CATION-SELECTIVE APICAL TRANSPORTERS MEDIATE AN INTESTINAL CYCLING MECHANISM OF METFORMIN THAT ENHANCES ITS PARACELLULAR ORAL ABSORPTION IN MICE

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ABSTRACT

CHESTER LEE COSTALES: Cation-selective Apical Transporters Mediate an Intestinal Cycling Mechanism of Metformin that Enhances its Paracellular Oral Absorption in Mice
(Under the direction of Dhiren R. Thakker, Ph.D.)

Metformin is the most widely prescribed drug for type 2 diabetes mellitus; yet its *in vivo* mechanism of oral absorption has not been elucidated. A pK_a of 12.4 and $logD_{pH6.0}$ of -6.13 suggest metformin is a hydrophilic cation at all physiologic pHs, limiting its ability to cross biological membranes. However, metformin is well-absorbed with an oral bioavailability ranging from ~40-60% in man. Previous *in vitro* studies conducted using the Caco-2 Transwell® model of intestinal absorption demonstrated efficient transporter-mediated metformin apical uptake and efflux and poor basolateral egress. Kinetic modeling of these results suggested absorptive transport is predominantly paracellular, and led to the development of a novel mechanism of absorption stating that during oral absorption of metformin, transporter-mediated apical uptake and a lack of basolateral efflux leads to intestinal drug accumulation. Changes in luminal drug concentration as a result of gastrointestinal transit leads to apical efflux of metformin and its enhanced paracellular absorption. Studies presented in this dissertation evaluate this novel metformin absorption mechanism in a mouse model.

Gene expression of the mouse orthologs of putative human metformin transporters, namely organic cation transporter 1–3 (mOct1–3), multidrug and toxin

extrusion 1 (mMate1), and plasma membrane monoamine transporter (mPmat), was characterized in mouse small intestine. Stable cell lines singly-expressing these transporters were generated, and metformin uptake kinetics for each transporter was determined. Pentamidine, quinidine, and desipramine, were identified as pan transporter inhibitors and were used in subsequent mouse studies. Absorptive transport of metformin in *ex vivo* experiments using mouse intestinal tissue was similar to results previously reported for Caco-2 cell monolayers, showing high transporter-mediated apical uptake compared to apical-to-basolateral transport. Metformin orally co-administered with pentamidine demonstrated that the intestinal accumulation and absorption of metformin is transporter-mediated. Attenuation of metformin apical efflux in the intestine after oral dosing showed a decreased metformin absorption rate, suggesting an important role for apical efflux of metformin during its oral absorption. Collectively, these studies provide strong circumstantial evidence that metformin is absorbed through the hypothesized mechanism, which can account for the intestinal accumulation and oral pharmacokinetics of metformin observed in human and animal studies.

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LIST OF ABBREVIATIONS

AMPK	5’ Adenosine monophosphate-activated protein kinase
AP	Apical
AUC	Area Under the Curve
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BL	Basolateral
CHO	Chinese Hamster Ovary
C_{max}	Peak plasma concentration
FBS	Fetal Bovine Serum
HBSS	Hank’s Balanced Salt Solution
HEK	Human Embryonic Kidney
IACUC	Institutional Animal Care and Use Committee
IP	Intraperitoneal
IV	Intravenous
KBR	Krebs-Bicarbonate Ringer
K_m	Concentration of Half-maximal Velocity (Michaelis-Menten Constant)
MATE	Multidrug and Toxin Extrusion
MPP^+	1-methyl-4-phenylpyridinium
OCT	Organic Cation Transporter

P_{app}	Apparent Permeability
PD	Pharmacodynamic
PK	Pharmacokinetic
PMAT	Plasma membrane monoamine transporter
PO	Per Os (oral)
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SLC	Solute Carrier (Family)
TEA	Tetraethylammonium
T_{max}	Time to reach C_{max}
V_{max}	Maximal velocity
V_{ss}	Steady-state volume of distribution

Chapter 1

INTRODUCTION

1.A. INTRODUCTION

Metformin (1,1-dimethylbiguanide) is an orally administered drug used to reduce blood glucose levels in patients with Type 2 diabetes mellitus, formally known as non-insulin dependent or adult-onset diabetes. As the first-line therapy for this global epidemic, metformin use is extensive with nearly 60 million prescriptions dispensed in 2011 in the United States alone (IMS Institute for Healthcare Informatics, National Prescription Audit). The biguande class of anti-diabetic drugs originated in the 1920s after extracts of French lilac (also known as goat's rue), a plant used to treat diabetes since medieval times, was found to contain guanidine (Bailey, 1992). Because guanidine was too toxic for clinical use, chemical derivatives including galegine, alkylguanidines, and biguanides were synthesized. However, these drugs were largely ignored, partly due to the introduction of insulin therapy for Type 1 and Type 2 diabetes in the early 20th century. Several biguanides, including metformin (Figure 1.1), phenformin, and buformin, were first reported in the literature in the 1950s to show their glucose lowering effects (Bailey, 1992). Although phenformin and buformin saw extensive clinical use based on potent glucose lowering

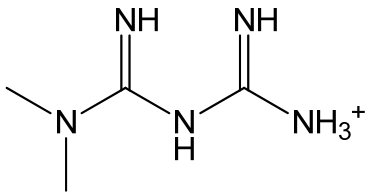

Figure 1.1: Structure of metformin

effects, both drugs were eventually withdrawn from the market due to a severe side effect, namely lactic acidosis. Because the risk of lactic acidosis associated with metformin was lower compared to other biguanides, its use was revitalized in Europe in the 1970s, and the FDA approved its use in the United States in the mid-1990s. Although the risk of lactic acidosis is still present with metformin therapy, it is much less common and typically avoidable when following generally prescribed guidelines (e.g., contraindicated for renal insufficiency).

Diabetes mellitus is a group of diseases characterized by elevated glucose levels in the blood (*e.g.*, fasting plasma glucose >125 mg/dl) and develops from the failure of the body to produce or utilize the peptide hormone, insulin. Type 1 diabetes, previously called juvenile diabetes, results from the inability of the beta cells of the pancreas to produce insulin. The other major and most common form of this disease is Type 2 diabetes. Although usually diagnosed later in life, it can occur at any age, typically developing in the setting of insulin resistance, where cells are unable to utilize insulin efficiently combined with insulin secretory defects.

The mechanism of action of metformin exhibits tissue-specific behavior (Figure 1.2). For instance, in the liver, which is the generally accepted major pharmacological target of metformin, glucose production is reduced by decreased

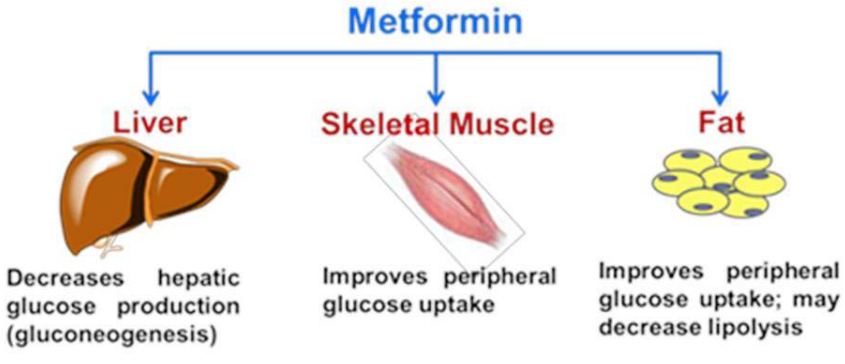

Figure 1.2 : Major sites of pharmacologic action of metformin

hepatic gluconeogenesis. In skeletal muscle tissue, increased metformin-mediated glucose uptake via an upregulation of glucose transporter 4 (GLUT4) in the plasma membrane helps reduce blood glucose levels (Hundal et al., 1992; Lee et al., 2011). 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., 2009). Although metformin is an insulin sensitizer, it does not increase insulin secretion, and therefore, hypoglycemia is rarely seen as a result of metformin monotherapy. In fact, insulin secretion is reduced in the setting of metformin treatment (Leclerc et al., 2004). In the small intestine, it has been proposed that blood glucose lowering by metformin occurs by decreased glucose absorption and increased glucose utilization (Wilcock and Bailey, 1991; Ikeda et al., 2000).

In 2001, Zhou *et al.* identified 5' adenosine monophosphate-activated protein kinase (AMPK) as the major intracellular target of metformin (Zhou et al., 2001). AMPK, a heterotrimeric enzyme that is well-conserved across most species, has been described as a cellular energy switch based on its regulatory control over a number of biosynthetic pathways, including glucose uptake and fatty acid oxidation. Modulation of these intracellular processes are effected in response to changes in the AMP:ATP ratio. In low energy states, ATP-dependent processes are reduced via direct phosphorylation of metabolic enzymes. While AMPK is known to mediate these processes, metformin is believed to activate AMPK by directly binding to various subunits of the enzyme, or indirectly through upstream processes such as inhibition of complex I of the respiratory chain in mitochondria (Owen et al., 2000).

1.B. METFORMIN PHARMACOKINETICS

1.B.1 Intravenous Pharmacokinetics of Metformin

Intravenous bolus dosing of metformin in human studies demonstrate that drug elimination from plasma is rapid with a terminal elimination half-life of 1.74 ± 0.11 hours (Pentikainen et al., 1979). Studies by Sirtori *et al.* (1994) or Pentikainen *et al.* (1979) and Tucker *et al.* (1981) show pharmacokinetic profiles of metformin that are consistent with two- and three-compartment models, respectively, suggesting initial distribution of metformin from the central compartment to peripheral tissues. Urinary recovery of unchanged metformin after intravenous dosing ranged from 78.9–99.9%, with no detectable metformin in the feces, suggesting that this drug is predominantly cleared via the kidneys. Estimated renal and plasma clearance of 454 ± 47 and 459 ± 6 ml/min, respectively, were obtained after a 500 mg intravenous dose of metformin (Pentikainen et al., 1979). Similar values were stated in the study by Sirtori *et al.* (1994) in which the half-life of metformin was assessed to be 1.52 ± 0.3 hours, while Tucker *et al.* (1981) reported a more prolonged terminal half-life ranging between 2.51–7.04 hours. Since the renal clearance of metformin is ~5-fold higher than creatinine clearance, active tubular secretion was implicated as the principal mechanism of its elimination. Analysis by two-dimensional thin-layer chromatography of urine samples after intravenous administration of metformin showed no evidence of its metabolism (Pentikainen et al., 1979). Protein binding of metformin is negligible based on equilibrium dialysis and ultrafiltration assays (Pentikainen et al., 1979; Tucker et al., 1981). In these studies, Pentikainen *et al.* (1979) reported the volume of distribution of metformin to be 69 ± 4.5 L, whereas Tucker *et al.* (1981) estimated a higher value of 276 ± 136 L.

1.B.2. Oral Pharmacokinetics of Metformin

Clinical pharmacokinetic studies show that metformin is well-absorbed, although incompletely (~20–30% in feces), with a reported oral bioavailability between 40–60% (Pentikainen et al., 1979; Tucker et al., 1981). The C_{max} of metformin ranges from ~1–3 μg/ml with an estimated T_{max} of ~2–3 hours. Higher metformin doses of 1.0 and 1.5 grams showed decreased bioavailabilities of 38% and 35%, respectively (Tucker et al., 1981). Similarly, non-linear increases in metformin C_{max} and AUC with increasing oral doses were observed by Sambol *et al.* (1996). Deconvolution analysis of metformin oral pharmacokinetic data suggests that its absorption is nearly complete within six hours, and is consistent with its absorption in the small intestine (Tucker et al., 1981). In these three separate studies, the terminal rate of elimination of metformin from plasma was slower following oral administration compared to intravenous administration, which is indicative of flip-flop kinetics. Collectively, these results suggest that the oral absorption of metformin is mediated by a saturable process in the intestine.

1.B.3. Metformin Metabolism

Although numerous studies suggest that metformin is not metabolized to any appreciable degree in humans (see section 1.B.1), less than 100% recovery has been reported during mass balance studies (Tucker et al., 1981). This finding was inconsistent with data reported by Pentikainen *et al.* (1979), showing ~100% of [^{14}C]metformin in urine as unchanged drug after intravenous administration. Despite the lack of direct evidence of detectable metabolites from any clinical study, metabolism of metformin by rat P450 enzymes was investigated, and ~80% of the drug was recovered in *in vitro* experiments using rat liver homogenates; these data suggest possible metabolic clearance

mechanisms for metformin (Choi et al., 2006). The authors also suggest that the 27% decrease in metformin plasma AUC in rats following its administration via the portal vein compared to delivery via the jugular vein was due to hepatic first pass metabolism. A subsequent study also conducted in rats assessed changes in non-renal (presumably metabolic) clearance of metformin in the presence of cytochrome P450 inhibitors (~24–79% lower clearance) and inducers (57% higher clearance) (Choi and Lee, 2006). Based on these changes in metformin clearance in the presence of P450 modulators, the authors proposed that rat Cyp2C11, 2D1, and 3A1/2 were involved in metformin metabolism. However, it should be noted that the use of P450 inhibitors such as quinine also significantly decreased calculated renal clearance of metformin by ~33–59%, a process mediated by drug transporters (Section 1.B.4). The effects of these specific inhibitors and inducers on transporter activity or expression have not been thoroughly investigated, although recent studies have begun to address this issue. For example, metformin has been shown to decrease P-glycoprotein (P-gp) expression by downregulating the multidrug resistance 1 (MDR1) gene in MCF-7 breast cancer cells (Kim et al., 2011). Collectively, these studies investigating metformin metabolism are inconclusive, having failed to detect a possible metabolite or show any evidence of metabolism by specific P450 isozymes through *in vitro* experiments.

