCTN2 is also expressed in the AP membranes of enterocytes (Elimrani et al., 2003; Kato et al., 2006), suggesting a potential role for this transporter in mediating the absorption of L-carnitine and cationic drug substrates. OCTN1 also mediates the renal excretion of an amino acid-type drug gabapentin, and genetic mutations of this transporter decrease the renal clearance of gabapentin in humans (Urban et al., 2007). OCTN2 also transports many drugs including β -lactam antibiotics, cephaloridine (Ganapathy et al., 2000) and the calcium channel blocker, verapamil (Grube et al., 2006).

1.B.3. Equilibrative Nucleoside Transporter (ENT4) (Plasma Membrane Monoamine Transporter (PMAT))

ENT4 was originally identified by genome database analysis and assigned to the equilibrative nucleoside transporter gene family (SLC29A4) based on gene sequence homology (Acimovic and Coe, 2002). Following more recent studies showing the involvement of ENT4 in facilitating the transport of organic cations such as TEA, MPP⁺ and metformin in a Na⁺-independent and membrane potential-sensitive manner (Engel and Wang, 2005; Zhou et al., 2007b), it was renamed as plasma membrane monoamine transporter (PMAT), recognizing its function as a polyspecific organic cation transporter. PMAT is expressed in human small intestine and localized in the AP membrane of the enterocytes (Zhou et al., 2007b), indicating that it may mediate the AP uptake of cationic drugs such as metformin in the intestine.

1.B.4. Multidrug and Toxin Extrusion Proteins (MATEs)

MATEs were assigned as the SLC47 family (SLC47A1: MATE1, SLC47A2: MATE2 or MATE2-K) (Terada and Inui, 2008). In humans, the MATE1 is strongly expressed in the liver, kidney and skeletal muscle and is also detected in the heart (Otsuka et al., 2005), whereas MATE2-K is predominantly expressed in the kidney (Masuda et al., 2006). MATEs exhibit AP localization in the hepatocytes and renal tubular cells (Masuda et al., 2006; Otsuka et al., 2005), presumptively forming vectorial transport systems with the basolaterally localized OCT1 and OCT2 in the liver and kidney. MATEs have similar substrate specificities with OCTs, transporting TEA, MPP⁺, guanidine, and some cationic drug molecules like metformin and verapamil (Masuda et al., 2006; Otsuka et al., 2005; Tanihara et al., 2007). However, uptake by

MATEs is independent of membrane potential and extracellular sodium concentration, and transport including uptake and efflux is stimulated by an oppositely directed proton gradient. Therefore, MATEs are considered as cation-proton antiporters that operate in both directions (Masuda et al., 2006; Otsuka et al., 2005; Tanihara et al., 2007). Besides organic cations, MATEs are capable of transporting anionic estrone sulfate, acyclovir, and ganciclovir (Tanihara et al., 2007).

1.B.5. Transporters of Endogenous Monoamines

Endogenous organic cations, such as monoamines, play an important role in molecular signaling in the central nervous system (CNS) as well as in peripheral organs. For example, 5-hydroxytryptamine (5-HT, serotonin) regulates mood, appetite, sleep, muscle contraction, cognitive functions, including memory and learning, as well as digestion and bowel movement (Berger et al., 2009; Bertrand and Bertrand, 2010; Mohammad-Zadeh et al., 2008; Ni and Watts, 2006). Other organic cationic molecules, such as dopamine, norepinephrine and choline, also play an important role in various physiological and pharmacological functions. Generally these cations cannot traverse biological membranes, due to the positive charge of amine at physiological pH (refer to structures in Table 1.1). Therefore, transporters for these endogenous amines contribute to the transportation and disposition of these molecules, and play a critical role in the functions of endogenous amines. These transporters are also popular targets for drug discovery and development for various diseases. Recent studies show that the transporters of endogenous monoamines and OCTs exhibit shared substrate specificities (Bacq et al., 2012; Duan and Wang, 2010; Grundemann et al., 1998; Hilber et al., 2005), suggesting that the endogenous monoamine transporters may have significant potential in

the pharmacology of many cationic drugs. However, their role in the transport of exogenous drugs has not been extensively investigated. The primary transporters of endogenous monoamines, and pharmacology and toxicity mediated by these transporters are discussed below.

Serotonin Reuptake Transporter (SERT)

SERT belongs to the SLC6A4 family (Gelernter et al., 1995; Nakamura et al., 2000). SERT is widely expressed in many organs and tissues, such as the brain, bone marrow, placenta, heart, platelets, lung, liver, kidney, intestine, thyroid gland, parathyroid gland, adrenal gland and pancreas (Gelernter et al., 1995; Gill et al., 2008; Mortensen et al., 1999; Qian et al., 1995; Talvenheimo et al., 1979; Wade et al., 1996). The endogenous substrate of SERT, serotonin (5-hydroxytryptamine, refer to structure in Table 1.1) is a small organic cation compound. The function of SERT is in the reuptake of extracellular serotonin (Bertrand and Bertrand, 2010; Reis et al., 2009). When serotonin is taken up into cells, it is metabolized and deactivated (Sjoerdsma et al., 1955). Therefore, SERT plays an important role in terminating and regulating the biological functions of serotonin.

SERT exhibits shared substrate specificities with other OCTs (Duan and Wang, 2010; Hilber et al., 2005), and it has been reported that knockout of the mouse serotonin reuptake transporter (mSert) results in up-regulation of mouse organic cation transporters (mOcts) *in vivo* (Baganz et al., 2008; Chen et al., 2001). The overlapping substrate specificities and interactions between OCTs and SERT suggest that SERT may play an important role in the pharmacology of several organic cation drugs.

Norepinephrine Transporter (NET)

NET, also called noradrenaline transporter (NAT), is encoded by the gene SLC6A2 (Kreek et al., 2005; Tellioglu and Robertson, 2001), and is a Na^+ - and Cl^- -dependent neurotransmitter transporter that transports the neurotransmitters norepinephrine and dopamine from the synapse back to cytosol (Bonisch et al., 1990). Norepinephrine and dopamine are both small organic cation compounds (refer to structures in Table 1.1) that control important biological functions. NET is mainly expressed in the CNS, and its function is to terminate noradrenergic signaling by rapid re-uptake of neuronally released norepinephrine and dopamine into presynaptic terminals, thus attenuating the function of the released norepinephrine and dopamine (Bonisch et al., 1990). NET controls many behavioral and physiological effects, such as mood, depression, feeding behavior, cognition, regulation of blood pressure and heart rate (Bonisch et al., 1990). Consequently, NET is a target of several drugs for the treatment of a number of CNS diseases, including depression, attention-deficit hyperactivity disorder (ADHS), substance abuse, neurodegenerative disorders (e.g., Alzheimer's disease and Parkinson's disease) (Blakely et al., 1994; Bonisch and Bruss, 2006; Kim et al., 2008).

Dopamine Transporter (DAT)

DAT, encoded by the gene SLC6A3 (Giros et al., 1992; Vandenberg et al., 1992), is a Na^+ - and Cl^- -dependent neurotransmitter transporter that transports dopamine from the synapse back to the cytosol. DAT is a plasma membrane protein expressed exclusively in dopamine synthesizing neurons in the CNS. It clears dopamine released in the synapse back into the cytosol to stop the function of dopamine; therefore, it plays an important role in regulating the amplitude and duration of dopamine signaling (Bannon et

al., 2001; Uhl, 2003). DAT also plays a critical role in the CNS physiology and pathology, and has been implicated in a number of dopamine-related disorders, including attention deficit hyperactivity disorder, Parkinson's disease, neurotoxicity, bipolar disorder, clinical depression, drug abuse and alcoholism, etc. (Bannon, 2005; Haenisch and Bonisch, 2011).

Choline Transporter System

Choline is a hydrophilic quaternary amine with a positive charge at all physiological pH values (refer to structure in Table 1.1). Since choline is not endogenously synthesized in cells, sufficient cellular uptake of exogenous choline is critical for normal functions in both the CNS and peripheral organs (Michel et al., 2006). Due to its positive charge and hydrophilicity, the transport of choline is mediated by transporters. Choline transporter system includes the high affinity choline transporter (CHT, belongs to SLC5A7 family), choline transporter-like proteins 1-7 (CTL1-7, belong to SLC44A1-7 family), and low affinity choline transport mediated by OCTs (Michel et al., 2006). CHT plays an important role in the uptake of choline in the CNS (Kus et al., 2003), and is also expressed in the AP membrane of colonic epithelial cells (Harrington et al., 2010). CTLs have a lower affinity for choline uptake and are also expressed in the intestine (Kouji et al., 2009; Traiffort et al., 2005). OCTs are also known to transport choline (Lee et al., 2009; Michel et al., 2006). This shared substrate specificity between choline transporters and OCTs indicate that CHT and CTLs may also play an important role in the transport of other organic cations.

1.C. CATION-SELECTIVE TRANSPORTERS IN DRUG-INDUCED TOXICITY IN ORGANS

1.C.1. Renal Toxicity

In the kidney, organic cations may be filtrated in the glomeruli or secreted in renal proximal tubules. Hydrophilic organic cations that do not bind to plasma proteins are readily ultrafiltrated and may be reabsorbed in the proximal tubule. Their reabsorption is dependent on the concentrations of the respective cation in the blood and the primary filtrate. Many endogenous cations and cationic drugs are bound to plasma proteins and are not filtrated efficiently, and several of them are secreted actively. Secretion and reabsorption of organic cations may occur in proximal tubules, distal tubules and collecting ducts; however, most investigations have focused only on secretion in the proximal tubule. In the first step of secretion, organic cations are translocated across the BL membrane in the proximal tubule. In humans, OCT2 and OCT3 are important for the BL uptake of cations. In the second step of secretion, organic cations are released across the luminal membrane by the proton/cation exchangers MATE1, MATE2-K and/or OCTN1. The proton/cation antiport mechanism used by these transporters helps to overcome membrane potential during cation efflux. Figure 1.3 shows the major cationic transporters involved in the drug elimination in the kidney (Koepsell et al., 2007).

Renal excretion appears to be one of the main determinants of the pharmacokinetics of hydrophilic cationic drugs. The transporters mentioned above play an important role in the renal clearance of organic cations in the proximal tubule which is a site for important clinical drug-drug interactions (DDIs) and nephrotoxicity. For example, cimetidine inhibits OCT2 and MATE1, which affects the renal elimination of procainamide and

metformin (Somogyi et al., 1983; Somogyi et al., 1987; Tsuda et al., 2009a; Tsuda et al., 2009b). Additionally, OCT2 and MATE1 play a critical role in the renal excretion of cisplatin, and are involved in cisplatin-induced nephrotoxicity (Iwata et al., 2012; Nakamura et al., 2010) (See discussion below in Section 1.D.).

1.C.2. Hepatotoxicity

Liver is an important organ for drug metabolism and elimination. Figure 1.3 shows the major cationic transporters involved in the drug transport in the liver (Koepsell et al., 2007). OCT1 and OCT3 are located in the sinusoidal membrane of the hepatocytes and take up organic cations from the blood (Chen et al., 2010; Faber et al., 2003; Nies et al., 2009). These transporters mediate the first step in biliary excretion of most cationic drugs; however, they can also mediate the release of organic cations from hepatocytes into the blood. The excretion of organic cations across the sinusoidal membrane into bile is mediated by MATE1 and/or by P-gp (Otsuka et al., 2005; Schinkel and Jonker, 2003). Decreased activity (by inhibition, polymorphisms or mutations) of OCT1 and OCT3 in the liver will decrease hepatic uptake and impair the biliary excretion of cationic drugs. Decreased activity of P-gp and/or MATE1 in the biliary membrane will result in increased intracellular concentrations of cationic drugs that are substrates of uptake transporters, which could lead to hepatic drug accumulation and hepatotoxicity.

1.C.3. Gastrointestinal (GI) Adverse Effects

Drug-induced toxicity in the GI tract is not commonly seen; however, organic cation drugs show different levels of GI adverse effects. For example, antidepressant drugs have common GI side effects such as diarrhea, nausea and vomiting (Barbui et al., 2004; Beasley et al., 2000; Trindade et al., 1998). These antidepressant drugs target and

inhibit SERT and could lead to an increase in serotonin in the intestine resulting in GI adverse effects (Bertrand et al., 2008). Other classes of organic cation drugs could also lead to GI side effects, such as metformin that induces GI adverse effects such as diarrhea, nausea, vomiting, bloating, and abdominal pain (Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009; Dandona et al., 1983). Currently, the mechanism underlying metformin's GI adverse effects is unknown. Considering that cationic transporters play an important role in the intestinal absorption and accumulation of metformin (Proctor et al., 2008), these transporters could also contribute to GI adverse effects. Figure 1.3 shows the major cation-selective transporters in the intestine (Koepsell et al., 2007).

1.C.4. Tumor Toxicity

Several cation-selective transporters have been detected in various tumors and/or tumor cells. These transporters translocate various cationic cytostatic drugs such as cisplatin that is transported by OCT2, oxaliplatin that is transported by OCT1 and OCT2, and Bamet-UD2 (cis-diammine-biursodeoxycholateplatinum(II) that is transported by OCT1 (Briz et al., 2002; Zhang et al., 2006). The role of these transporters may be critical in reaching therapeutic concentrations of chemotherapeutic agents that target tumors. Furthermore, metformin has been shown to exhibit anti-cancer efficacy over a broad spectrum of cancers, including breast, endometrial, ovarian, prostate, colon, gastric, cervical and lung cancer (Ashinuma et al., 2012; Bodmer et al., 2012; Chlebowski et al., 2012; Hanna et al., 2012; He et al., 2011; Kato et al., 2012; Tseng, 2012; Wu et al., 2012;

Xiao et al., 2012). Studies in our laboratory showed that cation-selective transporters are required for the intracellular uptake of metformin in breast cancer cell lines in order to have antiproliferative efficacy (Zhang et al., 2013).

1.D. CATION-SELECTIVE TRANSPORTERS IN TOXICITY OF THERAPEUTIC AGENTS

Various therapeutic drugs that are organic cations exhibit toxicities and cation-selective transporters play significant roles in mediating drug-induced toxicity. These compounds and the transporters involved in their toxicity are discussed below.

1.D.1. Antibiotics

Pentamidine and Furamidine (Anti-parasitic)

Aromatic diamidines are used in the clinic against early-stage human African trypanosomiasis (Barrett et al., 2007). Carrier mediated uptake and accumulation in target parasites is crucial for the antiparasitic activity of aromatic diamidines (Barrett et al., 2007). A recent study showed that aromatic diamidines, pentamidine and furamidine, are good substrates for OCT1 and potent inhibitors for multiple OCTs (Ming et al., 2009a). OCT1-mediated transport potentiated the cytotoxicity of diamidines. In humans, pentamidine is mainly distributed into the liver (Thomas et al., 1997) and eliminated via biliary secretion (Conte, 1991). Parenteral pentamidine therapy caused adverse drug reactions including nephrotoxicity and hepatotoxicity (O'Brien et al., 1997). These results suggest that OCT1 could promote the accumulation of aromatic diamidines in human tissues, and may play an important role in diamidine-induced cytotoxicity in organs that express OCT1, such as liver. Recently, 4',6-diamidino-2-phenylindole (DAPI), an aromatic diamidine, was identified as a substrate for MATE1 and MATE2-K (Yasujima et al., 2010), which suggested that MATEs may also transport other diamidines and mediate the renal uptake and accumulation of these diamidine drugs.

β -lactam antibiotics

Cephaloridine, a β -lactam antibiotic, causes carnitine deficiency in humans. *In vitro* studies showed that this zwitterionic drug is a substrate and inhibitor of OCTN2. It mediates AP uptake of carnitine in renal tubular cells and plays an important role in renal reabsorption of carnitine. Competitive inhibition of OCTN2 by cephaloridine may decrease carnitine reabsorption and cause its deficiency. Other β -lactam antibiotics, cefoselis, cefepime, and cefluprenam, also inhibit OCTN2, but at a lower potency (Ganapathy et al., 2000).

1.D.2. Antidiabetics

Metformin is a biguanide drug that is widely used for the treatment of type 2 diabetes mellitus. Metformin and other biguanide drugs are associated with lactic acidosis, a lethal side effect. OCTs mediate the hepatic uptake renal clearance of metformin, and are involved in the lactic acidosis. GI adverse effects are also common for metformin. A detailed discussion about metformin's adverse effects and toxicity is in Section 1. E.

1.D.3. Anti-tumor Agents

Platinum-based chemotherapeutic drugs are widely used to treat various types of solid tumors of prostate, bladder, colon, lung, testis and brain (Boulikas and Vougiouka, 2003). Among the platinum agents, cisplatin causes severe nephrotoxicity, which is a major hurdle in limiting its clinical application (de Jongh et al., 2003). The proximal tubule of the kidney, where multiple OCTs are expressed, is the major site of cisplatin-induced renal injury (Dobyan et al., 1980). Carrier-mediated transport of platinum agents in the renal tubular cells was suggested as a possible mechanism for nephrotoxicity.

OCT2 plays an important role in the BL uptake of cisplatin (Ciarimboli et al., 2005; Ludwig et al., 2004; Okuda et al., 1999; Tanihara et al., 2009). Secretion of cisplatin from the tubular cells into urine is believed to be ineffective because MATE1 and MATE2-K do not transport cisplatin (Tanihara et al., 2009; Yokoo et al., 2007); thus the accumulation of cisplatin causes nephrotoxicity. However, MATEs transport the less nephrotoxic oxaliplatin (Yokoo et al., 2007), which may indicate that these transporters lower renal accumulation of oxaliplatin, and subsequently alleviate nephrotoxicity. Nafamostat mesilate (NM), a serine-protease inhibitor that accumulates in the kidney, may be responsible for the serious side effect of NM-induced hyperkalemia. Transport studies indicate that NM is a substrate for OCT1 and OCT2; thus renal accumulation of NM can be mediated by OCTs (Li et al., 2004).

1.D.4. Antidepressants

Antidepressants are a family of a large number of compounds that are organic cations. In this drug family, there are mainly three classes of antidepressants that target SERT, NET and DAT, namely, tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and selective serotonin reuptake inhibitors (SSRIs). These drugs often induce different levels of adverse effects. Since the targets of these antidepressants are SERT, NET and DAT, these transporters could potentially play an important role in the adverse effects of these drugs.

TCAs are heterocyclic chemical compounds used primarily as antidepressants. They are named after their chemical structure, which contains three rings. TCAs can be used for the treatment of major depression, obsessive compulsive disorder (OCD), anxiety, chronic pain, and other depressive disorders (Gillman, 2007). These compounds

can inhibit SERT, NET and DAT, which may lead to side effects of the CNS, such as fatigue, dizziness, lightheadedness, headaches, confusion, agitation, insomnia, nightmares, increased anxiety, and seizures (Gillman, 2007). Recently, TCAs were largely replaced in clinical use in most parts of the world by newer classes of antidepressants (which typically have more favorable side-effects profiles) such as SNRIs and SSRIs.

SNRIs are a class of antidepressant drugs used in the treatment of severe depression and other mood disorders. Two neurotransmitters in the brain are known to play an important role in mood, namely, serotonin and norepinephrine. SNRIs can increase the concentration of these two molecules in the brain, by inhibiting their reuptake transporters SERT and NET, respectively, leading to antidepressive effects (Dell'Osso et al., 2006; Kolevzon et al., 2006). The inhibition of these transporters may also result in common adverse effects of SNRIs including loss of appetite, weight and sleep; drowsiness, dizziness, fatigue, headache, mydriasis, nausea/vomiting, sexual dysfunction, and urinary retention (Dell'Osso et al., 2006; Kolevzon et al., 2006). Serotonin syndrome is a potentially life-threatening adverse drug reaction that may occur following therapeutic drug use. It is a consequence of excess serotonergic activity at CNS and peripheral serotonin receptors due to the inhibition of the transporter SERT (Boyer and Shannon, 2005; Isbister et al., 2007).

SSRIs are a class of compounds used as antidepressants in the treatment of depression, anxiety disorders, and some personality disorders. SSRIs are believed to increase the extracellular level of the neurotransmitter serotonin by inhibiting its reuptake transporter SERT, thus increasing the level of serotonin in the synaptic cleft available to bind to the postsynaptic receptor (Mandrioli et al., 2012). Typical SSRIs have a high

affinity for SERT and low affinity for NET and DAT (Mandrioli et al., 2012). Almost all SSRIs are known to cause one or more of these adverse effects, possibly due to the inhibition of SERT: anhedonia, apathy, nausea, vomiting, drowsiness, somnolence, headache, bruxism, tinnitus, extremely vivid or strange dreams, dizziness, fatigue, mydriasis (pupil dilation), urinary retention, changes in appetite, insomnia and/or changes in sleep, weight loss/gain, increased risk of bone fractures and injuries, sexual dysfunctions, higher risk of suicidal thoughts (Landen et al., 2005; Olfson et al., 2006).

1.D.5. Environmental Toxins

MPP⁺, the prototypical organic cation extensively used in studies of organic cation transporters, is the metabolite of a neurotoxin, namely, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). It causes selective degeneration of neurons in humans and other primates, and is widely used to create animal models of Parkinson's disease. MPTP is first metabolized into MPP⁺ by monoamine oxidase B and uptake of MPP⁺ into neurons is a key step in the neurotoxicity of MPTP and MPP⁺, which is primarily mediated by DAT. Another toxin, paraquat, is a widely used herbicide and can cause major organ toxicities in the lung, liver, and kidney. Results from transporter-transfected cells suggested that OCT2 and MATE1 mediate transport and enhance cytotoxicity of paraquat, and may play an important role in the excretion and toxicity of paraquat in the kidney (Chen et al., 2007).

In summary, cation-selective transporters mediate the transport of various cationic compounds in different organs and tissues. The uptake, distribution and accumulation of these cations in organs may lead to toxicity and adverse effects.

1.E. METFORMIN

Metformin, or 1,1,-dimethylbiguanide, is a small, hydrophilic molecule (MW129.16; logD at pH7.4 of -6.13) that contains a net positive charge (pKa 12.4) at physiological pH (Saitoh et al., 2004) (refer to structure in Table 1.1). Because of its hydrophilic physicochemical properties, it cannot traverse cell membranes; however, its high oral bioavailability suggests that cation-selective transporters play a role in its intestinal absorption. Because metformin has been used as a model cationic compound to study the functions of cationic transporters, this dissertation work is focused on this molecule to study the role of cationic transporters in its pharmacokinetics, pharmacodynamics and adverse effects.

Metformin was first synthesized by Werner and Bell in 1921 (Werner and Bell, 1921), and was introduced as an oral treatment for Type II diabetes by Dr. Jean Sterne in 1957 in France (Bailey and Day, 1989). Commercial use of metformin first occurred in the United Kingdom as an oral antihyperglycemic agent for treatment of mature onset diabetes in 1958 (Hadden, 2005). In Europe, metformin was widely used from 1970s (Bailey and Day, 2004). This drug was approved for use in Canada in 1972 and was introduced in the United States in 1994 (Lucis, 1983). Today, metformin is widely prescribed worldwide. In 2011, there were 59 million metformin prescriptions issued in the US (Report by the IMS Institute for Healthcare Informatics, 2012).

1.E.1. Mechanism of Action

Metformin is an antihyperglycemic agent which improves glucose tolerance and lowers plasma glucose in patients with type 2 diabetes (Hermann et al., 1994; Jackson et al., 1987; Lord et al., 1983; Sambol et al., 1996b). Its glucose lowering effect in healthy

patients was not significant (Sambol et al., 1996b), and treatment did not cause clinical hypoglycemia or hyperinsulinemia (Bailey et al., 1989), suggesting that metformin is an insulin sensitizer rather than a hypoglycemic drug. The mechanism of its effects include decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization.

The liver is the major site of metformin action, where it inhibits hepatic gluconeogenesis. Metformin reduced hepatic glucose output and lactate gluconeogenesis in patients with type II diabetes (Hundal et al., 2000; Jackson et al., 1987; Stumvoll et al., 1995). The intestine also mediates a significant portion of the overall glucose lowering effects of metformin, and published reports suggest that it increases systemic glucose uptake in the intestine (Bailey et al., 1994; Gontier et al., 2008; Walker et al., 2005; Wilcock and Bailey, 1991). A study by Stepensky et al. reported that portal administration of metformin significantly reduced overall blood glucose-lowering effects in relation to intraduodenal administration of the drug (Stepensky et al., 2002). Therefore, the intestine appears to play a role in the pharmacology of metformin. The skeletal muscle is another major organ responsible for metformin-mediated glucose lowering effects. Metformin stimulates recruitment of glucose transporters (in particular GLUT4) to the plasma membrane of skeletal muscle (Garvey et al., 1988; Garvey et al., 1998) and increases glucose utilization (Johnson et al., 1993; Musi et al., 2002). Additionally, in adipose tissue, metformin decreases lipolysis, which lowers the concentration of free fatty acids in the plasma and attenuates insulin resistance (Bourron et al., 2010; Palanivel and Sweeney, 2005; Ren et al., 2006).

The main cellular target of metformin is 5'-adenosine monophosphate-activated protein kinase (AMPK) (Zhou et al., 2001), which plays an important role in cellular energy control. Published reports suggest that metformin mediates its pharmacologic action through indirect activation of AMPK, involving the cellular signaling complex I of the respiratory chain in mitochondria (Anedda et al., 2008; Hawley et al., 2002; Lenhard et al., 1997; Owen et al., 2000).

1.E.2. Clinical Pharmacokinetics

Metformin is an orally administered anti-hyperglycemic agent that is dosed as an immediate release formulation GLUCOPHAGE[®] Tablets and Extended-Release GLUCOPHAGE[®] XR Tablets, from 500 to 1000 mg (Bristol-Myers-Squibb, 2009). Maximal daily doses for immediate and extended formulations are 2550 mg and 2000 mg, respectively (Bristol-Myers-Squibb, 2009). The drug does not bind to plasma proteins *in vivo* or *in vitro* (Pentikainen et al., 1979; Tucker et al., 1981). Furthermore, metformin undergoes negligible metabolism (e.g. < 20%, mainly by hepatic metabolism) and is excreted primarily unchanged (Tucker et al., 1981).

Intravenous Administration

Metformin administered as a bolus intravenous dose is rapidly cleared from the body with an intravenous plasma half-life ($t_{1/2,P}$) of approximately 2 hours (Pentikainen et al., 1979). The majority of metformin is excreted in the urine unchanged with the percent of dose recovered ranging from 80-100% (Pentikainen et al., 1979; Tucker et al., 1981). Renal clearance represented total clearance of metformin following intravenous administration, and was approximately 5-fold higher than creatinine clearance

(Pentikainen et al., 1979; Tucker et al., 1981) indicating active tubular secretion in its elimination.

Oral Administration

Oral administration of single doses ranging from 0.25 to 1.5 g has been investigated. The maximal plasma concentration (C_{\max}) of metformin for 0.25 g and 1.5 g tablets was 0.59 ± 0.24 and 3.10 ± 0.93 $\mu\text{g/mL}$, respectively, and the oral plasma half-life ($t_{1/2,P}$) of metformin ranged between 2 and 6 hours, with a mean half-life of 4 hours (Somogyi et al., 1987; Tucker et al., 1981). Determined by deconvolution analysis, the absorptive half-life ($t_{1/2,abs}$) for a 0.5 g oral dose was significantly greater for the dose equivalent intravenous elimination half-life ($t_{1/2,\beta}$) (Pentikainen et al., 1979), indicating that the elimination rate during oral administration likely represented the rate of absorption. Thus metformin undergoes “flip-flop” kinetics, where absorption is the rate-limiting step to its elimination following oral administration.

Intestinal Absorption

The GI absorption of metformin is high considering its net positive charge and significant hydrophilicity at physiological pH values (refer to structure in Table 1.1). Absolute oral bioavailability of metformin ranges between 40-60% (Karttunen et al., 1983; Noel, 1979; Pentikainen, 1986; Pentikainen et al., 1979; Tucker et al., 1981). In addition, metformin absorption was dose-dependent, with greater percent of the drug being absorbed at low dose in relation to high dose (Noel, 1979; Sambol et al., 1996a; Sambol et al., 1996b; Tucker et al., 1981). Furthermore, Vidon et al. reported that metformin requires the entire length of the intestine for its absorption (Vidon et al., 1988). Proctor et al. studied the absorption mechanism of metformin in Caco-2 cell

monolayers, a cellular model of human intestinal epithelium, and found that AP uptake and efflux of metformin are saturable and efficient, while the BL uptake and efflux are linear and inefficient, and that metformin is predominately absorbed through the paracellular pathway (Proctor et al., 2008). However, compared to mannitol, which is exclusively absorbed by the paracellular route and has a bioavailability of 16% (Artursson and Karlsson, 1991), metformin shows an unusually high bioavailability of ~60%. To explain metformin's unusually high bioavailability, it was hypothesized by Proctor et al. that the bidirectional cation-selective transport mechanism(s) on the AP membrane of Caco-2 cell monolayers mediates efficient absorption of metformin by a cycling mechanism between the extracellular AP compartment and the intracellular space; this cycling provides the drug repeated opportunities to be absorbed through the paracellular route (Proctor et al., 2008) (Figure 1.4). However, the transporters involved in mediating the passage of metformin across the AP membrane of the intestinal epithelium have thus far not been identified. The dissertation work discussed in Chapter 2 focuses on the identification of specific AP uptake transporters of metformin in enterocytes.

Distribution and Elimination

In humans, metformin accumulates in the small intestine (Bailey et al., 2008) and in the liver (Shu et al., 2008). In mice, metformin is known to accumulate mostly in the stomach, liver, small intestine, and kidneys and to a lesser extent the heart, skeletal muscle, and white adipose tissue (Wang et al., 2002; Wilcock and Bailey, 1994; Wilcock et al., 1991). The reported values of the volume of distribution of metformin are highly variable ranging between 60 and 280 L (Noel, 1979; Pentikainen et al., 1979; Tucker et

al., 1981), which is high considering its hydrophilic physicochemical properties. Metformin is primarily excreted unchanged in the urine by active tubular secretion (Noel, 1979; Pentikainen et al., 1979; Sambol et al., 1996b; Tucker et al., 1981). Cation-selective transporters play an important role in its distribution and elimination, especially in the liver and kidney. The transporters involved in metformin's absorption, distribution and elimination are illustrated in Figure 1.5.

1.E.3. Drug-Drug Interactions (DDIs)

DDIs with metformin therapy are mainly mediated by transporters. It has been reported that cimetidine, a H₂-receptor antagonist, and cephalexin, a first generation cephalosporin antibiotic, could significantly increase metformin exposure and reduce the renal clearance of metformin (Jayasagar et al., 2002; Somogyi et al., 1987), suggesting cimetidine and cephalexin inhibiting cation transporters responsible for metformin active tubular secretion in the kidney (Tsuda et al., 2009b). DDIs are not commonly seen in the absorption of metformin.

1.E.4. Toxicity and Adverse Events

Lactic Acidosis

Metformin treatment could lead to a rare but fatal adverse effect, namely lactic acidosis, in approximately 2-10 incidents per 100,000 patient-years, with a mortality rate of approximately 50% (Brown et al., 1998; Misbin et al., 1998). Metformin-induced lactic acidosis could lead to severe metabolic acidosis (serum pH < 7.35), hyperlactataemia (serum lactate >5mM), and high serum lactate/pyruvate ratio (Assan et al., 1977; Seidowsky et al., 2009). Even though the mechanisms of metformin-induced lactic acidosis are not fully understood, the accumulation of this drug in the liver and

intestine leading to increased lactate production could be implicated in this phenomenon (Bailey et al., 2008; Wang et al., 2003).

GI Adverse Effects

Common adverse effects of metformin are in the GI tract, such as diarrhea, nausea, abdominal discomfort (Bailey and Nattrass, 1988; Bristol-Myers-Squibb, 2009). Other side effects have been reported to be metallic taste and altered absorption of vitamin B₁₂ and folic acid, possibly due to the GI stress and malfunction (Bailey and Nattrass, 1988). These GI adverse effects of metformin are considerably common. For example, diarrhea occurs in more than 50% of the patients taking GLUCOPHAGE[®], nausea/vomiting in approximately 25% of the patients (Bristol-Myers-Squibb, 2009). Other events such as flatulence, indigestion, abdominal discomfort were seen in more than 5% of the patients, and Table 1.2 summarizes these adverse effects of GLUCOPHAGE[®] during a clinical study in patients with type 2 diabetes (Bristol-Myers-Squibb, 2009). Due to the wide usage of metformin and the high occurrence rate of the intestinal adverse effects, millions of patients suffer from metformin-mediated GI distress. In severe cases, about 5% of the patients, these adverse effects lead to discontinuation of metformin treatment (Bailey, 1992; Dandona et al., 1983; Krentz et al., 1994).

To date, there is no direct evidence that explains the underlying mechanism of the GI adverse effects of metformin. Several hypotheses have been proposed to explain the mechanism of the intestinal adverse effects of metformin, such as alteration of bile salt metabolism and circulation (Carter et al., 2002; Carter et al., 2003; Caspary et al., 1977; Scarpello et al., 1998); change in the level of ghrelin (Doogue et al., 2009; Kadoglou et al., 2010; Kusaka et al., 2008); change of glucagon-like peptide-1(GLP-1) (Cuthbertson

et al., 2011; Mannucci et al., 2001; Mannucci et al., 2004); and increase of serotonin release (Cubeddu et al., 2000). However, these studies were inconclusive or contradictory; hence the mechanisms underlying the GI adverse effects of metformin remain unclear. As mentioned earlier in Section 1.B., transporters could potentially play a role in the adverse effects and toxicity of the drug. However, this area has not been thoroughly investigated. In this dissertation, the role of SERT in metformin-mediated adverse effects was studied, and the results are presented in Chapter 5.

1.F. RATIONALE AND OVERVIEW OF PROPOSED RESEARCH

Cation-selective transporters play an important role in the pharmacokinetics, pharmacology, toxicity and adverse effects of cationic drugs, as discussed earlier in this chapter. As a typical small organic cation, metformin has been investigated as a model drug of the research of cation-selective transporters. However, even though it is widely used in clinic and investigated in research, the mechanisms underlying its intestinal absorption and adverse effects are still unclear.