1.B.4 Drug Transporters Involved in Metformin Disposition

Organic cation transporter 1 (OCT1; SLC22A1) was the first OCT to be cloned following its identification in rat in 1994 (Grundemann et al., 1994). In these studies, complementary DNA was isolated from rat kidney encoding a 556 amino acid, 12 transmembrane domain protein that was distinct from ATP-dependent multidrug

transporting proteins (also known as ATP-binding cassette (ABC) transporters). OCT2 (SLC22A2) and OCT3 (SLC22A3) were subsequently cloned in 1996 (Okuda et al., 1996) and 1998 (Kekuda et al., 1998), respectively. OCT1 is primarily expressed in the liver, OCT2 is shows highest expression in the kidneys, while OCT3 is more widespread with high expression in liver, heart, skeletal muscle, and placenta (Koepsell et al., 2007) (Figure 1.3; Table 1.1). OCT3 is also known as the extraneuronal monoamine transporter (EMT) due to its role as a transporter of neurotransmitters such as dopamine, serotonin, and norepinephrine (Wu et al., 1998). OCT1-3 are driven in an electrogenic manner independent of sodium and proton gradients and can translocate substrates across membranes in either direction. Because they have the ability to transport a variety of endogenous and exogenous organic cations, these transporters are also described as polyspecific transporters.

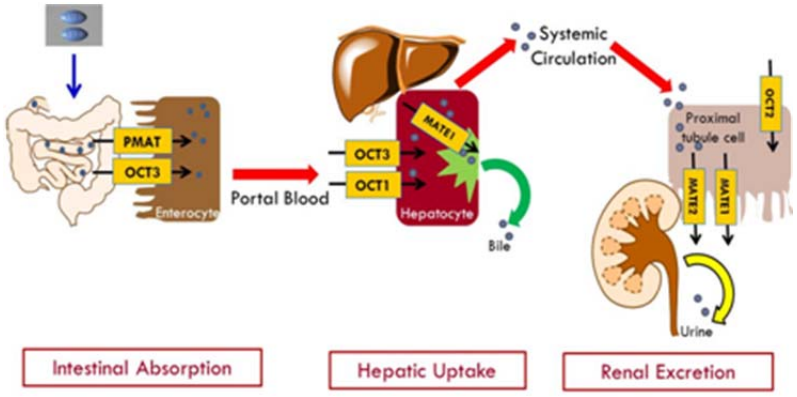

Figure 1.3: Transporters involved in the disposition of metformin. Adapted from Zolk 2009.

Metformin was first identified as an OCT substrate by Wang *et al.* (2002) through studies conducted in rOCT1 transfected chinese hamster ovary (CHO) cells and Oct1 knockout mice (Wang et al., 2002). Phenformin and buformin, two other biguanides evaluated in this study, showed transporter affinities that were >7-fold higher than that of metformin. Mouse studies on the tissue distribution of metformin showed an ~30-fold

higher accumulation of the drug in the liver of normal mice compared to Oct1 knockout animals, and small intestinal accumulation of metformin was ~3–7-fold higher in normal mice after intravenous dosing (Wang et al., 2002). A lack of difference in kidney accumulation of metformin suggest the presence of other uptake transporters of metformin in the basolateral membrane of renal proximal tubule cells which may be normally expressed in kidney tubules or upregulated as a compensatory mechanism. Studies conducted with Oct1/Oct2 double knockout mice demonstrated increased steady-state concentrations of the prototypical cation-selective transporter substrate, tetraethylammonium (TEA), providing strong evidence that Oct2 also mediates metformin uptake in the mouse kidney (Jonker et al., 2003).

More recently, the plasma membrane monoamine transporter (PMAT; SLC29A4) was cloned in 2004 (Engel et al., 2004). Although this transporter belongs to the equilibrative nucleoside transporter (ENT) family, its broad cationic substrate specificity yet poor ability to transport a majority of naturally occurring nucleosides and nucleobases suggest that it is better classified as a polyspecific organic cation transporter (Zhou et al., 2010). Transporter activity by PMAT showed a strong pH-dependence, where the uptake of the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) was greatly increased when the pH of the extracellular buffer was lowered from 7.4 to 6.6 (Xia et al., 2007). Furthermore, uptake activity was virtually abolished when the extracellular pH was increased to 8.4. In 2007, Zhou *et al.* demonstrated that metformin is a substrate for PMAT, and that this transporter is expressed in the apical membrane of the human small intestine.(Zhou et al., 2007). These results were the first evidence to identify a potential apical uptake transporter of metformin from the intestinal lumen using both localization

and functional data. However, these data are circumstantial since direct uptake of metformin via these transporters in either intestinal tissue or cell-based models has not been demonstrated.

The multidrug and toxin extrusion (MATE) proteins are the most recent transporters identified in the literature to show metformin substrate activity. Cloned in 2005, MATE1 (SLC47A1) was identified as the long-hypothesized mammalian proton/organic cation exchanger responsible for excretion of cationic toxins and endogenous compounds into urine and bile (Otsuka et al., 2005). A year later, a report identifying and characterizing the human kidney specific MATE2-K (SLC47A2) was published (Masuda et al., 2006). Uptake of metformin was first demonstrated by MATE2-K, and MATE1 soon thereafter, suggesting that these transporters mediate the apical efflux of metformin from liver and kidney cells (Masuda et al., 2006; Tanihara et al., 2007) (Figure 1.3).

Parallel studies to this dissertation work in the Thakker laboratory have identified two additional SLC transporters showing metformin substrate activity. Using the choline transporter specific inhibitor hemicholinium-3, Han *et al.* (2013) have shown evidence that the high affinity choline transporter (CHT1; SLC5A7) plays a role in the apical uptake of metformin in Caco-2 cell monolayers, a cellular model which mimics the human small intestine. Additionally, the involvement of the serotonin transporter (SERT; SLC6A4) in mediating the uptake of metformin was demonstrated using a singly-expressed serotonin transporter cell line. Like CHT1, SERT also mediates apical uptake of metformin into Caco-2 cell monolayers.

The ABC transporters, P-gp and breast cancer resistance transporter (BCRP), were reported to transport metformin based on studies using pooled inside-out vesicles prepared from human placental tissue (Hemauer et al., 2010). Evidence provided by Hemauer *et al.* (2010) for P-gp-mediated transport showed decreased vesicular uptake in the presence of the P-gp inhibitor, verapamil. However, at the concentration used in this study, verapamil (200 μM) could also inhibit organic cation transporters (i.e., OCT1 = 1.2 – 2.9 μM; OCT2 = 13.4 – 85 μM; MATE1 = 28 μM; MATE2-K = 32 μM). KO143 (25 nM and 1 μM) was also utilized to inhibit other ABC transporters suggesting that BCRP is another efflux transporter of metformin, although the inhibitory effect of this inhibitor on OCT- and MATE- mediated transport has not been investigated (Hemauer et al., 2010). However, studies by Proctor *et al.* (2008) using the P-gp selective inhibitor 1 μM GW918 showed no effect on metformin efflux from metformin preloaded Caco-2 cell monolayers. Additionally, in single-pass intestinal perfusion (SPIP) studies in rats, effective permeability (P_{eff}) of metformin was not affected by duodenal co-perfusion of 400 μg/ml (~880 μM) of verapamil, suggesting that P-gp does not efficiently transport metformin (Song et al., 2006). Future studies in singly-expressed P-gp or BCRP will aid in confirming these conflicting results.

Table 1.1. Putative Transporters of Metformin

Transporter Name	Transporter Abbreviation	Gene	Amino Acids (Human / Mouse)	Highest Tissue Expression
Organic Cation Transporter 1	OCT1/Oct1	SLC22A1	554 / 556	Liver
Organic Cation Transporter 2	OCT2/Oct2	SLC22A2	555 / 553	Kidney
Organic Cation Transporter 3	OCT3/Oct3	SLC22A3	556 / 551	Liver Skeletal Muscle Placenta Heart
Plasma Membrane Monoamine Transporter	PMAT/Pmat	SLC29A4	530 / 528	Brain Skeletal Muscle
Multidrug and toxin extrusion protein 1	MATE1/Mate1	SLC47A1	570 / 566	Liver Kidney
Multidrug and toxin extrusion protein 2	MATE2-K /Mate2	SLC47A2	602 / 573	Kidney
High affinity choline transporter 1	CHT1/Cht1	SLC5A7	580 / 580	Brain
Sodium-dependent Serotonin Transporter	SERT/Sert	SLC6A4	630 / 630	Lung Placenta Small Intestine

1.C. CLINICALLY OBSERVED TRANSPORTER-MEDIATED DRUG-DRUG INTERACTIONS (DDIS) WITH METFORMIN

Because metformin does not undergo extensive metabolism, DDIs with metformin are most likely to occur when cation-selective transporter substrates are co-administered with metformin. The first reported interaction of this nature was with the histamine H_2-receptor antagonist, cimetidine (Somogyi et al., 1987). Based on prior research investigating the effect of cimetidine on the renal clearance of procainamide and its active metabolite (Somogyi et al., 1983), a process that is mediated by an active tubular secretion mechanism, the authors hypothesized that the elimination of metformin could also be reduced by cimetidine. Metformin AUC in the presence of cimetidine was increased by an average of 50% in seven human subjects and the renal clearance of

metformin was decreased by 27% over 24 hours. These interactions of metformin and procainamide were previously attributed to renally expressed OCT2 despite the authors acknowledging that the IC_{50} value of cimetidine for metformin transport (73 μM) was considerably higher than estimated C_{max} concentrations (2.3–6.8 μM) following a 200 mg oral dose to patients with normal renal function (Kimura et al., 2005). The authors suggested that the metformin-cimetidine interaction mediated by OCT2 played a more significant role in the elderly and other individuals with renal dysfunction, and elevated cimetidine plasma concentrations in these populations.

With the identification of the MATE1 transporter in 2005, it was demonstrated that cimetidine (10 μM) was a potent inhibitor of MATE1-mediated efflux, decreasing TEA efflux by 45% (Otsuka et al., 2005). Subsequent studies indicated that cimetidine is a good substrate for MATE1 and MATE2-K with K_m values of 170 and 120 μM, respectively (Tanihara et al., 2007). In a separate study, K_m values for cimetidine were reported to be even lower, at 8 and 18 μM for MATE1 and MATE2-K, respectively, clearly indicating that cimetidine interactions with organic cations may in fact be due to inhibition of the MATE transporters (Ohta et al., 2009). In 2009, Tsuda *et al.* provided convincing *in vitro* evidence to support this hypothesis when apparent K_i values of cimetidine were reported to be 1.1 and 7.3 for MATE1 and MATE2-K, respectively, which was within the estimated effective plasma concentration range for cimetidine. These authors also conducted *in vitro* basolateral-to-apical transporter experiments in double-transfected MDCK-hOCT2/hMATE1 cells. Using this cell model, they showed that high cimetidine concentrations inhibited uptake of metformin via OCT2, whereas a

low cimetidine concentration reflective of normal plasma concentrations only affected efflux of metformin via MATE1.

Similar to the MATE-mediated cimetidine DDI, the MATE-specific inhibitor, pyrimethamine (Ito et al., 2010) was investigated by co-administration with a microdose (100 μg) or therapeutic dose (250 mg) of metformin in a single crossover study (Kusuhara et al., 2011). Metformin renal clearance was decreased by 23% and 35% with the microdose and therapeutic dose, respectively. At the therapeutic dose, C_{max} and AUC of metformin were significantly increased. Results from this study, as well as the growing body of work with cimetidine suggest that DDIs with metformin and other organic cations that occur at the renal level are most likely due to MATE inhibition. In 2011, the International Transporter Consortium (ITC) published a Nature Review on membrane transporters in drug development which suggested the use of cimetidine to investigate the clinical effects of OCT2 (Giacomini et al., 2010). Because this Transporter White Paper was influential in the preparation of the FDA DDI draft guidance, these DDI studies exemplify the importance of understanding the role of transporters at both apical and basolateral membranes of polarized cells that mediate drug disposition (FDA, 2012).

1.D. MECHANISMS OF INTESTINAL DRUG ABSORPTION

The small intestine is structurally complex and is well-built to serve its major function of nutrient absorption. Because the gut lumen is essentially a continuum of the outside world, the small intestine must perform this task while also restricting the free passage of xenobiotics and pathogens into the systemic blood circulation. The lumen of the intestine exhibits extensive folding along with the presence of finger-like projections

known as villi. These features greatly increase the surface area of the intestine, allowing for enhanced nutrient absorption. While the intestine comprises several layers of different cell types, the major barrier between the gut lumen and portal blood circulation is a single layer of polarized enterocytes, otherwise known as the intestinal epithelium. To further increase surface area and absorption, the apical membrane of these columnar-shaped enterocytes feature microvilli.

The polarized nature of enterocytes is due to the presence of a protein complex known as the tight junctions. Although the outer leaflets of plasma membranes of neighboring cells were originally believed to be fused together, freeze-fracture analysis showed that these "kisses-in-the-dark" were actually formed by transmembrane protein interactions (Furuse et al., 1993; Furuse et al., 1998). The cell-to-cell adhesions formed by the tight junctions are a hallmark of epithelial cells, and their functionality has been likened to both a gate and a fence (Schneeberger and Lynch, 2004) as they create (1) the distinct membranes by restricting the movement of membrane-bound proteins within the plasma membrane, and (2) restrict the passage of compounds through the paracellular space found between adjacent cells, respectively. According to the fluid mosaic model of the cell membrane, proteins and other molecules embedded in the phospholipid bilayer can move within the plasma membrane. However, the tight junctional proteins restrict the movement of molecules between the apical and basolateral membranes (Schneeberger and Lynch, 2004), including transporter proteins such as those responsible for the absorption of metformin. Therefore, the localization of various drug transporters, which is partially determined by tight junction formation, clearly influences the ability of

transporter substrates to cross the apical and basolateral membranes in the intestine and other organs.

The first transmembrane protein identified in the tight junction was occludin and was originally believed to be responsible for the limited transport of compounds through the paracellular space (Furuse et al., 1993). However, it was later shown by the Tsukita laboratory that another group of four transmembrane domain proteins, the claudin family, was responsible for this functionality (Furuse et al., 1998). To date, more than 20 claudins have been identified and intracellular interactions of these proteins are known to possess different charge selectivities conferred by specific amino acid residues within the extracellular loops of claudins. Although the physiological function of these pores are to regulate ion movement across epithelial and endothelial cell layers, small chemical drugs are able to permeate via this route.

The major mechanisms of drug movement across the intestinal epithelium involve either paracellular or transcellular transport (Figure 1.4) (Borchardt et al., 2006). Paracellular transport is typically restricted to small hydrophilic compounds due to the size of the paracellular space and the narrow pores formed by the tight junctions. Passive diffusion through epithelial cells is the predominant mechanism of transport for highly lipophilic compounds, as they are capable of partitioning into the phospholipid bilayers of the plasma membrane. The

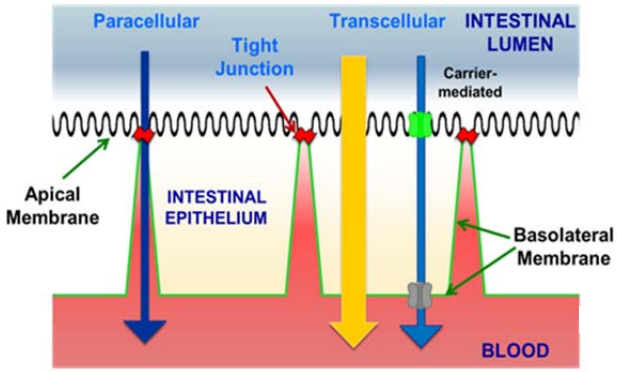

Figure 1.4: Drug transport mechanisms across the intestinal epithelium

concentration gradients formed across apical and basolateral membranes drive drug transport. The transcellular process, however, is energetically unfavorable for hydrophilic compounds, since they must break the hydrogen bonds formed with water molecules in order to cross biological membranes. Nevertheless, transcellular transport is possible for hydrophilic drugs that are substrates for transporters. This carrier-mediated process can involve transporter proteins at both the apical or basolateral membranes of enterocytes to facilitate the movement of drug across membranes.

1.D.2. Methods Used to Study Intestinal Drug Transport

Numerous experimental models exist to study drug transport across intestinal epithelia. A commonly used and well-established cellular model of intestinal transport is the Caco-2 cell monolayer grown in Transwell® plates that features two compartments separated by a semi-permeable membrane support (Hidalgo et al., 1989) (Figure 1.5A). Although these cells were derived from human colon carcinoma cells, they exhibit many features and behaviors of the small intestine. Upon reaching cellular confluence, the Caco-2 cell monolayers form tight junctions and express many of the relevant drug transporters. Similar to the Transwell® model, the Parallel Artificial Membrane Permeability Assay (PAMPA) uses lipid solutions infused into porous filters to create an artificial membrane

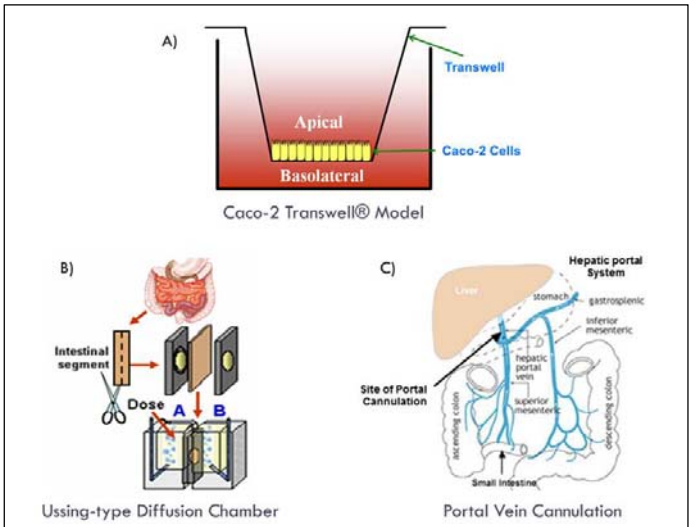

Figure 1.5: Intestinal absorption models used in the current study. Adapted from Dufek 2011.

that mimics biological lipid bilayers of cellular membranes. Permeability results obtained from PAMPA experiments do not account for active transport mechanisms and therefore represent the passive diffusion component of transcellular transport. *Ex vivo* models of intestinal absorption utilizing intestinal tissue include everted sac methods and Ussing-type diffusion chambers (Figure 1.5B), while the single pass intestinal perfusion technique is a commonly used *in situ* method. *In vivo* techniques such as portal vein cannulation (Figure 1.5C), often used in rodents, enables the direct measurement of drug concentrations in the portal vein before the drug reaches the liver and is subjected to possible hepatic distribution and/or first-pass metabolism (Dufek, 2011).

1.E. METFORMIN ABSORPTION ACROSS THE INTESTINAL EPITHELIUM

1.E.1. Rationale for the Proposed Study

The complex transport processes that govern the intestinal absorption of metformin have not been elucidated to date. Metformin is a small (MW = 129 daltons), highly water-soluble drug, with a logD of -6.14 @ 6.0 (Saitoh et al., 2004). Its pK_a of 11.5 suggests that >99.99% of metformin exists as a positively charged species at all physiologic pH values. The small size and hydrophilic nature of metformin would suggest that it is absorbed predominantly via the paracellular space. However, the high bioavailability reported for metformin belies this conjecture, and suggests the involvement of carrier-mediated drug transport across the intestinal epithelium. Several transporters, namely OCT3 and PMAT on the apical membrane, and OCT1 on the BL membrane, have been postulated as the transporters responsible for the intestinal absorption of metformin (Zhou et al., 2007; Graham et al., 2011; Zolk, 2011; Gong et al., 2012). However, contradictory to some published reports, including the 2010 ITC

Transporter White Paper and the 2012 FDA Draft Guidance on Drug Interaction Studies (Wang et al., 2002; Jonker et al., 2003; Muller et al., 2005; Giacomini et al., 2010; FDA, 2012; Han et al., 2013), the Thakker laboratory has convincingly shown the apical localization of OCT1/Oct1 in human and mouse intestinal tissue, as well as in Caco-2 cell monolayers (Han et al., 2013) using transporter-mediated uptake studies of the OCT1 substrate pentamidine and confocal imaging. These results by Han *et al.* (2013), combined with examples of other cationic drug transport studies, suggest a lack of polyspecific organic cation transporters on the basolateral membrane of enterocytes. For example, studies in the Thakker laboratory investigating the transport properties of the histamine H_2-receptor antagonist ranitidine across the basolateral membrane of Caco-2 cell monolayers have shown saturable basolateral uptake of ranitidine with a K_m of 66.9 mM by a mechanism which is not mediated by TEA-sensitive transporters (i.e., OCTs or the carnitine organic cation tranporters (OCTNs) (Lee et al., 2002). Subsequent studies showed that OCT1 is a high affinity transporter of ranitidine with a K_m of 70 ± 9 μM providing further evidence that this transporter is not in the basolateral membrane of Caco-2 cell monolayers (Bourdet et al., 2005).

Similar to results from studies with ranitidine and pentamidine, detailed kinetic studies in the Caco-2 cell monolayers also suggested that transporters of metformin are present on

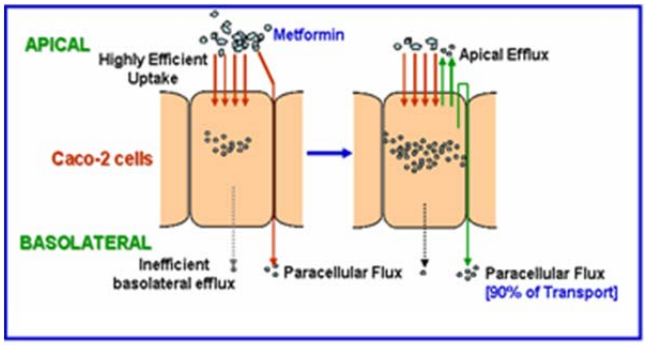

Figure 1.6: Metformin uptake and transport in Caco-2 cell monolayers

the apical membrane of these cells, whereas basolateral transporters are not involved with poor transport across this membrane (Proctor et al., 2008) (Figure 1.6). In this set of experiments, the apical and basolateral efflux of metformin were evaluated in Caco-2 cells pre-loaded with metformin. Apical efflux was ~7-fold higher than basolateral efflux. The addition of GW918, an inhibitor of P-gp, had no effect on metformin efflux suggesting that the efficient efflux across the apical membrane of Caco-2 cell monolayers is not mediated by this transporter. However, the OCT inhibitor quinidine (200 μM) reduced metformin efflux by ~80% compared to control, while OCT substrates TEA, MPP^+, and metformin trans-stimulated efflux. Proctor *et al.* (2008) also evaluated metformin apical-to-basolateral transport and uptake, and determined that the apparent permeability (P_{app}) of metformin was comparable to that of the prototypical paracellular probe, mannitol. A kinetic modeling approach, originally utilized to study ranitidine transport (Bourdet et al., 2006), was applied to data on the transport and apical uptake of metformin over time to estimate the relative contribution of paracellular and transcellular routes of transport. In agreement with the P_{app} of metformin, this modeling strategy suggested that the apical-to-basolateral transport of metformin occurs predominantly (>90%) via the paracellular route. This result was also similar to estimates of 88% paracellular transport reported by Saitoh *et al*. (2004), using data on metformin transport in Caco-2 cell monolayers and results from PAMPA studies to determine the relative contributions of these routes of transport. Interestingly, the percent of mannitol dose absorbed is reported to be ~16% (Artursson and Karlsson, 1991), whereas metformin dose absorbed ranges from 40–60% (Graham et al., 2011). This striking discrepancy between two seemingly similar compounds (e.g., small hydrophilic drugs), combined

with the uptake and efflux properties of metformin across the apical and basolateral membranes of Caco-2 cells, prompted the development of the "sponge" intestinal absorption mechanism of metformin, and the central hypothesis of this dissertation project (Figure 1.7), as stated below:

The high intestinal accumulation of metformin is mediated by efficient apical uptake and efflux of this drug combined with a lack of efficient BL efflux. As an oral metformin dose travels distally through the gastrointestinal tract, cycling of metformin between the gut lumen and enterocytes augments its intestinal absorption by increasing access of metformin to the paracellular space, the major route by which it crosses the intestinal epithelium.

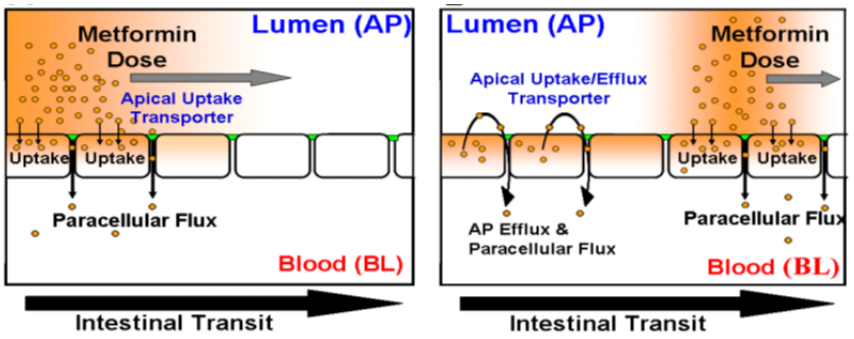

Figure 1.7: Proposed Intestinal Sponge Absorption Mechanism of Metformin. (Proctor et al. 2008)

1.E.2. Specific Aims

Because this hypothesis was developed in a static, *in vitro* cell culture model, yet suggested the involvement of changes in drug concentration due to intestinal transit, an *in vivo*-based approach was used to test this hypothesis. Mouse was chosen as the *in vivo* model, and as such, characterization of metformin transporters in this species was necessary prior to conducting studies. Therefore, the ***first aim*** of this project was to identify mouse intestinal transporters of metformin and determine their role in metformin uptake and efflux in mouse intestinal tissue. Doing so required the generation of mouse transporter-expressing cell lines to characterize metformin transport by the orthologs of the putative human transporters of metformin. This also enabled the identification of potential metformin transporter inhibitors for use in future *in vivo* studies.

The ***second aim*** of this dissertation work was to demonstrate that the transporters identified in the first aim play a role in the intestinal absorption of metformin in mice. Various pharmacokinetic studies were conducted to understand the role of drug transporters in the intestinal absorption of metformin, and specifically to demonstrate the involvement of apical transporters in the high intestinal accumulation and absorption of metformin via the paracellular route.

Specific Aim 1: Identify mouse intestinal transporters of metformin and determine their role in metformin uptake and efflux in mouse intestinal tissue.

A. Determine uptake and efflux of metformin in transporter-expressing cell systems.
B. Determine the role of transporters in metformin uptake and efflux across mouse intestinal tissue using transporter inhibitors.

Specific Aim 2: Using inhibitors of metformin transporters identified in Aim 1, demonstrate that apical intestinal transporters indirectly enhance metformin absorption via the paracellular route.

A. Determine the portal exposure of metformin in the presence/absence of a pan organic cation transporter inhibitor using a portal vein cannulated mouse model.
B. Assess systemic bioavailability of metformin in the presence/absence of metformin transporter inhibitors, and relate it to portal exposure.
C. Demonstrate that increased transporter-mediated absorption of metformin is not transcellular, and therefore paracellular, based on the distribution of metformin in intestinal tissue and its absorption pharmacokinetic behavior in the presence/absence of inhibitors.

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Chapter 2

CHARACTERIZATION OF CATION-SELECTIVE TRANSPORTERS OF METFORMIN IN THE MOUSE SMALL INTESTINE

2.A. OVERVIEW

The high bioavailability of the orally administered anti-diabetic drug metformin, despite its hydrophilic nature and positive charge at all physiological pH values, suggests the involvement of intestinal drug transporters. Because the mouse is a commonly used preclinical model for investigating metformin disposition and pharmacology, the goal of this study was to characterize the intestinal mouse orthologs of human putative metformin transporters, and evaluate their potential role in metformin uptake from the gut lumen. Gene expression studies of polyspecific organic cation transporters, namely organic cation transporter (Oct) 1-3, plasma membrane monoamine transporter (Pmat), and multidrug and toxin extrusion (Mate) 1 and 2, in the mouse small intestine showed that mOct1 and mMate1 were highly expressed in the intestinal mucosa. Stable cell lines expressing mOct1-3, mPmat, and mMate1 were generated to characterize metformin uptake kinetics and inhibitory potencies of a panel of transporter inhibitors toward metformin uptake. Metformin was found to be a substrate for all five transporters, with K_m values similar to those of their respective human orthologs. However, IC_{50} values of several inhibitors for metformin uptake were significantly different from those of the

corresponding human orthologs. Desipramine (10 μM) and pyrimethamine (1 μM) were identified as selective inhibitors of mOct1 and mMate1, respectively. A higher concentration of desipramine (1 mM) was used as a pan inhibitor in *ex vivo* studies, and reduced metformin uptake by 40% from the apical side of mouse intestinal tissue. This is the first report showing that cation-selective transporters at the luminal membrane of mouse small intestine mediate metformin uptake, and first study to characterize the kinetics of metformin mOct3- and Pmat-mediated uptake into the mouse intestinal tissue. Involvement of these transporters could explain the high oral bioavailability and dose-dependent absorption of metformin observed in the clinic.