Previous studies from the Thakker laboratory have shown that metformin traverses predominantly paracellularly across Caco-2 cell monolayers (Proctor et al., 2008). However, its oral bioavailability is significantly higher than that of the paracellular probe mannitol (40-60% vs ~16%) (Artursson and Karlsson, 1991; Karttunen et al., 1983; Noel, 1979; Pentikainen, 1986; Pentikainen et al., 1979; Tucker et al., 1981). This discrepancy was resolved by a hypothesis proposed by Proctor et al. (2008) that the functional synergy between AP transporters and paracellular transport significantly enhanced the overall absorption of metformin (Proctor et al., 2008). The transporters involved in the AP uptake of metformin have not been identified (Figure 1.6). Identifying the specific transporters involved in this process and determining their cellular localization could enable us to further investigate the intestinal absorption mechanisms that allow for an unexpectedly high oral bioavailability of a hydrophilic cationic drug. This knowledge will significantly improve our understanding of the oral absorption mechanism of metformin, and could be applied to the oral absorption mechanisms of other cationic drugs. In addition, the mechanisms underlying the common GI adverse effects of metformin are currently unknown. Identifying the intestinal transporters of metformin

will also enable us to investigate whether specific intestinal transporters are involved in the GI adverse effects of metformin. Therefore, the present study aims to identify these cation-selective transporters and determine the role of transporters in the intestinal adverse effects of metformin, based on the hypotheses that:

(1) Specific cation-selective transporter(s) mediates the AP uptake of metformin in Caco-2 cell monolayers and enterocytes, and plays a role in the oral absorption of metformin.

(2) The intestinal adverse effects of metformin, such as diarrhea, are caused by metformin-mediated inhibition of SERT, which decreases serotonin reuptake thus increasing intestinal serotonin concentration; the increased intestinal concentrations of serotonin causes increased GI motility and accompanying side effects like diarrhea and GI discomfort.

The following specific aims were designed to test these hypotheses:

Specific Aim 1: Determine the AP transporters involved in metformin uptake in Caco-2 cell monolayers.

1a. Develop a new chemical inhibition strategy using specific and non-specific inhibitors of cation-selective transporters.

1b. Using the novel chemical inhibition scheme developed in Aim 1a, identify specific transporters of metformin in Caco-2 cell monolayers.

1c. Using shRNA technology, confirm the findings of the chemical inhibition studies about the role of transporters in the AP uptake of metformin in Caco-2 cell monolayers.

Specific Aim 2: Determine the cellular localization of OCT1 (mOct1) in Caco-2 cell monolayers, and human and mouse intestinal tissues.

2a. Assess the functional activity of OCT1 in the AP and BL membrane by studying uptake of the OCT1-specific substrate, pentamidine, in Caco-2 cell monolayers, and in human and mouse intestinal tissues.

2b. Determine the localization of OCT1 (mOct1) in all three systems, using immunochemical staining and confocal microscopy.

Specific Aim 3: Investigate the role of SERT in metformin oral absorption in a mouse model.

3a. Determine the inhibitory profile of paroxetine towards mouse cation-selective transporters; and using the selective inhibitory concentration of paroxetine toward mSert, investigate the role of mSert in the intestinal absorption of metformin in mice.

3b. Confirm the role of mSert in the intestinal absorption of metformin using mSert knockout mice.

Specific Aim 4: Determine the role of SERT (mSert) in the intestinal adverse effects of metformin.

4a. Determine the inhibitory profile of metformin towards SERT, using SERT-expressing cells.

4b. Assess inhibitory potency of metformin toward serotonin AP uptake in Caco-2 cell monolayers.

4c. Demonstrate the effect of the inhibition of SERT-mediated uptake of serotonin by metformin on intestinal motility and water retention in mice.

Table 1.2. Most common adverse reactions (>5.0 %) in a placebo-controlled clinical study of GLUCOPHAGE[®] monotherapy (Bristol-Myers-Squibb, 2009).

Adverse Reaction	GLUCOPHAGE [®] Monotherapy (n=141)	Placebo (n=145)
	% of Patients	% of Patients
Diarrhea	53.2	11.7
Nausea/Vomiting	25.5	8.3
Flatulence	12.1	5.5
Asthenia	9.2	5.5
Indigestion	7.1	4.1
Abdominal Discomfort	6.4	4.8
Headache	5.7	4.8

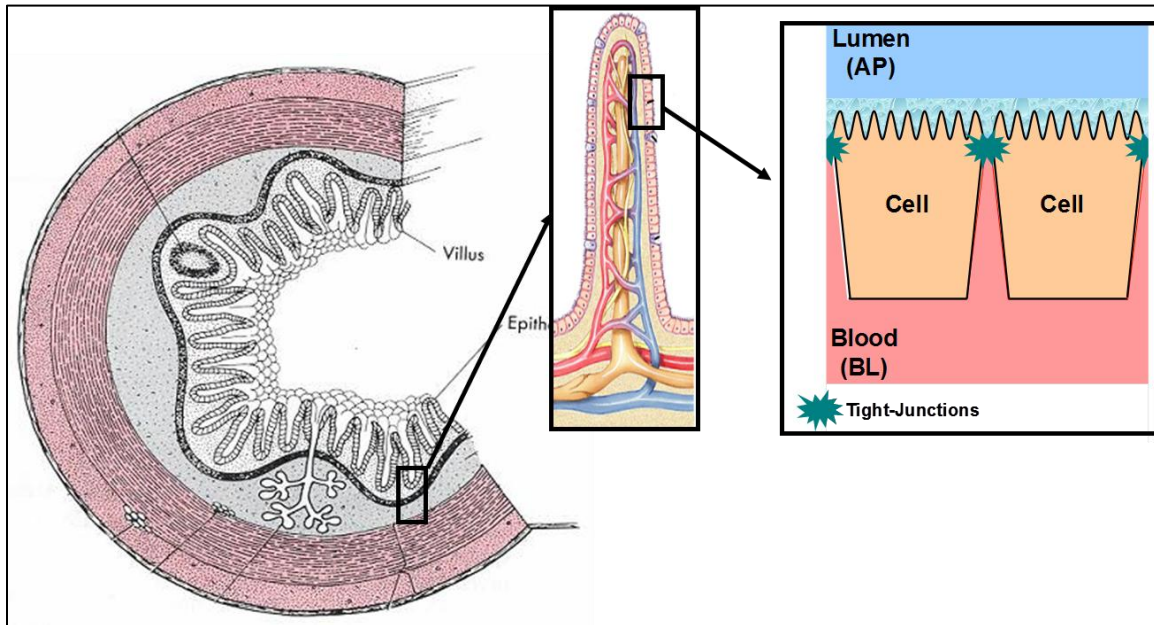


Figure 1.1. Intestinal epithelial monolayer is the major barrier for absorption of drugs and nutrients. (Adapted from <http://www.abbysenior.com/biology>).

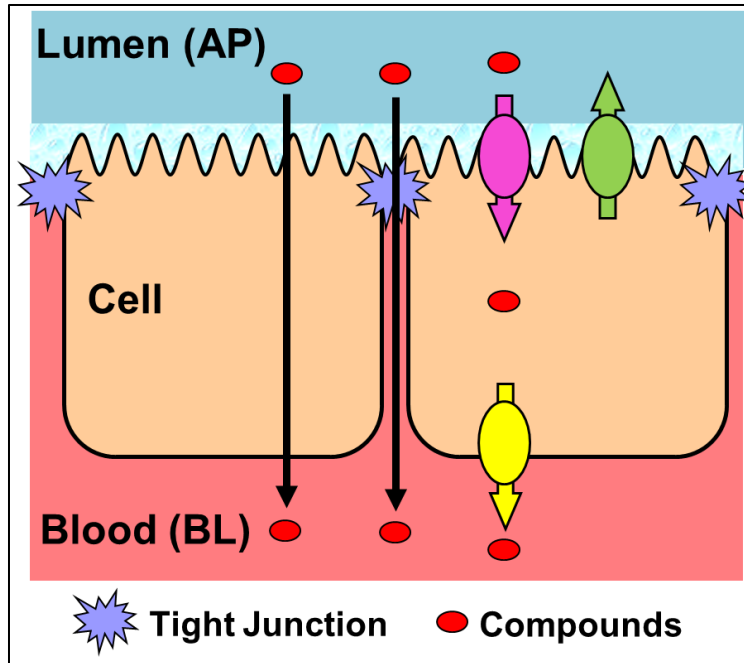


Figure 1.2. Intestinal absorption pathways. Paracellular transport (through tight junctions) and transcellular transport (passive diffusions and transporter-mediated transport).

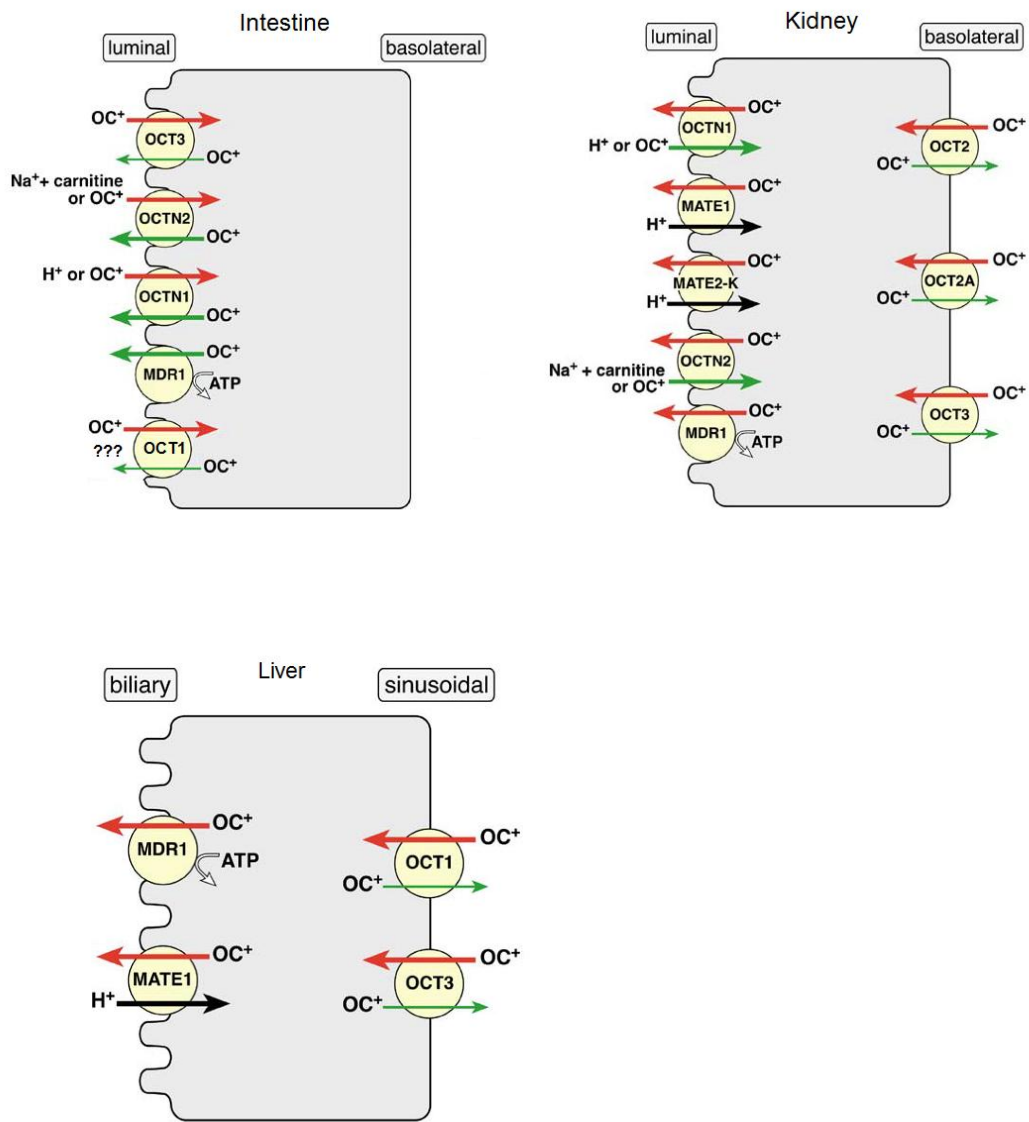


Figure 1.3. Cation-selective transporter expression in the intestine, liver, and kidney. Adapted from (Koepsell et al., 2007).

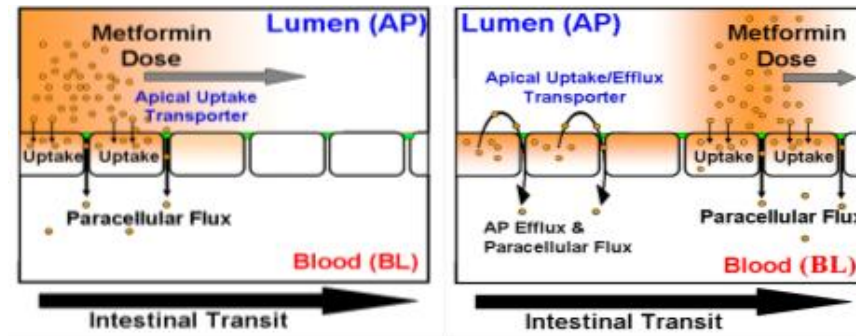


Figure 1.4. “Sponge hypothesis” proposed by Proctor et al. (2008). It states that the functional synergy between AP transporters and paracellular transport enhances the overall intestinal absorption of metformin (Proctor et al., 2008).

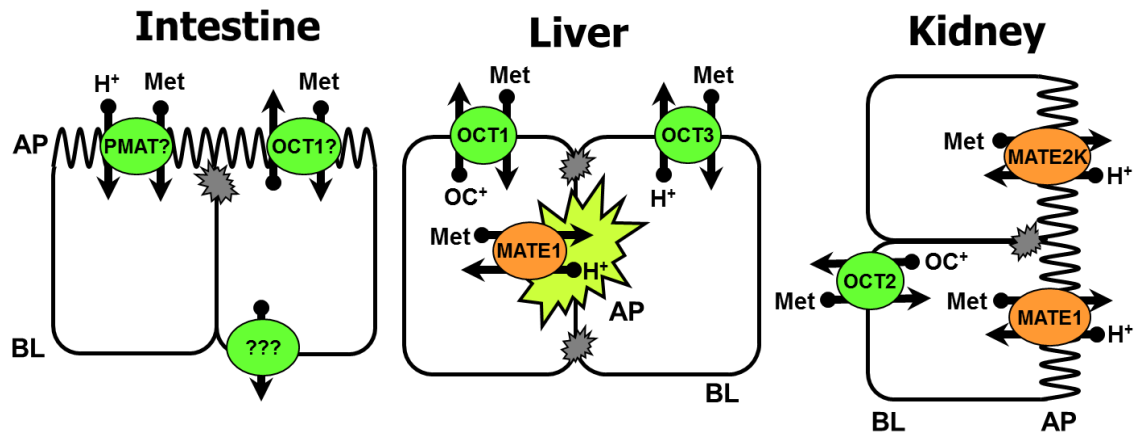


Figure 1.5. Metformin transporters in the intestine, liver and kidney. (Kimura et al., 2005; Nies et al., 2009; Tsuda et al., 2009b; Zhou et al., 2007a).

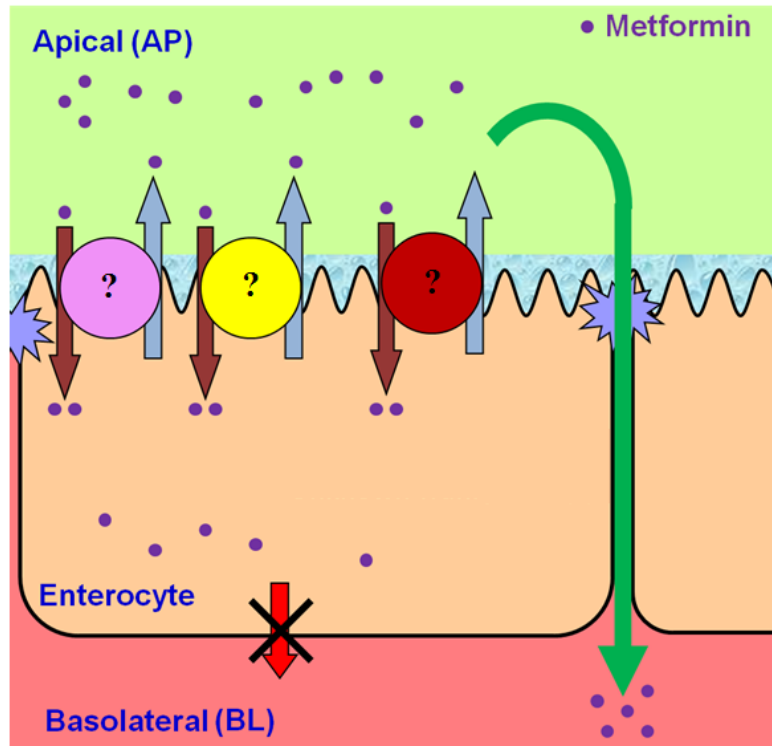


Figure 1.6. Unknown metformin transporters involved in its AP uptake and efflux in the intestine, and their contributions to overall absorption.

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CHAPTER 2

FOUR CATION-SELECTIVE TRANSPORTERS ARE INVOLVED IN THE APICAL UPTAKE AND ACCUMULATION OF METFORMIN IN CACO-2 CELL MONOLAYERS

2.A. OVERVIEW

Metformin is the frontline therapy for Type II diabetes mellitus. The oral bioavailability of metformin is unexpectedly high, about 60%, given its hydrophilicity and positive charge at all physiologic pH values. Previous studies in Caco-2 cell monolayers, a cellular model of the human intestinal epithelium, showed that although metformin traverses predominantly (90%) via the paracellular route, cation-selective transporters contribute to its cellular apical (AP) uptake (Proctor et al., 2008). Based on these findings, Proctor et al. proposed a “sponge hypothesis” which states that the functional synergy between AP transporters and paracellular transport enhances the intestinal absorption of metformin (Proctor et al., 2008), and subsequently, its oral bioavailability. Since the major AP intestinal transporters of metformin have not been determined, this study aimed at identifying these transporters in the Caco-2 cell monolayers.

[¹⁴C]metformin AP uptake and chemical inhibition studies revealed that the organic cation transporter 1 (OCT1) and the plasma membrane monoamine transporter (PMAT) are two major metformin AP transporters in the Caco-2 cell monolayers. Functional studies in stable OCT1-knockdown and PMAT-knockdown Caco-2 clones,

generated by sequence-specific shRNA technology, confirmed the role of these two transporters in metformin AP uptake. Two additional cation-selective transporters, namely, the serotonin transporter (SERT) and choline transporter (CHT) were also investigated for their contributions to metformin AP uptake in the Caco-2 cell monolayers. A novel and complete chemical inhibition scheme confirmed that OCT1, PMAT, SERT and CHT contribute to ~25%, ~20%, ~20% and ~15% of metformin AP uptake in Caco-2 cell monolayers, respectively.

2.B. INTRODUCTION

Metformin is a widely prescribed anti-hyperglycemic agent for the treatment of Type II diabetes mellitus. Despite its reputation as the front line anti-diabetic agent, little is known about the intestinal absorption mechanism of this very hydrophilic and charged drug. Metformin is a polar hydrophilic molecule (logD at pH7.4 of -6.13) that contains a net positive charge (pKa 12.4) at physiological pH (Saitoh et al., 2004). Consequently, the physiochemical properties of metformin prohibit efficient membrane permeability by passive diffusion, which should result in poor intestinal absorption unless its intestinal absorption is mediated by transporters. Metformin exhibits high but variable oral bioavailability 40-60% (Pentikainen et al., 1979; Tucker et al., 1981b) and undergoes “flip-flop” kinetics, where absorption is the rate-limiting step in its elimination following oral administration (Pentikainen et al., 1979; Tucker et al., 1981a). Further, oral absorption of metformin is dose-dependent (Karttunen et al., 1983; Noel, 1979; Pentikainen, 1986; Pentikainen et al., 1979; Tucker et al., 1981a). Hence, it appears that oral absorption of metformin is mediated by transporters.

Mechanisms of the intestinal absorption of metformin have been studied in the well-established model of intestinal epithelium, the Caco-2 cell monolayers (Proctor et al., 2008). The study showed that metformin was taken up across the AP membrane of the Caco-2 cells efficiently by bidirectional cation-selective transporter(s) during absorptive transport. However, metformin efflux across the basolateral (BL) membrane of Caco-2 cell monolayers was inefficient, resulting in the accumulation of metformin in the Caco-2 cells. The cellular kinetic studies revealed that absorptive transport of metformin was predominantly ($\geq 90\%$) via the paracellular route (Proctor et al., 2008).

However, the oral bioavailability of metformin is significantly higher than that of the paracellular probe mannitol (16%) (Artursson and Karlsson, 1991), suggesting that the absorption of metformin may be enhanced by other mechanisms. It was hypothesized that the bidirectional cation-selective transport mechanism(s) on the AP membrane of Caco-2 cell monolayers mediates efficient absorption of metformin by creating a cycling mechanism between the extracellular AP compartment and the intracellular space that provides the drug repeated opportunities to be absorbed through the paracellular route (Proctor et al., 2008). Thus the transporters that mediate the movement of metformin across the AP membrane of the intestinal epithelium play an indirect role in the absorptive transport of metformin by facilitating its uptake and accumulation in the intestinal epithelium as well as its cycling between enterocytes and intestinal lumen.

The Caco-2 cell monolayers, like the human intestine, express several cation-selective transporters that are potentially capable of transporting metformin. The present study was undertaken to identify the cation-selective transporters that facilitate AP uptake in the Caco-2 cell monolayers and human intestinal epithelium and to determine their relative contribution toward metformin AP uptake. To achieve this, a combination of chemical inhibitors were employed, individually and as a cocktail, at concentrations that selectively inhibit transporter activity of one or more transporters suspected to be involved in the uptake of metformin. Using this simple and cost effective technique, two known transporters of metformin, organic cation transporter (OCT) 1, and plasma membrane monoamine transporter (PMAT) were identified as important contributors of metformin AP uptake. In addition, two previously unidentified transporters of metformin, serotonin re-uptake transporter (SERT) and a choline high affinity transporter (CHT),

were found to be involved in the uptake of the drug across the AP membrane of Caco-2 cell monolayers. The findings presented here advance our understanding of the processes responsible for the absorption of metformin and potentially other hydrophilic drugs with permanent charge at physiologic pH values, and help identify potential sources of variability observed in the pharmacokinetics and pharmacodynamics of metformin. Additionally, these studies help refine our understanding of the transporters of cationic drugs that are active in the commonly employed Caco-2 cell model and the intestine.

2.C. MATERIAL AND METHODS

Materials

Eagle's minimum essential medium (EMEM) with Eagle's salts and L-glutamate, F-12 Nutrient Mixture, HEPES (1M), penicillin-streptomycin-amphotericin B solution (100x), non-essential amino acids (100x), geneticin, SuperScript[®] III First-Strand Synthesis SuperMix, TaqMan[®] Gene Expression Master Mix, and TaqMan[®] Gene Expression Assays for OCT1, 2, 3, PMAT, MATE1, 2, SERT, CHT, CTL1, 2, 3, 4, 5 were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Hank's balanced salt solution (HBSS) with calcium and magnesium was purchased from Mediatech, Inc. (Manassas, VA, USA). Hygromycin B solution was obtained from Roche Applied Science (Indianapolis, IN, USA). Total RNA from normal human intestinal tissue was purchased from Zyagen (San Diego, CA, USA). Fetal bovine serum (FBS), Dialyzed FBS (MWCO 10,000), trypsin-EDTA (1X), and all chemicals (unless mentioned otherwise) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [¹⁴C]Metformin was purchased from Moravsek Biochemicals and Radiochemicals (Brea, CA, USA). [³H]Serotonin was purchased from PerkinElmer (Waltham, MA, USA). OCT1-3, OCTN2, and mock transfected Chinese Hamster Ovarian (CHO) cells were previously generated and characterized in our laboratory (Ming et al., 2009). The Caco-2 (HTB-37) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). SERT transfected Human Embryonic Kidney 293 (HEK 293) cells were provided by Dr. Randy Blakely from Vanderbilt University (Nashville, TN, USA). Control HEK 293 cells were purchased from the Tissue Culture Facility at the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA).

Methods

Culturing of OCT Expressing CHO cells, Caco-2 Cells, and SERT-expressing HEK

293 Cells

Chinese Hamster Ovary (CHO) cells stably expressing OCT1, OCT2, OCT3, OCTN2, and vector-control cells (mock) were cultured and transport experiments were performed as described previously (Ming et al., 2009). Briefly, cells were cultured in F12 Nutrient Mix supplemented with 10% FBS, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ amphotericin B with additional 500 $\mu\text{g/ml}$ geneticin for OCT1-3 and mock-CHO cells or 200 $\mu\text{g/ml}$ hygromycin B for OCTN2-CHO cells. The cells were passaged following 90% confluency using trypsin-EDTA, and plated at a 1:20 ratio in 75-cm² T-flasks.

Caco-2 cells were cultured as described previously (Proctor et al., 2008). Briefly, the cells (passage numbers 30 to 40) were seeded at a density of 60,000 cells/cm² on polycarbonate membranes of TranswellsTM. The culture medium was changed the day following seeding and every other day thereafter. Cell monolayers with transepithelial electrical resistance greater than 300 $\Omega\cdot\text{cm}^2$ were used for experimentation at 21-28 days post seeding.

SERT-transfected HEK 293 cells and control parental HEK 293 lines were maintained in monolayer cultures in 75-cm² flasks in an atmosphere of 5% CO₂ at 37 °C as described previously (Qian et al, 1997). Both cell lines were grown in Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum (MWCO: 10,000; from Sigma), 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin within 10 passages. Culture medium for the transfected cell line was

supplemented with geneticin (250 mg/ml). Cells were seeded on Poly-D-lysine coated 24-well plates at 100,000 cells/well 48-72 hr before experimentation.

Metformin Transport Experiments in CHO Cells

Transport experiments using CHO cells were performed as described previously (Ming et al., 2009). Briefly, CHO cells were seeded at 100,000 cells/cm² in sterile 24-well polycarbonate plates and transport experiments were performed between days 5-7 post seeding. CHO cell monolayers were preincubated with transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES, pH 7.2) for 30 min at 37 °C. Uptake experiments were initiated by replacing the buffer solution with 300 µL of dosing solution. Uptake was terminated at the indicated time points by aspirating the donor solution and washing the monolayer 3X with 1 mL of 4 °C transport buffer. The cell monolayers were allowed to dry and 500 µL of lysis buffer (0.1 N NaOH with 0.1% SDS) was added to each well. Plates were shaken for 3 hr to ensure total lysis of the cell monolayer. Protein content of the cell lysate was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Radiolabeled compound in the cell lysate was analyzed by liquid scintillation spectrometry and the rate of initial uptake of each compound was determined. The uptake rate for metformin uptake into mock CHO cells was subtracted from the uptake rates obtained in each OCT-expressing cell line to obtain a corrected uptake rate reflective of only the carrier-mediated transport of metformin.

Metformin AP Uptake in Caco-2 Cell Monolayers

Initial AP uptake of metformin was performed using methods outlined previously (Proctor et al., 2008) with minor deviations. Inhibition of initial [¹⁴C]metformin

[0.15 μ Ci/mL, 10 μ M, 5 min] AP uptake in Caco-2 cells was performed in the absence (control) or presence of cimetidine [5 μ M], mitoxantrone [25 μ M], corticosterone [150 μ M], desipramine [200 μ M], MPP⁺ [5mM], paroxetine [0.1 μ M], and hemicholinium-3 (HC3) [1 μ M]. Cell monolayers were preincubated for 30 min in transport buffer in the presence of inhibitors outlined above or vehicle control bathing both the AP and BL compartments. AP uptake experiments were initiated by replacing the buffer solution in the AP donor compartment with transport buffer containing the substrate in the presence of an inhibitor or vehicle control. Uptake was terminated during the initial linear uptake range in Caco-2 cell monolayers [5 min] by washing the cell monolayers with 0.75mL of 4 $^{\circ}$ C transport buffer three times in each compartment. The cell monolayers were allowed to dry, excised from the insert, lysed and analyzed as above, and the rate of initial uptake of metformin was determined.

SERT-HEK 293 Cell Transport Experiments

Uptake of [¹⁴C]metformin by SERT was determined at indicated time in SERT-transfected HEK 293 cells and corrected for [¹⁴C]metformin uptake in control HEK 293 cells. Following uptake, the cells were washed 3X with 4 $^{\circ}$ C transport buffer, and 500 μ L of lysis buffer was added to the wells and incubated for 3 hr with shaking, and metformin apparent kinetic parameters were determined (K_m ~463 mM and V_{max} ~400 nmol/min/mg protein). To determine whether this low affinity was due to surface binding of metformin, a [¹⁴C]metformin surface binding assay was performed. The cells were washed with 1M unradiolabeled metformin following the 4 $^{\circ}$ C transport buffer wash, and the amount of surface bound [¹⁴C]metformin released into the wash solution was quantified. After washing with metformin solution, the cells were lysed as

described above and [^{14}C]metformin intrinsic cellular uptake was quantified by scintillation spectrometry, and the intrinsic kinetic parameters were determined.

Generation of OCT1- and PMAT-Knockdown Caco-2 Clones

Three OCT1-specific small interfering RNA (siRNA) sequences (Sigma) were evaluated for their ability to silence OCT1 gene expression. Sequence 1 (sense strand: 5'-GCUAUGAAGUGGACUGGAA-3'; antisense strand: 5'-UCCAGUCCACUUCAUAGC-3') and Sequence 3 (sense strand: 5'-CCAUCUGUGUGGGCAUCGU-3'; antisense strand: 5'-ACGAUGCCCACACAGAUGG-3') had the highest gene silencing activity (data not shown). The PMAT-specific siRNA sequence (sense strand: 5'-CAGCUUCAUCACGGACGUG-3'; antisense strand: 5'-CACGUCCGUGAUGAAGCUG-3') was obtained from published reports (Engel et al., 2004). The antisense and sense oligonucleotides were linked together with a hairpin loop, and annealed with the respective complementary DNA oligonucleotides. Each resulting double-stranded DNA was inserted into the BamHI and HindIII sites of the pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ) to generate the short hairpin RNA (shRNA) plasmid (Figure 2.S.1.). Caco-2 cells at 90% confluence were transfected with the shRNA plasmid, using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol. Transfectants were selected with 0.2 mg/ml hygromycin B for 3 weeks, and screened by quantitative real-time PCR for OCT1 or PMAT expression. Three clones from each sequence with the lowest gene expression, normal morphology and growth rate were chosen for further functional studies.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The mRNA expression of human OCT1-3, PMAT, MATE1-2, CHT, and CTL1-5 relative to 18s rRNA (18s) in Caco-2 cells and human intestine was determined using qRT-PCR analysis. qRT-PCR experiments were conducted using established methods (Holmes et al., 2006) with minor deviations. Total RNA was isolated from Caco-2 cell monolayers and human intestinal tissue using RNeasy Plus Mini Kit. cDNA was synthesized from total RNA (5 µg) using Superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). An equal amount of RNA was included in a No-RT control for each separate RNA sample. Real-time PCR was performed with 1:20 dilutions of the cDNA (in triplicate). Quantitative PCR reactions (25 µL total volume) were performed using TaqMan[®] Gene Expression Assays for quantitative PCR with primer pairs at 0.75 µM final reaction concentration, and 5 µL of cDNA or No-Template negative control. RT-PCR amplification was performed in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Inc. Foster City, CA, USA). All gene products were amplified above the fluorescent threshold by cycle 45 in the cDNA sample. The expression of the 18s housekeeping gene was determined in each RT-PCR experiment and served as the normalization control. cDNA preparation, fluorescent threshold, and PCR conditions remained constant in order to calculate the expression of transporter genes relative to 18s rRNA. Relative expression values were calculated by $2^{-\Delta C_t}$, where $\Delta C_t = (C_{t, 18s} - C_{t, gene})$.

Data Analysis

A Michaelis-Menten equation with one saturable component was fit to the corrected uptake rate data obtained in CHO cell experiments, which represented only the

carrier-mediated transport, described by the following expression:

$$V = \frac{(V_{\max} * C)}{(K_m + C)} \quad (1)$$

where C is the metformin concentration, V_{\max} is the maximal velocity, and K_m is the Michaelis-Menten constant.

Inhibitory potency (e.g. IC_{50} value) was determined for each inhibitor across the transporter expressing cell line. The following equation was fit to the corrected uptake data:

$$V = \frac{V_o}{\left[1 + \left(\frac{I}{IC_{50}}\right)^n\right]} \quad (2)$$

where V is the uptake rate in the presence of inhibitor $[I]$, V_o is the uptake rate in the absence of inhibitor, IC_{50} is the inhibitor concentration to achieve 50% inhibition, and n is the Hill coefficient. Uptake kinetic model and IC_{50} curve model estimates were obtained by non-linear regression analysis by GraphPad Prism 5 (La Jolla, CA, USA). IC_{50} data for transporter expressing cells and Caco-2 uptake data were reported relative to the control. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test unless otherwise noted. Data represent mean \pm S.D; $n=3$ unless otherwise noted, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control, # $p<0.05$ compared to each other.