2.B. INTRODUCTION

The biguanide, metformin, is the first-line therapy for the management of type 2 diabetes. In addition to its widespread use as an anti-hyperglycemic agent, it has been increasingly prescribed off-label to treat other diseases such as polycystic ovarian syndrome (Palomba et al., 2009), gestational diabetes (Wensel, 2009), and is currently being evaluated for its anti-cancer effects (Alimova et al., 2009; Jiralerspong et al., 2009). While this drug has been used in clinic for decades worldwide for increasingly varied indications, its mechanism of action and disposition are still under active investigation (Sakar et al., 2010; Kusuhara et al., 2011; Lee et al., 2011).

Physicochemical properties of metformin (Figure 2.1), namely a pK_a of 12.4 and predicted logD of -6.13 at pH 6.0 (Saitoh et al., 2004), suggest that it is highly water soluble and exists almost exclusively as a charged species at all physiological pH values. Despite the hydrophilic nature of metformin that implies a limited passive diffusion component in its oral absorption, metformin is well absorbed with a bioavailability ranging from 40 – 61% (Pentikainen et al., 1979; Scheen, 1996), which suggests the potential involvement of cation-selective drug transporters. The group of polyspecific organic cation transporters comprises proteins from the solute carrier (*SLC*) 22, *SLC29*, and *SLC47* families of transporters. Members of this group include OCT1, 2, and 3 (*SLC22A1-3*), PMAT/ENT4 (*SLC29A4*), and MATE1 and 2 (*SLC47A1-2*), all of which have the common ability to transport structurally diverse endogenous and exogenous cationic compounds. This broad substrate specificity enables these transporters to play a major role in the disposition of numerous drugs and toxins. Substrate activity of metformin has been confirmed for human OCTs, PMAT, and the MATE transporters

(Kimura et al., 2005; Zhou et al., 2007; Nies et al., 2009; Proctor, 2010; Ito et al., 2012; Han, 2013) in single transporter-transfected cell lines.

Two transporters, OCT3 and PMAT, have been postulated as the primary candidates that mediate the intestinal absorption of metformin, based on protein expression and their ability to transport this drug *in vitro* (Zhou et al., 2007; Nies et al., 2009; Graham et al., 2011). However, functional evidence is lacking in the literature to substantiate this assumption. In this chapter, a comprehensive assessment was conducted to characterize metformin transporter kinetics for mouse orthologs of the putative human metformin transporters using singly-transfected mouse transporter expressing cell lines. To our knowledge, this is the first report on the kinetics of mouse Oct3– and Pmat–mediated metformin uptake. Additionally, preliminary studies performed in mouse intestinal tissue implicate apically localized cation-selective transporters in the oral absorption of metformin. These findings are significant as they enhance our ability to interpret the *in vivo* pharmacokinetic (PK) and pharmacodynamic (PD) behavior of metformin in the mouse, a well-established and commonly employed experimental model of drug disposition and pharmacology.

2.C. METHODS

Materials

The CHO-K1 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Ham's F-12 Nutrient mixture, penicillin-streptomycin-amphotericin B solution (100X), and geneticin were obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA), and Hank's balanced salt solution (HBSS) with calcium and magnesium was purchased from Cellgro (Manassas, VA, USA). Metformin, quinidine, 1-methyl-4-phenyl pyridinium (MPP^+), tetraethylammonium (TEA), desipramine, corticosterone, mitoxantrone, pyrimethamine, and D-(+) glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{14}C]Metformin (107 mCi/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA).

Cell Culture and Generation of Stable Cell Lines

CHO-K1 cells (ATCC® CCL-61) were transfected with plasmids containing full-length mOct1, mOct2, mPmat (Origene, Rockville, MD, USA), mOct3, mMate1a or mMate2 (Open Biosystems/Thermo Scientific, Waltham, MA, USA) cDNAs by Lonza (Walkersville, MD, USA) nucleofection according to the manufacturer's optimized protocol for this cell line (Cell Line Nucleofector Kit T: high efficiency program (U-023)). Co-transfection or subcloning into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) was performed for plasmids lacking appropriate selectable markers or promoters. 婚 婜 婢 婲 婴 存 孺 孽 宂 宖 官 忆 忠 恶 忪 忔 怀 愄 愼 愬 愲 慂 慄 桖 柏 概 榮 椿 榶 樀 槈 沢 泄 泆 泌 泠 波 涮 淄 淆 淌 淘 淚 激 潰 澥 for further studies. Parental cells were grown in F-12 Nutrient Mixture (Ham's) with

10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Culture media for transfected cell lines were supplemented with 0.25 mg/ml geneticin. All cells were grown at 37ºC in a humidified atmosphere with 5% CO_2.

Quantitative Real Time-PCR (qRT-PCR)

Total RNA was extracted from transfected cell lines or mouse intestinal mucosa from male C57BL/6J (Jackson Laboratories, Bar Harbor, ME, USA) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified using the RNeasy Plus kit (Qiagen, Valencia, CA, USA) with slight modification. cDNA was reverse transcribed by FirstStrand Synthesis supermix kit (Invitrogen, Carlsbad, CA, USA) using equal amounts of total RNA for each sample. Relative mRNA levels of cation-selective transporters in mouse small intestine were determined using Taqman® assays validated for each individual transporter utilizing an Applied Biosystems 7300 RT-PCR system. Data were normalized to the 18s rRNA eukaryotic housekeeping gene by the $2^{-\Delta\Delta C_T}$ method.

***In vitro* Uptake Studies in CHO Cell Lines**

Transfected and control cells were seeded into 24-well plates at a density of 100,000 cells/cm^2. Growth medium was changed on alternate days and the day prior to experimentation. Uptake experiments were conducted 5–7 days post-seeding. Cells were pre-incubated with transport buffer (HBSS with 10 mM HEPES and 25 mM glucose, pH 7.2) for 30 minutes at 37ºC. Assays were initiated by replacing the pre-incubation buffer with transport buffer containing varying concentrations of [^{14}C]metformin (0.1 µCi/ml) in the presence of chemical inhibitors or appropriate vehicle controls. [^{14}C]Metformin uptake was terminated by aspirating the metformin solution. For the determination of

mMate1 kinetic parameters, the extracellular pH (of transport buffer) was adjusted to pH 8.0 to increase [^{14}C]metformin uptake. Cells were washed 3X with 4ºC transport buffer, and lysed in 500 µl of 0.1 N NaOH/0.1% SDS for 4 h with shaking. Cellular accumulation of [^{14}C]metformin was determined by liquid scintillation spectrometry. Protein concentration was measured by the BCA protein assay (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin as a standard.

Metformin Transport and Uptake Studies in Mouse Intestinal Tissue

Male C57BL/6J mice (overnight fast) (Jackson Laboratories, Bar Harbor, ME, USA) were anesthetized by an intraperitoneal (IP) injection of ketamine (140 mg/kg) and xylazine (15 mg/kg). Intestinal tissue was excised from the proximal jejunum and immediately washed in 4ºC Krebs-Bicarbonate Ringer (KBR) buffer plus protease inhibitors. A glass rod was gently inserted through a 2 cm intestinal segment. The segment was then cut longitudinally and mounted between two halves of a diffusion chamber insert that was placed between two side-by-side diffusion chambers. KBR buffer (37ºC) was added to each chamber for 30 minutes prior to experimentation, and bubbled with 95%/5% oxygen/carbon dioxide to maintain viability. Uptake studies were initiated by replacing apical KBR buffer with [^{14}C]metformin diluted in buffer. To determine metformin uptake in the presence or absence of transporter inhibitors, the tissue was removed after the designated incubation period, washed three times with 4ºC KBR and dissolved with 2N NaOH. Samples were neutralized with HCl and [^{14}C]metformin concentrations were determined by liquid scintillation spectrometry.

Data Analyses

For kinetic studies, uptake of [^{14}C] metformin was evaluated in transporter-transfected cell lines. Nonspecific cell-associated radioactivity was determined by measuring drug uptake in parental cell lines. These values were subtracted from data obtained from transporter-transfected cells to obtain the kinetic parameters K_m and V_{max} (Michaelis constant and maximal uptake velocity, respectively) by modeling metformin concentration-dependent uptake data in Winnonlin 5.3 (Pharsight, Mountain View, CA, USA) using equation 1.

Equation 1:

$$V = \frac{V_{max} + [S]}{K_m + [S]}$$

where V is the uptake rate in the presence of varying concentrations of metformin, S.

Inhibitory potency (IC_{50}) was determined for each inhibitor by fitting equation 2 to the uptake data,

Equation 2:

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

where V is the uptake rate in the presence of the inhibitor, I, V_o is the rate in the absence of the inhibitor, IC_{50} is the inhibitor concentration required for 50% inhibition, and n is the Hill Coefficient.

All data are expressed as mean ± S.D. from three measurements unless otherwise noted. Statistical significance was determined by a Student's *t* test.

2.D. RESULTS

Expression of Mouse Cation-Selective Transporters in the Intestine of C57BL/6J Mice

The expression of cation-selective transporters in mouse small intestine is shown in Figure 2.2. mOct1 and notably, mMate1 were the most highly expressed genes in all regions of the small intestine, followed by mOct3, mMate2, and mPmat, while mOct2 was poorly detected. A trend showing highest gene expression in the proximal jejunum for all transporters was observed, except for mOct3 and mPmat which showed highest expression in the ileum.

Metformin Substrate Activity for Mouse Organic Cation Transporters

Metformin is a known substrate for human OCT1, 2, and 3, MATE1 and -2, as well as PMAT (Wang et al., 2002; Kimura et al., 2005; Masuda et al., 2006; Zhou et al., 2007; Sato et al., 2008; Proctor, 2010). To assess if the mouse orthologs of these human transporters show similar or different substrate activity for metformin, single transporter-expressing CHO cell lines (*i.e.*, mOct1-3, mPmat, and mMate1) were generated. Following confirmation of mouse transporter gene expression by qRT-PCR analysis, functional activity of the expressed transporters was determined by uptake assays of probe substrates (*e.g.*, TEA). Attempts to reproduce previously reported (Hiasa et al., 2007) functional uptake of probe substrates by mMate2 transfected cell lines were unsuccessful, and thus metformin uptake kinetics for mMate2 was not evaluated in the present study.

The linear time range for initial uptake of metformin was determined by evaluating its time-dependent uptake up to 30 minutes (Figure 2.3A). A single time point

of 5 minutes (within the linear range for uptake) was selected for all subsequent studies with transporter-transfected cell lines, except for studies with mOct2-expressing CHO cells where a shorter time of 30 seconds was required. Metformin uptake in transporter-transfected cell lines compared to parental cells was 3–20-fold higher, confirming substrate activity of metformin for the mouse orthologs of human metformin transporters (Figure 2.3A). Concentration-dependent uptake of metformin was saturable in all transporter-transfected cell lines (Figure 2.3B). Estimates of the Michaelis constant, K_m, ranged from 0.3 mM for mMate1 to 4.3 for mPmat (Table 2.1), indicating that the affinity of metformin for each transporter was in the following rank order: mMate1 > mOct1 > mOct2 > mOct3 > mPmat.

In Vitro Chemical Inhibition of Metformin Uptake

To identify transporter-selective chemical inhibitors, the inhibitory potencies toward the cellular uptake of metformin were evaluated for a panel of known chemical inhibitors of human metformin transporters (Figure 2.1). The prototypical cation-selective transporter inhibitor, MPP^+, inhibited metformin uptake via mOct1-3 and mMate1 with IC_{50} values <25 μM and ~180 μM for mPmat, whereas quinidine was a weaker pan inhibitior with IC_{50} values between 120 – 760 μM for mOct2, mOct3, mPmat, and mMate1; IC_{50} for mOct1 was 29 μM. Additionally, since previous studies in the Thakker laboratory demonstrated that specific concentrations of mitoxantrone (25 μM) and corticosterone (150 μM) selectively inhibit OCT1 and OCT1-3, respectively, and that desipramine (200 μM) is a potent inhibitor of OCT1-3, PMAT, and MATE1, these chemical inhibitors were also evaluated for their inhibitory potencies towards mouse orthologs of the human transporters (Han, 2013) . Mitoxantrone was less potent

for mOct1 than with OCT1, with an IC_{50} >20-fold higher (Table 2.2), while corticosterone (30 μM) inhibited both mOct2 and mOct3 by approximately 80%. A low concentration of desipramine (10 μM) was shown to be a strong inhibitor of mOct1, decreasing metformin uptake by 85% (Figure 2.4). Pyrimethamine, a selective inhibitor of human and mouse MATE transporters (Ito et al., 2010; Kusuhara et al., 2011), strongly inhibited mMate 1 at 1μM, while only weakly inhibiting mOct2 (~30%). Desipramine, at a higher concentration of 1 mM, inhibited >80% of metformin uptake by all five mouse transporters investigated. IC_{50} values of the inhibitors for each transporter are reported in Table 2.2.

Metformin Uptake in Mouse Intestinal Tissue

To provide evidence that cation-selective transporters mediate the apical uptake of metformin in mouse intestinal tissue, metformin uptake was evaluated utilizing an Ussing-type diffusion chamber. Results showed that metformin was taken up into the intestinal tissue from the apical side, and that 1 mM desipramine significantly decreased ($p < 0.01$) metformin uptake in mouse intestinal tissue by 40%, a surprising finding given the non-specific and potent inhibition of metformin uptake (>90%) observed by desipramine at this concentration in singly-expressed mouse transporter cell lines (Figure 2.5).