2.D. RESULTS

Inhibition of OCT1-, OCT2-, and OCT3-mediated Metformin Uptake in CHO Cells by a Panel of Cation-Selective Inhibitors

The K_m values for cellular uptake of metformin via the most common cation-selective transporters, OCT1-3 and OCTN2 were estimated. Using OCT1-, OCT2-, and OCT3-(singly) expressing CHO cells, metformin was shown to be a substrate for all three transporters with apparent K_m values of 3.1 ± 0.3 , 0.6 ± 0.03 , and 2.6 ± 0.2 mM, respectively (Figure 2.S.2.) (Proctor, 2010). This confirms previous reports outlining substrate affinities of these transporters for metformin (Koepsell et al., 2007). In contrast, metformin uptake into OCTN2-expressing CHO cells was inefficient and not significantly different from uptake into the mock CHO cells (Figure 2.S.2.)(Proctor, 2010), thus providing evidence that metformin is not a substrate for OCTN2. Therefore, this transporter was omitted in the subsequent studies evaluating the role of cation-selective transporters in the AP uptake of metformin into Caco-2 cell monolayers. The IC_{50} curves generated for inhibition of metformin uptake by the OCT inhibitors, mitoxantrone, corticosterone, cimetidine and desipramine, into OCT1-, OCT2-, and OCT3-expressing CHO cells are shown in Figure 2.1A-D, and the IC_{50} values are summarized in Table 2.1. Uptake (initial rate) of metformin into the three OCT-expressing cells was inhibited in a concentration-dependent manner by the four inhibitors. Mitoxantrone, reported to be an OCT1-specific inhibitor (Koepsell et al., 2007), was a potent inhibitor of OCT1 with an IC_{50} value of 3.0 ± 0.8 μ M, which is 40- to 60-fold lower than the estimated IC_{50} values for OCT2 and OCT3 (Figure 2.1A, Table 2.1). Corticosterone and desipramine were also strong inhibitors of OCT1-

mediated metformin transport at the same relative inhibitory potency (e.g. IC_{50} values < 10 μ M), although not as selective for OCT1 as mitoxantrone. Corticosterone and desipramine were the most potent OCT2 inhibitors with IC_{50} values estimated to be less than 3 μ M (Figure 2.1B and D, Table 2.1). The IC_{50} values for inhibition of OCT1, 2 and 3 by cimetidine, a potent inhibitor of yet another cation transporter, multidrug and toxin extrusion (MATE)1 transporter, was 10- to 20-fold lower than the reported IC_{50} value for MATE1 ($1.1 \pm 0.3 \mu$ M) (Figure 2.1C, Table 2.1) (Proctor, 2010; Tsuda et al., 2009).

Identification of AP Transporters of Metformin Using a Novel Chemical Inhibition Scheme in Caco-2 Cell Monolayers

The experimentally derived inhibition data for OCT1-3 by mitoxantrone, cimetidine, corticosterone and desipramine (Figure 2.1A-D), as well as previously reported inhibition data for MATE1 (Tsuda et al., 2009) and PMAT (Engel and Wang, 2005) enabled the selection of inhibitors and the development of a scheme to use these transporters singly or as a cocktail to estimate the relative contributions of these transporters in the AP uptake of metformin in Caco-2 cell monolayers. The concentration selected for each inhibitor was at least 4-fold greater than the estimated IC_{50} value for metformin uptake by that transporter, and at least 2-fold less than the IC_{50} values for other metformin transporters. At the selected inhibitor concentrations, the target transporters were inhibited by >80% with no significant (<20%) inhibition of the other transporters. Figure 2.1E depicts the chemical inhibition scheme employed in elucidating the involvement of specific cation-selective transporters in the AP uptake of metformin in Caco-2 cell monolayers (Proctor, 2010).

Utilizing the novel chemical inhibition scheme, metformin AP uptake in Caco-2 cells was evaluated in the presence and absence of inhibitors and presented in Figure 2.1F. Inhibition by cimetidine had no effect on the AP uptake of metformin in Caco-2 cells, which eliminated the contribution of MATE1 in mediating AP uptake of metformin. Mitoxantrone significantly decreased the AP uptake of metformin to $74 \pm 8\%$ ($p < 0.01$) of the control, suggesting that OCT1 contributes to ~25% of the AP uptake of metformin. Similarly, corticosterone inhibited AP uptake of metformin by ~25%, reducing the AP uptake to $73 \pm 3\%$ ($p < 0.01$). The inhibitory effects of mitoxantrone and corticosterone on metformin uptake into Caco-2 cell monolayers were not significantly different, suggesting that OCT2 and 3 were likely not involved in the AP uptake of metformin. Desipramine reduced the AP uptake of metformin by ~65% ($p < 0.001$), which is an additional ~40% reduction in metformin AP uptake to the ~25% reduction observed with corticosterone. These data suggest that PMAT also contributes to the AP uptake of metformin in Caco-2 cell monolayers (Figure 2.1 D and E). MPP⁺ which inhibits all transporter-mediated uptake, reduced metformin uptake by ~80%, indicating that non-specific binding or uptake via passive diffusion may contribute to ~20% of the signal for metformin AP uptake. The significant difference ($p < 0.05$) in the inhibitory effects of desipramine and MPP⁺ suggests that there may be another unknown transporter(s) involved in the AP uptake of metformin in Caco-2 cell monolayers (Proctor, 2010). In summary, OCT1 and PMAT were identified as major contributors to the AP uptake of metformin in Caco-2 cell monolayers, and a possible role of an unknown transporter(s) in the AP uptake of metformin was uncovered.

Serotonin Reuptake Transporter (SERT) and Choline High-affinity Transporter (CHT) Contribute to the AP Uptake of Metformin in Caco-2 Cell Monolayers

To determine which transporter(s) was involved in the AP uptake other than OCT1 and PMAT, metformin's affinity for the serotonin reuptake transporter (SERT) was evaluated, as SERT is known to be able to transport organic cations and expressed in Caco-2 cells (Hilber et al., 2005; Martel et al., 2003; Seidel et al., 2005). Time-dependent and concentration-dependent uptake of metformin in SERT singly-transfected HEK 293 cells (SERT-HEK 293) was determined relative to metformin uptake in control HEK 293 cells. Metformin uptake in SERT-HEK 293 cells showed linearity up to 2 min following which the uptake was saturable, while metformin uptake in control HEK 293 cells was significantly lower and linear up to 30 min (Figure 2.2A). Concentration-dependent uptake of metformin by SERT indicated that metformin was a substrate of SERT with an apparent K_m value of approximately 4.0 mM (Figure 2.2B).

To determine whether SERT contributes to the AP uptake of metformin in Caco-2 cell monolayers, inhibition of metformin uptake in SERT-HEK 293 cells was examined in the presence or absence of varying concentrations of paroxetine, a serotonin selective reuptake inhibitor (SSRI) with a reported K_i for SERT of 0.8 nM (Owens et al., 1997). To determine whether paroxetine can also inhibit other transporters, metformin uptake in singly transfected cells expressing OCT1, 2, 3, or SERT was determined in the presence of varying concentrations of paroxetine (Figure 2.2C). The IC_{50} values for OCT1, 2, 3 and SERT are $0.99 \pm 0.16 \mu\text{M}$, $11.92 \pm 1.24 \mu\text{M}$, $6.43 \pm 1.27 \mu\text{M}$, and $5.96 \pm 0.60 \text{ nM}$, respectively. In subsequent experiments, a

paroxetine concentration of 0.1 μM was used to selectively inhibit SERT, without inhibiting OCTs (Table 2.1).

The contribution of SERT to metformin AP uptake in Caco-2 cell monolayers was evaluated using chemical inhibitors at fixed concentrations outlined in the modified chemical inhibition scheme (Figure 2.2D) with results presented in Figure 2.2E. Similar to the results presented in Figure 2.1F, mitoxantrone inhibited AP uptake of metformin by ~25%, suggesting that OCT1 was responsible for ~25% of metformin AP uptake. However, if mitoxantrone inhibits SERT, the contribution of OCT1 toward metformin AP uptake into Caco-2 cell monolayers would be overestimated. Therefore, metformin uptake into SERT-expressing cells was measured in the presence of varying concentrations of mitoxantrone. Our results showed that the IC_{50} value estimated for mitoxantrone was $977.2 \pm 1.2 \mu\text{M}$ (Figure 2.S.3., Table 2.1), suggesting that mitoxantrone at 25 μM likely inhibited only OCT1 and did not significantly inhibit SERT. The selective inhibitor of SERT, paroxetine, decreased metformin AP uptake into Caco-2 cell monolayers by ~20%, which suggests that SERT is responsible for ~20% of metformin AP uptake. In light of the nearly 20% contribution by SERT toward metformin AP uptake into the Caco-2 cell monolayers, it was necessary to re-evaluate our results in Figure 2.1F, which showed that desipramine at 200 μM inhibited OCT1, 2, 3 and PMAT. Published reports indicate that desipramine also inhibits SERT with a K_i value of $54 \pm 4.2 \text{ nM}$. (Barker et al., 1994). Therefore, the ~65% decrease in metformin AP uptake by desipramine suggests that OCT1, PMAT and SERT together are responsible for ~65% of metformin AP uptake, with relative contributions of ~25%, ~20%, and ~20%, respectively. However, since ~15% of the AP uptake of metformin

was unaccounted for based on the inhibition studies with MPP^+ , it lead us to conclude that yet another unknown transporter(s) was likely involved in the AP uptake of metformin in Caco-2 cell monolayers.

Based on the structural similarity of metformin to choline (permanently charged nitrogen functionality plus small hydrophilic molecule), choline transporter system was considered as a possible candidate. Choline, an essential nutrient, is taken up by the choline transporter system, which includes the choline high-affinity transporter (CHT) and choline transporter-like proteins (CTL1-5). The choline high-affinity transporter (CHT) plays an important role in the uptake of choline in the CNS (Kus et al., 2003), and is also expressed in the AP membrane of colonic epithelial cells (Harrington et al., 2010). Studies have shown that a selective CHT inhibitor, hemicholinium-3 (HC3) can inhibit CHT with higher potency ($IC_{50} = 4 \text{ nM}$) than CTLs ($\sim 100 \mu\text{M}$) (Apparsundaram et al., 2000; Lockman and Allen, 2002). To determine whether HC3 can also inhibit OCTs and SERT, the inhibitory profiles of HC3 on OCTs and SERT were investigated. Figure 2.3A shows the inhibitory effects of HC3 on OCT1-3 and SERT. The IC_{50} values for OCT1, 2, 3 and SERT are $112.20 \pm 1.45 \mu\text{M}$, $131.83 \pm 1.41 \mu\text{M}$, $524.81 \pm 1.65 \mu\text{M}$, $70.80 \pm 1.19 \mu\text{M}$, respectively (Table 2.1). Therefore, $1 \mu\text{M}$ HC3 can be used to selectively inhibit CHT without affecting the other transporters. A modified chemical inhibition scheme (Figure 2.3B) was implemented to examine if choline transporters were involved in the AP uptake of metformin in Caco-2 cells. HC3 caused a $\sim 15\%$ decrease in the AP uptake of metformin relative to control cells (Figure 2.3C). Combining desipramine and HC3 inhibited $\sim 80\%$ of the AP uptake of metformin, which was not significantly different from the inhibitory effect of 5mM MPP^+ (Figure 2.3C).

These results strongly suggested the involvement of CHT, with a relative contribution of ~15% in the AP uptake of metformin, which combined with OCT1, PMAT, and SERT accounts for all of the carrier-mediated transport of metformin across the AP membrane in Caco-2 cell monolayers.

Stable Knockdown of OCT1 and PMAT by shRNA Reduced AP Uptake of Metformin in Caco-2 Cell Monolayers

Since the contribution of PMAT toward metformin AP uptake into the Caco-2 cell monolayers has been determined “by difference”, and not directly by measuring inhibition of metformin uptake with a PMAT-selective transporter, the PMAT (shRNA)-knockdown Caco-2 clones were developed to evaluate the reduction of metformin uptake associated with the reduction in PMAT expression. OCT1-knockdown clones were also generated to provide a positive control, since inhibition of metformin uptake by reduced OCT1 expression can be directly compared with the inhibition of uptake due to an OCT1-selective inhibitor. Metformin uptake was studied in OCT1- and PMAT-knockdown Caco-2 clones. Three OCT1 knockdown clones, namely construct 1 clone 43 (1-43) and construct 3 clones 21 and 27 (3-21 and 3-27), and three PMAT knockdown clones (2, 9, 24) were selected for functional studies based on the extent of transporter-knockdown, normal morphology, and growth rate. OCT1 and PMAT mRNA expression in the respective knockdown clones, as determined by qRT-PCR, was significantly attenuated (Figure 2.4A and B). AP uptake of metformin in the OCT1- and PMAT-knockdown Caco-2 clones was decreased by ~20% and ~40%, respectively (Figure 2.4C and D). These results confirm the involvement of OCT1 and PMAT in the AP uptake of metformin in Caco-2 cell monolayers.

Transporter mRNA Expression in Caco-2 Cell Monolayers and Human Intestinal Tissue

The gene expression levels of OCT1, PMAT, SERT and CHT, as well as other cation-selective transporters in Caco-2 cells and human intestinal tissue were evaluated by qRT-PCR. Results are shown in Figure 2.5A-B. In Caco-2 cells, CTLs and SERT are the most highly expressed transporters relative to other cation-selective transporters examined, with PMAT and OCT3 also highly expressed. OCT1, OCT2, MATEs, and CHT have low but detectable levels of expression. In human intestinal tissue, OCT3 and PMAT are highly expressed relative to the other transporters. SERT and CTLs are also expressed although their expression levels are not as high as that observed in Caco-2 cell monolayers. OCT2, MATE2 and CHT mRNA expression was not detected in human intestine. OCT1 is expressed at low levels in both Caco-2 cell monolayers and human intestinal epithelium.

2.E. DISCUSSION

Previous studies have shown that cation-selective transporter(s) are involved in the AP uptake and subsequent accumulation of metformin in the well-established human intestinal epithelial cell model, the Caco-2 cell monolayers (Proctor et al., 2008). It was hypothesized that the bidirectional transport processes on the AP membrane of Caco-2 cells and inefficient BL efflux work synergistically with the paracellular transport to enhance the overall absorption of this widely prescribed anti-diabetic drug metformin.

OCT1-3 and OCTN1-2, as well as other cation-selective transporters such as PMAT and MATE1 could be potential contributors to the intestinal absorption of metformin. The goal of this study was to identify the specific transporter(s) responsible for the uptake of metformin across the AP membrane of the established intestinal cell model, Caco-2 cell monolayers, and to estimate their relative contribution to the AP uptake of metformin. However, the wide substrate specificity of cation-selective transporters towards metformin and the potential for multiple transporter expression on the AP membrane in Caco-2 cells presented a challenge to elucidating the specific transporter(s) responsible for AP uptake of metformin. To address this problem, a novel chemical inhibition approach was implemented to estimate the relative contribution of each candidate transporter to the AP uptake of metformin in Caco-2 cell monolayers (Figure 2.1E). Chemical inhibition in the traditional or more classical sense was employed when possible, where one specific inhibitor is used to assess the function of one candidate transporter. However, this approach is often not feasible, especially in the case of cation-selective transporters where the inhibition curves often overlap across different isotypes or in cases with newly cloned transporters where the substrate or

inhibitor profiles are not widely established (e.g., PMAT). Briefly, here is how the inhibition scheme works: mitoxantrone at 25 μM should selectively inhibit OCT1, whereas cimetidine at 5 μM should selectively inhibit MATE1 without inhibiting other transporters; and corticosterone at 150 μM should inhibit OCT1, 2, and 3. The difference between metformin uptake in the presence of corticosterone or mitoxantrone was used to determine the contributions of OCT2 and 3. Since desipramine at 200 μM can inhibit all the listed candidate transporters, the contribution of PMAT can be elucidated by assessing the difference in metformin uptake in the presence of desipramine or corticosterone, assuming that OCT1-3 and PMAT account for all of the metformin AP uptake. Finally, studies with MPP^+ at 5mM were performed to completely abolish all carrier-mediated transport (Sato et al., 2008); therefore, the difference in inhibition of metformin uptake by MPP^+ and desipramine would indicate the contribution of other unknown transporters, if any, in the AP uptake of metformin. Through this novel use of both single inhibitors and combinations of inhibitors, it was demonstrated that two known cation-selective transporters of metformin, OCT1 and PMAT, and two previously unidentified transporters of metformin, SERT and CHT, were responsible for the AP uptake of metformin in Caco-2 cells. Gene expression data demonstrated that each of the four transporters identified above were expressed in both Caco-2 cells as well as human intestine (Figure 2.5). However, as is the case often, gene expression of each transporter poorly correlated with transporter activity. Further quantitative analysis of these transporters in Caco-2 cells and intestine is warranted to accurately assess the expression in relation to functional activity.

The first step to determine the AP transporters involved in uptake of metformin in Caco-2 cells was to generate a list of candidate transporters that have been previously reported to transport metformin or similar cation transporters that are likely candidates as metformin transporters and that are expressed in Caco-2 cells and in the intestine. These initially included OCT1-3, OCTN2, MATE1, and PMAT (Figure 2.1E); however uptake studies with metformin in OCTN2-expressing CHO cells revealed that metformin is not a substrate for this transporter (Figure 2.S.2.). This novel finding is highly significant in that it rules out metformin transport by one of the most highly expressed AP cation-selective transporters present in both Caco-2 cells and human intestine (Elimrani et al., 2003) as well as the heart, liver, and kidney (Hilgendorf et al., 2007; Tamai et al., 1998; Wu et al., 1999). Inhibition studies with carefully selected concentrations of cimetidine, mitoxantrone, corticosterone, and desipramine indicated that OCT1 and PMAT were involved in the AP uptake of metformin, ruling out candidates OCT2, OCT3, and MATE1 (Figure 2.1F).

Initially it was assumed that the five candidate transporters depicted in Figure 2.1E would account for the majority of the carrier-mediated transport of metformin across the AP membrane in Caco-2 cells. However, the chemical inhibition scheme (Figure 2.1E) revealed that there was a significant carrier-mediated uptake unaccounted for (Figure 2.1F), suggesting that there were other transporter(s) involved. That led us to look at other proteins known to transport cations, such as the serotonin reuptake transporter (SERT, SLC6A4), as a potential transporter involved in the AP uptake of metformin. SERT is expressed on both the AP and BL membranes, but the expression on the AP side is predominant in the intestine and Caco-2 cells (Wade, Chen et al. 1996;

Martel, Monteiro et al. 2003; Gill, Pant et al. 2008), and functions in the inward (e.g. uptake) direction (Nelson and Rudnick, 1979). As its name implies, the endogenous substrate of SERT is serotonin. However, recent studies showed that SERT and other organic cation transporters have shared substrate specificities. Duan and Wang showed that in OCT3- and PMAT-transfected HEK 293 cells, OCT3 and PMAT can uptake serotonin (Duan and Wang, 2010). Additionally, Hilber et al. showed that in SERT-transfected HEK 293 cells, SERT can uptake several organic cations, such as tyramine, MPP⁺, para-chloroamphetamine (Hilber et al., 2005). Based on its AP localization in the intestine and similar substrate affinities to known transporters of metformin, the role of SERT in AP uptake of metformin was investigated.

In this report, metformin was demonstrated to be a substrate for SERT with an apparent K_m of approximately 4 mM, which is approximately the same affinity as that of OCT1 and OCT3 for metformin. To the best of our knowledge, this is the first report of metformin being transported by SERT. To determine the role of this transporter in the AP uptake of metformin in Caco-2 cells, transport experiments were performed in the presence of paroxetine, a potent serotonin selective reuptake inhibitor (SSRI), at a concentration that did not inhibit OCT1-3 (Figure 2.2C and Table 2.1). Lacking the PMAT singly transfected cell line, the inhibition of paroxetine on PMAT remains unknown. However, studies in mouse Pmat (mPmat) singly transfected cells showed that paroxetine can inhibit mPmat with an IC_{50} of ~25 μ M (data not shown). Based on 87% sequence homology between mouse and human PMAT (from BLAST analysis at National Center for Biotechnology Information), it is a reasonable assumption that 0.1 μ M paroxetine would not inhibit PMAT function significantly. Furthermore,

mitoxantrone inhibited SERT only at a high concentration ($IC_{50} \sim 1\text{mM}$, Figure 2.S.3. and Table 2.1). Therefore, mitoxantrone can still be used to selectively inhibit OCT1 in the presence of other metformin transporters including SERT. A study by Barker et al. reported that desipramine inhibited SERT with K_i of $54 \pm 4.2\text{ nM}$. (Barker et al., 1994). Therefore, studies with desipramine represented not only inhibition of OCT1 and PMAT, but also SERT (Table 2.1). By employing a modified chemical inhibition scheme (Figure 2.2D), it was shown that SERT accounted for $\sim 20\%$ of the AP uptake of metformin (Figure 2.2E). Taking into account the effects of desipramine on SERT function, the contribution of PMAT was estimated to be 20% by subtracting the contributions of OCT1 (25%) and SERT (20%) from the 65% reduction in overall transport relative to control (Figure 2.2E). Furthermore, a significant difference ($\sim 15\%$) in metformin uptake between desipramine and MPP^+ indicated that there may yet be another unknown transporter(s) involved in the AP uptake of metformin in Caco-2 cells.

Choline, a quaternary amine with a permanent charge, is an essential nutrient that is absorbed through the intestine via passive and carrier-mediated processes (Herzberg and Lerner, 1973; Kamath et al., 2003; Kuczler et al., 1977; Sanford and Smyth, 1971). The choline transporter system, which includes choline high-affinity transporter (CHT) and choline transporter-like proteins (CTLs), plays a significant role in the absorption process of choline and may play a role in the AP uptake of metformin. A specific inhibitor of CHT, hemicholinium-3 (HC3) (IC_{50} value of 4 nM (Apparsundaram et al., 2000), was used to estimate the contribution of CHT in metformin AP uptake in Caco-2 cells. The inhibition profiles of HC3 on the other transporters involved were assessed to ensure that the concentration selected was specific for CHT and not for OCTs or

SERT (Figure 2.3A). HC3 can inhibit mPmat with $IC_{50} \sim 100\mu\text{M}$ (data not shown); therefore it is likely that PMAT should not be inhibited by low concentrations of HC3. Including CHT into the new chemical inhibition scheme (Figure 2.3B), the AP uptake of metformin in Caco-2 cells was examined. It was found that HC3 reduced the AP uptake of metformin by $\sim 15\%$, supporting a role of CHT in metformin transport. In addition, inhibition with the mixture of desipramine and HC3 completely abolished the entire carrier-mediated uptake of metformin in Caco-2 cells, reducing the uptake to the level of MPP^+ . Direct evidence for CHT involvement in metformin transport is still lacking; however, the inhibition data by HC3 strongly suggests the involvement of this transporter in the AP uptake of metformin into the Caco-2 cell monolayers. In addition to CHT, there also are low affinity choline transporter-like proteins (CTL1-5) that are highly expressed in the intestine; however these proteins are not as sensitive to inhibition by HC3 (IC_{50} values ranging in the $100\mu\text{M}$ range (Lockman and Allen, 2002); thus these transporters likely are not involved in metformin AP uptake. Future studies on metformin uptake using CHT singly transfected cells will conclusively demonstrate that metformin is capable of being transported by CHT.

Of the four transporters implicated, only PMAT was determined based on exclusion without the use of a selective inhibitor to directly assess its role in the AP uptake of metformin in Caco-2 cell monolayers. To confirm the involvement of PMAT and the accuracy of our chemical inhibition scheme, stable PMAT (shRNA)-knockdown Caco-2 clones were generated using an established siRNA sequence (Engel et al., 2004). OCT1 (shRNA)-knockdown Caco-2 clones were also generated and used as positive control. In both OCT1- and PMAT-knockdown cells, the AP uptake of

metformin was decreased by ~20% and ~40%, respectively (Figure 2.4C-D). The latter finding is novel in that it is the first report of PMAT expression and function in Caco-2 cells. Together, these studies confirm the chemical inhibition data demonstrating OCT1 and PMAT are two contributors to the cation-selective AP uptake of metformin in Caco-2 cells. The contribution of OCT1 in the AP metformin uptake into the Caco-2 cell monolayers as determined by selective inhibition of OCT1 by mitoxantrone was similar to that determined using the OCT1-knockdown Caco-2 cell clone. However, the contribution of PMAT was overestimated in the PMAT (shRNA)-knockdown Caco-2 cells in relation to the contribution estimated using chemical inhibition (e.g. 40% vs. 20%). This was likely due to single clone differences in relation to the heterogeneous population of the control Caco-2 cells in the function of transporters other than PMAT. This further highlights the utility of chemical inhibition in regards to identifying transporters involved in Caco-2 cells or intestinal enterocytes.

In conclusion, the data presented in this report support the involvement of OCT1, PMAT, SERT and CHT in the AP uptake of metformin in Caco-2 cells. A schematic representation of the transporters involved in the AP uptake of metformin in Caco-2 cell is presented in Figure 2.6. This is the first report of as many as four transporters being involved in the AP uptake of metformin into the Caco-2 cell monolayers, and by inference into the intestinal epithelium. This is a remarkable finding considering that metformin is in clinical use for decades without any recognition that its intestinal absorption is likely to be mediated by any intestinal transporter, let alone four transporters. Identification of the intestinal transporters that facilitate metformin uptake will provide insight into the mechanisms responsible for the dose-dependent and

variable absorption (Noel, 1979; Tucker et al., 1981a), intestinal accumulation, and associated pharmacology (Bailey et al., 2008; Stepensky et al., 2002) of metformin. A striking finding in this report is the presence of several metformin transporters present on the AP membrane of Caco-2 cells and conceivably in the intestine, which may be a reason that there are not many known intestinal drug-drug interactions involving metformin absorption. However, the high dose (e.g. $\geq 800\text{mg}$) of metformin administered may result in intestinal DDIs for other cationic drugs or nutrients with more selective affinities for the transporters identified here. Finally, the novel chemical inhibition scheme outlined here could be used to elucidate the contributions of cation-selective transporters involved in metformin or other promiscuous substrates in other cell lines, tissues, or organs and can help identify, as shown here, previously overlooked or unknown transporters. The use of a cocktail of inhibitors in a chemical inhibition scheme that provided an estimate of the relative contribution of four metformin transporters, including that of PMAT for which no selective inhibitor is available, is a significant contribution of this work. It opens up the possibility of elucidating the role of individual transporters in the uptake of drugs in tissues that express multiple transporters of a drug even when selective inhibitors for all the transporters involved are not known or available.

Table 2.1. Experimental and literature reported IC_{50} values for chemical inhibitors.

	IC_{50} Values					
	Mean \pm S.D. (μ M)					
Inhibitor	OCT1	OCT2	OCT3	SERT	CHT	CTLs
Mitoxantrone	3.0 ± 0.8	135 ± 12	174 ± 19	977.2 ± 1.2	N/A	N/A
Cimetidine	20.9 ± 1.4	16.6 ± 1.3	9.8 ± 1.3	N/A	N/A	N/A
Corticosterone	3.2 ± 0.5	1.3 ± 0.1	0.15 ± 0.05	N/A	N/A	N/A
Paroxetine	0.99 ± 0.16	11.92 ± 1.24	6.43 ± 1.27	0.00596 ± 0.0006	N/A	N/A
Desipramine	2.2 ± 0.4	2.4 ± 0.2	3.8 ± 0.3	0.054 ± 0.0042 ^[1]	N/A	N/A
HC3	112.20 ± 1.45	131.83 ± 1.41	524.81 ± 1.65	70.80 ± 1.19	0.004 ^[2]	~ 100 ^[3]

N/A: Not available. References: [1] (Barker et al., 1994). [2] (Apparsundaram et al., 2000). [3] (Lockman and Allen, 2002).

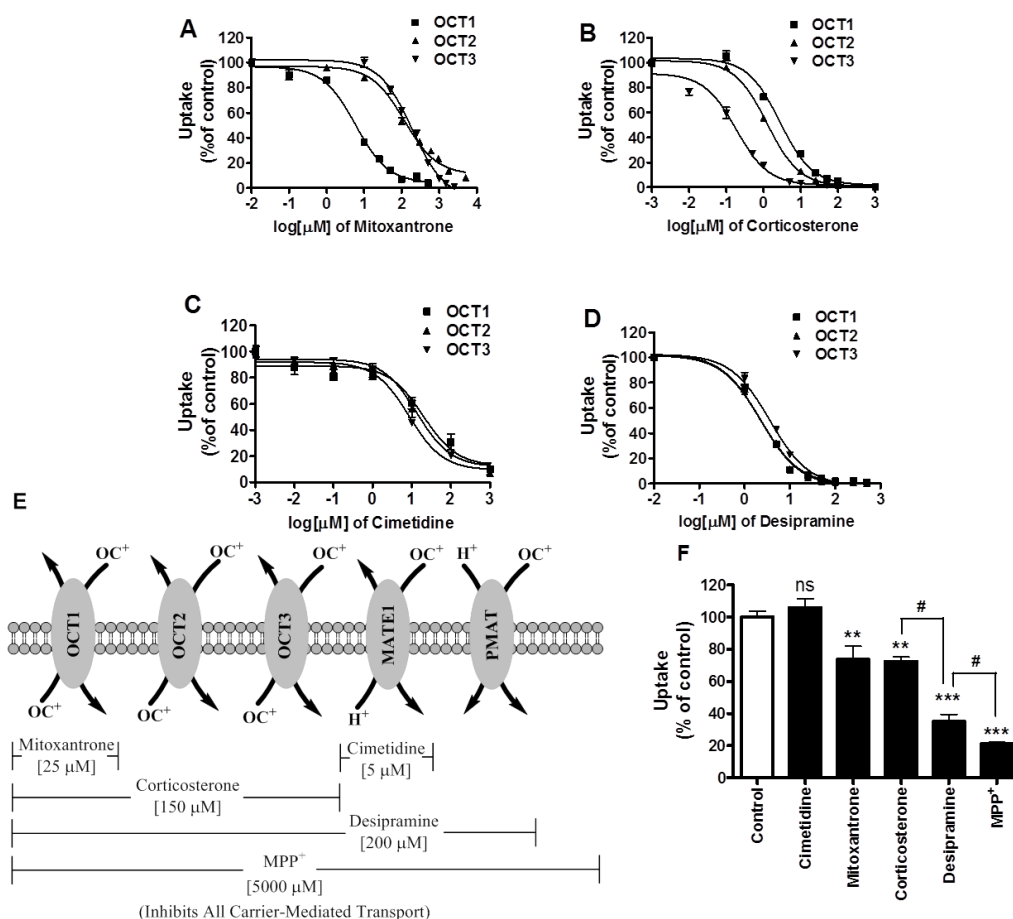


Figure 2.1. Elucidation of metformin AP uptake transporter(s) through a novel chemical inhibition scheme. Inhibition profiles (IC_{50} curves) of mitoxantrone (A), corticosterone (B), cimetidine (C) and desipramine (D) on metformin uptake [10 μM, 5 min] by OCT1-3 in transporter singly-expressing CHO cells. Data represent mean \pm S.D., n=3. IC_{50} curves were fit to corrected uptake data in the presence of varying concentrations of each inhibitor. (E) Chemical inhibition scheme to determine the contributions of transporters to metformin AP uptake in Caco-2 cells. (F) Inhibition of metformin [10 μM] initial AP uptake [5 min] in the presence of chemical inhibitors outlined in E. Data represent mean \pm S.D., n=3. **p<0.01, ***p<0.001 compared to control. #p<0.05 compared to each other (Proctor, 2010).

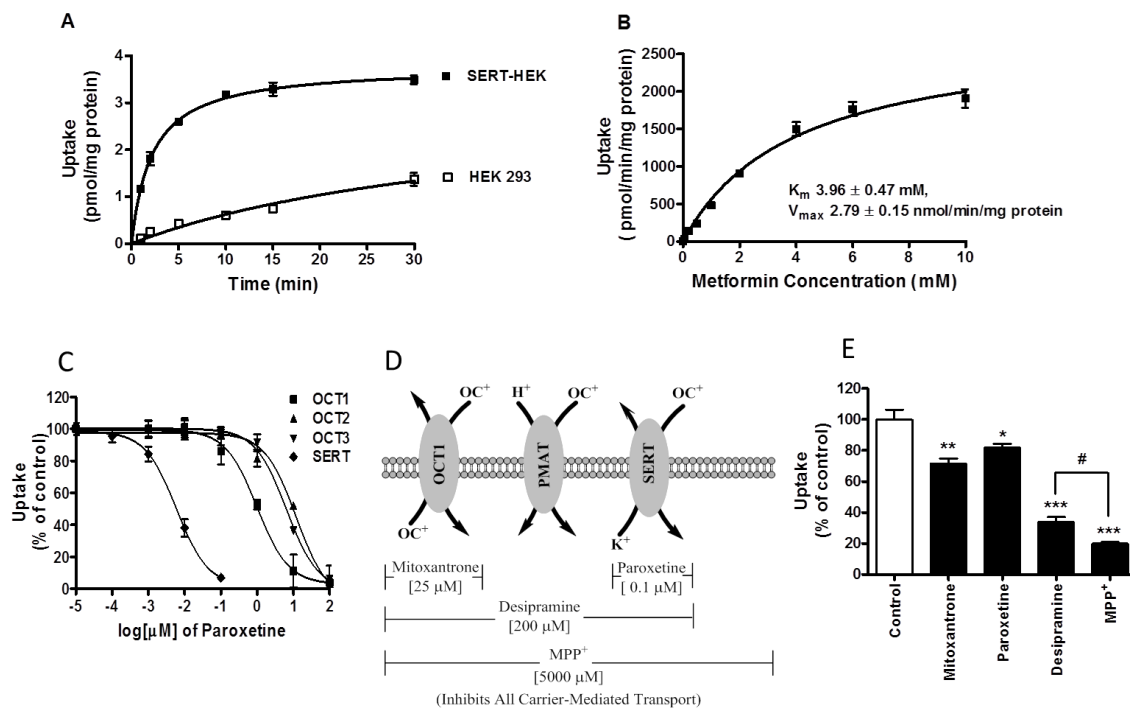


Figure 2.2. SERT is a metformin transporter and contributes to the AP uptake of metformin in Caco-2 cell monolayers. (A) Time-dependent uptake of metformin [1 μ M] in SERT- transfected HEK 293 cells (closed symbols) and HEK 293 control cells (open symbols). (B) Concentration-dependent uptake of metformin [2 min] in SERT-HEK 293 cells, corrected for surface binding and uptake of metformin in control HEK 293 cells. A Michaelis-Menten equation with one saturable component was fit to the corrected uptake rate data obtained in CHO cell experiments and the estimated K_m values are presented. (C) Inhibition profiles (IC₅₀ curves) of paroxetine on metformin uptake [10 μ M, 5 min] by OCTs and SERT. (D) Modified chemical inhibition scheme for OCT1, PMAT and SERT. (E) Inhibition of metformin AP uptake [10 μ M, 5 min] in the presence of inhibitors outlined in E. Data represent mean \pm S.D., n=3. *p<0.05, **p<0.01, ***p<0.001 compared to control. #p<0.05 compared to each other.

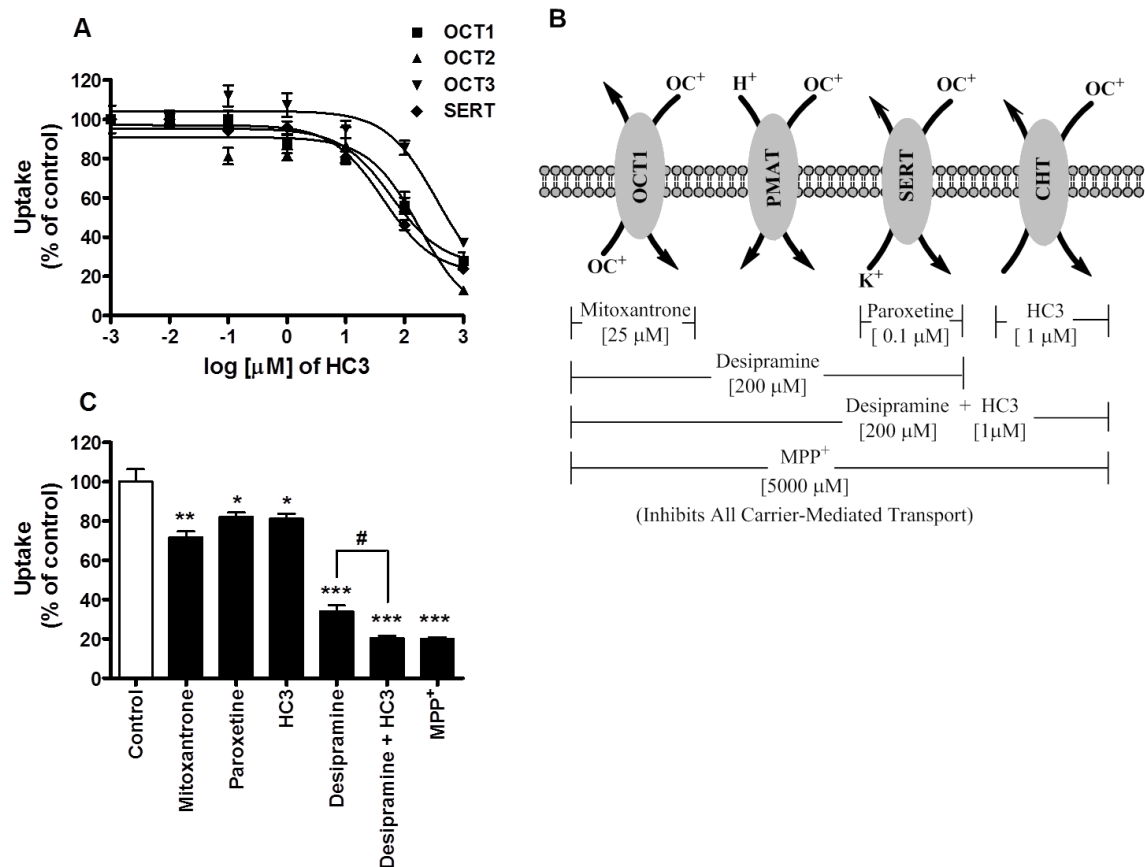


Figure 2.3. Inhibition of HC3 reduced the initial AP uptake of metformin. (A) Inhibition profiles (IC_{50} curves) of HC3 with respect to OCT1-3 and SERT. **(B)** Modified chemical inhibition scheme for OCT1, PMAT, SERT and CHT. **(C)** Inhibition of metformin AP uptake [10 μM , 5 min] by inhibitors used in B. Data represent mean \pm S.D., $n=3$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control. # $p<0.05$ compared to each other.

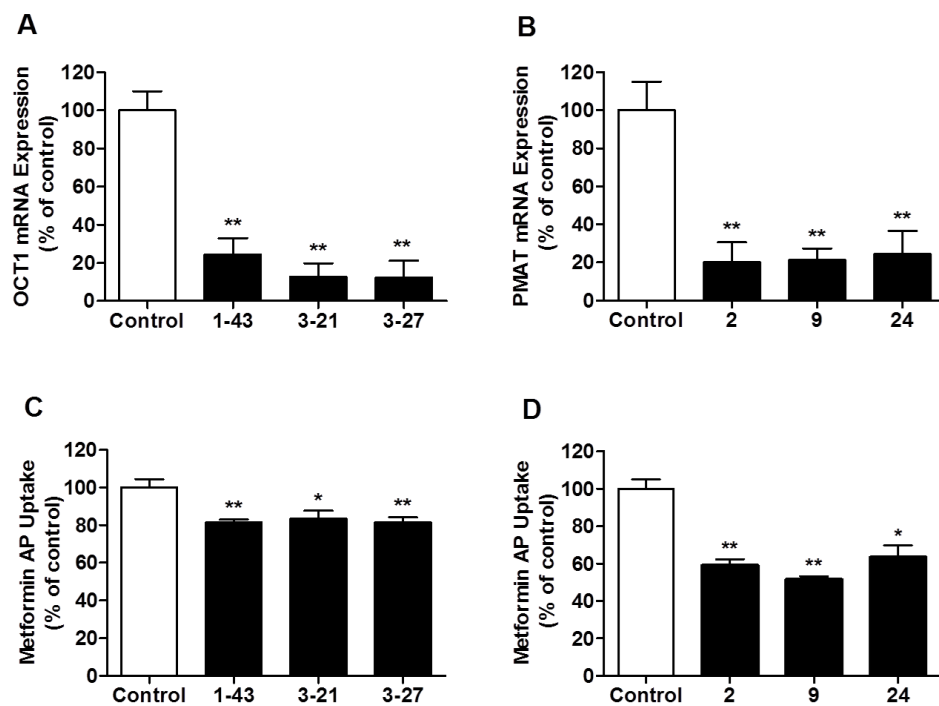


Figure 2.4. OCT1- and PMAT-knockdown Caco-2 cells. **A-B:** mRNA expression levels of OCT1 (A) and PMAT (B) in knockdown clones of Caco-2 cells relative to control (wild type Caco-2 cells), n=1. **C-D:** Metformin AP uptake (10 μ M, 5 min) in OCT1 (C) and PMAT (D) knockdown clones relative to control (wild type Caco-2 cells). Data represent mean \pm S.D., n=3. *p<0.05, **p<0.01 compared to control.

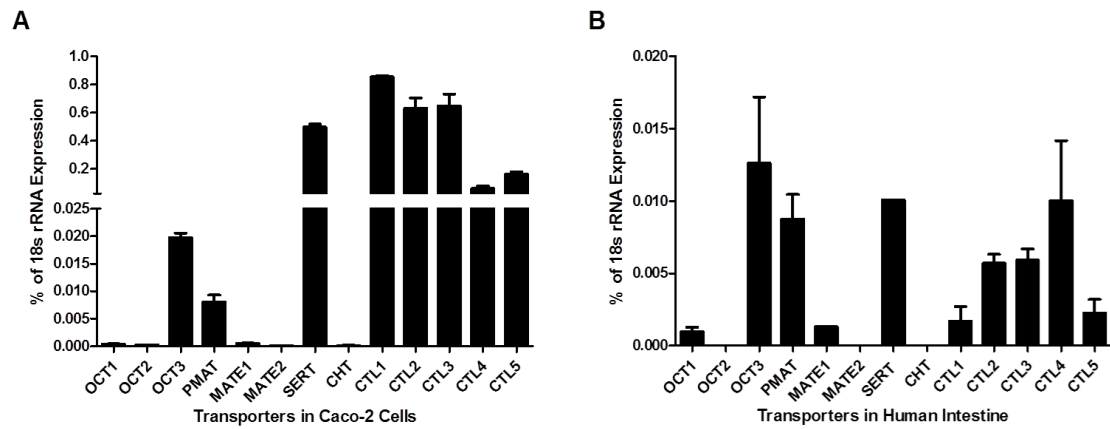


Figure 2.5. mRNA expression levels of cation-selective transporters in Caco-2 cell monolayers and human intestinal tissue. (A) Caco-2 cell monolayers, (B) human intestinal tissue; data represent mean \pm S.D., n=3.

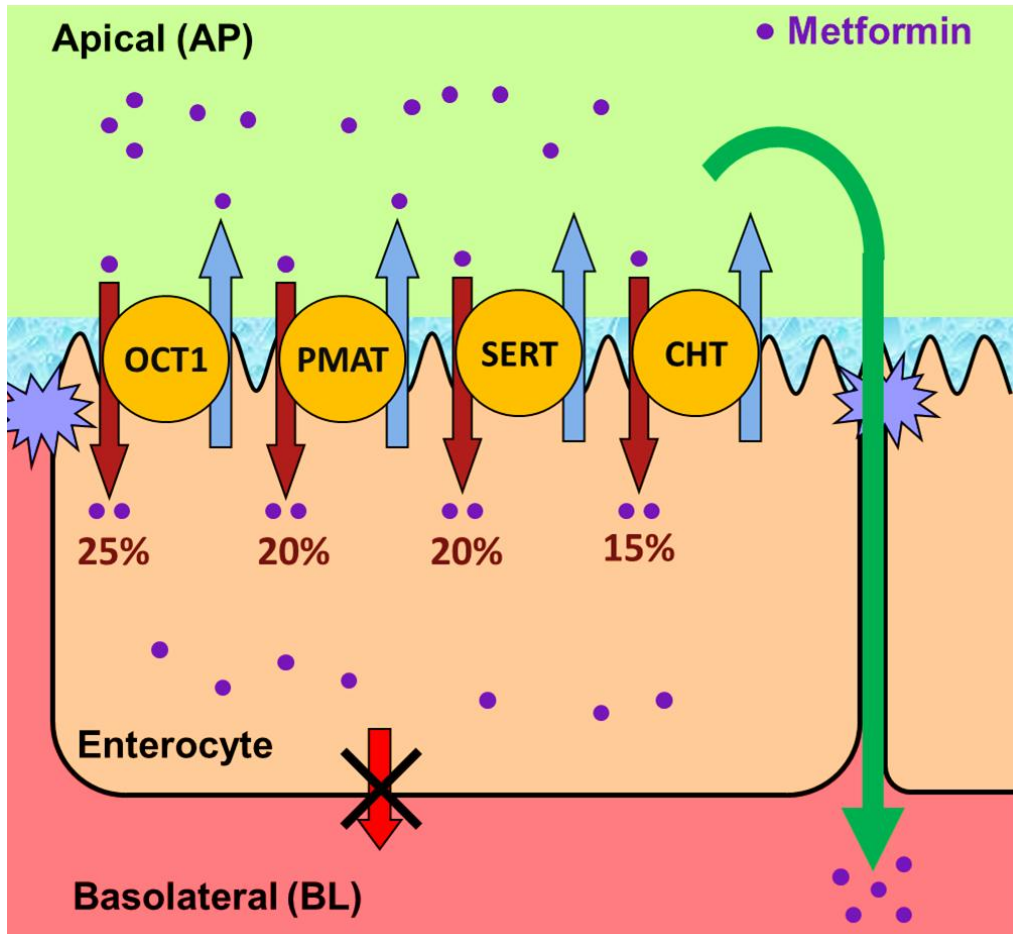


Figure 2.6. Contributions of cation selective transporters to metformin AP uptake in Caco-2 cell monolayers. OCT1, PMAT, SERT and CHT are responsible for 25%, 20%, 20% and 15% of the AP uptake of metformin in Caco-2 cell monolayers, respectively. Passive processes and non-specific binding likely contribute to the remaining ~20% of the AP uptake of metformin.

Sigma siRNA sequences

hOCT1 Seq#1

5' GATCCCGCTATGAAGTGGACTGGAATTGATATCCGTTCCAGTCCACTTCATAGCTTTTTTCCAAA 3'
5' AGCTTTTGGAAAAAAGCTATGAAGTGGACTGGAACGGATATCAATTCCAGTCCACTTCATAGCGG 3'

GATCCCGCTATGAAGTGGACTGGAATTGATATCCGTTCCAGTCCACTTCATAGCTTTTTTCCAAA
GGCGATACTTCACCTGACCTTAACTATAGGCAAGGTCAGGTGAAGTATCGAAAAAAGGTTTTCGA

hOCT1 Seq#3

5' GATCCCGCCATCTGTGTGGGCATCGTTTGATATCCGACGATGCCACACAGATGGTTTTTTCCAAA 3'
5' AGCTTTTGGAAAAAACCATCTGTGTGGGCATCGTCGGATATCAAACGATGCCACACAGATGGCGG 3'

GATCCCGCCATCTGTGTGGGCATCGTTTGATATCCGACGATGCCACACAGATGGTTTTTTCCAAA
GGCGGTAGACACACCCGTAGCAAACCTATAGGCTGCTACGGGTGTGTCTACCAAAAAAGGTTTTCGA

hPMAT Sequence

5' GATCCCGCAGCTTCATCACGGACGTGTTGATATCCGCACGTCCGTGATGAAGCTGTTTTTTCCAAA 3'
5' AGCTTTTGGAAAAAACAGCTTCATCACGGACGTGCGGATATCAACACGTCCGTGATGAAGCTGCGG 3'

GATCCCGCAGCTTCATCACGGACGTGTTGATATCCGCACGTCCGTGATGAAGCTGTTTTTTCCAAA
GGCGTCGAAGTAGTGCCTGCACAACTATAGGCGTGCAGGCACTACTTCGACAAAAAAGGTTTTCGA

Figure 2.S.1. OCT1- and PMAT-specific shRNA sequences.

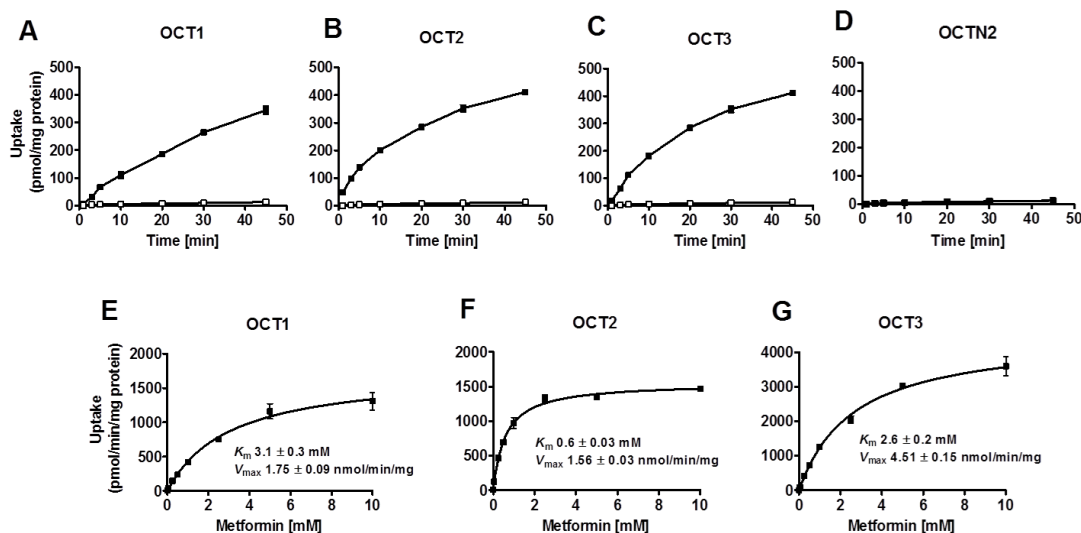


Figure 2.S.2. Metformin is a Substrate for OCT1-3 and not OCTN2. (A-D) Time profiles of metformin [10 μ M] uptake in OCT1, OCT2, OCT3 and OCTN2 expressing CHO cells (solid symbols) and their mock-transfected CHO control (open symbols). (E-G) Concentration profiles of metformin uptake in OCT1- [5 min], OCT2- [30 sec], and OCT3- [5 min] expressing CHO cells, respectively. Uptake data is reported as active transport for each transporter, where the uptake rate from the mock-transfected CHO cells was subtracted from each data point. A Michaelis-Menten equation with one saturable component was fit to the corrected uptake rate data obtained in CHO cell experiments and the estimated K_m values are presented. Metformin is a substrate of OCT1, OCT2, and OCT3, with apparent K_m values of 3.1 ± 0.3 , 0.6 ± 0.03 , and 2.6 ± 0.2 mM, respectively. But metformin is not a substrate of OCTN2. Data represent mean \pm S.D., n=3 (Proctor, 2010).

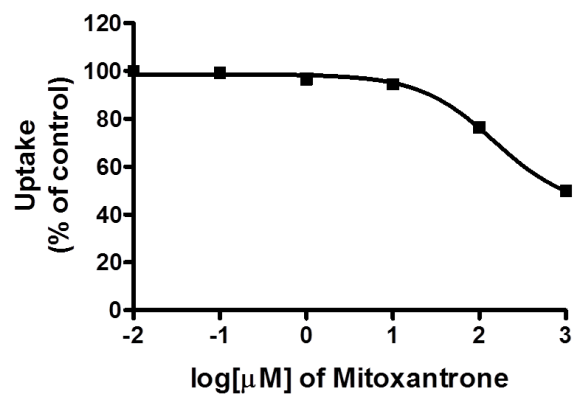


Figure 2.S.3. Serotonin uptake by SERT in the presence of mitoxantrone. Data represent mean \pm S.D., n=3. IC_{50} value is $977.2 \pm 1.2 \mu\text{M}$.

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CHAPTER 3

ORGANIC CATION TRANSPORTER 1 IS LOCALIZED IN THE APICAL MEMBRANE OF CACO-2 CELL MONOLAYERS AND ENTEROCYTES

3.A. OVERVIEW

Organic cation transporters (OCTs) are members of the solute carrier 22 (SLC22) family of transporter proteins that involved in absorption, distribution, and excretion of organic cations. While OCT3 is localized in the apical (AP) membrane of enterocytes, the literature is ambiguous about OCT1 (mOct1) localization, with some evidence suggesting basolateral (BL) localization in human and mouse enterocytes. This is contrary to our preliminary findings showing AP localization of OCT1 in Caco-2 cell monolayers, an established model of human intestinal epithelium. Therefore, this study aims at determining the localization of OCT1 (mOct1) in Caco-2 cells, and human and mouse enterocytes. Functional studies using OCT1-specific substrate pentamidine showed transporter-mediated AP but not BL uptake in Caco-2 cells, human and mouse intestinal tissues. OCT1 inhibition decreased AP uptake of pentamidine by ~50% in all three systems, with no effect on BL uptake. shRNA-mediated OCT1 knockdown in Caco-2 cells decreased AP uptake of pentamidine by ~50%, but did not alter BL uptake. Immunostaining and confocal microscopy in all three systems confirmed AP localization of OCT1 (mOct1). Our studies unequivocally show AP membrane localization of OCT1 (mOct1) in Caco-2 cells, human and mouse intestine. These results are highly significant

as they will require re-interpretation of previous drug disposition and drug-drug interaction studies where conclusions were drawn based on BL localization of OCT1 in enterocytes. Most importantly, these results will require revision of the regulatory guidance for industry in the USA and elsewhere since it has stated that OCT1 is basolaterally localized in enterocytes.

3.B. INTRODUCTION

The intestine plays a critical role in the absorption of nutrients and drugs. The intestinal epithelial cells (enterocytes) are polarized by the presence of tight junctions, and form a monolayer with the apical (AP) membrane facing the luminal side and the basolateral (BL) membrane facing the serosal side. Intestinal epithelium is the major barrier in the intestine, which controls the absorption of nutrients and drugs. Lipophilic compounds can cross this barrier by passive diffusion across AP and BL membranes of epithelial cells. However, hydrophilic and charged compounds can cross the epithelial barrier either via a paracellular route, which is highly inefficient, or transcellularly with the assistance of one or more transporters that are present in epithelial cell membranes.

Epithelial cells in the intestine, liver and kidney have specific transporters localized in either AP or BL membrane to facilitate directional transport of compounds and ensure their absorption by the intestine and excretion by the kidney and/or liver. Organic cation transporters (OCTs) belong to the solute carrier family 22 (SLC22) of polyspecific transporter proteins that are expressed in the liver, kidney, intestine, and other organs, transporting organic cationic compounds such as nutrients, endogenous amines, and many cationic drugs. OCTs are expressed as three different isoforms, namely OCT1, 2, and 3, and exhibit organ-specific distribution. Like other transporters, OCTs are expressed in specific cell membranes in epithelial tissues. For example, it is well established that OCTs, such as OCT1 and 3 are localized in the sinusoidal (BL) membrane of human hepatocytes, and are responsible for the uptake of cations from blood into the hepatocytes (Chen et al., 2010; Faber et al., 2003; Nies et al., 2009); similarly in the kidney, OCT2 is localized in the BL membrane of human renal proximal

tubules and is responsible for the uptake of cations from the blood (Karbach et al., 2000; Motohashi et al., 2002; Okuda et al., 1996). In the intestine, OCT3 is expressed in the AP membrane of enterocytes, and mediates the uptake of cations from the lumen into the enterocytes (Koepsell et al., 2007; Muller et al., 2005). However, OCT1, which belongs to the same family of transporters as OCT3, has been reported to be localized in the BL membrane (Giacomini et al., 2010; Jonker et al., 2001; Koepsell et al., 2007; Muller et al., 2005; Wang et al., 2002; Watanabe et al., 2002a; Watanabe et al., 2002b). This is surprising considering that other members of the OCT family, when expressed in the intestinal epithelium, are expressed in the AP membrane. It can be argued that OCT1 is expressed in the BL membrane to take up circulating organic cations into enterocytes; however, the affinities of OCTs for most substrates are low (e.g. apparent K_m values $>500\mu\text{M}$) (Koepsell et al., 2007) and hence very high expression of the transporter would be needed to play a role in the serosal uptake and intestinal excretion of circulating organic cations.

Previous functional studies in our laboratory suggested that OCT1 is not localized in the BL membrane of Caco-2 cell monolayers. The organic cation drug ranitidine, which is a substrate for OCTs, showed saturable AP uptake into Caco-2 cell monolayers with egress across the BL membrane via passive diffusion processes (Bourdet et al., 2006; Bourdet et al., 2005). BL uptake of ranitidine was mediated by a transporter with a very high apparent K_m value of 67 mM (Lee et al., 2002); this kinetic behavior is not consistent with the presence of OCT1 in the BL membrane of Caco-2 cell monolayers. Another organic cation drug metformin, also a substrate for OCTs (Kimura et al., 2005), showed saturable AP uptake and non-saturable inefficient BL uptake into Caco-2 cell

monolayers (Proctor et al., 2008). These data suggest, but do not provide definitive evidence that OCT1 is apically localized in Caco-2 cell monolayers; however, the results seem to rule out BL localization of the transporter in this cell model. Preliminary immunolocalization studies in Caco-2 cell monolayers suggested that OCT1 was localized in the AP membrane (Figure 3.S.1.) (Ng, 2002). The present study was conducted to investigate the cellular localization of OCT1 in Caco-2 cell monolayers as well as in the mouse and human intestinal epithelia so as to resolve the contradiction between ambiguous reports in the literature suggesting BL localization of this transporter and the preliminary findings in our laboratory suggesting AP localization of OCT1 in Caco-2 cell monolayers.

3.C. MATERIAL AND METHODS

Materials

Minimum essential medium (MEM) with Eagle's salts and L-glutamate, HEPES (1M), penicillin-streptomycin-amphotericin B solution (100x), non-essential amino acids (100x), geneticin, SuperScript[®] III First-Strand Synthesis SuperMix, TaqMan[®] Gene Expression Master Mix, and TaqMan[®] Gene Expression Assays for OCT1 were obtained from Life Technologies Corporation (Grand Island, NY, USA). Restore[®] Western Blot Stripping Buffer and SuperSignal[®] West Dura Extended Duration Substrate Kit were purchased from Thermo Scientific (Rockford, IL, USA). Rabbit anti human OCT1 antibody was purchased from Sigma-Aldrich (Cat# AV41516, St. Louis, MO, USA). Alexa Fluor[®] 568-goat anti rabbit IgG (A11036), Alexa Fluor[®] 488-donkey anti goat IgG (A11055), Normal Goat Serum, Nitrocellulose Membrane Filter Paper Sandwich, Nupage[®] Western Blot buffers, Bis-Tris gels and other Western blotting reagents were purchased from Invitrogen (Carlsbad, CA, USA). RIPA lysis buffer system, rabbit anti-human P-gp antibody (sc-8313), rabbit anti-human Na⁺/K⁺ ATPase antibody (sc-28800), goat anti-human villin antibody (sc-7672), goat anti-rabbit IgG-CFL488 (sc-362262), and goat anti-rabbit IgG-HRP (sc-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human and mouse jejunum paraffin sections were purchased from Zyagen (San Diego, CA, USA). VECTASHIELD[®] mounting medium with DAPI (4',6-diamidino-2-phenylindole) was purchased from Vector Laboratories (Burlingame, CA, USA). [3H]pentamidine (4,4'-[pentane-1,5-diylbis(oxy)]dibenzenecarboximidamide) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA), [14C]tetraethylammonium ([14C]TEA) was

purchased from American Radiolabeled Chemical Inc. (St. Louis, MO, USA). Mitoxantrone (1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethylamino]-anthracene-9,10-dione), desipramine (3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N-methylpropan-1-amine) and quinidine ((S)-(6-methoxyquinolin-4-yl)((2R,4S,8R)-8-vinylquinuclidin-2-yl)methanol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Caco-2 (HTB-37) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA).

Methods

Caco-2 Cell Culture

Caco-2 cells were cultured as described previously (Proctor et al., 2008). Briefly, the cells (passage numbers 30 to 40) were seeded at a density of 60,000 cells/cm² on polycarbonate membranes of Transwells™. The culture medium was changed the day following seeding and every other day thereafter. Cell monolayers with transepithelial electrical resistance greater than 300 Ω·cm² were used for experimentation at 21-28 days post seeding.

Generation of OCT1-Knockdown Caco-2 Clones

Three OCT1-specific small interfering RNA (siRNA) sequences (Sigma) were evaluated for their ability to silence OCT1 gene expression. Sequence 1 (sense strand: 5'-GCUAUGAAGUGGACUGGAA-3'; antisense strand: 5'-UUCCAGUCCACUUCAUAGC-3') and Sequence 3 (sense strand: 5'-CCAUCUGUGUGGGCAUCGU-3'; antisense strand: 5'-ACGAUGCCCACACAGAUGG-3') had the highest gene silencing activity (data not shown). The antisense and sense oligonucleotides were linked with a hairpin loop, and

annealed with the respective complementary DNA oligonucleotides. Each resulting double-stranded DNA was inserted into BamHI and HindIII sites of the pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ, USA) to generate a short hairpin RNA (shRNA) plasmid. Caco-2 cells at 90% confluency were transfected with the shRNA plasmid, using the Nucleofector System (Amaxa, Gaithersburg, MD, USA) according to the manufacturer's protocol. Transfectants were selected by treatment with 0.2 mg/ml hygromycin B for 3 weeks, and screened by quantitative real-time polymerase chain reaction (qRT-PCR) for OCT1 expression. Three clones (sequence 1 clone 43, sequence 3 clones 21 and 27, i.e., 1-43, 3-21, 3-27) with the lowest gene expression, normal morphology and growth rate were chosen for functional studies.

qRT-PCR for OCT1 Gene Expression in Caco-2 Cells

mRNA expression of human OCT1 relative to 18s rRNA (18s) was determined by qRT-PCR analysis. qRT-PCR experiments were conducted using established methods (Holmes et al., 2006) with minor deviations. Total RNA was isolated from Caco-2 cell monolayers using RNeasy Plus Mini Kit (Qiagen, Valencia CA, USA). cDNA was synthesized from total RNA (5 µg) using Superscript III reverse transcriptase. An equal amount of RNA was included in a No-Reverse Transcriptase control for each separate RNA sample. qRT-PCR was performed using 1:20 dilutions of the cDNA. PCR reactions (45 cycles) were conducted using TaqMan[®] Gene Expression Assays in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). The expression of the 18s housekeeping gene was determined in each RT-PCR experiment and served as the normalization control (n=1).

Functional Uptake Studies

In Caco-2 cell monolayers, initial AP and BL uptake of [^{14}C]TEA and [^3H]pentamidine was quantified using methods outlined previously (Proctor et al., 2008) with minor deviations. Uptake of [^{14}C]TEA [5 μM , 0.05 $\mu\text{Ci/mL}$, 10 min] across the AP and BL membranes was measured in the absence or presence of 500 μM unlabeled TEA or 500 μM quinidine. [^3H]pentamidine [0.1 μM , 0.05 $\mu\text{Ci/mL}$, 5 min] uptake was performed in control and OCT1-knockdown cells. Mitoxantrone [25 μM] was used as the OCT1-specific inhibitor (Han et al., 2012; Koepsell et al., 2007), and 500 μM quinidine was used as OCTs pan inhibitor.

Human intestinal tissues collected from gastric bypass surgical patients were procured from the University of North Carolina Hospitals (Chapel Hill, NC, USA), and all investigational experiments were performed in accordance with approval from the IRB at the Office of Human Research Ethics at the University of North Carolina at Chapel Hill and in compliance with federal regulations. The tissues were stripped of the exterior seromusculature and serosa layers by sharp resection and the intestinal epithelium was mounted between two halves of a diffusion chamber insert. The entire procedure was completed within 1 hour from collection so as to preserve viability of the tissues. The inserts were placed between two side-by-side diffusion chambers. KBR (3 ml) at 37 $^{\circ}\text{C}$ was added to the AP and BL chambers and bubbled with oxygen/carbon dioxide (95:5) gas to maintain tissue viability (Johnson et al., 2002). For AP uptake studies, three intestinal epithelial samples were preincubated for 30 min at 37 $^{\circ}\text{C}$ with 3 ml of KBR buffer in the AP and BL compartments. Following equilibration, the transepithelial electrical resistance (TEER) was measured to test the epithelial integrity

of the intestinal tissue. KBR buffer from the BL chamber was removed and replaced with fresh buffer; while buffer in the AP chamber was replaced with [^3H]pentamidine [0.1 μM , 0.05 $\mu\text{Ci/ml}$] in KBR, or [^3H]pentamidine [0.1 μM , 0.05 $\mu\text{Ci/ml}$] with 25 μM mitoxantrone or 500 μM quinidine for inhibition studies. The intestinal epithelial segments were incubated for 5 min at 37 $^{\circ}\text{C}$, the buffer from both chambers was aspirated, and the tissues were washed 10X in ice-cold KBR buffer, digested for 10 min with 0.2 ml of 2N NaOH at 50 $^{\circ}\text{C}$, and neutralized with 0.2 ml of 2N HCl and 0.2 ml of KBR. The fully digested tissue solution was analyzed by liquid scintillation spectrometry, and the rate of initial uptake of [^3H]pentamidine was determined.

For mouse studies, male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were housed according to approved Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC) requirements and protocols and in compliance with The Animal Welfare Act public laws. Mice were fasted overnight and then anesthetized with an intraperitoneal (IP) injection of urethane (1.2-1.5 g/kg). A 10 cm segment of jejunum was dissected and flushed with 10 ml ice-cold KBR with 50% complete EDTA-free protease inhibitors. The intestinal segment was separated into 2 cm segments and mounted in a diffusion chamber and [^3H]pentamidine uptake in mouse intestinal tissue was determined as described above, with the exception that a mOct1 specific inhibitor, desipramine [10 μM] was used instead of mitoxantrone (Costales et al., 2011). Quinidine [500 μM] was used as a pan inhibitor.

Western Blot Analysis

The specificity of the primary rabbit anti-human OCT1 antibody was confirmed by Western blot analysis. Chinese Hamster Ovary (CHO) cells were cultured as described previously (Ming et al., 2009). CHO cells singly transfected with human OCT1-3 or mouse mOct1-3, parental CHO K1 cells, Caco-2 cells (including wild-type and OCT1 knockdown Caco-2 cells), human liver, mouse liver, and mouse kidney specimens were lysed and homogenized in RIPA buffer system according to the manufacturer's protocol (Santa Cruz Biotechnology). The homogenized lysates were centrifuged at 14,000g for 15 min at 4°C. The pellets were discarded and protein content in the supernatants was measured with BCA protein assay kit (Pierce, Rockford, IL) and bovine serum albumin as a standard. Proteins were denatured with Nupage[®] loading buffer and reducing agent, and heated at 70°C for 10 min. After cooled to RT, protein samples (20 µg each lane) were loaded to 4-12% (gradient) Bis-Tris gel, and separated by electrophoresis (200 V, 160 mA for 1 hour) and transferred to nitrocellulose membranes (30 V, 160 mA for 1 hour). The membranes were probed with rabbit anti-human OCT1 antibody followed by goat anti-rabbit IgG-HRP secondary antibody. Positive protein bands were detected with SuperSignal[®] West Dura Extended Duration Substrate Kit and imaged by Versa Doc[™] Imaging System Model 1000 (Bio-Rad, Hercules, CA, USA). After imaging, the membrane was stripped, washed and analyzed for GAPDH.