2.E. DISCUSSION

Results in the present study provide the first direct evidence that transport of the anti-diabetic drug, metformin, across the apical membrane of mouse intestine is mediated by polyspecific organic cation transporters. Because metformin is not subjected to metabolism or significant protein binding, functionally active transporters in key cellular membranes dictate its overall disposition and pharmacology. The major transporters of metformin that facilitate its uptake into organs such as liver and kidney from the systemic blood circulation in humans are the OCTs, with OCT1 and OCT2 predominantly expressed in the liver and kidney, respectively, and OCT3 having a broader tissue expression (Gorboulev et al., 1997; Wu et al., 2000; Motohashi et al., 2002). The MATE transporters are believed to play a substantial role in the excretion of cations from the kidney (MATE2) and liver (MATE1) into the urine and bile, respectively. PMAT is also highly expressed in the brain and central nervous system (Engel et al., 2004; Dahlin et al., 2007), although its role in metformin disposition remains unclear. While studies have been conducted to determine the major metformin transporters in elimination organs such as the liver and kidney, a lack of information on the membrane localization of these transporters in the intestine limits our current understanding of metformin oral absorption.

Although the gene expression of mouse Octs, Pmat, and Mates in the intestine has been reported in the literature (Alnouti et al., 2006; Lickteig et al., 2008), a comprehensive data set comparing the relative expression of all putative metformin transporters in the mouse intestine is lacking. In agreement with previously published data (Alnouti et al., 2006), the present study showed that mOct1 was highly expressed in

the small intestine (Figure 2.2). Surprisingly, given its relatively low expression in mouse enterocytes compared to other tissues (Lickteig et al., 2008), mMate1 was expressed at levels similar to mOct1. mOct3, mPmat and mMate2 were also expressed in the mouse intestine, whereas mOct2 was poorly detected. Although OCT3 and PMAT are believed to localize predominantly at the apical membrane in the human small intestine (Muller et al., 2005; Zhou et al., 2007), some published reports suggest the basolateral localization of OCT1 in human and mouse enterocytes, and in Caco-2 cell monolayers, a well-established model of human intestinal epithelia (Jonker et al., 2001; Wang et al., 2002; Muller et al., 2005). Contrary to these reports, recent functional uptake data from the Thakker laboratory showed that OCT1 is localized at the apical membrane of mouse and human intestinal epithelia, and Caco-2 cell monolayers (Han et al., 2013). In the same study, immunostaining and confocal microscopy provided visual evidence that substantiated the apical localization of OCT1 in Caco-2 cell monolayers, and in mouse (mOct1) and human small intestinal tissues. While gene expression levels of transporters do not necessarily correlate to their functional protein levels, results from the current study suggest the likely presence of all putative metformin transporters in mouse intestinal tissue except for mOct2 (Figure 2.2). Furthermore, in addition to OCT1, preliminary evidence in the literature suggests that OCT3 and PMAT exist at the apical membrane in human and/or mouse intestine. Studies to confirm the precise polarized localization of Oct3, Pmat, as well as Mate1 in mouse intestine, while not a part of this investigation, are important in understanding the intestinal absorption mechanisms of metformin and other cationic drugs.

In addition to investigating the expression profile of metformin transporters in mouse intestine, an understanding of metformin uptake kinetics is beneficial for studying and predicting its overall disposition. Although metformin kinetics have been characterized for all major human metformin transporters (Kimura et al., 2005; Tanihara et al., 2007; Zhou et al., 2007; Nies et al., 2009; Han, 2013) and some mouse transporters (Ito et al., 2012; Toyama et al., 2012), to our knowledge, metformin kinetics for mouse Oct3 and Pmat have not been reported. The K_m of metformin for each transporter, determined in single transporter-expressing CHO cells, is critical for assessing and understanding differences between metformin disposition in humans and the preclinical mouse model that is extensively used for investigating metformin PK and PD. The K_m values of metformin for all mouse transporters investigated were within a similar range to those previously reported in the literature (± 4-fold) for the human orthologs (Nies 2011) (Table 2.1). It is interesting to note that metformin has a higher affinity for mOct1 compared to mOct2 (0.7 *versus* 1.3 mM, respectively), which is not consistent with its affinity for the human orthologs of these two transporters (3.1 vs 0.6 mM, respectively) (Kimura et al., 2005). This observation in mouse corroborates with recent data from transporter-transfected HEK293 cell lines where K_m values of metformin were 1.8, 2.9, and 0.3 mM for mOct1, mOct2, and mMate1, respectively, (Toyama et al., 2012), and substantiates extrapolated results reported by Ito *et al.* (2011). Although attempts to generate stable cell lines expressing mMate2 were unsuccessful in the present study, limited data in the literature characterizing cation substrate activity for mMate2 suggest relatively inefficient TEA uptake (Hiasa et al., 2007). Because the sequence homology

between mMate2 and MATE2 is limited (45% by BLAST analysis), whether metformin is transported efficiently by the mouse ortholog of MATE2 remains to be determined.

To address the lack of information from *in vivo* and *ex vivo* uptake studies to support intestinal transporter expression data, potential chemical inhibitors of mouse metformin transporters were evaluated using singly-transfected transporter cell lines. MPP^+, a well-established pan inhibitor of human cation transporters, was found to be a potent inhibitor of metformin uptake by a majority of the transporters investigated in this study (Table 2.2A). However, quinidine, another commonly used pan cation-selective transporter inhibitor, showed a lower potency for inhibition of metformin uptake by most mouse metformin transporters compared to their corresponding human orthologs (Table 2.2B), with IC_{50} values >100 μM. Ito *et al.* (2010) demonstrated that pyrimethamine was a strong inhibitor of mMate1-mediated TEA uptake with an IC_{50} of 145 nM, while IC_{50} values of mOct1-2 were in the micromolar range. Results presented in the current study also support the previous finding that pyrimethamine is a potent inhibitor of mMate1 but does not significantly inhibit mOct3 or mPmat at nanomolar concentrations. Desipramine (1 mM) was identified as a general inhibitor of the five mouse transporters of metformin. Understanding the extent of inhibition by a chemical inhibitor at specific concentrations in combination with transporter expression patterns and levels in different organs will allow us to evaluate the contributions of individual transporters in specific organs to the disposition of cationic drugs.

OCT3 and PMAT have been proposed as major transporters that mediate metformin uptake from the lumen of the gut into the enterocytes in humans (Graham et al., 2011; Zolk, 2011). Additionally, as previously mentioned, recent studies in the

Thakker laboratory demonstrate that OCT1 is also localized in the apical membrane in Caco-2 cells, and mouse and human intestinal tissues. However, functional uptake data confirming that transporters mediate the intestinal absorption of metformin are lacking in the literature. The inhibition of metformin apical uptake into mouse intestinal tissue by desipramine (Figure 2.5) provides the first direct evidence for the involvement of cation-selective transporters in metformin uptake from the gut lumen in the mouse. However, uptake inhibition of metformin by desipramine was only 40%, which was less than expected. This suggests the possibility of other transporters of metformin in mouse small intestine which are not sensitive to desipramine. Alternatively, interactions of metformin with secreted proteins found in the extracellular matrix such as mucins, which are negatively charged due to extensive glycosylation, or non-specific cell surface binding (Han, 2013) may overestimate the transporter-mediated uptake of metformin. However, additional studies with selective transporter inhibitors and/or knockout animals are required to identify the major metformin transporters and determine their relative contributions to the intestinal absorption of metformin.

In conclusion, the data presented here indicate that transporters localized in the apical membrane of mouse small intestine mediate the uptake of metformin from the gut lumen into the enterocytes. By defining the kinetics of metformin uptake in singly-transfected transporter cell lines, it was shown that the affinity of metformin for specific cation-selective mouse transporters was similar to that observed in the corresponding human orthologs. This is a critical step in being able to use mouse model for the study of metformin intestinal absorption as gross differences between mouse and human could lead to misinterpretation of data due to poor correlation. Although metformin uptake by

the mouse transporters was relatively similar to uptake by the human orthologs, large differences were observed in the inhibitory potencies of some metformin transporter-selective chemical inhibitors, such as quinidine and mitoxantrone. The kinetic and inhibitory potency parameters determined in this study can improve our understanding of specific transporters that may be important contributors in the major organs of drug disposition in the mouse.

2.F. TABLES AND FIGURES

Table 2.1. Uptake kinetic parameters of mouse cation-selective transporters for metformin

	mouse		*human*	
Transporter	K_m	V_{max}	K_m	V_{max}
	mM	*nmol/min/mg protein*	*mM*	*nmol/min/mg protein*
OCT1	0.7 ± 0.1	4.2 ± 0.2	3.1 ± 0.3[1]	1.75 ± 0.09[1]
OCT2	1.3 ± 0.2	20 ± 0.1	0.6 ± 0.03[1]	1.56 ± 0.03[1]
OCT3	2.4 ± 0.2	21.3 ± 0.5	2.6 ± 0.2[1]	4.51 ± 0.15[1]
MATE1	0.3 ± 0.06	1.7 ± 0.09	0.78 ± 0.1[2]	4.46 ± 0.59[2]
PMAT	4.3 ± 0.8	3.8 ± 0.3	1.32 ± 0.11[3]	16.68 ± 0.94[3]

[1]Han 2013, [2]Tanihara 2007, [3]Zhou 2007

Table 2.2. **Inhibitory potencies of organic cations for mouse and human transporters**

A) Inhibitory potencies of organic cations for mouse transporters expressed in CHO cell lines

Inhibitor	mOct1 IC_{50}	mOct2 IC_{50}	mOct3 IC_{50}	mMate1 IC_{50}	mPmat IC_{50}
	μM	μM	μM	μM	μM
MPP^+	9.0 ± 1.4	3.1 ± 1.2	23 ± 1.1	175.9 ± 1.2	17 ± 1.8
Quinidine	29 ± 1.2	190 ± 1.3	120 ± 1.3	760 ± 2.1	260 ± 1.6
Corticosterone	72 ± 1.3	2.3 ± 1.3	3.1 ± 1.2	>300	>300
Desipramine	1.2 ± 1.1	11 ± 1.3	84.3 ± 1.1	282.4 ± 1.4	39.3 ± 1.3
Mitoxantrone	67.1 ± 1.1	260 ± 1.2	345 ± 1.2	168 ± 1.3	>1000
Pyrimethamine	3.6 ± 1.2	3.0 ± 1.4	5.4 ± 1.3	0.087 ± 1.3	43.5

B) Inhibitory potencies of organic cations for human transporters; data taken from Nies 2011, Engel 2005, and Han 2013. Values in ( ) indicates K_m values, N.A. = not available.

Inhibitor	OCT1 IC_{50}	OCT2 IC_{50}	OCT3 IC_{50}	MATE1 IC_{50}	PMAT IC_{50}
	μM	μM	μM	μM	μM
MPP^+	15 - 32	2.4 - 54	(47 - 83)	(100)	(33)
Quinidine	5.4 - 114	7.1 - 446	14 - 124	29	25.3
Corticosterone	7 - 22	5.4 -34	0.12 - 0.29	>20	450.5
Desipramine	5.4 - 57	16	14	56	32.6
Mitoxantrone	3	135	174	N.A.	N.A.
Pyrimethamine	3.8	10	N.A.	0.077	N.A.

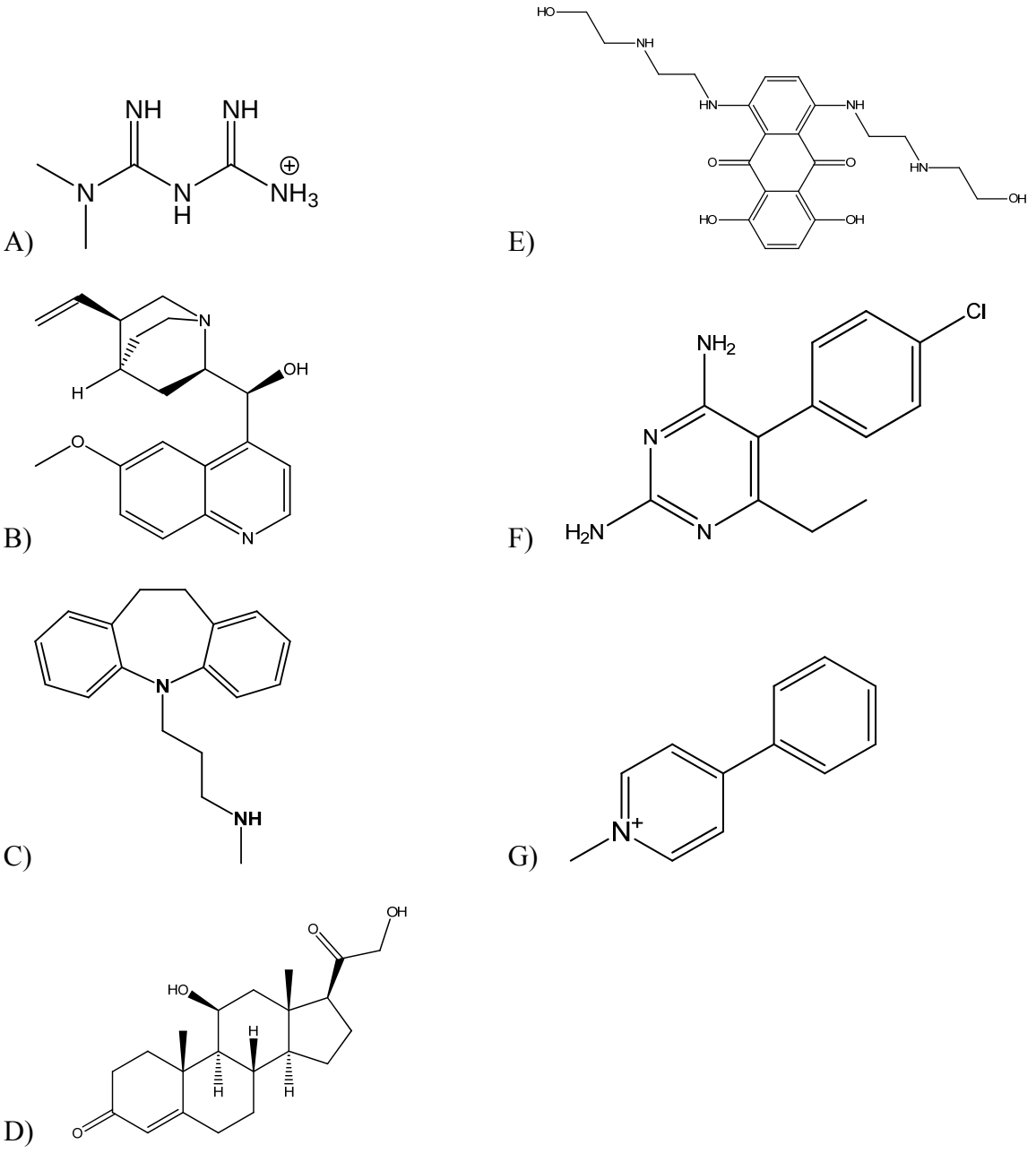

Figure 2.1. Structure of metformin and chemical inhibitors of metformin transporters. A) Metformin; B) Quindine; C) Desipramine; D) Corticosterone; E) Mitoxantrone; F) Pyrimethamine; G) MPP^+