Laser Scanning Confocal Microscopy

Caco-2 Cell Monolayers - Caco-2 cells grown on Transwells[™] for 21-28 days were washed 3X in phosphate buffered saline (PBS), frozen in Tissue-Tek Cryo-OCT Compound, cut longitudinally into 5 µM thick sections, and fixed on glass slides with the

AP side of the Caco-2 cells facing up so as to readily identify the polarity of the cells. The sections were rinsed with PBS, and permeabilized for 10 min in 0.5% Triton X-100 in PBS. The sections were blocked for 30 min with PBS containing 5% normal goat serum (NGS) followed by incubation with rabbit anti-human OCT1 primary antibodies (1:75 dilution) or rabbit anti-human P-gp primary antibodies (1:100 dilution) for 1 h at RT. The slides were rinsed 3X in PBS and then incubated with a secondary antibody (Alexa Fluor 568-conjugated goat anti-rabbit IgG for OCT1 or Alexa Fluor 488-conjugated goat anti-rabbit IgG for P-gp) for 1 h at RT in the dark. After washing 3X in PBS, mounting medium with DAPI was applied for nuclear staining, and confocal images were obtained with a Leica SP2 AOBS laser scanning confocal microscope. To visualize the localization of a BL biomarker against which AP localization of OCT1 can be compared, rabbit anti- Na^+/K^+ ATPase primary antibody (1:100 dilution) was used to detect Na^+/K^+ ATPase which is known to be confined to the BL membrane of Caco-2 cells and enterocytes. Alexa Fluor 568-conjugated goat anti-rabbit IgG was used as a secondary antibody.

Human and Mouse Intestinal Tissue: Slides containing paraffin fixed human or mouse jejunum tissue sections were deparaffinized in xylene 3X for 5 min, rehydrated in serial ethanol dilutions (2X for 3 min in 100% ethanol, 1 min in 95%, 1 min in 80%, then rinsed in distilled water), and transferred to PBS. Slides were incubated in citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0) at 95-100 °C for 30 min as antigen retrieval, and cooled down to RT. The tissues were permeabilized with 0.5 % Triton X-100 in PBS for 10 min, transferred back to PBS, blocked for 30 min with PBS containing 5 % normal goat serum (NGS) followed by incubation with rabbit anti-human OCT1 primary

antibodies (1:75 dilution), rabbit anti-human P-gp primary antibodies (1:100 dilution), or rabbit anti-Na⁺/K⁺ ATPase primary antibody (1:100 dilution) for 1h at RT. The slides were rinsed 3X in PBS and then incubated with the secondary antibody (Alexa Fluor 568-conjugated goat anti-rabbit IgG for OCT1 and Na⁺/K⁺ ATPase; or Alexa Fluor 488-conjugated goat anti-rabbit IgG for P-gp) for 1 h at RT in the dark. After three washes in PBS, mounting medium containing DAPI was applied and confocal images were obtained with a Leica SP2 AOBS laser scanning confocal microscope.

To demonstrate co-localization of OCT1(mOct1) with villin in Caco-2 cells and intestinal tissues, rabbit anti-human OCT1 and goat anti-human villin primary antibodies were applied together for 1h at RT, washed, and Alexa Fluor 568-goat anti-rabbit IgG and Alexa Fluor-488 donkey anti-goat IgG secondary antibodies were applied together to the cells or tissue slides. After washing, mounting medium with DAPI was applied and confocal images were obtained with a Leica SP2 AOBS laser scanning confocal microscope.

3.D. RESULTS

Functional Evidence for the Absence of Cation-selective BL Transporters in Caco-2 Cell Monolayers and Enterocytes

TEA, a substrate for cation-selective transporters, was not taken up across the BL membrane of Caco-2 cell monolayers via a transporter-mediated process. This was evidenced by the result that the BL uptake of [^{14}C]TEA was not inhibited by unlabeled TEA at a 100-fold greater concentration or by the potent inhibitor of organic cation transporters, quinidine (Figure 3.1 A) (Ng, 2002). In contrast, unlabeled TEA and quinidine inhibited the uptake of [^{14}C]TEA across the AP membrane of Caco-2 cell monolayers by approximately 50% and 90%, respectively (Figure 3.1 A) (Ng, 2002). These results suggest that the BL membrane of Caco-2 cell monolayers does not express cation-selective transporters that can facilitate the transport of TEA, whereas one or more cation-selective transporters in the AP membrane mediate TEA uptake. Similarly, our results demonstrate that uptake of pentamidine, a selective substrate for OCT1 over OCT2 or 3 (Ming et al., 2009), was not mediated by OCT1 at the BL membrane of Caco-2 cell monolayers, because the uptake was not inhibited by the selective OCT1 inhibitor mitoxantrone (Han et al., 2012; Koepsell et al., 2007) or the cationic transporter pan inhibitor, quinidine. In contrast, ~50% of pentamidine uptake across the AP membrane was inhibited by mitoxantrone or quinidine (Figure 3.1 B). Collectively, these results provided strong evidence that the BL membrane of Caco-2 cell monolayers is devoid of OCT transporters, and that these transporters, including OCT1, are functional in the AP membrane. Similar results were obtained with human intestinal epithelium and mouse intestinal tissue, where uptake of pentamidine across the BL membrane was not inhibited

by the selective inhibitor of OCT1 (mitoxantrone) and mOct1 (desipramine 10 μ M; IC_{50} values for mOct1 = 1.2 ± 1.1 μ M , mOct2 = 11 ± 1.3 μ M, mOct3 = 84 ± 1.1 μ M (unpublished data)) (Costales et al., 2011), or the pan inhibitor quinidine, whereas these inhibitors inhibited pentamidine uptake across the AP membrane by ~50% (Figure 3.1 C-D). These data imply that functionally active OCT1 localizes in the AP membrane of Caco-2 cell monolayers, and human and mouse intestinal tissues, and is absent in the BL membrane.

AP and BL Uptake of Pentamidine in OCT1 Knockdown Caco-2 Clones

To confirm results from chemical inhibition studies which provided evidence for functional localization of OCT1 in Caco-2 cells monolayers, pentamidine uptake was evaluated in Caco-2 cell monolayers in which OCT1 expression was down-regulated by OCT1-specific shRNA. Three clones in which OCT1 expression was reduced by 70% or more were selected for experimentation (Figure 3.2 A). Uptake of pentamidine across the AP membrane was decreased by 50% in OCT1-knockdown clones compared to control Caco-2 cell monolayers (Figure 3.2 B). Similar to results from chemical inhibition studies, the BL uptake of pentamidine in the OCT1-knockdown clones was not significantly different from that of control cells (Figure 3.2 C). Data from chemical inhibition and OCT1-knockdown studies provided definitive evidence that OCT1 in Caco-2 cell monolayers is not localized in the BL membrane, but is present in the AP membrane.

Specificity of the Rabbit Anti-human OCT1 Antibody for OCT1 and mOct1

In order to use the rabbit anti-human OCT1 antibody to assess the cellular localization of OCT1 and mOct1, it was important to demonstrate by Western blot

analysis that the antibody detects OCT1 and does not cross-react with OCT2 or OCT3, but does cross-react with mOct1. Protein extracts from CHO cells that are singly transfected with OCT (mOct) transporters (OCT1-CHO, OCT2-CHO, OCT3-CHO, mOct1-CHO, mOct2-CHO, mOct3-CHO), parental CHO K1 cells, wild-type Caco-2 cells, OCT1 knockdown Caco-2 cells (Clone 3-27), human liver, mouse liver and mouse kidney were probed with the rabbit anti-human OCT1 antibody. The antibody specifically bound to a ~60 kDa protein, which is the expected size of OCT1 (mOct1), in the protein extracts of the OCT1-CHO cells, mOct1-CHO cell, wild-type Caco-2 cell, human and mouse liver homogenates (positive controls). In contrast, the antibody either did not yield a band or yielded a faint band with the protein extracts of the OCT2-3 CHO cells, mOct2-3 CHO cells, CHO K1 cells, OCT1 knockdown Caco-2 cells and mouse kidney (negative control) (Figure 3.3). These results confirm that the rabbit anti-human OCT1 antibody detects OCT1 and mOct1 without producing a false-positive signal with OCT2-3 (mOct 2-3).

Immunolocalization of OCT1 by Confocal Imaging in Caco-2 Cells, and Human and Mouse Intestinal Tissues

Immunostaining and confocal microscopy studies in Caco-2 cell monolayers revealed that OCT1 is localized in the AP membrane (Figure 3.4 A). The efflux transporter, P-gp, which is known to be exclusively localized in the AP membrane of Caco-2 cells, was found to be localized in the same membrane as OCT1 (Figure 3.4 B), thus confirming the AP expression of OCT1. Further, these studies confirmed that OCT1 is not expressed in the membrane where the BL membrane marker Na^+/K^+ ATPase was immunostained (Figure 3.4 C). Thus, the distinct difference in the polarized localization

of OCT1 and Na⁺/K⁺ ATPase, and the unmistakable overlap in the membrane localization of OCT1 and P-gp unequivocally confirm that OCT1 is localized in the AP membrane of Caco-2 cells. Similarly, OCT1 (mOct1) was found to be localized in the same membrane as P-gp in human (Figure 3.4 D-F) and mouse enterocytes (Figure 3.4 G-I), confirming AP localization of OCT1 (mOct1) in these tissues. Finally, because co-localization of OCT1 (mOct1) with P-gp in the same cell/tissue preparation did not produce good signal due to lack of appropriate secondary antibodies, co-localization of OCT (mOct1) with another apically localized protein, villin, was investigated by immunostaining and confocal microscopy (Figure 3.5). When images of antibody-labeled OCT1 (mOct1) (red fluorescence) and villin (green fluorescence) were merged, co-localization of these two proteins in Caco-2 cell as well as in human and mouse intestinal tissues was evidenced by a yellow color, thus unequivocally confirming the AP localization of OCT1 (mOct1).

3.E. DISCUSSION

The results presented here contradict several reports that concluded that OCT1 (mOct1) is localized in the BL membrane of Caco-2 cell monolayers and enterocytes. The first suggestion that OCT1 (mOct1) is localized basolaterally in the intestinal epithelium came from studies by Jonker et al. (2001) based on their observation that approximately twice the amount of [^{14}C]TEA was found in the intestinal content of gall bladder-cannulated wild type mice compared to mOct1 (-/-) mice at 60 min post intravenous injection. However, it should be noted that the amount of TEA found in the intestinal content represented only 0.7% and 1.3% of the total excretion in mOct1 (-/-) and wild type mice, respectively, and that distribution of TEA in the liver was reduced by 4-fold in mOct1 (-/-) mice compared to the wild type mice. Further, the intestinal excretion of another mOct1 substrate, MPP^+ , was not significantly different in mOct1 (-/-) and wild type mice. This was consistent with the findings of Bleasby et al. (2000) which showed that in Caco-2 cells, OCT1 does not play a role in the BL uptake of MPP^+ . Thus, the conclusion by Jonker et al. (2001) that mOct1 was basolaterally localized in the intestinal epithelium based on their results was tenuous. Later, Wang et al. (2002) showed that the amount of metformin associated with the duodenum, jejunum, and ileum was 3- to 7-fold higher in wild type mice than in mOct1 (-/-) mice. The authors interpreted these results to be suggestive of the presence of mOct1 in the BL membrane of the intestinal epithelium since this membrane faces the serosal blood capillaries. However, they appropriately indicated that this hypothesis should be tested with further experiments. Using functional studies of sulpiride uptake, Watanabe et al. suggested that OCT1 is localized at the BL membrane of Caco-2 cells (Watanabe et al., 2002a;

Watanabe et al., 2002b). Subsequently, in Caco-2 cell monolayers that are widely used to study intestinal drug absorption and that exhibit low but detectable levels of OCT1 mRNA (Englund et al., 2006; Muller et al., 2005; Seithel et al., 2006), BL localization of OCT1 was reported by Muller et al. (2005) based on immunostaining and confocal microscopy studies. They also reported BL localization of OCT1 in human intestinal tissue. However, the immunohistochemical images in this paper show weak signals and widespread cellular distribution of OCT1, including the cytoplasm and entire cell membrane. This is likely due to the use of a rat Oct1 antibody in this study that was not sufficiently selective for the human OCT1 transporter. Taken together, this body of work did not provide conclusive evidence for BL localization of OCT1 (mOct1) in the intestinal epithelium, and needs to be re-interpreted in light of the results reported here.

The results reported here are highly significant because all the experimental observations since 2001 involving the intestinal absorption or secretion of cationic compounds have been interpreted based on the above mentioned studies that reported BL localization of OCT1 (mOct1). Our results warrant that the literature reports since 2001 on intestinal absorption and disposition of cationic drugs be re-evaluated. Our findings could impact not only the results involving the intestinal absorption of a single drug, but also reports on drug-drug interactions of co-administered drugs that implicated intestinal OCT1 (mOct1). It is important to note that several reviews and the US Food and Drug Administration (FDA) guidance for industry (Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, 2012, FDA, US) state that OCT1 is localized in the BL membrane of the enterocytes. The FDA

guidance impacts numerous drug development decisions involving compounds that are likely or known substrates/inhibitors of OCT1. Clearly, a re-evaluation and revision of the FDA guidance is warranted.

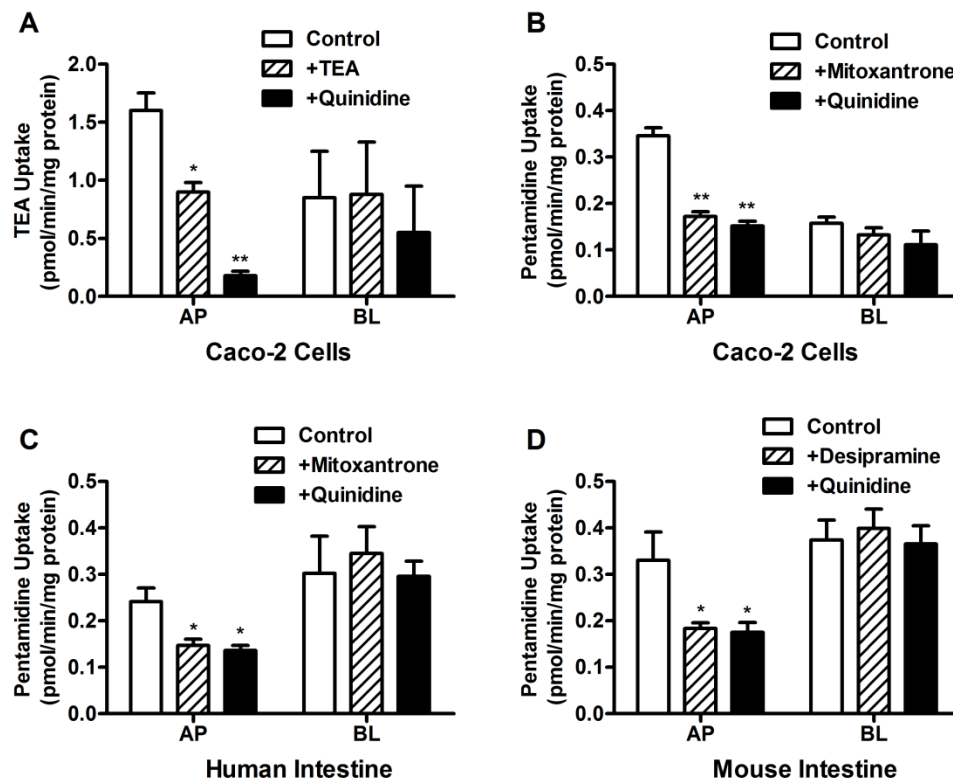


Figure 3.1. AP and not BL uptake of TEA and pentamidine is reduced by OCT1 inhibitors in Caco-2 cell monolayers, human intestinal epithelium, and mouse intestinal tissue. A: Initial uptake (10 min) of [14 C]TEA [5 μ M, 0.05 μ Ci/mL] across the AP and BL membranes of Caco-2 cells was measured in the absence (open bars) or presence of unlabeled TEA [500 μ M] (hatched bars) or quinidine [500 μ M] (solid bars) (Ng, 2002). Initial uptake (5 min) of [3 H]pentamidine [0.1 μ M, 0.05 μ Ci/mL] in Caco-2 cell monolayers (B), human intestinal epithelium (C), and mouse intestinal tissue (D) across AP and BL membrane in the absence (open bars) and presence of mitoxantrone [25 μ M] for OCT1 or desipramine [10 μ M] for mOct1 (hatched bars), or quinidine [500 μ M] (solid bars). Data represent the mean \pm SD (n = 3). *p < 0.05, **p < 0.01, compared to control.

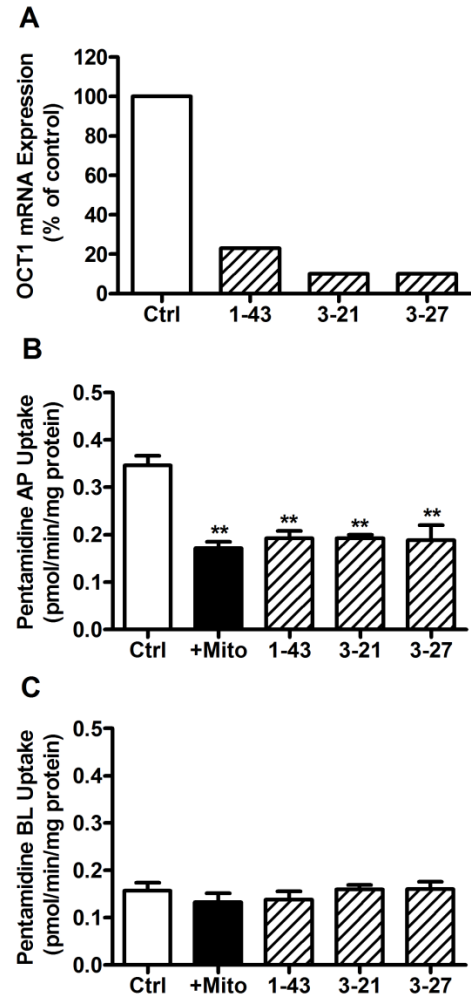


Figure 3.2. AP and not BL uptake of pentamidine is reduced in OCT1 knockdown Caco-2 clones compared to wild type Caco-2 cells. A: OCT1 mRNA expression in control Caco-2 cells (open bar) and OCT1 knockdown Caco-2 clones 1-43, 3-21, and 3-27 (hatched bars) (n=1). AP (B) and BL (C) uptake of [3 H]pentamidine [0.1 μ M, 0.05 μ Ci/mL] (5 min) in the absence (open bars) and presence (solid bars) of mitoxantrone [25 μ M] in control Caco-2 cells, and in OCT1 knockdown Caco-2 clones 1-43, 3-21, and 3-27 (hatched bars). In B and C, data represent the mean \pm SD (n = 3), **p< 0.01 compared to control.



Figure 3.3. Western blot analysis shows the specificity of rabbit anti-human OCT1 antibody for OCT1 and cross-reactivity with mOct1. Lanes 1-12: 1- OCT1-CHO, 2- OCT2-CHO, 3- OCT3-CHO, 4- mOct1-CHO, 5- mOct2-CHO, 6- mOct3-CHO, 7- parental CHO K1 cells, 8- wild-type Caco-2 cells, 9- OCT1-knockdown Caco-2 cells, 10- human liver homogenate, 11- mouse liver homogenate, 12- mouse kidney homogenate. Anti-human OCT1 antibody specifically bound to a protein with an apparent molecular mass of ~60 kDa as shown. GAPDH was used as loading control.

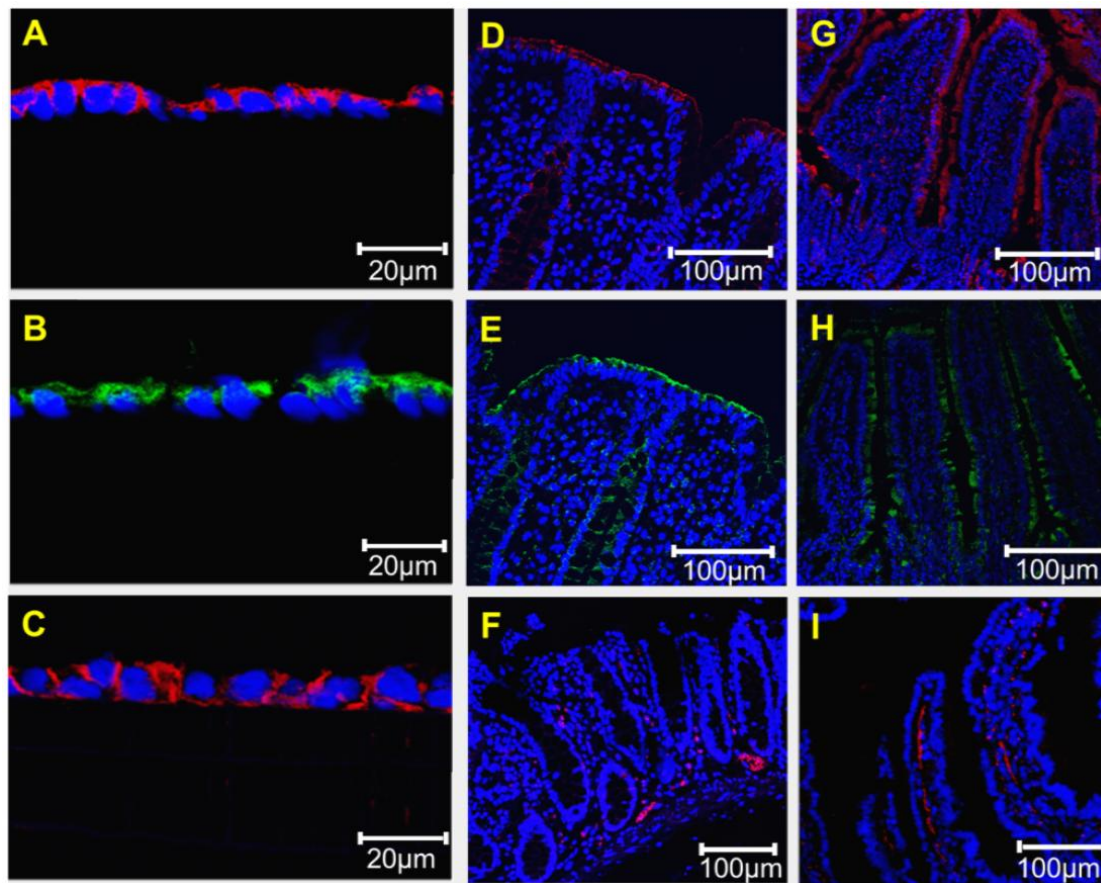


Figure 3.4. Immunocytochemical localization of OCT1 (mOct1) in Caco-2 cell monolayers, and in human and mouse intestinal tissues. OCT1 (A), P-gp (B) and Na^+/K^+ ATPase (C) localization in Caco-2 cell monolayers. The orientation of the monolayers shows the AP side facing up and the basal side facing down. OCT1 (D), P-gp (E) and Na^+/K^+ ATPase (F) localization in human intestinal tissues; mOct1 (G), P-gp (H) and Na^+/K^+ ATPase (I) localization in mouse intestinal tissues. Blue fluorescence represents nuclear staining with DAPI.

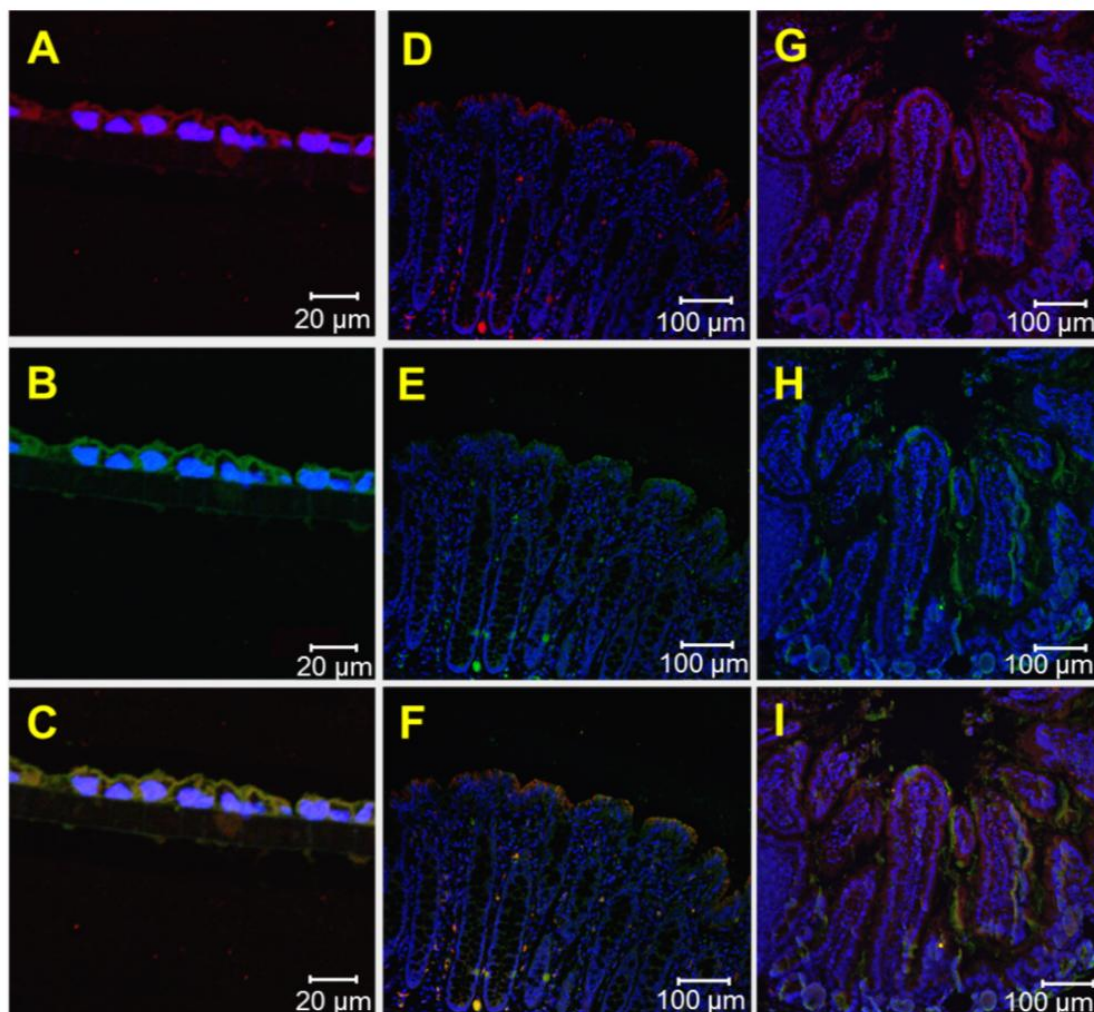


Figure 3.5. Immunocytochemical co-localization of OCT1 (mOct1) with villin in Caco-2 cell monolayers, and in human and mouse intestinal tissues. OCT1 (A), villin (B) and co-localization of OCT1 and villin (C) in Caco-2 cell monolayers. The orientation of the monolayers shows the AP side facing up and the basal side facing down. OCT1 (D), villin (E) and co-localization of OCT1 and villin (F) in human intestinal tissues; mOct1 (G), villin (H) and co-localization of mOct1 and villin (I) in mouse intestinal tissues. Blue fluorescence represents nuclear staining with DAPI.

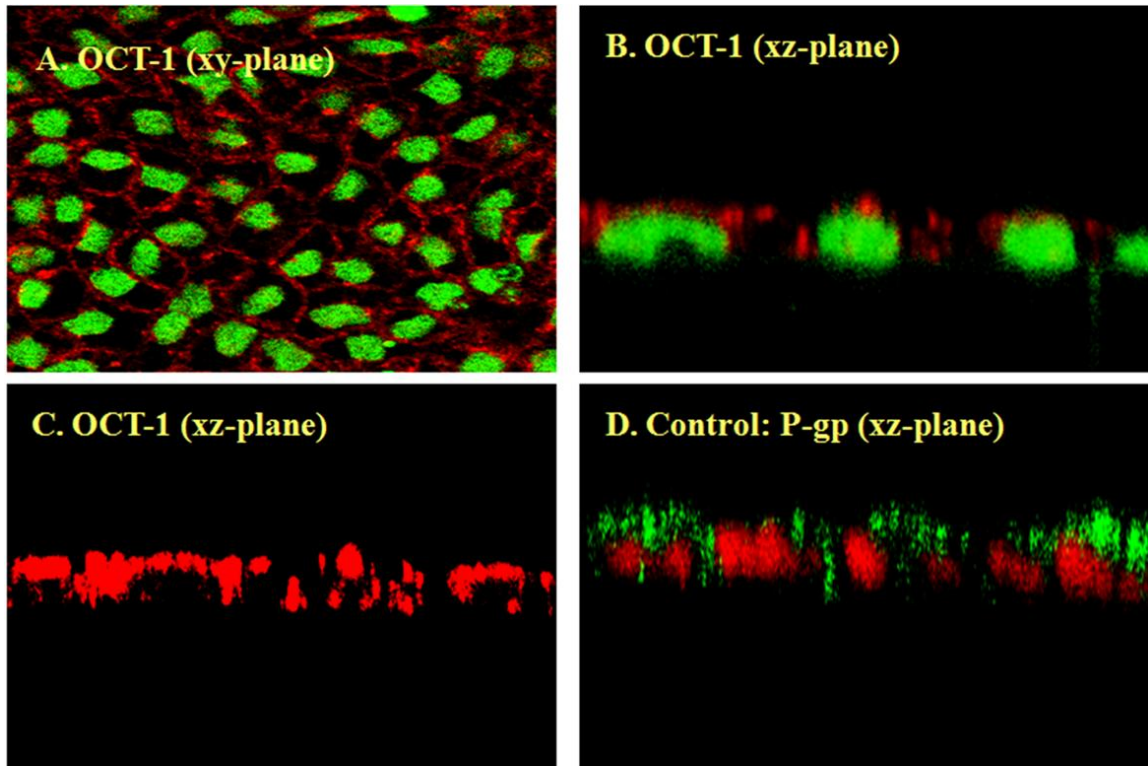


Figure 3.S.1. Immunocytochemical localization of OCT1 in Caco-2 cell monolayers.

Immunostaining shows nuclei in green and OCT1 in red (A-C). Confocal images in the XY-plane on the AP side (A), in the XZ-plane with nuclei (B) and without nuclei (C) show that OCT1 is localized in the apical membrane. As a reference, XZ-image of P-gp (P-gp stained in green, and nuclei in red) is shown in D; as expected, P-gp is localized in the apical membrane of Caco-2 cells. Comparison of frame D with frames B and C shows that OCT1 and P-gp are localized in the same (i.e. AP) membrane (Ng, 2002).

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CHAPTER 4

ROLE OF SEROTONIN RE-UP TAKE TRANSPORTER IN THE ORAL ABSORPTION OF METFORMIN IN MICE

4.A. OVERVIEW

Studies in Chapter 2 have shown that cation-selective transporters, including the serotonin re-uptake transporter (SERT), mediate the apical (AP) uptake of metformin in Caco-2 cell monolayers, and that metformin is a substrate of SERT. In studies using a mouse model, it was demonstrated that mouse serotonin re-uptake transporter (mSert) contributes to the intestinal absorption of metformin. [^{14}C]metformin was administered orally (with or without the mSert-specific inhibitor, paroxetine [$1\text{ }\mu\text{M}$]) to C57BL/6 mice, and portal and systemic plasma concentrations of [^{14}C]metformin were quantified. Paroxetine decreased portal and systemic $\text{AUC}_{0-4\text{h}}$ of metformin by approximately 10% ($p<0.05$), and reduced portal and systemic $\text{AUC}_{0-2\text{h}}$ (in the early absorption phase) by 15% ($p<0.05$). To rule out any confounding effects of the anesthetic agent, urethane, similar studies were conducted in conscious mice. Paroxetine decreased metformin systemic $\text{AUC}_{0-6\text{h}}$ by 9% ($p<0.05$) in conscious wild-type mice and by 6% ($p<0.05$) in mSert $^{-/-}$ mice compared to wild-type mice. These data provide the first direct evidence for the involvement of mSert in the intestinal absorption of metformin in mice.

4.B. INTRODUCTION

Metformin is the front line therapy for Type II diabetes mellitus, and has been widely used in the United States and worldwide for decades. Even though this drug is widely used, the mechanism underlying its intestinal absorption is not clear. The oral bioavailability of metformin is unexpectedly high, about 50-60% (Tucker et al., 1981), given its hydrophilicity and positive charge at all physiologic pH values (Saitoh et al., 2004). Previous studies in Caco-2 cell monolayers, a cellular model of the human intestinal epithelium, showed that although metformin traverses predominantly (90%) via the paracellular route, cation-selective transporters contribute to its cellular AP uptake (Proctor et al., 2008). Based on these findings, Proctor et al. proposed a “sponge hypothesis” which states that the functional synergy between AP transporters and paracellular transport enhances the intestinal absorption of metformin, and subsequently, its oral bioavailability (Proctor et al., 2008).

In Chapter 2, four transporters, namely, organic cation transporter 1 (OCT1), plasma membrane monoamine transporter (PMAT), SERT and choline high-affinity transporter (CHT) were identified as mediators of the AP uptake of metformin in Caco-2 cell monolayers. Since this is the first report implicating SERT as a transporter of metformin *in vitro*, this chapter addresses the role of mSert, the mouse ortholog of SERT, in the oral absorption of metformin using a mouse model. SERT is the re-uptake transporter of the neurotransmitter serotonin, an important monoamine that controls various functions in the central nervous system (CNS), cardiovascular system and gastrointestinal system (Berger et al., 2009; Bertrand and Bertrand, 2010; Mohammad-Zadeh et al., 2008; Ni and Watts, 2006). Re-uptake of serotonin by SERT from the

extracellular space into the enterocyte via SERT terminates the function of serotonin (Bertrand and Bertrand, 2010; Blakely et al., 1991; Hoffman et al., 1991). SERT is expressed in both the AP and BL membranes of Caco-2 cells and intestine, with predominant AP expression (Chen et al., 2001; Gill et al., 2008; Martel, 2006; Martel et al., 2003; Wade et al., 1996), and functions in the inward (e.g. uptake) direction (Nelson and Rudnick, 1979). As its name implies, the endogenous substrate of SERT is serotonin. However, recent studies showed that SERT and other cationic selective transporters exhibit shared substrate specificities (Duan and Wang, 2010; Hilber et al., 2005). In Chapter 2, it was clearly demonstrated that metformin is a substrate of SERT and that SERT mediates the AP uptake of metformin in Caco-2 cells. In this chapter, the role of mSert in the intestinal absorption was investigated.

4.C. MATERIALS AND METHODS

Materials

Minimum essential medium (MEM) with Eagle's salts and L-glutamate, F-12 Nutrient Mixture, HEPES buffer (1M), Hank's Balanced Salt Solution (HBSS), penicillin-streptomycin-amphotericin B solution (100x), non-essential amino acids (100x), geneticin, and fetal bovine serum (FBS) were purchased from Life Technologies Corporation (Grand Island, NY, USA). [14C]metformin was purchased from Moravsek Biochemicals and Radiochemicals (Brea, CA, USA). Paroxetine was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Caco-2 (HTB-37) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Chinese Hamster Ovary (CHO) cells stably expressing mouse organic cation transporter 1 (mOct1), mOct2, mOct3, mouse plasma membrane monoamine transporter (mPmat), mouse multidrug and toxin extrusion protein (mMate1), and vector-control (mock transfected) CHO cells were previously generated and characterized in our laboratory (Costales et al., 2013).

Methods

Cell Culture and Transport Experiments in CHO Cells

Chinese Hamster Ovary (CHO) cells were cultured as described previously (Ming et al., 2009). Briefly, cells were cultured in F12 Nutrient Mix supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B with additional 500 µg/ml geneticin. The cells were passaged following 90% confluency using trypsin-EDTA, and plated at a 1:20 ratio in 75-cm² T-flasks. Transport experiments using CHO cells were performed as described previously (Ming et al., 2009) and in Chapter 2. Briefly, CHO cells were seeded at 100,000 cells/cm²

density in sterile 24-well polycarbonate plates and transport experiments were performed between days 5-7 post seeding. CHO cell monolayers were pre-incubated with transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES, pH 7.2) for 30 min at 37 °C. [¹⁴C]metformin [5 µM, 0.2 µCi/mL] initial uptake (30 second for mOct2-CHO Cells, and 5 minutes for the remaining cells) in various concentrations of paroxetine was studied. Experiments were initiated by replacing the buffer solution with 300 µL of dosing solution. Uptake was terminated at the indicated time points by aspirating the donor solution and washing the monolayer 3X with 1 mL of 4 °C transport buffer. The cell monolayers were allowed to dry and 500 µL of lysis buffer (0.1 N NaOH with 0.1% SDS) was added to each well. Plates were shaken for 3 hours to ensure total lysis of the cell monolayer. Protein content of the cell lysate was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Radiolabeled compound in the cell lysate was analyzed by liquid scintillation spectrometry and the rate of initial uptake of each compound was determined. The uptake rate of metformin into mock CHO cells was subtracted from its uptake rates into individual transporter-expressing cell lines to obtain a corrected uptake rate reflective of only carrier-mediated transport of metformin. *IC*₅₀ curves of paroxetine of each transporter were generated, and analyzed by GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) to obtain the *IC*₅₀ values.

Animals

Male C57BL/6 mice and male mSert^{-/-} mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed according to approved

Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC) requirements and protocols. The animal housing facility was under the care, supervision, and animal husbandry of the University of North Carolina at Chapel Hill's Division of Laboratory Animal Medicine, in compliance with the Animal Welfare Act public laws. All animals were maintained under a normal 12-hour day/night schedule with lights on at 8:00 am.

Portal Vein Cannulation of Mouse

Portal vein cannulation was performed as described previously (Dufek et al., 2013). Briefly, C57BL/6 mice were fasted overnight and anesthetized with an intraperitoneal (IP) injection of urethane (1.2-1.5g/kg). The depth of anesthesia was monitored throughout the surgical procedure and the experiment by toe pinch reflex. When appropriate, a half dose of urethane was administered intramuscularly in the hind limb to maintain the anesthetic state. A 2 cm abdominal midline incision was made and the intestines were pushed to the side in order to expose the portal vein. A silastic catheter (0.025" OD x 0.012" ID) (Braintree Scientific, Braintree, MA) filled with saline, with a 26 1/2 ga. needle tip (Becton, Dickinson Biosciences, Franklin Lakes, NJ, USA) attached to the end, was inserted into the portal vein and secured to the surrounding tissue with a micro-serrefines vascular clamp (FST, Foster City, CA). Body temperature of the animals was monitored throughout the experiment by placement of a rectal thermometer (Fisher Scientific, Pittsburgh, PA, USA) and maintained at 37 °C on surgical board (VWR, Radnor, PA), which was maintained at 37 °C with a heating pad (Jarden Corporation, New York, NY, USA). [^{14}C]metformin [1mM, 30 $\mu\text{Ci/mL}$, 1.3mg/kg] in the

presence or absence of mSert specific inhibitor paroxetine [1 μ M, 3.3 μ g/kg] was administered by oral gavage. Portal blood samples (20 μ l each sample) were withdrawn through the portal cannula over four hours (at 5, 15, 30, 60, 120, and 240 min), and simultaneously, systemic blood samples were withdrawn through the tail vein of the same animal. Each blood sample was replaced with an equal volume of 10 U heparinized saline. The blood samples were placed in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA, USA), and stored on ice until the completion of the experiment. Following centrifugation at 9,000 g at 4 $^{\circ}$ C for 10 min, plasma was collected, and plasma samples were analyzed by liquid scintillation spectrometry.

Metformin Pharmacokinetics (PK) in Conscious Mice

Mice were fasted overnight before the experimentation. Doses of [14 C]metformin [0.5mM, 15 μ Ci/mL, 0.65mg/kg] in the presence and absence of paroxetine [1 μ M, 3.3 μ g/kg] in wild type C57BL/6 mice, and in the absence of paroxetine in mSert $^{-/-}$ mice, were administered by oral gavage. After oral administration of metformin, mice were placed back in the cage with *ad libitum* to water. Systemic blood samples (20 μ l each sample) were withdrawn through the tail vein by tail nick over six hours (at 5, 15, 30, 60, 120, 240 and 360 min). The blood samples were placed in heparinized capillary tubes and stored on ice until the completion of the experiment. Following centrifugation at 9,000 g at 4 $^{\circ}$ C for 10 min, plasma was collected, and plasma samples were analyzed by liquid scintillation spectrometry.

Statistical Analysis

Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test unless otherwise noted. Data represent mean \pm S.D; n=6 unless otherwise noted, *p<0.05, compared to the control.

4.D. RESULTS

Inhibition of mOct1-, mOct2-, mOct3-, mPmat-, mMate1-mediated Metformin Uptake in CHO Cells by Paroxetine

To determine the role of mSert in the oral absorption of metformin using a mouse model, it was imperative to first identify a selective inhibitor (and its inhibitory concentration) of mSert that would have minimal or no effect on other cationic transporters at the dose of the inhibitor used in the oral absorption studies. This was accomplished by determining the IC_{50} values for one of the most potent inhibitors of SERT (mSert), namely paroxetine (Figure 4.1). IC_{50} values for mOct1, mOct2, mOct3, mPmat, and mMate1 in singly-expressing transporter CHO cells were 4.3 ± 1.5 , 5.2 ± 1.2 , 82.3 ± 1.4 , 24.0 ± 1.7 , 1165.5 ± 98.1 μ M, respectively, whereas the literature reports an IC_{50} value of 40 ± 3 nM of paroxetine for mSert (Chang et al., 1996). Based on this inhibition profile of paroxetine, 1 μ M of paroxetine was chosen to specifically inhibit mSert *in vivo* in the current study.

Portal and Systemic Plasma Concentrations of Orally Administered Metformin in Urethane Anesthetized Mice

In Chapter 2, it was demonstrated that metformin is a substrate of SERT with a K_m of approximately 4 mM in SERT-expressing HEK cells. In addition, it was shown that SERT plays a role in the AP uptake of metformin in Caco-2 cell monolayers. In order to determine whether SERT is involved in the intestinal absorption of metformin *in vivo*, [14 C]metformin (1mM, 1.3mg/kg) was administered orally to portal vein cannulated mice in the absence and presence of the selective SERT (mSert) inhibitor, paroxetine, at a dose (3.3 μ g/kg) that would achieve ~ 1 μ M concentration in the

intestinal lumen, and plasma concentrations of metformin in the portal circulation were measured as a function of time. The portal plasma concentrations of [^{14}C]metformin over 4 hours post oral administration were lower in paroxetine-treated mice compared to untreated mice (Figure 4.2). Paroxetine decreased metformin area under the curve of portal concentration-time profile over 4 hours ($\text{AUC}_{\text{portal},0-4\text{h}}$) by approximately 10% ($p<0.05$) following oral administration of the two compounds (Figure 4.3). Similarly, there was a decrease in the systemic concentration of [^{14}C]metformin when co-administered with paroxetine, with approximately 10% decrease ($p<0.05$) in $\text{AUC}_{\text{systemic},0-4\text{h}}$ in the paroxetine-treated group (Figure 4.3).

As shown in Figure 4.2, metformin portal and systemic concentrations reached plateau 2 hours post metformin administration and did not decline up to four hours post dose, which could be due to the likely impairment of intestinal motility and metformin elimination by the anesthetic agent, urethane. However, portal and systemic AUCs evaluated over the first two hours (in the early absorption phase) following oral administration of the drug were found to be decreased by approximately 15% ($p<0.05$) upon co-administration of paroxetine (Figure 4.4). These results provide strong evidence for the involvement of mSert in the intestinal absorption of metformin in mouse.

Systemic Plasma Concentration of Orally Administered Metformin in Conscious Mice

To determine the role of mSert in the oral absorption of metformin without the confounding effect of the anesthetic agent, the influence of paroxetine on metformin absorption was evaluated in conscious mice. [^{14}C]metformin systemic concentrations

were determined (over a 6 hour period) following oral administration of [^{14}C]metformin (0.5mM, 0.65mg/kg) alone and with paroxetine (1 μM , 3.3 $\mu\text{g/kg}$). No significant differences were observed in systemic concentrations of metformin between the two treatment groups at each time point assessed (Figure 4.5), although paroxetine decreased metformin $\text{AUC}_{\text{systemic } 0-6\text{h}}$ by 9% ($p<0.05$) (Figure 4.6). Systemic concentrations of orally administered [^{14}C]metformin were also assessed in conscious mSert $^{-/-}$ mice over a 6-hour period. The results show no significant difference in systemic plasma concentrations of metformin between mSert $^{-/-}$ and wild-type mice at each time point assessed (Figure 4.5), although $\text{AUC}_{\text{systemic } 0-6\text{h}}$ in mSert $^{-/-}$ mice was decreased by 6% ($p<0.05$) compared to wild-type mice (Figure 4.6).

4.E. DISSCUSSION

In Chapter 2, it was demonstrated that four transporters, namely OCT1, PMAT, SERT and CHT, are involved in the AP uptake of metformin in Caco-2 cells; and that SERT contributed to approximately 20% of the total AP uptake of metformin. In the present study, the contribution of mSert in the oral absorption of metformin was investigated in a mouse model. The portal vein cannulated mouse model, implemented for the study of the oral absorption of drugs (Dufek et al., 2013), was used with minor modification in the current study. This modified experimental design involved collecting and analyzing portal blood (withdrawn from portal cannula) and systemic blood (withdrawn from the tail vein) from the same portal-vein cannulated mouse. Implementing this strategy enabled the comparison of portal and systemic metformin concentrations within the same animal, allowing a more rigorous comparison of the metformin plasma concentration and exposure in the portal and systemic circulation. In addition, a parallel study in conscious mice ensured that the effect of mSert on metformin concentrations and exposure in the systemic circulation of the portal vein cannulated mice was not affected by the anesthetic used in that model.

The results in the present study show that paroxetine caused approximately 10% decrease in metformin $AUC_{portal,0-4h}$. Since the concentrations were measured in the portal circulation, prior to the compound entering the liver, this decrease must reflect the effect of mSert on the intestinal absorption of metformin. Interestingly, the systemic concentrations in the same mice were found to be somewhat lower than the corresponding portal concentrations. This may be due to distribution of metformin into the liver tissue. Interestingly, the systemic concentrations in the same study also showed

approximately 10% decrease in AUC_{0-6h} attributed to inhibition of mSert by paroxetine. Thus paroxetine inhibitory effect on mSert appears to be localized to the intestinal tissue. In addition, the systemic concentration of metformin is lower than the portal concentration of metformin at each time point in the same animal, which indicates the distribution of metformin into liver. Paroxetine also decreased metformin systemic exposure (AUC_{0-6h}) by approximately 9% in conscious mice. Furthermore, the systemic AUC_{0-6h} of metformin in mSert^{-/-} mice following oral administration was 6% lower compared to that in the wild-type mice. Although it has been reported that mOcts are up-regulated in mSert^{-/-} mice (Baganz et al., 2008; Chen et al., 2001), the decrease in metformin absorption in mSert^{-/-} compared to wild-type mice was similar to the decrease in metformin absorption caused by inhibition of mSert with paroxetine. Overall these results provide strong evidence that mSert contributes to a small percentage (10% or less) of metformin intestinal absorption.

The results suggest that SERT inhibitors would not affect oral absorption of metformin by much. In fact, because metformin is transported by as many as four transporters, it is unlikely that oral absorption of metformin would be affected by inhibitors of any of the metformin transporters. However, metformin could act as a perpetrator drug, as high concentrations of metformin in the intestine could inhibit several transporters and affect the intestinal absorption of the substrates of these transporters. Since the literature is sparse on information related to metformin-mediated drug-drug interactions, data on the intestinal absorption of several cationic drugs that are co-administered with metformin should be re-evaluated in the context of the findings from this study.

This is the first direct *in vivo* evidence showing an interaction between the endogenous monoamine transporter (i.e., SERT) and metformin. These data are highly significant as they demonstrate that a transporter for an endogenous monoamine could also play a role in the absorption of an exogenous drug and/or interact with it. This finding raises the possibility that metformin and other cationic drugs could interfere with the homeostasis of endogenous monoamine transmitters. The novel discovery in the current study demonstrating an interaction between SERT and metformin led to the investigation of the involvement of serotonin in metformin's pharmacology. Since common adverse effects of metformin, such as diarrhea, nausea and vomiting may be caused by excess serotonin (Bertrand and Bertrand, 2010; Cirillo et al., 2011; Crowell et al., 2004; De Ponti, 2004); an extensive study on the intestinal interactions between serotonin and metformin was conducted. Details of this study are discussed in Chapter 5.

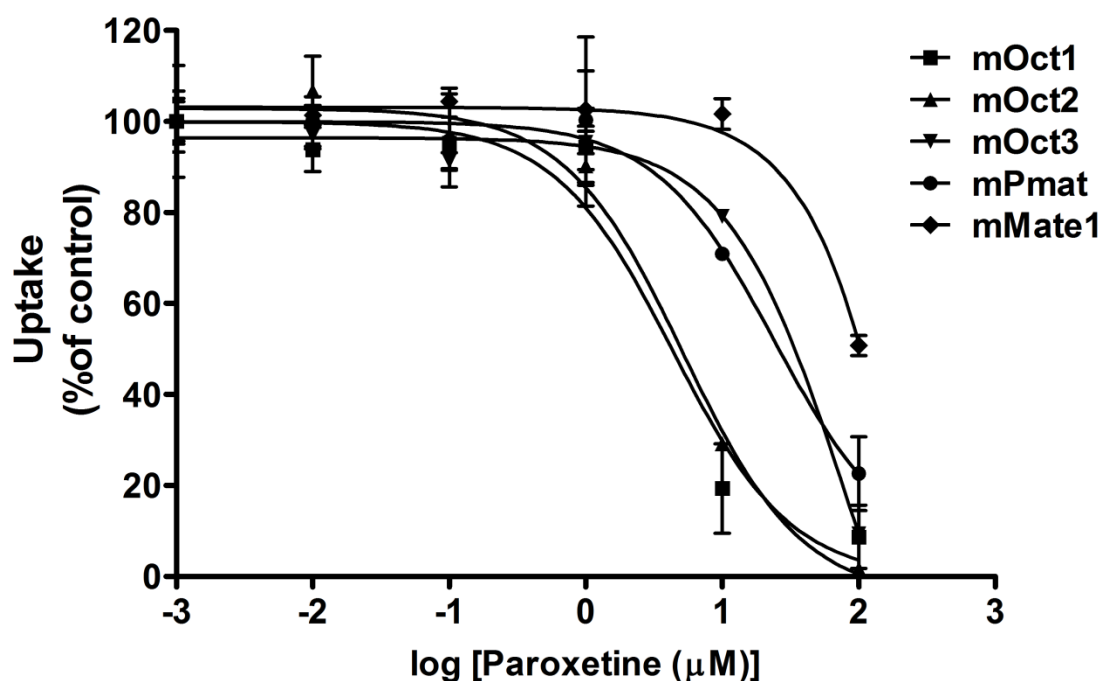


Figure 4.1. Inhibition of metformin uptake by paroxetine in mOct1-, mOct2-, mOct3-, mPmat-, mMate1-expressing CHO cells. [^{14}C]metformin [5 μM , 0.2 $\mu\text{Ci/mL}$] initial uptake was measured for 30 second (mOct2-CHO cells) or 5 minutes (remaining cells) in the presence of various concentrations of paroxetine [1 nM-100 μM]. The uptake rate of metformin into mock CHO cells was subtracted from its uptake rates into individual transporter-expressing cell lines to obtain a corrected uptake rate reflective of only carrier-mediated transport of metformin. Data represent mean \pm SD, $n=3$. IC_{50} curves of paroxetine of each transporter were generated and analyzed by GraphPad Prism 5. IC_{50} values of mOct1, mOct2, mOct3, mPmat, and mMate1 are 4.3 ± 1.5 , 5.2 ± 1.2 , 82.3 ± 1.4 , 24.0 ± 1.7 , 1165.5 ± 98.1 μM , respectively.

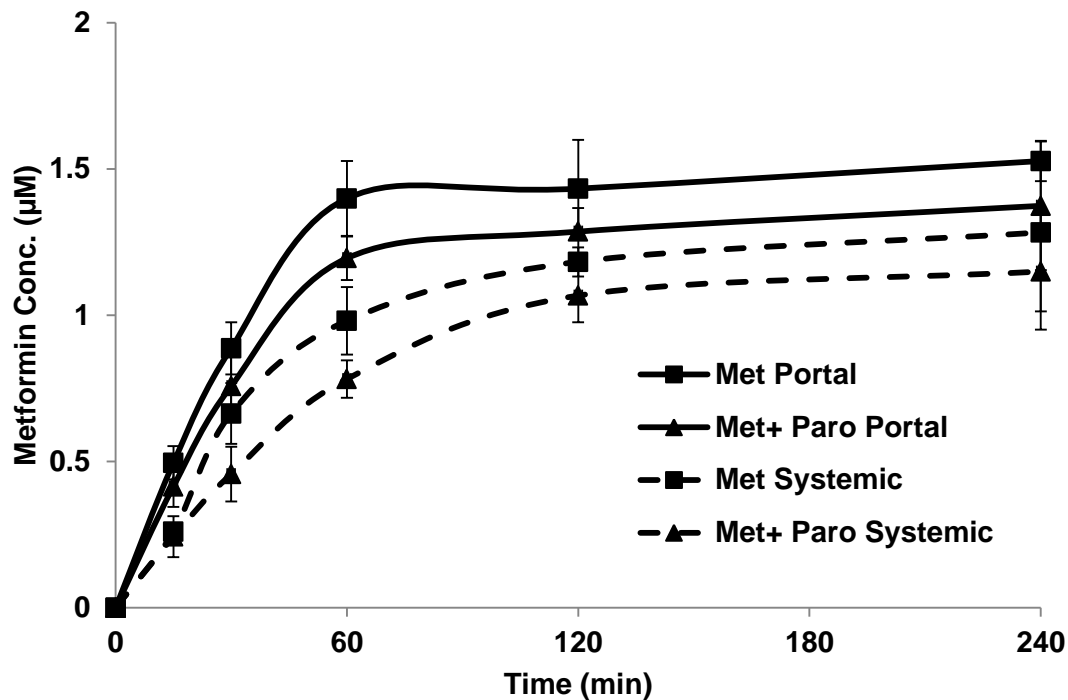


Figure 4.2. Portal and systemic plasma concentrations of metformin in anesthetized (urethane) mice after oral administration of the drug with or without paroxetine. [^{14}C]metformin [1mM, 30 $\mu\text{Ci/mL}$, 1.3mg/kg] was administered via oral gavage to C57BL/6 mice that were anesthetized with urethane, with or without paroxetine [1 μM , 3.3 $\mu\text{g/kg}$]. Portal and systemic concentrations of metformin were measured as a function of time. Data represent mean \pm SD, n=6. Met represents metformin. Paro represents paroxetine.

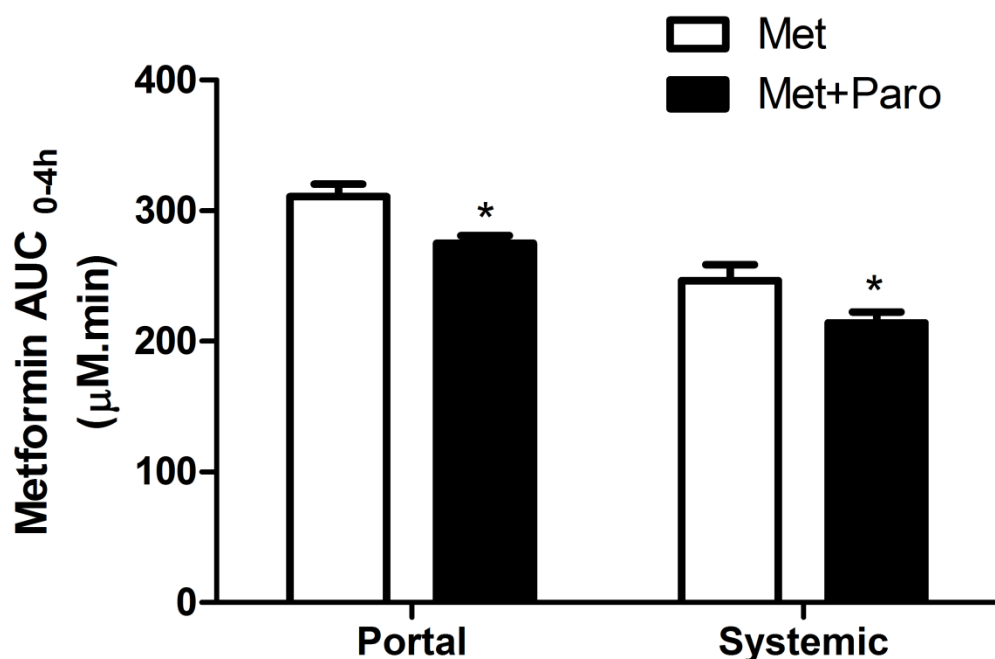


Figure 4.3. Portal and systemic AUC_{0-4h} of metformin upon oral administration with or without paroxetine. AUC_{0-4h} of metformin was calculated for each of the concentration *versus* time profiles of metformin in Figure 4.2 and is represented as open bar (metformin) or solid bar (metformin plus paroxetine). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data represent mean \pm S.D; n=6, *p<0.05, compared to the control. Met represents metformin. Paro represents paroxetine.

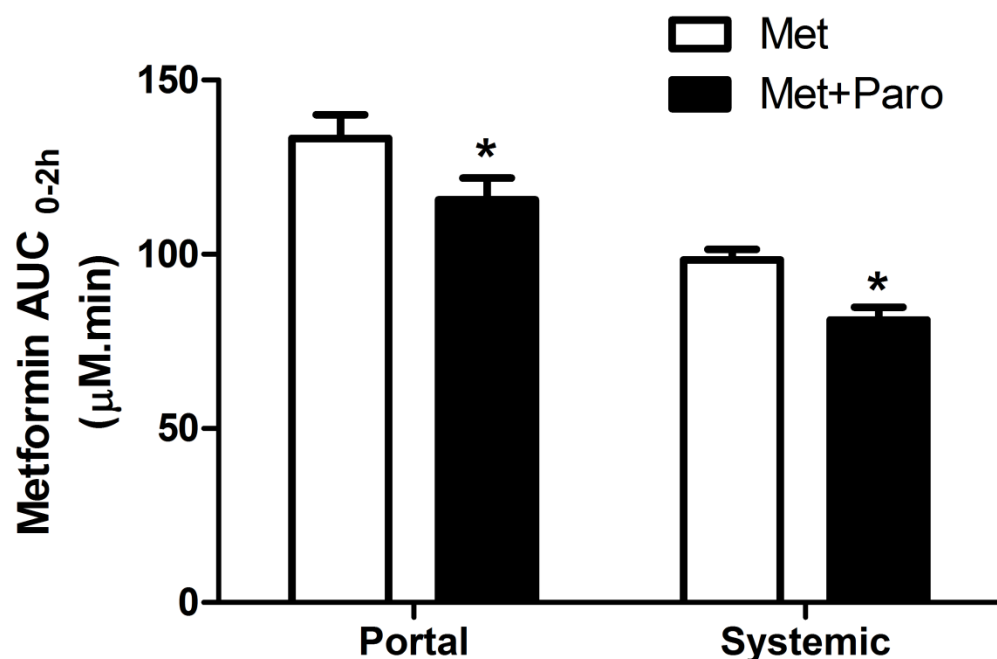


Figure 4.4. Portal and systemic AUC_{0-2h} of metformin upon oral administration with or without paroxetine. The AUC_{0-2h} was calculated from the concentration versus time profiles shown in Figure 4.2 and represented as open bar (metformin) and solid bar (metformin plus paroxetine). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data represent mean \pm S.D; n=6, *p<0.05, compared to the control. Met represents metformin. Paro represents paroxetine.

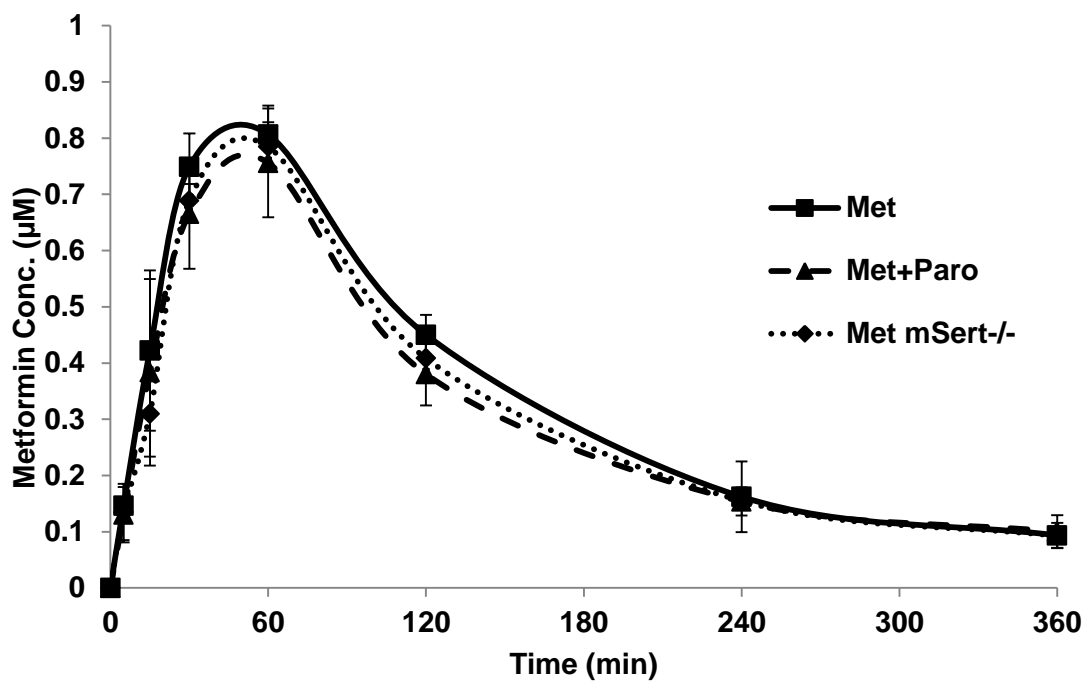


Figure 4.5. Systemic plasma concentrations of metformin as a function of time upon oral administration of the drug with or without paroxetine in conscious mice. [^{14}C]metformin [0.5mM, 15 $\mu\text{Ci/mL}$, 0.65mg/kg] was administered via oral gavage to conscious C57BL/6 mice with or without paroxetine [1 μM , 3.3 $\mu\text{g/kg}$]. [^{14}C]metformin [0.5mM, 15 $\mu\text{Ci/mL}$, 0.65mg/kg] was also administered to mSert $^{-/-}$ mice. Data represent mean \pm SD, n=6. Met represents metformin. Paro represents paroxetine.

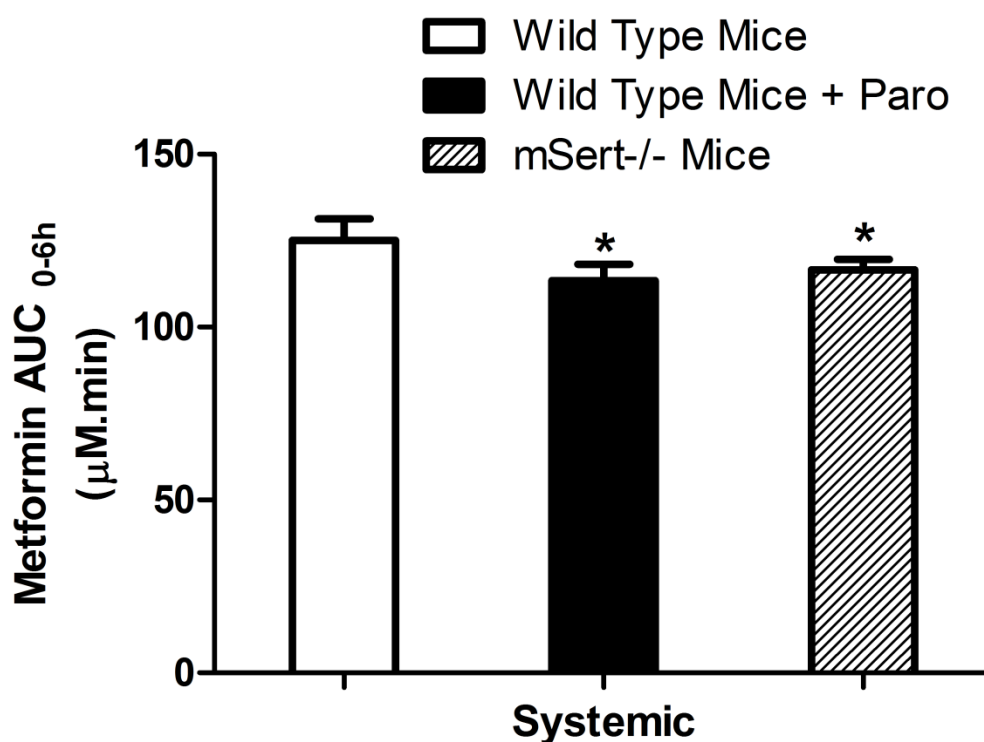


Figure 4.6. Systemic AUC_{0-6h} of metformin in conscious mice upon oral administration to C57BL/6 mice with or without paroxetine or to mSert^{-/-} mice without paroxetine. Systemic AUC_{0-6h} of metformin was calculated from the plasma concentration *versus* time profile in Figure 4.5 and represented as open bars (metformin), solid bar (metformin plus paroxetine), and hatched bar (metformin in mSet^{-/-} mice). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data represent mean \pm S.D; n=6, *p<0.05, compared to the control. Met represents metformin. Paro represents paroxetine.

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CHAPTER 5

ROLE OF SEROTONIN REUPTAKE TRANSPORTER IN THE INTESTINAL ADVERSE EFFECTS OF METFORMIN

5.A. OVERVIEW

Metformin is a widely used drug for Type II diabetes mellitus, which causes common gastrointestinal (GI) adverse effects such as diarrhea, nausea, and vomiting. Although metformin has been used widely for decades, the mechanisms involved in these adverse effects are unknown. Previous studies have shown that the serotonin reuptake transporter (SERT), mediates apical (AP) uptake of metformin in Caco-2 cell monolayers (Chapter 2), and plays a modest role in the oral absorption of this drug in mice (Chapter 4). In this study, the role of SERT in GI adverse effects induced by metformin was investigated by using a selective serotonin reuptake transporter inhibitor (SSRI) paroxetine, as well as the serotonin receptor 4 (5HT₄) agonist cisapride and antagonist GR113808. The results presented in this chapter show that metformin inhibits SERT-mediated intestinal reuptake of serotonin resulting in increased intestinal motility and water retention, as assessed by intestinal transit of fluorescently-labeled dextran and magnetic resonance imaging (MRI), respectively. Metformin increased intestinal motility by 4-fold ($p < 0.001$), which was comparable to paroxetine- and cisapride-mediated increase in intestinal movement, and was attenuated by the 5HT₄ antagonist GR113808. Metformin, paroxetine and cisapride enhanced water retention in the large

intestine by ~50% ($p < 0.05$) compared to saline controls; this increase in water content was partially blocked by GR113808. This is the first direct evidence that metformin-induced GI adverse effects involve metformin-mediated inhibition of intestinal serotonin reuptake by SERT, leading to increased intestinal motility and water retention in the large intestine.

5.B. INTRODUCTION

Metformin has been widely used for decades in the United States and worldwide for the treatment of Type II diabetes mellitus. In 2011, there were 59 million metformin prescriptions issued in the US (Report by the IMS Institute for Healthcare Informatics, 2012). Although it is considered to be relatively safe and well tolerated, it induces common GI adverse effects, such as diarrhea (in ~50% of patients), nausea and vomiting (~25%), bloating, and abdominal pain (~5%) (Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009; Dandona et al., 1983). Due to the wide usage of metformin and the high incidence of its GI adverse effects, millions of patients suffer from metformin-induced GI distress. In severe cases (~5%), these adverse effects lead to discontinuation of metformin treatment (Bailey, 1992; Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009; Dandona et al., 1983; Krentz et al., 1994).