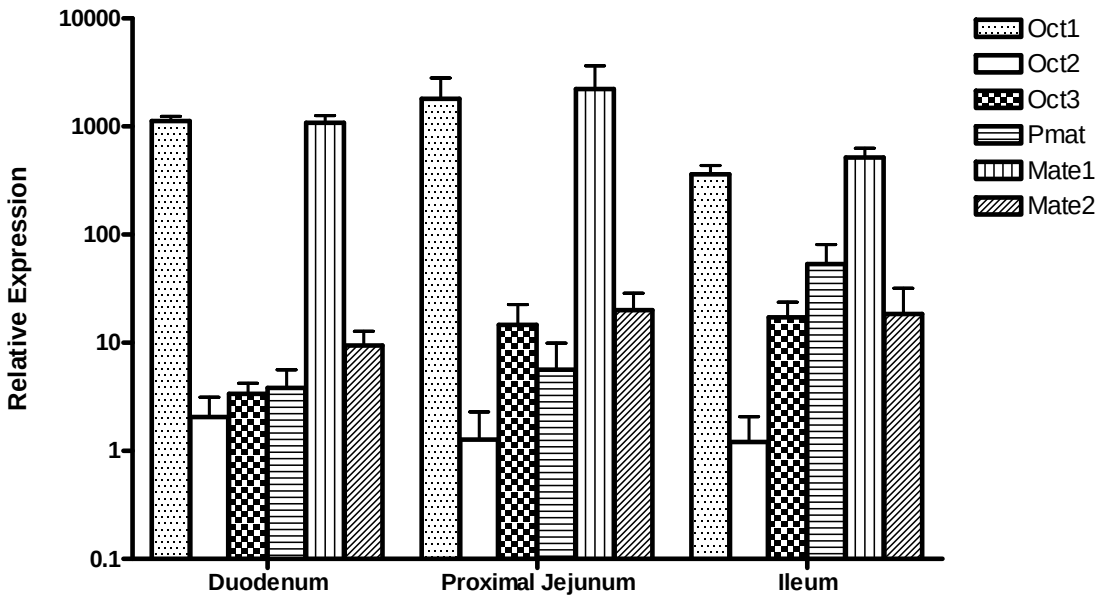

Figure 2.2. Cation-selective transporter gene expression in the small intestine of C57BL/6J mice. Intestinal expression of mOct1–3, mPmat, and mMate1 in the duodenum, proximal jejunum, and ileum of mouse small intestine. Data are expressed as levels relative to the lowest detected transporter, mOct2.

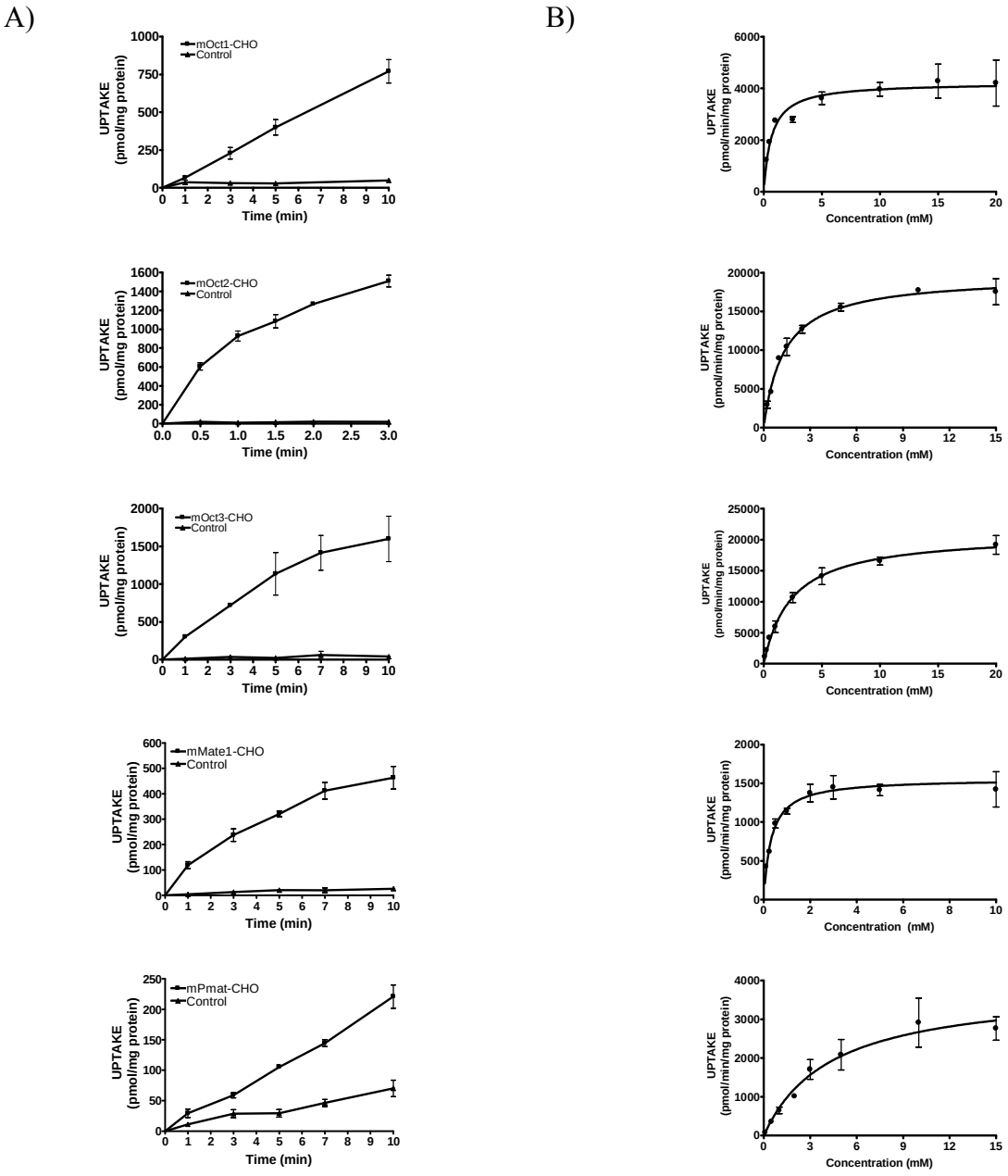

Figure 2.3. Kinetics of metformin uptake by mOct1–3, mMate1, and mPmat. A) CHO cells stably transfected with mOct1, mOct2, mOct3, mMate1, or mPmat were incubated with [^{14}C]metformin (50 μM) at 37ºC for the indicated time. B) Uptake of [^{14}C]metformin (indicated concentrations) was determined in the transfected or untransfected cells. Nonspecific cell-associated radioactivity was determined by

measuring the compound uptake in control cells and these values were subtracted from values obtained in the transporter-transfected cell lines. Data represent mean ± S.D. of experiments conducted in triplicate.

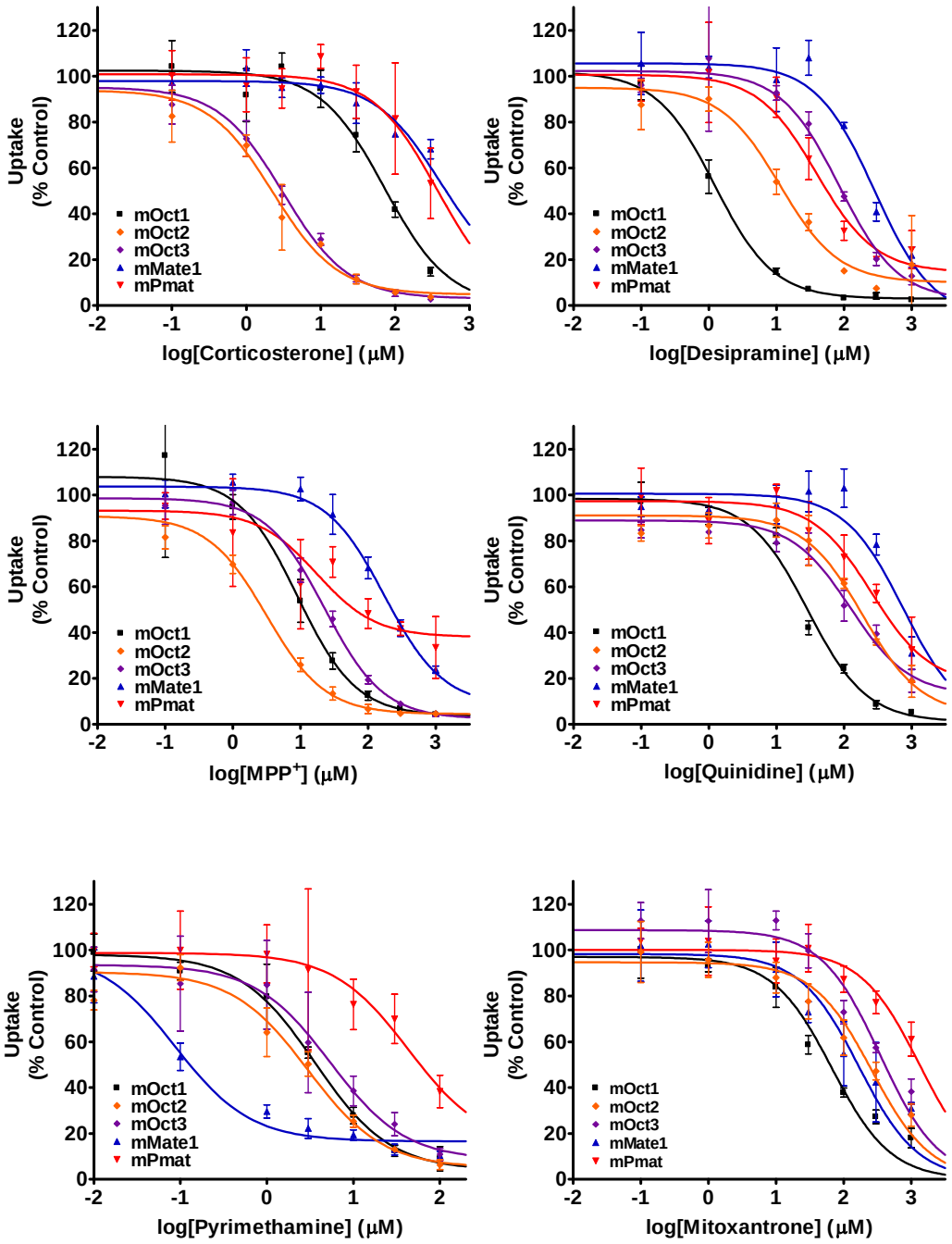

Figure 2.4. Inhibition of mOct1-, mOct2-, mOct3-, mMate1-, or mPmat-mediated [^{14}C]metformin (50 μM) uptake by cationic inhibitors of these transporters in the respective mouse transporter-expressing CHO cell lines. Data represent mean ± S.D. of experiments conducted in triplicate.

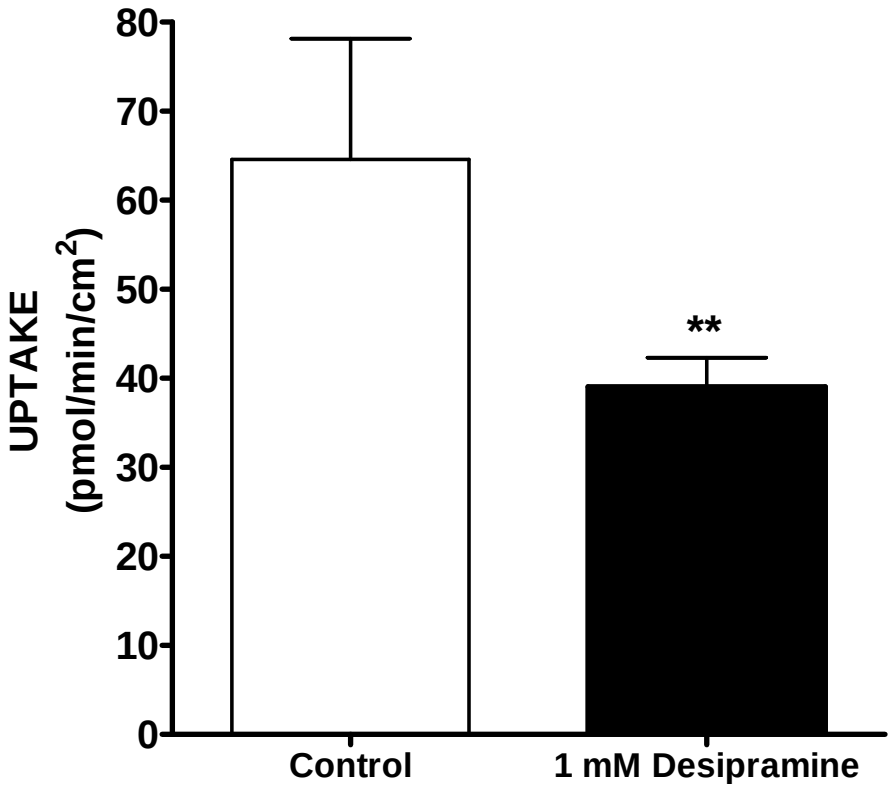

Figure 2.5. Metformin apical uptake in mouse intestinal tissue in the presence or absence of desipramine. Metformin (50 μM) uptake across the apical membrane of mouse intestinal tissue over 5 minutes in the presence (dark bar) or absence (open bar) of the cation-transporter inhibitor desipramine. Data represent mean ± S.D. of experiments conducted in triplicate. **$p<0.01$.