To date, there is no direct evidence that explains the mechanistic basis of these adverse effects, although several hypotheses have been proposed. Firstly, metformin was reported to alter bile salt metabolism and circulation, which could cause diarrhea (Carter et al., 2002; Carter et al., 2003; Caspary et al., 1977; Scarpello et al., 1998). However, results from these studies are inconclusive, and the effect of metformin on bile salts remains unclear. Secondly, it was suggested that metformin alters the level of ghrelin (Doogue et al., 2009; Kadoglou et al., 2010; Kusaka et al., 2008), a gastric peptide which plays an important role in regulating GI motility and gastric emptying (Asakawa et al., 2011; Chen and Tsai, 2012; Ogiso et al., 2011). However, the literature is ambiguous on the effect of metformin on ghrelin; Doogue et al. reported that metformin increases plasma ghrelin concentrations (Doogue et al., 2009), Kusaka et al. suggested that

metformin decreases ghrelin levels (Kusaka et al., 2008), and Kadoglou et al. reported that metformin did not significantly alter the levels of this peptide (Kadoglou et al., 2010). Thirdly, it was reported that metformin increased the concentration of glucagon-like peptide-1 (GLP-1), a peptide hormone, which plays a role in digestive functions (Cuthbertson et al., 2011; Mannucci et al., 2001; Mannucci et al., 2004), and could therefore potentially contribute to the intestinal adverse effects of metformin, although direct evidence is still needed to draw any firm conclusions. Finally, metformin was suspected to interact with serotonin, due to its structural similarity with a 5HT₃ agonist, chlorophenylbiguanide which is also a biguanide compound (Sepulveda et al., 1991). Serotonin is excreted by the enterochromaffin cells of the intestinal epithelium, and upon release, it activates the serotonin receptors, which affect various functions, including GI motility (Bertrand and Bertrand, 2010; Mohammad-Zadeh et al., 2008). Cubeddu et al. reported that in human intestinal tissues, metformin treatment increased the extracellular concentration of serotonin, indicating that serotonin could mediate the GI adverse effects of metformin (Cubeddu et al., 2000). In the same study, the authors also demonstrated that metformin is not an agonist of 5HT₃, and that the increase in serotonin is independent of this receptor (Cubeddu et al., 2000). Others have reported that in clinical studies, ondansetron, a 5HT₃ antagonist, could not reduce metformin-induced intestinal adverse effects (Hoffmann et al., 2003). Therefore, based on these data, it is likely that 5-HT₃ is not involved in metformin-induced GI side effects.

The studies mentioned above did not investigate the effects of metformin on SERT which is the re-uptake transporter of the neurotransmitter serotonin. Serotonin reuptake by SERT from the intestinal extracellular space into the enterocyte decreases the

extracellular concentration of serotonin and terminates the function of serotonin (Bertrand and Bertrand, 2010; Blakely et al., 1991; Hoffman et al., 1991). Although metformin does not directly activate serotonin receptors, it has been established that metformin is a substrate of SERT (Chapter 4). Based on these data, it has been hypothesized that metformin inhibits SERT and increases the extracellular concentration of serotonin in the intestine, thus causing GI adverse effects such as diarrhea, nausea and vomiting. *In vitro* and *in vivo* studies were conducted to test this hypothesis and thus assess the role of serotonin and SERT in the intestinal adverse effects of metformin. As reported in this chapter, metformin increases GI motility and colonic water content. These effects are mediated by 5HT₄ receptor, but metformin causes these effects indirectly by increasing serotonin interactions with the receptor by inhibiting SERT-mediated attenuation of its local GI serotonergic effects.

5.C. MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) buffer (1M), Hank's Balanced Salt Solution (HBSS), penicillin-streptomycin-amphotericin B solution (100x), non-essential amino acids (100x), geneticin, and fetal bovine serum (FBS), Alexa Fluor® 680-Dextran (10,000 MW) were purchased from Life Technologies Corporation (Grand Island, NY, USA). Dialyzed FBS (MWCO 10,000), D-glucose, pargyline, ascorbic acid, metformin, and paroxetine were purchased from Sigma-Aldrich (St. Louis, MO, USA). [3H]serotonin was purchased from Perkin Elmer (Waltham, MA, USA). The Caco-2 (HTB-37) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). SERT transfected Human Embryonic Kidney 293 (HEK 293) cells were provided by Dr. Randy Blakely from Vanderbilt University (Nashville, TN, USA). Control HEK 293 cells were purchased from the Tissue Culture Facility at the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA).

Methods

Culturing of SERT-Transfected HEK 293 Cells and Caco-2 Cell Monolayers

SERT-transfected and control parental HEK 293 cell lines were maintained in monolayer cultures in 75-cm² flasks in an atmosphere of 5% CO₂ at 37 °C as described previously (Qian et al., 1997). Both cell lines were grown in Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum (MWCO: 10,000), 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin within 10 passages. Culture medium for the transfected cell line was supplemented with geneticin (250

mg/ml). Cells were seeded 48-72 hr before experimentation on poly-D-lysine coated 24-well plates at a density of 100,000 cells/well.

Caco-2 cell monolayers were cultured as described previously (Proctor et al., 2008). Briefly, the cells (passage numbers 30 to 40) were seeded at a density of 60,000 cells/cm² on polycarbonate membranes of Transwells™. The culture medium was changed the day following seeding and every other day thereafter. Cell monolayers with transepithelial electrical resistance greater than 300 Ω·cm² were used for experimentation at 21-28 days post seeding.

Uptake Studies with SERT-Transfected HEK 293 Cells

Serotonin uptake into SERT-HEK cells was performed as described previously (Qian et al., 1997). Cells were pre-incubated with transport buffer (HBSS with 25 mM D-glucose and 10 mM HEPES, pH 7.2) containing 100 μM pargyline and 100 μM ascorbic acid for 30 min before the transport experiments. [³H]serotonin [0.1 μM, 0.1 μCi/mL, 5 min] uptake in the absence and presence of various concentrations of metformin was studied. Experiments were initiated by replacing the buffer solution with 300 μL of dosing solution. Uptake was terminated at the indicated time point by aspirating the donor solution and washing the monolayer 3X with 1 mL of 4 °C transport buffer. The cell monolayers were allowed to dry and 500 μL of lysis buffer (0.1 N NaOH with 0.1% SDS) was added to each well. Plates were shaken for 3 hr to ensure total lysis of the cell monolayer. Protein content of the cell lysate was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Radiolabeled compound in the cell lysate was analyzed by liquid scintillation spectrometry and the rate of initial uptake of each compound was

determined. The uptake rate of serotonin into parental HEK cells was subtracted from the uptake rates in the SERT-HEK cell line to obtain a corrected value reflective of only SERT-mediated transport of serotonin. Inhibition curves were generated and analyzed by GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) to obtain the IC_{50} value.

Serotonin AP Uptake in Caco-2 Cell Monolayers

AP uptake of serotonin was performed using methods outlined previously (Proctor et al., 2008) with minor deviations. Caco-2 cell monolayers were pre-incubated with transport buffer containing 100 μ M pargyline and 100 μ M ascorbic acid for 30 min prior to the initiation of transport experiments. AP uptake of [3 H]serotonin [0.1 μ M, 0.1 μ Ci/mL, 5 min] in Caco-2 cell monolayers was assessed under four experimental conditions: (1) serotonin dose in the absence of metformin (2) serotonin dose with metformin [10 mM] (3) serotonin dose without metformin but metformin pre-loaded in Caco-2 cell monolayers (in the pre-incubation buffer 10 mM for 30min) and (4) with metformin in serotonin dose and pre-loaded in Caco-2 cell monolayers. AP uptake experiments were initiated by replacing the buffer solution in the AP donor compartment with transport buffer containing the doses as indicated above. Uptake was terminated within the initial linear uptake range in Caco-2 cell monolayers [5 min] by washing the cells with 0.75mL of 4 $^{\circ}$ C transport buffer 3X in each compartment. The cells were dried, excised from the insert, lysed and analyzed as above, and the rate of initial uptake of metformin was determined.

Animals

Male C57BL/6 and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed according to approved Association for

Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC) requirements and protocols. The animal housing facility was under the care, supervision, and animal husbandry of the University of North Carolina at Chapel Hill's Division of Laboratory Animal Medicine, in compliance with The Animal Welfare Act public laws. All animals were maintained under a normal 12-hour day/night schedule with lights on at 8:00 am.

Biodistribution of Alexa Fluor 688-conjugated Dextran

Male BALB/c mice were used for all fluorescent imaging studies, and the mice were given Teklad 2018 Alfalfa-free diet for at least 7 days before experimentation in order to reduce the background and auto-fluorescent signals (Harlan Laboratories, Madison, WI, USA). Animals were fasted overnight before experimentation. Orally administered Alexa Fluor 688-conjugated Dextran (10kD) was used as a dye to assess intestinal transit and motility. In order to verify that this dye will not be absorbed into the blood stream, biodistribution of the fluorescent dye was determined. At 45 min post oral administration of the dye, the mouse was sacrificed under anesthesia, and major organs, blood and urine were collected, and imaged in IVIS® Kinetic Optical Imaging System (Perkin Elmer, Washington, DC), with excitation/emission wavelength at 675/720 nm.

Intestinal Transit Study

Male BALB/c mice were fasted overnight prior to experimentation. Metformin and other gut motility modulating compounds (see below) were given to mice by oral gavage 30 min before oral administration of Alexa Fluor 688-conjugated Dextran (10kD). Treatment groups included: saline control, metformin [15mg/kg, 50µL], paroxetine

[1 μ M, 50 μ L], 5HT₄ agonist cisapride [100 μ M, 50 μ L], 5HT₄ antagonist GR113808 [25 μ M, 50 μ L], metformin plus GR113808 (dosed together, 30 min before dextran), paroxetine plus GR113808 (dosed together, 30 min before dextran), cisapride plus GR113808 (GR113808 was administered orally 15min before cisapride oral dose, and dextran was administered 30 min after cisapride treatment). Mice were kept in cages under normal conditions (without anesthetics) after treatment until sacrifice. At 5min, 10min, 15min, 30 min and 60min post treatment with the fluorescent dye, animals were sacrificed using isoflurane followed by cervical dislocation. Stomach and intestines were harvested and fluorescent imaging was performed as described above, to quantify the intestinal transit of fluorescent dextran. The distance from the distal region of the stomach to the anterior most region of the fluorescent signal in the intestine was designated as the intestinal transit of dextran. To represent this value as a percentage of the length of the intestine (i.e., both total length including small and large intestine (40 cm) or only the length of the small intestine (34 cm)), the intestinal transit was divided by either 40 or 34. The motility of the small intestine was determined by dividing the percentage of dextran transit in the small intestine by the time required to travel that distance (i.e., percentage of small intestinal dextran transit per minute). Three mice were used per time point. Statistical significance of small intestine motility was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test unless otherwise noted.

Determination of Large Intestinal Water Content

Male C57BL/6 mice were used for evaluating intestinal water content. The animals were fasted overnight prior to experimentation. MRI was utilized for analysis

of water content. Treatment groups included: saline control, metformin [15mg/kg, 50 μ L], paroxetine [1 μ M, 50 μ L], 5HT₄ agonist cisapride [100 μ M, 50 μ L], 5HT₄ antagonist GR113808 [25 μ M, 50 μ L], metformin plus GR113808 (dosed together), paroxetine plus GR113808 (dosed together), cisapride plus GR113808 (GR113808 was administered orally 15min before cisapride oral dose). Sixty min post treatment, the animals were placed in a temperature-controlled restraining chamber in the MRI instrument with isoflurane as inhalational anesthesia throughout imaging. Breathing rate, heart beat rate and body temperature of the animals were monitored. MRI imaging was performed in BRUKER Bio Spec 94/30 USR System (Billerica, MA, USA), with field at 9.40 Tesla, and current at 190 A. The abdominal region of the animals was scanned (3 scans for each animal \times 3 animals), and the large intestinal lumen at the junction of the transverse colon and descending colon was selected for imaging. The proton content in the selected area was quantified and the spectra for water present in this area were generated. These spectra were analyzed by MatLab[®] software (MathWorks Inc., Natick, MA, USA) to generate a water peak. The area under the curve (AUC) of this water peak was used as an indicator of water content in the selected area of the large intestine. The specific area selected for imaging was based on its biological similarity in all animals used, as well as for technical accuracy. Water content-time profiles of metformin- and saline (control)-treated mice were generated over eight hours following metformin oral administration. Three animals (single scan) were used per time point. Relative water content (percentage of control) was compared between groups. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test unless otherwise noted.

5.D. RESULTS

Metformin Inhibits Serotonin Uptake into SERT-expressing HEK 293 Cells and Caco-2 Cell Monolayers

[³H]Serotonin uptake in SERT-expressing HEK 293 cells in the presence of several concentrations of metformin was investigated, and our results showed that metformin inhibits serotonin uptake with an IC_{50} value of 50.1 ± 1.4 mM (Figure 5.1); thus metformin is a weak inhibitor of SERT.

AP uptake of [³H]serotonin in Caco-2 cell monolayers was decreased by approximately 40% ($p < 0.01$) when metformin was in the donor solution. Pre-loaded metformin in Caco-2 cell monolayers decreased [³H]serotonin uptake by about 20% ($p < 0.05$). The presence of metformin in both the donor compartment and in pre-loaded Caco-2 cell monolayers decreased the [³H]serotonin AP uptake by approximately 50% ($p < 0.001$) (Figure 5.2). These results demonstrate that metformin inhibits SERT-mediated uptake of serotonin in HEK 293 cells, and presumably also in Caco-2 cell monolayers.

Biodistribution and Intestinal Transit of Alexa Fluor 688-conjugated Dextran after Oral Dose

In order to validate that Alexa Fluor 688-conjugated Dextran is not absorbed into the blood stream, biodistribution of this dye was assessed after oral administration. Our results clearly demonstrate that this fluorescent dye stays exclusively in the GI tract, with no fluorescent signal in the lung, heart, spleen, liver, kidneys, blood or urine (Figure 5.3). The intestinal transit of Alexa Fluor 688-conjugated dextran was assessed as a measure of intestinal motility. Thus, by measuring the transit time of the

fluorescent dextran after an oral dose of metformin, one can evaluate if metformin causes increased intestinal motility. Figure 5.4 shows the intestinal transit of the fluorescent dextran in control (saline-treated) and metformin-treated mice. In metformin-treated mice, the fluorescent dextran reached the large intestine (cecum) within 15 min of administration, whereas in the control group, it required 60 min to traverse the same distance (Figure 5.4). It is hypothesized that the 4-fold increase ($p < 0.001$) in the intestinal motility caused by an oral dose of metformin is due to inhibition of SERT by metformin, and subsequent increase in the intestinal concentration of serotonin. Paroxetine, a selective inhibitor of SERT, also caused a similar increase in intestinal motility (Figure 5.5 A-B, Figure 5.6). The role of serotonin in increased intestinal motility was clearly demonstrated by an increase in the intestinal motility by 5HT₄ receptor agonist, cisapride (Figure 5.5 A-B, Figure 5.6). The 5HT₄ antagonist, GR113808, alone did not alter intestinal motility compared to the control group, but when used in combination with metformin, paroxetine or cisapride, it attenuated the increase in intestinal movement induced by the other drugs (Figure 5.5 A-B, Figure 5.6). Collectively, these results provide definitive evidence that metformin causes increased intestinal motility by increasing the intestinal serotonin concentration through inhibition of SERT.

Role of Metformin in Reducing Absorption of Water in the Mouse Colon

The GI adverse effects of metformin include diarrhea, characterized by increased water content in the feces, caused by decreased water absorption in the colon. Hence, to quantify this effect of metformin, the water content in the colon was measured by MRI after an oral dose of metformin to mice. Metformin, as well as paroxetine and cisapride

increased water content by 40-50% compared to the control group ($p < 0.05$) as measured by the area under the water peak obtained in the NMR spectra of a specific region of the large intestine. Interestingly, the 5HT₄ receptor antagonist, GR113808, attenuated this increase (Figure 5.7), providing evidence that the effect of metformin on water retention in the large intestine is mediated by the serotonin-5HT₄ interactions.

Pharmacodynamics of Metformin-induced Water Content Increase in the Large Intestine

Water content in the large intestine of saline (control)- and metformin-treated mice was studied over 8 hours. It can be seen in Figure 5.8 that water retention is delayed compared to metformin pharmacokinetics. Water content reached the highest level around 60-120 min after oral administration of metformin, while T_{\max} of metformin plasma concentration occurs between 30-60 min (Chapter 4).

5.E. DISCUSSION

Metformin was first synthesized by Werner and Bell in 1921 (Werner and Bell, 1921) and introduced into humans as an oral treatment for Type II diabetes in 1957 in France (Bailey and Day, 1989). In Europe, metformin achieved widespread use in late 1970s (Bailey and Day, 2004). The drug was approved for use in Canada in 1972 (Lucis, 1983) and was introduced in the United States in 1994 (Alexander et al., 2008). Currently, metformin is widely used in the US and worldwide. In 2011, there were 59.1 million prescriptions of metformin in the US (IMS institute The Use of Medicines in the United States: Review of 2011). It has been reported that metformin can cause intestinal adverse effect, such as diarrhea, in about 20% of patients with chronic use (Dandona et al., 1983), and about 50% in patients who start the medication or increase the dose (Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009). Considering the large number of patients on metformin therapy, its adverse effects pose an enormous problem in public health and often lead to non-compliance where in severe cases, some (5%) diabetic patients are compelled to terminate metformin use, and choose a second-line treatment, which is not ideal for the disease (Bailey, 1992; Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009; Dandona et al., 1983; Krentz et al., 1994).

In this chapter, the mechanism underlying the intestinal adverse effects of metformin was investigated. This is the first study that demonstrates that the interaction between SERT and metformin in the GI tract leads to GI distress such as diarrhea. This finding has major scientific and medical significance, because metformin-mediated intestinal adverse effects have affected and continue to affect millions of patients on metformin therapy, leading to non-compliance.

The current study addresses a long-standing historical mystery, and for the first time provides a mechanistic basis for the intestinal adverse effects of metformin. In previous chapters, it has been shown that metformin is a substrate of SERT, and that SERT plays a role in the AP uptake of metformin in Caco-2 cell monolayers, and its oral absorption in mice *in vivo*. *In vitro* studies have shown that metformin is a weak inhibitor of SERT in SERT singly-transfected HEK 293 cells. However, taking into account the high dose of metformin prescribed to diabetic patients (850-1000mg per day), the intestinal lumen concentration of metformin could be as high as 30 mM, if administered with 250mL of water. In addition, based on the sponge mechanism of the intestinal absorption of metformin proposed in our laboratory (Proctor et al., 2008), and on results from our studies on the *in vivo* absorption of metformin in mice (Costales et al., 2013), metformin accumulates in the intestine, and hence its intracellular concentration can exceed 70 mM. Therefore, although metformin is a weak inhibitor of SERT, it is likely that at these elevated intestinal concentrations, it can mediate SERT inhibition. This weak inhibitory effect of metformin on SERT could also account for a decrease in the prevalence of GI adverse effects observed with low dose or slow-release formulation of metformin (Bristol-Myers-Squibb, 2009). Moreover, the weak inhibition of SERT by metformin could result in varying extents of GI adverse effects based upon different levels of expression and functional activity of SERT or tolerance of serotonin, thus providing a mechanistic explanation for the high inter-individual variability of these adverse effects. Furthermore, the inhibition of SERT by metformin and subsequent decrease of serotonin reuptake was investigated in Caco-2 cell monolayers, a well-

established model for human intestinal epithelium. In these *in vitro* studies, it was found that a 10mM metformin concentration significantly decreases the reuptake of serotonin.

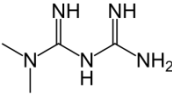
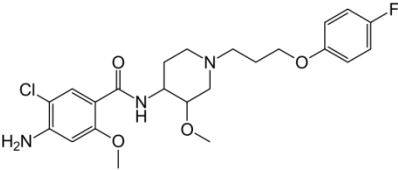
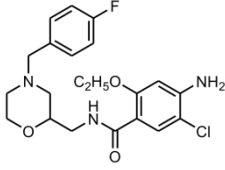
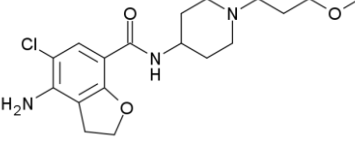
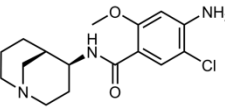
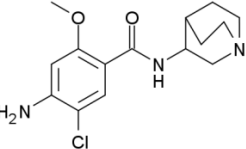
In vivo evidence on the role of SERT in metformin-induced GI adverse effects was provided from studies elucidating the effect of metformin on intestinal transit and water content in mice. The results demonstrate that metformin increases intestinal transit and water retention. In order to clearly demonstrate the role of serotonin in these GI adverse effects, a comparison was made between the GI effects of SSRI paroxetine and 5HT₄ agonist cisapride, and metformin. Interestingly, all three compounds have similar effects on GI motility and water retention. Paroxetine inhibits the reuptake of serotonin, increasing its extracellular concentration and causing increased activation of 5HT₄, while cisapride directly activates 5HT₄. Previous reports suggest that metformin is unable to activate serotonin receptor (Cubeddu et al., 2000; Hoffmann et al., 2003), and our results showed its ability to inhibit SERT. Clearly, metformin acts similar to paroxetine in that it also inhibits reuptake of serotonin and thus causes the GI effects through increased activation of the 5HT₄ by the elevated amount of serotonin in the intestine. Furthermore, when GR113808 was used to block the activity of 5HT₄, the effects of metformin, paroxetine and cisapride were diminished. This is the first study to show that a serotonin receptor antagonist blocks metformin-induced GI adverse effects. As mentioned above, metformin is not an agonist of 5HT₃ (Cubeddu et al., 2000), and the antagonist of 5HT₃, ondansetron, could not attenuate metformin-induced intestinal adverse effects (Hoffmann et al., 2003). Therefore, the results presented here suggest that another serotonin receptor expressed in the intestine, namely 5HT₄ (Tong et al., 2011), is involved in GI motility and water retention following an increase in the extracellular concentration of

serotonin caused by metformin-mediated inhibition of SERT. It could be argued that metformin may be an agonist of 5HT₄ (even though it is not a 5HT₃ agonist), and thus mediates GI adverse effects by directly activating this receptor. However, due to structural dissimilarities between metformin and 5HT₄ agonists, this is highly unlikely (Table 5.1) (Baeyens et al., 1984; Werner and Bell, 1921) (Bhandari and Andrews, 1991; Bouras et al., 1999; Kato et al., 1991; Staniforth and Pennick, 1990). In addition, the delay in metformin-induced water retention compared to metformin pharmacokinetics suggests an indirect effect of metformin on this receptor. Together, the results in the current study unequivocally provide a mechanistic basis for metformin-induced GI adverse effects that involves inhibition of SERT in the intestine, followed by a subsequent decrease in serotonin reuptake and increase in serotonin concentration in the GI tract, which activates 5HT₄ and results in increased intestinal motility and water retention.

This current study provides a novel approach to investigate GI adverse effects (i.e., diarrhea) *in vivo*. Intestinal transit of the fluorescent dye and quantification of water content are easy and accurate methods for assessing diarrhea. A non-invasive direct imaging to quantify the intestinal movement of the fluorescent dye was attempted in live mice; however, due to the small size of the abdominal region of mice and limited resolution of the imaging instrument, this attempt was unsuccessful. Nevertheless, intestinal transit could be studied in humans by imaging the movement of fluorescent dextran as a function of time as the abdominal region is considerably larger. Water retention assessment by MRI can also be utilized in human subjects to quantify a relatively small increase in water content with greater sensitivity and accuracy, which may not be detected by other methods.

Importantly, this is the first direct evidence that establishes a link between an exogenous drug, metformin, and an endogenous monoamine, serotonin. Endogenous monoamines are generally important molecules, such as hormones and neurotransmitters, and their homeostasis is critical to biological functions. The interactions between these endogenous cations and exogenous drugs could lead to profound effects. The mechanisms discussed in this chapter could be extrapolated to other cationic drugs with similar adverse effects that may share an identical mechanism. Although it is known that cationic drugs often induce gastrointestinal stress and that numerous cationic drugs are in the market (Neuhoff et al., 2003), the basis for this adverse effect has not been thoroughly investigated. Thus, the mechanisms discussed in this chapter could provide important information and new guidelines for the development of drugs with reduced adverse effects.

Table 5.1. Structures of metformin and 5HT₄ agonists.

Name	Class	Structure	References
Metformin	SERT Inhibitor		(Werner and Bell, 1921)
Cisapride	5HT ₄ Agonist		(Baeyens et al., 1984)
Mosapride	5HT ₄ Agonist		(Kato et al., 1991)
Prucalopride	5HT ₄ Agonist		(Bouras et al., 1999)
Renzapride	5HT ₄ Agonist		(Staniforth and Pennick, 1990)
Zacopride	5HT ₄ Agonist		(Bhandari and Andrews, 1991)

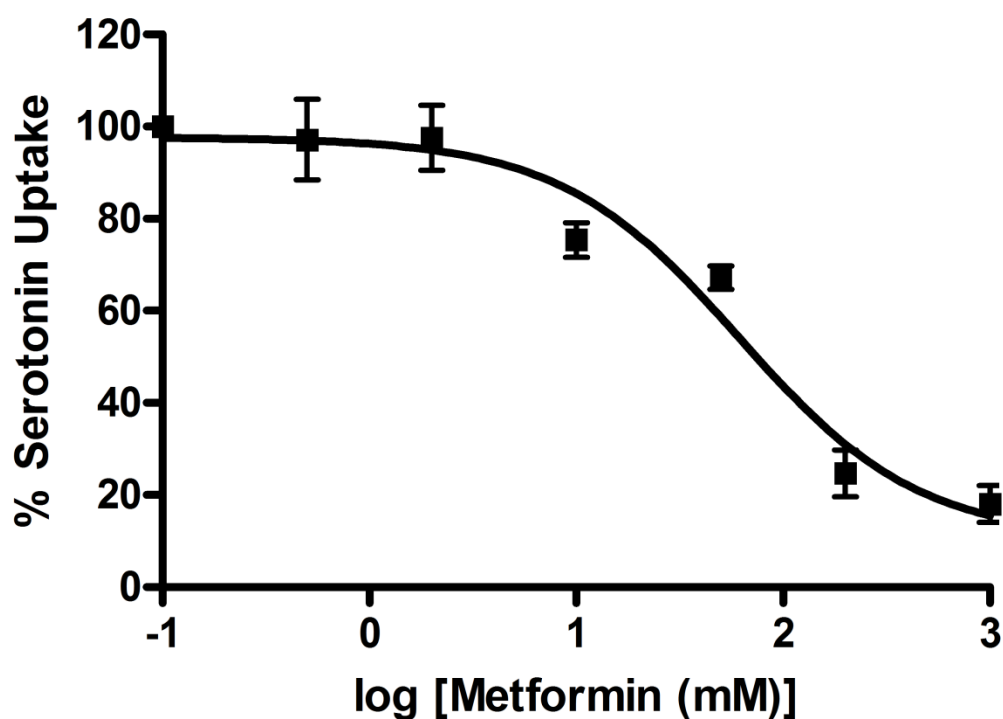


Figure 5.1. Inhibition by metformin on serotonin uptake by SERT-expressing HEK 293 cells. [^3H]serotonin [$5\text{ }\mu\text{M}$, $0.2\text{ }\mu\text{Ci/mL}$] uptake (2min) in the presence of several concentrations of metformin. The uptake rate of serotonin into parental HEK cells was subtracted from the uptake rates in the SERT-HEK cell line to obtain a corrected value reflective of only SERT-mediated transport of serotonin. Data represents mean \pm S.D., $n=3$. IC_{50} curves were fit to corrected uptake data in the presence of varying concentrations of the inhibitor. IC_{50} value is $50.1 \pm 1.4\text{ mM}$. Data represent mean \pm SD; $n=3$.

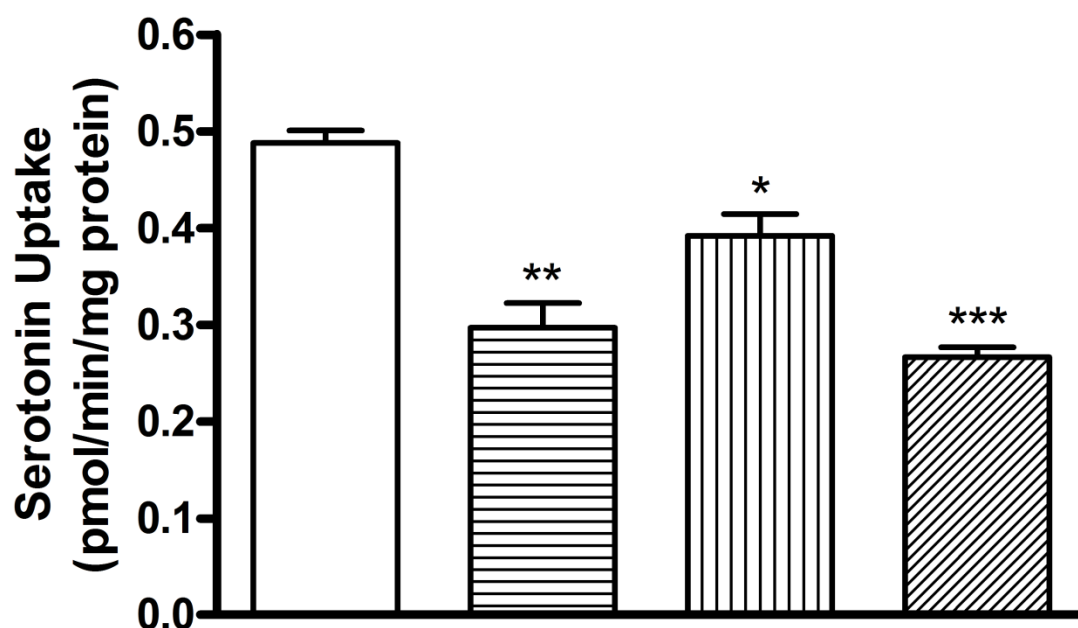


Figure 5.2. Metformin inhibits serotonin uptake in Caco-2 cell monolayers. AP uptake of [^3H]serotonin in Caco-2 cell monolayers was assessed in the absence of metformin (open bar), in the presence of metformin [10 mM] applied concurrently with serotonin (horizontal hatched bar), in the presence of metformin that is pre-loaded in Caco-2 cells (in the pre-incubation buffer 10 mM for 30 min but not applied concurrently with serotonin) (vertical hatched bar), or in the presence of metformin both applied concurrently with serotonin and pre-loaded in cells (angled hatched bar). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data represent mean \pm SD; $n=3$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control.

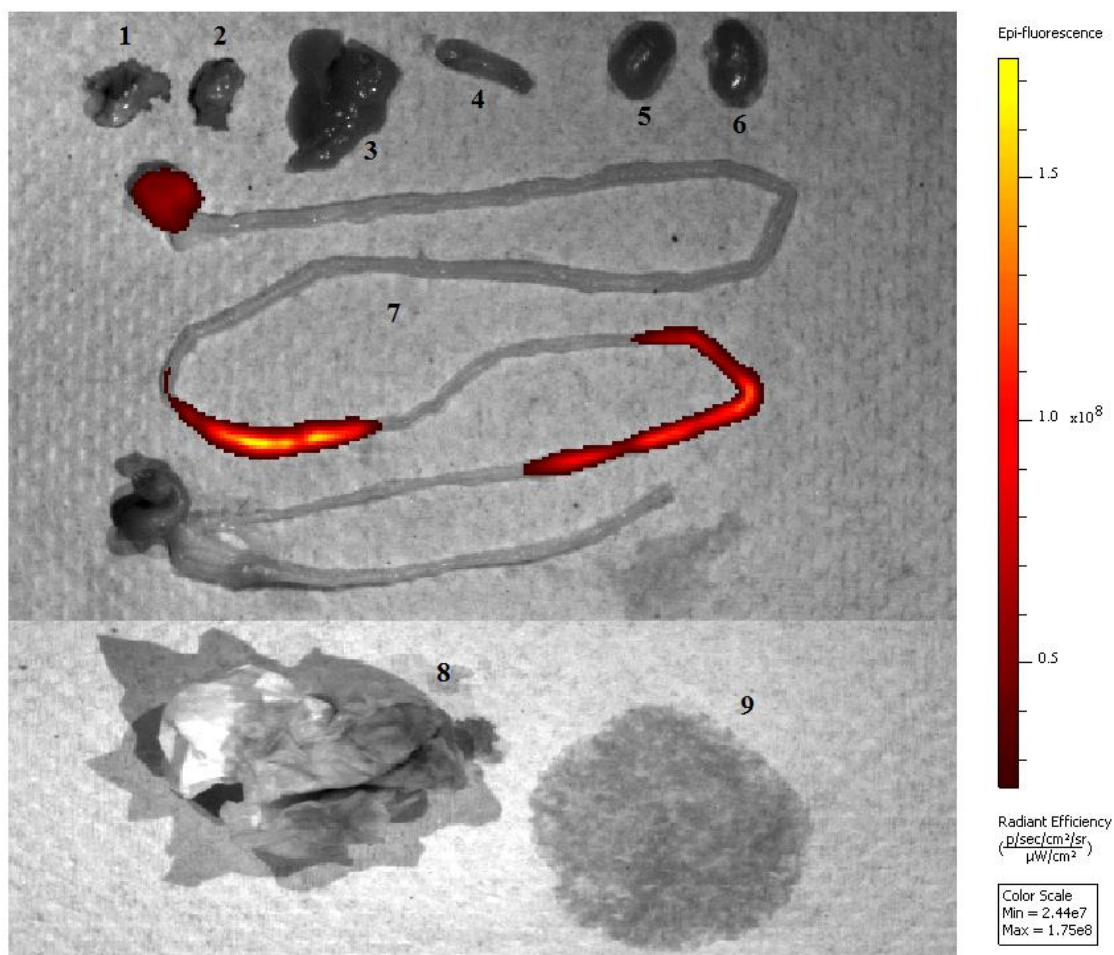


Figure 5.3. Biodistribution of Alexa Fluor 688-conjugated dextran after oral dose.

Alexa Fluor 688-conjugated Dextran [10kD, 50 μ M, 50 μ L] was administered by oral gavage to a male BALB/c mouse. Forty five min later, the animal was sacrificed under anesthesia, and major organs, namely, lung (1), heart (2), liver (3), spleen (4), kidneys (5, 6), stomach and the entire intestine (7), as well as blood (8) and urine (9) were collected and fluorescent signal was measured. The fluorescent dextran was detected only in the stomach and the intestine.

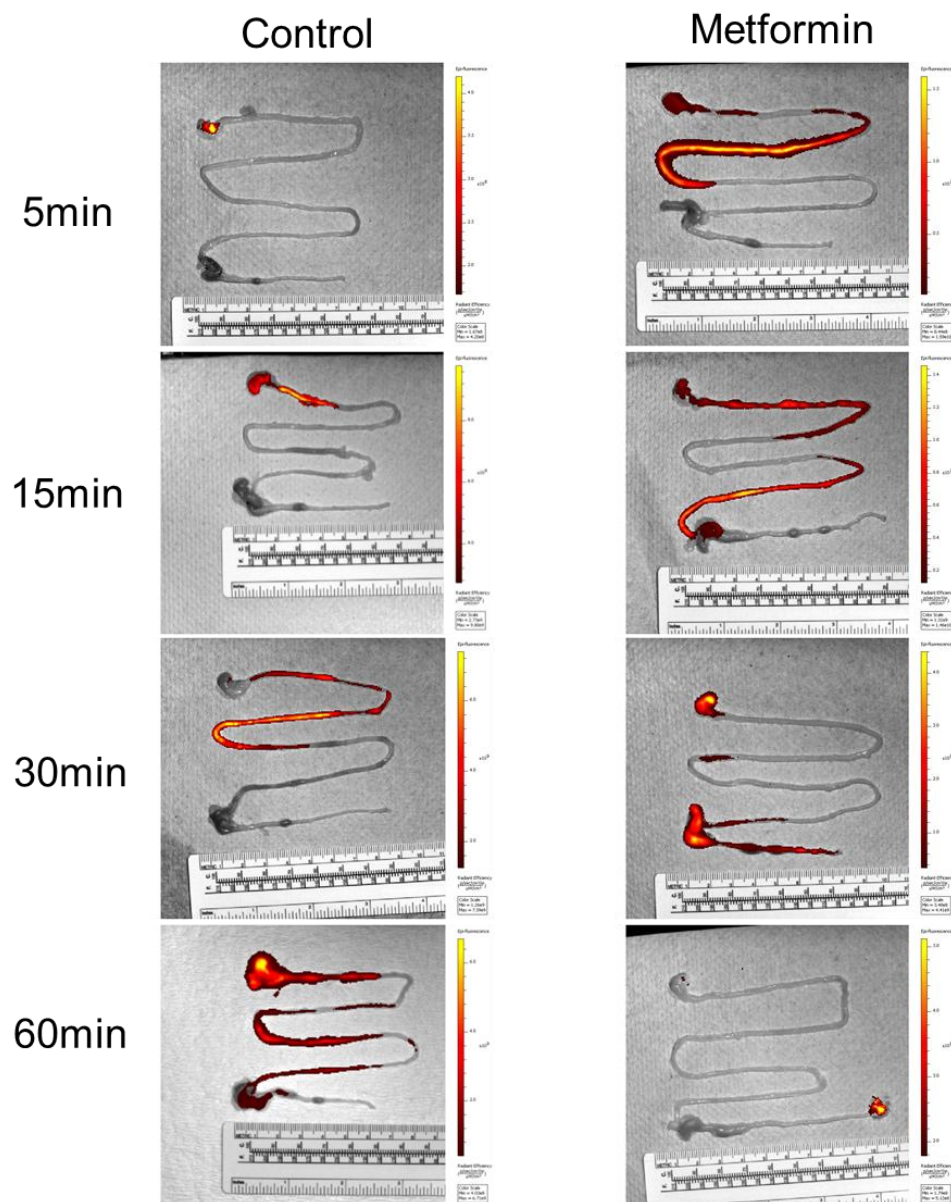


Figure 5.4. Intestinal transit of fluorescent dextran in control (saline-treated, left panel) and metformin-treated (right panel) mice. Saline [50 μ L] or metformin [15mg/kg, 50 μ L] was administered orally to mice 30 min before the oral dose of Alexa Fluor 688-conjugated Dextran [10kD, 50 μ M, 50 μ L]. At each time point, animal was sacrificed, and the stomach and the entire intestine were collected and imaged. The intestinal transit of fluorescent dextran over time is shown.

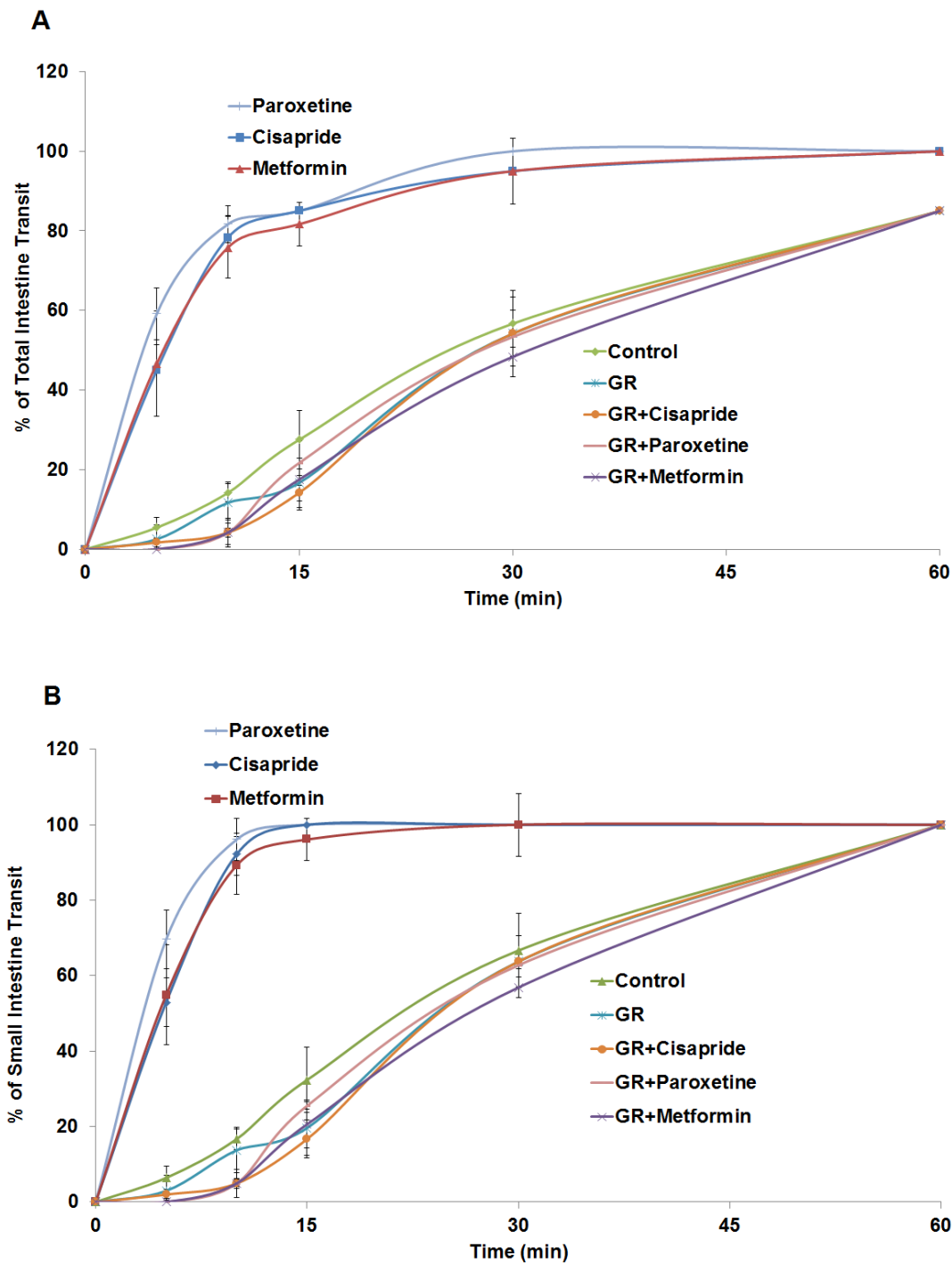


Figure 5.5. Quantification of the intestinal transit of fluorescent dextran. The distance from the distal region of the stomach to the anterior most region of the fluorescent signal in the intestine was designated as the intestinal transit of dextran. To

represent this value as a percentage of the total length of the intestine (~40 cm), the intestinal transit was divided by 40 (A). To represent this value as a percentage of the length of the small intestine (~34 cm), the intestinal transit was divided by 34 (B). Percentage of the intestinal transit of fluorescent dextran over time after different treatment groups are shown in the figure. GR represents GR 113808. Data represent mean \pm SD; n=3.

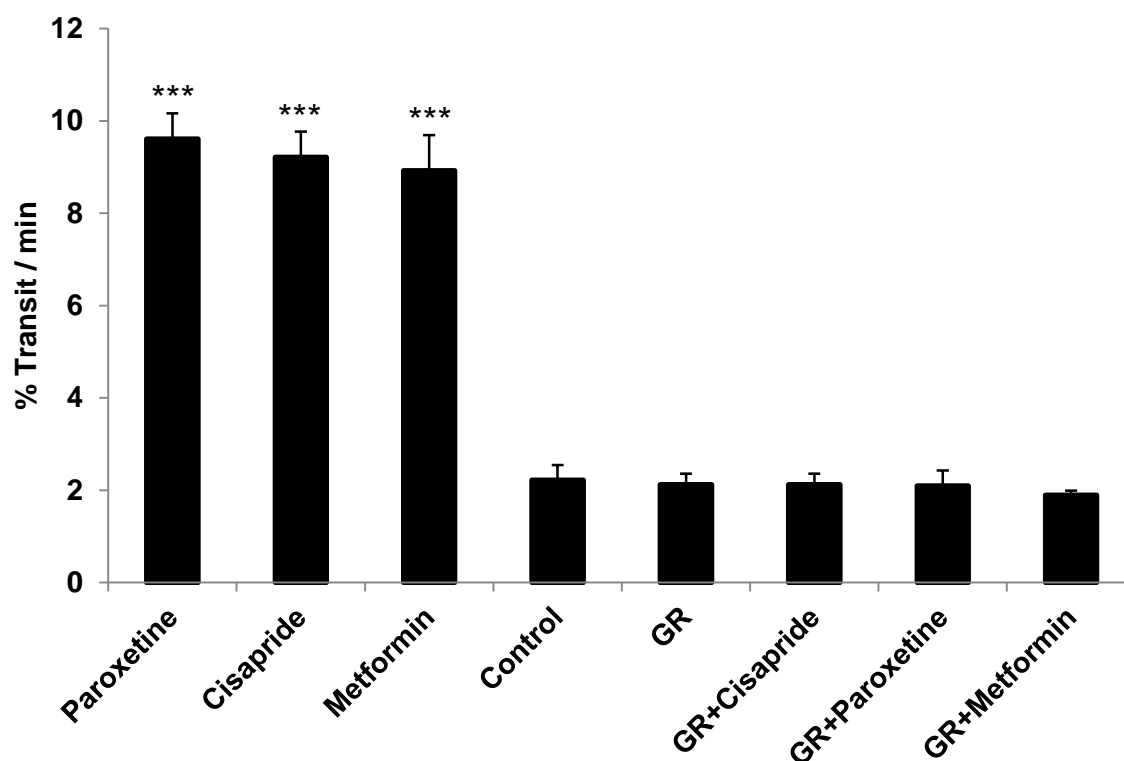


Figure 5.6. Small intestine motility. The motility of the small intestine was determined by dividing the percentage of dextran transit in the small intestine by the time required to travel that distance (i.e., percentage of small intestinal dextran transit per minute). Motility of the small intestine after different treatment groups are shown in the figure. GR represents GR 113808. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data represent mean \pm SD; n=3. ***p<0.001 compared to the control.

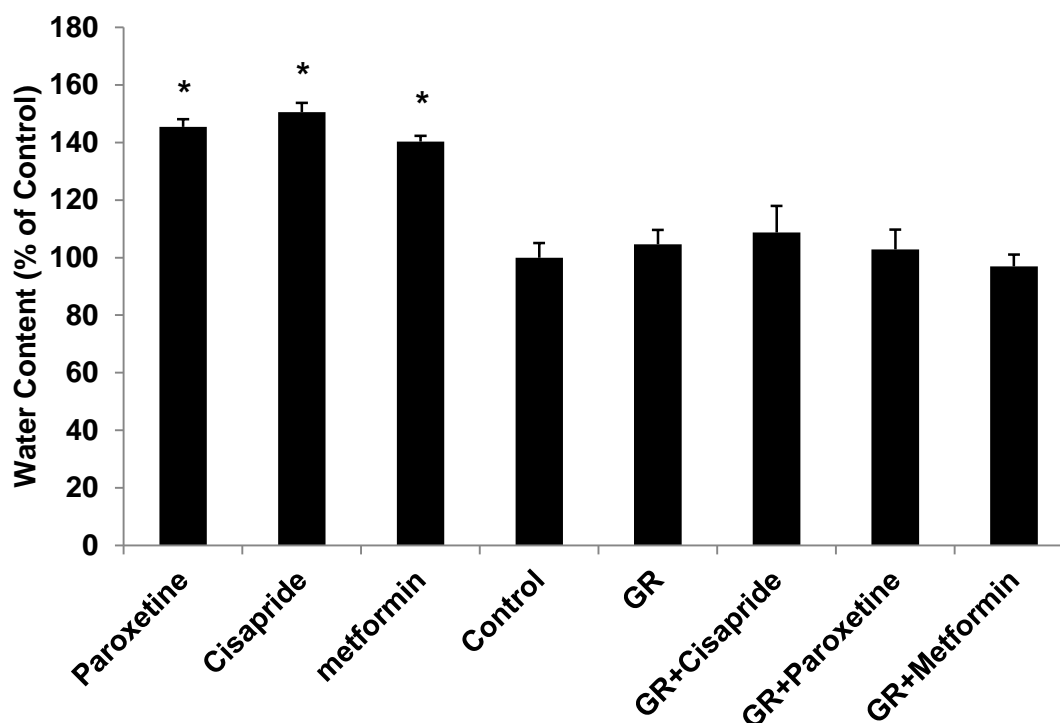


Figure 5.7. Descending colon water content in mice after oral administration of metformin, paroxetine, and cisapride. Sixty min post treatment, the abdominal region of the animals was scanned, and the large intestinal lumen at the junction of the transverse colon and descending colon was selected for imaging. The proton content in the selected area was quantified and the spectra for water present in this area were generated. These spectra were analyzed by MatLab[®] software to generate a water peak. The area under the curve (AUC) of this water peak was used as an indicator of water content in the selected area of the large intestine. Water content (percentage of control) in each treatment group is shown. GR represents GR 113808. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Data represent mean \pm SD; n=9 (3 scans for each animal \times 3 animals), *p<0.05 compared to the control.

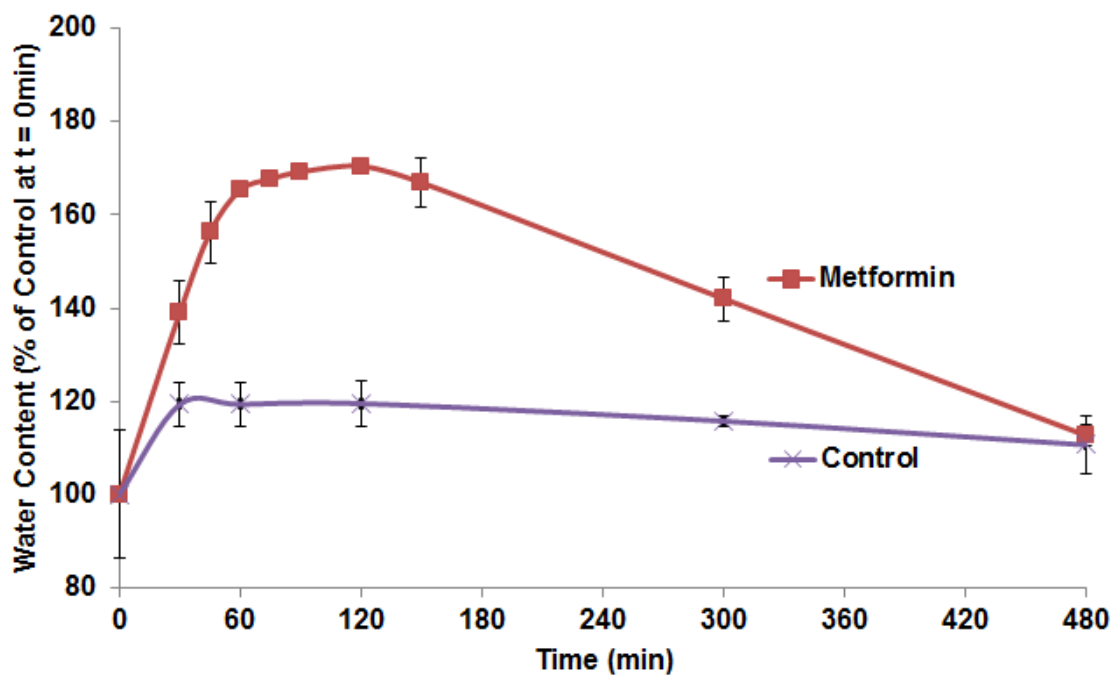


Figure 5.8. Metformin-induced water content increase in the large intestine of mice as a function of time. Water content was quantified using similar method as described in Figure 5.7. Water content as a function of time in metformin- and saline (control)-treated mice were generated over eight hours following metformin oral administration. Water content (percentage of control at $t = 0$ min) in each treatment group is shown in the figure. Data represent mean \pm SD; $n=3$.

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CHAPTER 6

CONCLUSIONS

Identifying the Intestinal Transporters of Metformin

The studies undertaken in this dissertation project have focused on elucidating the role of transporters in the intestinal absorption and adverse effects of metformin. Metformin, or 1,1-dimethylbiguanide, is a small, hydrophilic molecule that contains a net positive charge at physiological pH (Saitoh et al., 2004), and is a substrate for several cation-selective transporters (Koepsell et al., 2007). These metformin transporters play significant roles in the absorption, distribution, and elimination of this drug, as well as its pharmacology and adverse effects, as discussed in previous chapters.

In Chapter 2, it was shown that four cation-selective transporters, namely, organic cation transporter 1 (OCT1), plasma membrane monoamine transporter (PMAT), serotonin reuptake transporter (SERT) and choline high-affinity transporter (CHT), mediate the apical (AP) uptake of metformin in Caco-2 cell monolayers, a well-established model of the human intestinal epithelium. The scientific significance of this finding is detailed below.

To the best of our knowledge, this is the first report demonstrating that endogenous monoamine transporters, namely SERT and CHT, transport metformin. SERT and CHT play important roles in the physiology of the central nervous system (CNS) and

peripheral organs such as the intestine, and mediate various pivotal normal biological functions. The interactions between metformin and these transporters, which have not been investigated, could profoundly impact the pharmacology of metformin, and could cause significant drug-drug interactions (DDIs). For example, in this dissertation work, in the process of investigating the interaction between metformin and SERT, the mechanism underlying the intestinal adverse effect of metformin was discovered (Chapter 5).

It is a remarkable finding that as many as four transporters are involved in the AP uptake of metformin in Caco-2 cells, and possibly in the intestine. This discovery significantly improves our understanding of the pharmacokinetics of metformin, and provides insights into potential DDIs involving metformin. The high dose of metformin (850-1000mg) administered to patients with type II diabetes could lead to a very high concentration of the drug in the intestinal lumen, which could potentially saturate intestinal cationic transporters. However, the four transporters identified in this research project, which exhibit varying affinities and capacities for metformin, could work together to mediate the AP uptake of metformin despite its high concentration in the intestine, and compensate for each other in mediating metformin uptake, if one of them is saturated or inhibited. Therefore, it is unlikely that metformin is a victim drug of intestinal transporter-mediated DDIs. However, one cannot ignore the possibility that the high dose of metformin administered could result in intestinal DDIs involving other co-administered cationic drugs that exhibit high affinities for the transporters identified here.

It is notable that some cation-selective transporters expressed in the intestine are functionally inactive in metformin uptake. For example, studies in Chapter 2

demonstrate that metformin is a substrate of OCT3, a transporter that is highly expressed in the AP membrane of Caco-2 cell monolayers; however, OCT3 does not play any role in mediating the AP uptake of metformin. Conversely, OCT1 and CHT, which are expressed at significantly lower levels compared to OCT3, play a significant role in the AP uptake of metformin (Chapter 2). A plausible explanation for the functional inactivity of OCT3 in metformin uptake in the intestine is unknown, and is worthy of further investigation in order to fully understand the physiology of cation transporters. Nonetheless, this discovery reiterates that one needs to exercise caution and cannot assume that the expression of a transporter assures its function in mediating transport of its substrates, and that gene expression levels do not necessarily translate into functional activity.

The novel chemical inhibition scheme developed in this dissertation work (Chapter 2) provides an economical and easy way to investigate the contributions of individual transporters to the uptake of substrates even when selective inhibitors are not available for all the transporters being investigated. While gene knockout or knockdown studies enable evaluation of the contribution of individual transporters, these studies are labor- and time-intensive, and sometimes in single transporter (mSert) knockout animals, other related transporters (mOcts) are up-regulated (Baganz et al., 2008; Chen et al., 2001). Furthermore, the methodology and strategy used to develop this novel chemical inhibition scheme significantly impact areas beyond cation transporters. For example, a similar strategy could be applied to other fields of research to resolve complex problems involving several players, such as drug metabolism by multiple enzymes, cell signaling pathways implicating several up- or down-stream factors, multiple cell receptors contributing to a biological function, etc.

AP Localization of OCT1

In Chapter 3, it was demonstrated that OCT1 (mOct1) is localized in the AP membrane of Caco-2 cells, and human and mouse intestines. This finding contradicts previous studies suggesting the BL localization of OCT1 (mOct1) in Caco-2 cells and enterocytes (Giacomini et al., 2010; Jonker et al., 2001; Koepsell et al., 2007; Muller et al., 2005; Wang et al., 2002; Watanabe et al., 2002a; Watanabe et al., 2002b). The evidence for localization of OCT1 (mOct1) in the AP membrane comprised functional studies as well as immunocytochemistry and confocal microscopy, which conclusively showed that OCT1 (mOct1) is localized in the AP membrane. This finding about the AP localization of OCT1 in Caco-2 cells and intestinal tissues is very significant because both the publication by the international transporter consortium and the regulatory guidance, based on previously published reports, suggested that OCT1 (mOct1) is in the BL membrane. The findings of the studies reported in Chapter 3 will change our understanding of this drug transporter, which would impact research, drug development, and regulatory guidance for industry as described below.

This finding warrants the re-evaluation and re-interpretation of previous drug disposition and DDI studies where conclusions were drawn based on BL localization of OCT1 in enterocytes. Since 2001, several studies have applied the notion that OCT1 is localized basolaterally in the intestine to interpret the pharmacokinetics of organic cations. Furthermore, several reviews on drug transporters, as well as the US Food and Drug Administration (FDA) guidance for industry on Drug Interactions state that OCT1 is localized in the BL membrane of the intestine. Since the FDA guidance impacts

numerous drug development decisions involving compounds that are likely or known substrates/inhibitors of OCT1, a re-evaluation and revision of this document is warranted.

The assumption of a BL localization of OCT1 underestimates DDIs of organic cations in the intestine. It is known that a large portion (~40%) of orally administered drugs are organic cations or weak bases at physiological pH (Neuhoff et al., 2003). Following oral administration, cationic transporters such as OCT1, play an important role in the intestinal absorption of these organic cation drugs, during which the luminal (AP) concentrations of the organic cations are significantly higher than their concentrations in the blood side (BL). Therefore, the potential to inhibit OCT1 resulting in OCT1-mediated DDIs for co-administered organic cations is higher if this transporter is localized in the AP membrane of enterocytes, and as such, the assumption that OCT1 is basolaterally localized could cause a substantial underestimation of OCT1-mediated DDIs in the intestine.

It is well-established that OCTs, such as OCT1 and 3 are localized in the sinusoidal (BL) membrane of human hepatocytes, and are responsible for the uptake of cations from the blood into the hepatocytes (Chen et al., 2010; Faber et al., 2003; Nies et al., 2009); similarly in the kidney, OCT2 is localized in the BL membrane of human renal proximal tubules and is responsible for the uptake of cations from the blood (Karch et al., 2000; Motohashi et al., 2002; Okuda et al., 1996). The location of OCTs in elimination organs such as liver and kidney clearly signify their biological functions as uptake transporters. Therefore, in an absorption organ such as the intestine, uptake transporters should be localized in the AP membrane of the enterocytes in order to take up substrates from the luminal side based on the concentration gradient of the substrates. For instance, OCT3 is

known to be localized in the AP membrane of enterocytes (Koepsell et al., 2007; Muller et al., 2005). Generally, transporters belonging to the same family are located in the same membrane in polarized tissues, which would suggest that OCT1, like OCT3, is also localized in the AP membrane of enterocytes. Although it can be argued that OCT1 is localized in the BL membrane to take up circulating organic cations into the enterocytes, the affinities of most OCTs for organic cations are too low (e.g. apparent K_m values $>500\mu\text{M}$) (Koepsell et al., 2007; Lee et al., 2002) for these transporters to play a role in this process.

The AP localization of OCT1 and other cation-selective transporters coupled with a lack of cation-selective transporters in the BL membrane reflects the important self-protective mechanism of the intestine. From an evolutionary point of view, the intestinal localization pattern of transporters is the result of natural selection. In nature, a wide range of substances, including many toxins and biologically active alkalines are organic cations. As the first barrier of oral absorption, the intestine needs to prevent the absorption of these toxins for survival of the organism. The presence of AP polyspecific cation-selective uptake transporters and the absence of BL efflux transporters ensure that toxins are either metabolized and/or effluxed back into the lumen, so as to prevent their absorption into the blood stream.

Role of Transporters of Endogenous Monoamines in Oral Absorption and Adverse Effects of Metformin

Studies in Chapter 4 and 5 have shown for the first time that a transporter of endogenous monoamine (SERT) can mediate the oral absorption and gastrointestinal (GI) adverse effects of an exogenous drug (metformin). Interactions between an endogenous

monoamine transporter and exogenous drugs, which is an unexplored area, could potentially explain many unknown mechanisms of the pharmacology of these drugs.

SERT plays a role in metformin pharmacokinetics, as demonstrated in mice where, using chemical inhibition or gene knockout studies, the mouse serotonin reuptake transporter (mSert) was shown to play a role in metformin oral absorption *in vivo* (Chapter 4). The contribution of mSert to the overall oral absorption of metformin was found to be ~10%, which is plausible, as in Caco-2 cells, the contribution of SERT to the AP uptake of metformin (not overall absorption) is ~20% (Chapter 2).

The substrate specificity of SERT toward metformin could have significance in the pharmacology of metformin in CNS. A recent study has reported that metformin traverses the blood-brain barrier (BBB) and accumulates in the rat brain following oral administration (Labuzek et al., 2010). However, the mechanism underlying metformin's permeability across the BBB has not been investigated. It was reported that SERT is expressed in the BBB, and localized in both the luminal and abluminal sides of mouse brain capillary endothelial cells (Wakayama et al., 2002), indicating that this transporter could play a role in enabling its substrate to cross the BBB. Therefore, the findings that metformin is a substrate of SERT presented in this dissertation work provides a mechanism that enhances the permeability of metformin across the BBB, although further studies are needed. This is a highly significant discovery, as the distribution and pharmacological effects of metformin in the CNS are poorly understood. Results from this dissertation work could provide new perspectives and insights into metformin's potential efficacy in brain tumors and other CNS diseases, and the role of SERT in mediating the transport of other cationic drugs across the BBB. This unexplored area

would have great impact on our current understanding of transporters and in developing new therapeutic approaches targeting the CNS.

This is the first study to show that metformin is an inhibitor of SERT. This inhibition leads to a decrease in serotonin reuptake and increase in serotonin concentration in the GI tract, resulting in GI adverse effects. This is also the first study to directly show the mechanism underlying metformin-mediated GI adverse effects. These adverse effects, including diarrhea (in ~50% of patients), nausea and vomiting (~25%) are commonly observed in clinic (Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009; Dandona et al., 1983). Due to the wide usage of metformin and the high incidence of its GI adverse effects, millions of patients suffer from metformin-induced GI distress. In severe cases (~5%), these adverse effects lead to non-compliance and discontinuation of metformin treatment (Bailey, 1992; Bouchoucha et al., 2011; Bristol-Myers-Squibb, 2009; Dandona et al., 1983; Krentz et al., 1994) (Chapter 5). This dissertation research has uncovered a previously overlooked relationship between the exogenous drug, metformin, and a transporter for an endogenous monoamine, SERT. Similar relationships between endogenous monoamine transporters and other cationic drugs could also result in toxicities and adverse effects for which the underlying mechanisms have not been explained.

The imaging strategy implemented in Chapter 5 (intestinal transit by fluorescent imaging and water content by magnetic resonance imaging (MRI)) provides novel and easy ways to study GI adverse effect, such as diarrhea. These methods can be utilized in other animals, and also applied to human clinical studies. In larger animals and human subjects where the imaging area will be larger than that in small rodents and the

resolution could be high enough to determine the movement of the dye, this imaging strategy for assessing intestinal transit could potentially be modified as a non-invasive method. Moreover, radiolabeled compounds could be used in human subjects, and imaging techniques such as computed tomography (CT), positron emission tomography (PET), and single photon emission computed tomography (SPECT), could clearly detect the accurate location of the compound to assess intestinal transit. Water content scanning is a sensitive and accurate method to determine diarrhea. Compared to other methods, such as water content in feces, which involves unpleasant procedures such as processing watery feces samples, this *in vivo* scanning method is a good alternative. This non-invasive and rapid MRI imaging method can be easily applied to other animals and human subjects. In summary, this dissertation work provides not only new mechanisms and insights into this unexplored research area, but also methodologies that are applicable in such areas of research.

Future Directions

This dissertation project has led to significant new findings in the area of metformin's pharmacokinetics, pharmacology and adverse effects, and has greatly improved our understanding of this widely used drug. However, there are several unanswered questions that need further investigation, such as: a likely rationale for the biological inactivity of highly expressed transporters (such as OCT3 that does not mediate metformin AP uptake in Caco-2 cells), as well as the contributions of other transporters (OCT1, PMAT and CHT) to the oral absorption of metformin *in vivo*.

Additionally, based on results generated in this study, metformin-mediated DDIs need to be further investigated. Although metformin is unlikely to be a victim drug of

DDIs during intestinal absorption, it could behave as a perpetrator drug that affects the absorption of other co-administered cationic drugs with high affinities for cation-selective transporters. Due to the wide use of metformin for the treatment of diabetes, it is highly likely that this drug is responsible for DDIs with other medications that are co-administered in diabetics, including but not limited to: (1) hypertension medications, such as acebutolol and prazosin; (2) H₂ receptor antagonists, such as ranitidine, cimetidine, and famotidine; (3) antibiotics and antiviral drugs, such as acyclovir, ritonavir, nelfinavir, and pentamidine; (4) antidepressants such as desipramine, and citalopram. These cationic drugs, which are known to interact with cationic transporters (Koepsell et al., 2007), could be used concomitantly with metformin in diabetic patients. Therefore, clinical studies are warranted to clearly demonstrate the effect of metformin on the intestinal absorption and exposure of co-administered drugs.

Since metformin exhibits anti-cancer efficacy over a broad spectrum of cancers, including breast, endometrial, ovarian, prostate, colon, gastric, cervical and lung cancer (Ashinuma et al., 2012; Bodmer et al., 2012; Chlebowski et al., 2012; Hanna et al., 2012; He et al., 2011; Kato et al., 2012; Tseng, 2012; Wu et al., 2012; Xiao et al., 2012), cation-selective transporters could be critical to its anti-cancer efficacy (Zhang et al., 2013). The identification of two new transporters of metformin in this dissertation work, namely SERT and CHT, which are both highly expressed in the CNS (and many other organs), could lead to new discoveries in the anti-cancer efficacy of metformin in cancers related to the CNS. Additionally, metformin is often used as a combination therapy in cancer along with other chemotherapeutic agents such as doxorubicin and cisplatin. Since cation-selective transporters play a role in the absorption and disposition of these

chemotherapeutic drugs, metformin could cause DDIs and affect their absorption and subsequent efficacy. Therefore, the potential for metformin-mediated DDIs in cancer therapy needs to be investigated in order to understand the anticancer effects of metformin and doxorubicin/cisplatin as combination therapies, and optimize their dosing regimens and chemotherapeutic efficacies.

Furthermore, results from the current study could be extrapolated to other cation drugs. (1) Interactions between other exogenous cationic drugs and endogenous monoamine transporters and the role of these transporters in the pharmacokinetics, pharmacodynamics and toxicity of various organic cationic drugs need to be evaluated. (2) Clinical studies should be conducted to determine the interactions between drugs and endogenous monoamine transporters, in order to clearly understand this interaction. (3) A better understanding of this interaction could provide new insights into drug development in pharmaceutical industry, so that drugs with optimized therapeutic efficacy and minimum adverse effects could be developed. These future investigations could significantly improve our understanding of various cation drugs. As mentioned above, this dissertation has provided scientific strategies as well as experimental approaches for future studies, which could benefit and guide future research to a new and fruitful avenue.

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