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Chapter 3

METFORMIN UPTAKE AND ACCUMULATION IN THE SMALL INTESTINE OF MOUSE IS MEDIATED BY APICALLY LOCALIZED TRANSPORTERS

3.A. OVERVIEW

Metformin transport across Caco-2 cell monolayers, as previously reported by Proctor *et al.* (2008), showed clear evidence of transporter-mediated apical uptake and efflux, and a lack of basolateral efflux. In the previous chapter, using *in vitro* and *ex vivo* methods, intestinal mouse orthologs of putative human metformin transporters were characterized, and chemical inhibitors of these transporters, such as desipramine and quinidine were identified. Although it is believed that transporters mediate the intestinal disposition of metformin, direct *in vivo* evidence to substantiate this speculation is lacking. Utilizing *ex vivo* and *in vivo* strategies, this study investigates (1) whether human and mouse intestinal tissues demonstrate similar kinetic behavior towards metformin as that observed in Caco-2 cell monolayers and (2) the role of cation-selective transporters in the enterocytic accumulation of orally administered metformin. *In vitro* chemical inhibition experiments were also used in the current study to identify a cation-selective transporter inhibitor with a low bioavailability and consequently higher concentration in the small intestine so as to induce a stronger inhibitory effect. The apical uptake clearance versus transport clearance of metformin showed a similar trend in mouse and human intestinal tissues and in Caco-2 cell monolayers, where metformin uptake was

high compared to its transport, suggesting rate-limiting basolateral efflux of metformin via the transcellular route. Pentamidine was identified as a potent inhibitor of mouse organic cation transporters 1-3 (Oct1-3), plasma membrane monoamine transporter (Pmat), and multidrug and toxin extrusion transporter 1 (Mate1); it decreased metformin uptake in transporter-transfected CHO cell lines by >90% at 1 mM concentrations. In metformin pre-loaded transporter-transfected cells, pentamidine inhibited metformin efflux via mOcts by >84% and via mPmat and mMate by 35% and 46%, respectively. At 3 mM, the pan cation transporter inhibitor, quinidine inhibited all transporter-mediated metformin efflux (except via mPmat) by >90%. Surprisingly, desipramine (a potent inhibitor of metformin uptake) trans-stimulated metformin efflux via mOct1, mOct3, and mMate1 by ~25–50%, and via mPmat by ~900%. In mouse intestinal tissue, basolateral efflux of metformin was unaffected by transporter inhibitors, quinidine and pentamidine. However, quinidine and pentamidine reduced the apical efflux of metformin by 33% and 24%, respectively, although the pentamidine-mediated decrease was not statistically significant. Orally administered metformin (0.65 mg/kg) in mice showed an intestinal accumulation as high as 25% at 15 minutes. Oral co-administration of pentamidine (3.4 mg/kg) reduced metformin accumulation in the promixal 9-cm region of the small intestine. In control mice, accumulated metformin decreased by ~80% between 15 – 60 minutes in the proximal region of the intestine, whereas the levels of accumulated metformin remained constant in pentamidine-treated animals. These results suggest that,

in mice, the intestinal accumulation of metformin is mediated by apical cation-selective transporters. Additionally, the interaction of metformin with transporters in the apical and basolateral membranes of mouse enterocytes is similar to that observed in Caco-2 cell monolayers and in human intestinal tissue.

3.B. INTRODUCTION

The biguanide metformin is the first-line therapy for the treatment of type 2 diabetes mellitus. The mechanisms of the anti-hyperglycemic effects of metformin vary in different organs. For example, in the liver metformin induces a reduction in gluconeogenesis, whereas in peripheral tissues such as muscle and fat, it has been reported to increase glucose uptake via upregulation of glucose transporter expression in the plasma membrane (Hundal et al., 1992; Grisouard et al., 2010; Lee et al., 2011). The intestine has also been identified as a pharmacological site of action where metformin is believed to reduce glucose absorption and increase glucose disposal (Caspary and Creutzfeldt, 1971; Wilcock and Bailey, 1991; Bailey et al., 1994; Proctor, 2010). High levels of intestinal accumulation of metformin observed in both mouse and human intestine (Wilcock and Bailey, 1994; Bailey et al., 2008) also suggest the likelihood of the small intestine as a pharmacologic target and possible source of increased lactate production, one of metformin's few but serious side effects. However, despite these reports that implicate the intestine as a pharmacological site of metformin's effects, the intestinal absorption of this drug remains poorly understood.

The oral absorption of metformin is extensive with its bioavailability reported to be as high as 60%, which decreases with increasing doses (Sambol et al., 1996). Flip-flop kinetics was also observed following oral dosing of metformin, suggesting that this drug exhibits rate-limiting intestinal absorption (Pentikainen et al., 1979). Significant research has been conducted to understand the disposition of metformin with respect to elimination organs such as the liver and kidney (Wang et al., 2002; Kimura et al., 2005a; Kimura et al., 2005b; Kusuhara et al., 2011). However, few reports have described the

mechanism of the intestinal absorption of this widely used drug. Proctor *et al*. (2008) described studies in Caco-2 cell monolayers which showed that during absorptive transport, efficient transporter-mediated uptake of metformin across the apical membrane and poor basolateral egress was observed. Metformin efflux across the apical membrane was ~7X higher than efflux across the basolateral membrane. Additionally, kinetic modeling of cellular uptake and absorptive transport data indicated that metformin is transported predominantly via the paracellular route, as was also suggested by Nicklin *et al.* and Saitoh *et al.* (Nicklin et al., 1996; Saitoh et al., 2004). Due to the small size of the paracellular space, transport via this route should typically account for only a small percentage of drug transport across the intestinal epithelial monolayer. However, metformin has a high fraction of dose absorbed despite being administered in gram quantities daily. This apparent discrepancy between *in vitro* and *in vivo* data was reconciled by proposing the "sponge" mechanism of oral absorption in which metformin absorption is enhanced by repeated access to the paracellular space by transporter-mediated cycling (i.e., uptake and efflux of metformin between the gut lumen and the enterocytes (Figure 1.7)).

Transporters of the solute carrier (SLC) family, including the OCTs (*SLC22A*), MATE proteins (*SLC47A*), as well as PMAT (*SLC29A*) possess metformin transport activity (Kimura et al., 2005a; Kimura et al., 2005b; Masuda et al., 2006; Tanihara et al., 2007; Zhou et al., 2007; Tzvetkov et al., 2009; Proctor, 2010). Both PMAT and OCT3 have been postulated as intestinal uptake transporters of metformin, yet functional data to support this notion thus far is speculative in nature (Zhou et al., 2007; Graham et al., 2011; Zolk, 2011). Additionally, based on a recent study conducted in mouse and human

intestinal tissues, and Caco-2 cells, OCT1 is shown to be localized in the apical membrane of these three systems (Han et al., 2013), and may mediate metformin uptake from the gut lumen.

The results in this current study show that, similar to observations in Caco-2 cell monolayers, metformin uptake, efflux across the apical membrane, and accumulation in mouse intestinal tissue are transporter-mediated. This important finding supports the novel intestinal absorption mechanism of metformin proposed by Proctor *et al.* (2008) and indicates that the mouse is an appropriate model for conducting *in vivo* studies that can assist in further elucidating the processes involved in the intestinal absorption of metformin.

3.C. METHODS

Materials

Previously generated (Chapter 2) transporter-transfected Chinese hamster ovary (CHO) cell lines were used. Ham's F-12 Nutrient mixture, penicillin-streptomycin-amphotericin B solution (100X), and geneticin were obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA), and Hank's balanced salt solution (HBSS) with calcium and magnesium was purchased from Cellgro (Manassas, VA, USA). Metformin, desipramine, pentamidine, quinidine, sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), and Krebs Bicarbonate Ringer (KBR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{14}C]Metformin (110 mCi/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). Ketamine (100 mg/mL) and xylazine (20 mg/ml) were purchased from Med-Vet International (Mettawa, IL, USA).

Cell Culture

Stably transfected Chinese hamster ovary (CHO) cells expressing mouse Oct1, Oct2, Oct3, Pmat, or Mate1 were grown in F-12 Nutrient Mixture (Ham's) with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 0.25 mg/ml geneticin. Cells were grown at 37ºC in a 90% humidified atmosphere with 5% CO_2. Transporter-transfected cells were seeded into sterile 24-well plates (Corning Life Sciences, Tewksbury, MA, USA) at a density of 100,000 cells/cm^2. Growth medium was changed on alternate days and the day prior to experimentation.

Metformin Uptake Studies in CHO Cell Lines

Uptake experiments were conducted 5–7 days post-seeding. Cells were pre-incubated with transport buffer (HBSS with 10 mM HEPES and 25 mM glucose, pH 7.2) for 30 minutes at 37ºC. Uptake assays were initiated by replacing the pre-incubation buffer with 400 µl of transport buffer containing 50 µM [^{14}C]metformin (0.1 µCi/ml) in the presence of pentamidine or vehicle control. [^{14}C]Metformin uptake was terminated by aspirating the metformin solution. Cells were washed 3X with 750 µl of 4ºC transport buffer, and lysed in 500 µl of 0.1 N NaOH/0.1% SDS for 4 hours with shaking to ensure complete lysis of cells. Cellular accumulation of [^{14}C]metformin was measured by liquid scintillation spectrometry and the uptake rate was assessed at time points previously determined to be within the linear range (5 minutes for all transporters except for mOct2 (30 seconds)). Protein concentration was measured by the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.

Metformin Efflux in CHO cells

[^{14}C]Metformin (0.5 mM; 0.2 µCi/ml) dissolved in transport buffer was incubated with cells overexpressing each of the mouse transporters of metformin for 30 minutes to preload the cells. Metformin solution was aspirated and the cells were washed 3X with ice cold transport buffer and replaced with 37ºC buffer in the presence or absence of inhibitors to initiate the experiment. The amount of extracellular metformin present in the buffer at 10 minutes was quantified by liquid scintillation spectrometry.

Animals

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and were 9-12 weeks of age at the time of experimentation. The animals were housed according to the requirements and approved protocols of the Association for

Assessment and Accreditation of Laboratory Animal Care and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. The animal housing facility was under the supervision, care, and husbandry of the University of North Carolina at Chapel Hill's Division of Laboratory Animal Medicine. All animals were maintained under a normal 12-hour day/night schedule.

Patients

Jejunal intestinal tissue, which is normally discarded following resection during gastric bypass surgery, was collected from an adult patient undergoing elective gastric bypass surgery at the University of North Carolina Hospitals at Chapel Hill (Chapel Hill, NC, USA). To preserve patient confidentiality and to obtain approval from the Biomedical Institutional Review Board (IRB), the age, gender, health, or other personal identifying information were not collected. The procurement of intestinal tissue from gastric bypass surgical patients and all investigational experiments were performed in accordance with the approval from the IRB of the Office of Human Research Ethics at the University of North Carolina at Chapel Hill and in compliance with federal regulations.

Diffusion Chamber Studies using Human and Mouse Intestinal Tissues

Intestinal Tissue Preparation

During the standard operational procedure for a gastric bypass surgery, ~10 cm segment of human jejunum is surgically resected during the creation of a gastrojejunostomy. Following surgical resection and excision, the intestinal tissue was immediately placed in 500 ml of oxygenated ice cold KBR with 50% complete EDTA-free protease inhibitor (Roche, Basel, Switzerland), and transported from the surgical

suite to the laboratory for experimentation. The tissue was "stripped" of the exterior seromusculature and serosa layers by sharp resection. The intestinal epithelium was mounted between two halves of a diffusion chamber insert. The entire procedure was completed in <1 hour from the time the tissue was excised from the patient.

To prepare mouse intestinal tissue for diffusion chamber studies, male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized by an intraperitoneal (IP) injection of ketamine (140 mg/kg) and xylazine (15 mg/kg). Intestinal tissue was excised from the proximal jejunum and immediately washed in 4ºC KBR buffer with 50% complete EDTA-free protease inhibitors (Roche, Basel, Switzerland), as described previously (Dufek et al., 2013). Briefly, a glass rod was gently inserted through a 2 cm intestinal segment, and the intestinal tissue was cut longitudinally and mounted between two halves of a diffusion chamber insert. The insert was placed between two side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA) and bubbled with oxygen/carbon dioxide gas (95%/5%) to maintain viability.

For metformin transport and uptake studies, intestinal tissue segments were preincubated with KBR buffer (37°C) for 30 minutes and initiated by replacing the buffer in the apical chamber with 3 ml of 50 μM [^{14}C]metformin (0.1 μCi/ml) diluted in KBR buffer in the presence of transporter inhibitors or vehicle controls. To determine metformin uptake, buffer containing metformin was aspirated after the designated incubation period and the tissue was quickly removed, washed 3X with ice cold KBR, and dissolved with 2N NaOH at 50°C for 1 hour. Samples were neutralized with 2 N HCl and [^{14}C]metformin concentrations were determined by liquid scintillation spectrometry. Clearance (CL) values for metformin uptake across the apical membrane

and transport from the apical to basolateral compartment in *ex vivo* studies were calculated using equation 3:

$$\mathrm{CL} = \frac{dX/dt}{C_o}$$

where *dX/dt* represents the mass of metformin (*X*) accumulated or transported over time (*t*) and C_o represents the initial concentration in the donor chamber.

Metformin Efflux Studies

Intestinal tissue segments were preloaded with 0.5 mM [^{14}C]metformin (0.3 μCi/ml) in KBR buffer (37ºC) was added to each chamber for 30 minutes prior to initiation of efflux experiments. [^{14}C]Metformin was removed and the intestinal tissue was quickly washed 3X with 4ºC KBR buffer. The experiment was initiated by adding 3 ml of warm KBR buffer (37ºC) to both apical and basolateral chambers in the presence or absence of inhibitors (1 mM pentamidine or 200 μM quinidine). Buffer samples (500 μl) from the apical and basolateral chambers were collected at designated time points with replenishment of equal amounts of buffer to maintain a constant volume.

Intestinal Accumulation of Metformin in Mouse Small Intestine Upon Oral Administration

[^{14}C]Metformin (0.65 mg/kg) in 0.9% NaCl was administered with or without pentamidine (3.4 mg/kg to achieve an intestinal lumen concentration of 1 mM) by oral gavage at a volume of 10 ml/kg to overnight fasted mice. Mice were sacrificed at 15, 30, and 60 minutes. Intestinal tissue was quickly harvested, rinsed and flushed with ice cold phosphate buffered saline, and divided into 9 cm segments. Each tissue segment was

dissolved in 2 N NaOH at 50ºC for 1 hour, neutralized with 2 N HCl, and the amount of [^{14}C]metformin was determined by liquid scintillation spectrometry.

Data Analysis

Significance between two test groups was determined using a Student's t-test using GraphPad Prism® version 4.03 for Windows (GraphPad Software Inc., La Jolla, CA, USA). All data are reported as mean ± S.D. The criterion for a significant difference in values was $p < 0.05$.

3.D. RESULTS

Apical Uptake and Absorptive Transport of Metformin in the Intestinal Tissue from Mice and Humans

The absorptive (apical-to-basolateral) transport of metformin in mouse intestinal tissue was compared to its apical uptake clearance to determine if these processes are similar between human and mouse enterocytes and whether the uptake and transport processes in the intestinal tissues mimic those in the Caco-2 cell monolayers (Proctor et al., 2008). The metformin uptake and transport studies in the Caco-2 cell monolayers suggest that apical uptake of metformin is efficient, the egress across the basolateral membrane is rate-limiting, and consequently apical cellular uptake of metformin was 4.6-fold higher than its transport into the basolateral compartment. The transcellular route plays a minor role in the transport of metformin across Caco-2 cells monolayers, and approximately 90% of the drug traverses the monolayers via the paracellular route. In the current study, a similar ratio of 4.6-fold higher metformin apical uptake *versus* absorptive transport was observed in mouse intestinal tissue, although in human intestinal tissue, the ratio of apical uptake to absorptive transport was 17 (Figure 3.2).

Inhibition of Metformin Uptake in Mouse Cation-selective Transporter-transfected CHO Cells by Pentamidine

In Chapter 2, inhibitors of metformin uptake by several cation-selective transporters were evaluated. However, preliminary *in vivo* results showed that the inhibitors, which were effective in attenuating uptake of metformin in an *in vitro/ex vivo* model, may not work well as *in vivo* inhibitors of metformin intestinal uptake/efflux because they may be absorbed too rapidly. Therefore, pentamidine was considered for an

in vivo inhibitor of metformin intestinal uptake/efflux because of its ability to partition into subcellular compartments and cellular retention. Metformin uptake into mouse transporter-transfected CHO cell lines was conducted in the presence of pentamidine at a range of concentrations (1–1000 μM) to determine the inhibitory potency of pentamidine towards mouse Oct transporters (Figure 3.3). A concentration of 10 μM pentamidine decreased mouse Oct1-, Oct2-, and Oct3-mediated metformin uptake by 90.3 ± 0.8, 97.2 ± 2.0, and 79.4 ± 1.1%, respectively, suggesting that these mouse transporters exhibit greater sensitivity towards pentamidine than their human orthologs (IC_{50} values of 10.6 – 16.4 μM) (Ming et al., 2009). Pentamidine at 10 μM inhibited mPmat- and mMate1-mediated metformin uptake by 67% and 54%, respectively. Greater than 90% inhibition of all transporter-mediated metformin uptake was observed with 1 mM pentamidine.

Inhibition of Metformin Efflux by Organic Cation Compounds in Transporter-transfected CHO Cells and Mouse Small Intestinal Tissue

When CHO cells that individually expressed mouse intestinal transporters Oct1-3 were preloaded with metformin and then incubated with 1 mM pentamidine, metformin efflux was inhibited by >84% (Figure 3.4). mPmat and mMate1 were less sensitive to pentamidine inhibition than Octs, as the inhibitor reduced metformin efflux by 35% and 46%, respectively. In Chapter 2, quindine and desipramine were shown to broadly inhibit uptake of metformin via mouse cation-selective transporters. In the current study, the inhibitory effects of quinidine (3 mM) on metformin efflux were comparable to that of pentamidine (i.e, ~80% for mOct1, mOct3, and mMate1; 68% for mOct2) with the exception of Pmat-mediated efflux, where no inhibition was observed. Unexpectedly, 3 mM desipramine, a compound previously shown to inhibit >90% metformin uptake via

the mouse transporters (Chapter 2) stimulated metformin efflux by approximately 25–50% via mOct1, mOct3, and mMate1 and 900% via mPmat. No inhibition or stimulation of Oct2 by desipramine was observed.

To evaluate the ability of inhibitors to block transporter-mediated metformin efflux in mouse intestinal tissue, the pan inhibitors quinidine and pentamidine were utilized. Desipramine was not evaluated due to the trans-stimulatory effect observed in the previous *in vitro* experiments. Following preloading of metformin into mouse intestinal tissue, its efflux across the apical membrane was found to be ~6.8-fold higher than its basolateral efflux (Figure 3.5A). In the presence of quinidine, the apical efflux of metformin decreased by 33%, with no significant difference in its basolateral efflux. Similar results were obtained with pentamidine, although the 24% decrease in metformin apical efflux in the presence of pentamidine did not reach statistical significance (Figure 3.5B).

Intestinal Accumulation of Orally Dosed Metformin With or Without Orally Dosed Pentamidine

The accumulation of metformin in mouse intestinal tissue after oral dosing was evaluated at 15, 30, and 60 minutes. As high as 25% of the metformin dose was associated with intestinal tissue at the earliest time point, and decreased to ~12% by 60 minutes (Figure 3.6). Metformin accumulation after oral dosing in the proximal 9-cm segment of small intestine was reduced when pentamidine was co-administered (Figure 3.7). Additionally, when metformin was administered alone, drug that had accumulated

at 15 minutes post metformin dose had decreased by ~75% by 60 minutes, with no corresponding change in pentamidine-treated animals. In the middle and distal regions of the small intestine, a reduction in metformin accumulation was not observed in the presence of pentamidine.

3.E. DISCUSSION

Intestinal transporters such as OCT1, OCT3, and PMAT have been postulated as mediators of metformin transport across the small intestine into the portal circulation (Zhou et al., 2007; Graham et al., 2011; Zolk, 2011). However, despite the speculation that transporters mediate the intestinal disposition of metformin, there is currently no direct *in vivo* evidence to support the involvement of cation-selective transporters in the intestinal absorption of metformin. In the present study, the uptake and transport clearance of metformin in intestinal tissue was compared to previously reported observations in Caco-2 cell monolayers (Figure 3.2). The results clearly indicate that metformin transport processes (*i.e.,* efficient apical uptake and poor basolateral egress) in mouse and human intestinal tissues were similar to the mechanisms observed in Caco-2 cell monolayers. These results showed that the use of a mouse model to study the mechanism of intestinal absorption of metformin as proposed, based on the use of the Caco-2 cell monolayers, was quite appropriate.

An important experimental approach that can demonstrate the involvement of transporters in oral absorption is to show that the absorptive transport is reduced by a known inhibitor of putative transporters implicated in the absorptive process. In previous studies conducted in mouse transporter-expressing CHO cells, several inhibitors were identified as pan inhibitors of metformin uptake, including desipramine and quinidine (Chapter 2). However, it became clear upon preliminary experimentation that these inhibitors may not be very effective in inhibiting the intestinal transporters *in vivo* because they may be absorbed too rapidly from the first part of the intestine (e.g. duodenum and anterior jejunum). Therefore, additional *in vitro* experiments were

conducted in the current study to identify cation-selective transporter inhibitors that would be retained longer in the intestinal lumen or epithelium, enabling them to be present at higher levels in the small intestine and to produce a robust inhibitory effect compared to a rapidly absorbed inhibitor. Pentamidine, a diamidine drug with two positive charges at physiologic pH, is used for the treatment of human African trypanosomiasis, pneumocystis pneumonia, and leishmaniasis, and is administered parenterally due to poor oral absorption. Metformin uptake studies conducted in the transporter-transfected cell lines showed that pentamidine was in fact a potent inhibitor not only of mOct1-3, as was the case for human orthologs, but also for mPmat and mMate1 (Figure 3.3). A pentamidine dose of 3.4 mg/kg (10 ml/kg), which results in an intestinal concentration of 1 mM required to inhibit intestinal uptake of metformin in mouse, was well below the reported oral LD_{50} value (lethal dose, 50%) of 300 mg/kg making this inhibitor suitable for use in *in vivo* studies (*Pentamidine* MSDS). Additionally, 1 mM pentamidine in the extracellular buffer was shown to trans-inhibit efflux of metformin from pre-loaded transporter-transfected cell lines. This is an important finding given that efflux of metformin across the luminal membrane of the enterocytes that line the small intestine is a key feature of its intestinal absorption mechanism proposed by Proctor *et al*. (2008).

In Chapter 2, diffusion chamber studies showed that the pan cation-transporter inhibitor, desipramine, attenuated apical uptake of metformin. Interestingly, the current studies showed that desipramine trans-stimulated the efflux of metformin that was pre-loaded in the mouse transporter-expressing cells, a phenomenon which can occur when the uptake of a competitive transporter inhibitor (*i.e.,* substrate) increases the efflux

activity of a bidirectional transporter (Proctor et al., 2008). In contrast, quinidine and pentamidine inhibited the efflux of preloaded metformin from CHO cell lines and mouse intestinal tissue (Figures 3.4 and 3.5). Although the 25% reduction in metformin efflux from mouse intestinal tissue by pentamidine was not significant, it demonstrates a trend towards inhibition of transporter-mediated apical efflux of metformin. Collectively, the *ex vivo* and *in vitro* efflux studies that show a weaker inhibitory effect of pentamidine (compared to quinidine) on mMate1-mediated efflux of metformin, indicate that mMate1 potentially contributes to the apical efflux of metformin in mouse intestinal tissue. Assuming that mMate1 is localized in the apical membrane of the enterocytes, the above conclusion regarding the role of mMate1 in the efflux of metformin seems reasonable based on its pH-dependent activity. Since MATE transporters are proton antiporters (Otsuka et al., 2005), the pH gradient in the upper region of the small intestine would enhance its activity as an efflux transporter. However, further experiments using transporter knockdown cell lines, selective chemical inhibitors, or transporter knockout animals are necessary to confirm this hypothesis. The results from the inhibition studies in mouse intestinal tissue and the lack of efflux inhibition of metformin across the basolateral membrane strongly support the findings of Han *et al.* (2013), which suggest that mOct1 is in the apical membrane of the small intestine. These data are also in agreement with results from studies conducted in Caco-2 cell monolayers that showed no evidence of transporter-mediated basolateral efflux of metformin (Proctor et al., 2008).

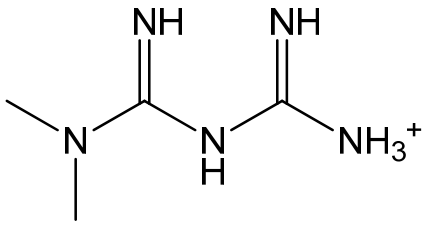
The percent of the metformin dose associated with the small intestinal tissue of mouse at 15 minutes was ~25% (Figure 3.6). This finding of high metformin accumulation in the intestine is consistent with studies reported by Bailey *et al.* (2008) in

which obese type 2 diabetes patients showed 30–300 times higher concentration of metformin in jejunum compared to plasma (Bailey et al., 2008). The effect of pentamidine on the intestinal accumulation of metformin after oral dosing is most prominent in the proximal region of the small intestine, where pentamidine reduced the accumulation by ~55% at both 15 and 30 min. At 60 min, however, metformin accumulation increased by >2-fold when pentamidine was co-administered. Because metformin accumulation was not measured at an earlier time point, it is conceivable that the maximum reduction of metformin accumulation by pentamidine is underestimated, since the control group shows a decline in accumulation between 15-60 minutes. Interestingly, pentamidine-treated mice show steady levels of metformin accumulation over time, suggesting that metformin efflux from the enterocytes may be inhibited. A lack of reduced metformin accumulation (in the presence of pentamidine) in the lower region of the small intestine is not surprising given that pentamidine concentrations are likely to decrease further down the gastrointestinal tract. The use of chemical inhibitors in *in vivo* studies can be challenging since the absorption kinetics of the inhibitor must be considered during data interpretation. While pentamidine is poorly bioavailable, it is a known substrate of human OCT1 and therefore presumably, like metformin, accumulates in the small intestinal tissue (Ming et al., 2009). The observed trend for higher accumulation of metformin in region 2 of the small intestine (Figure 3.7) in the presence of pentamidine can be explained by higher amounts of metformin in the gut lumen due to uptake inhibition in the upper portion of the intestine. However, confirmation of this would require measuring the luminal concentration of metformin in the various regions of the intestine.

In summary, it has been shown that accumulation of metformin in the gut wall is mediated by apical cation-selective transporters combined with a lack of efficient basolateral egress. Based on studies utilizing the transporter inhibitors, pentamidine and quinidine, cation-selective transporters also mediate the efficient efflux of metformin across the apical membrane of mouse small intestine. Collectively, these studies show that the mouse intestine behaves similar to the Caco-2 cell monolayers in terms of apical uptake and efflux as well as lack of basolateral efflux. Therefore, mouse can be used to test the mechanism of the intestinal absorption of metformin as proposed by the "sponge hypothesis" mentioned in the introduction, based on the transport and uptake behavior of metformin in the static Caco-2 cell monolayer model.

3.F. FIGURES

A)



B)

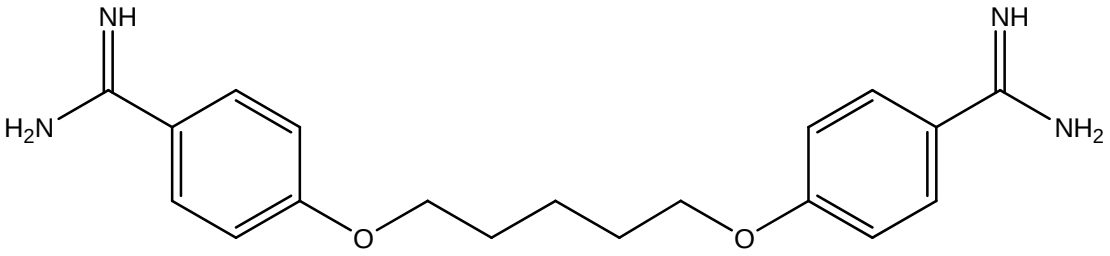


Figure 3.1. Chemical structures of A) metformin and B) pentamidine.

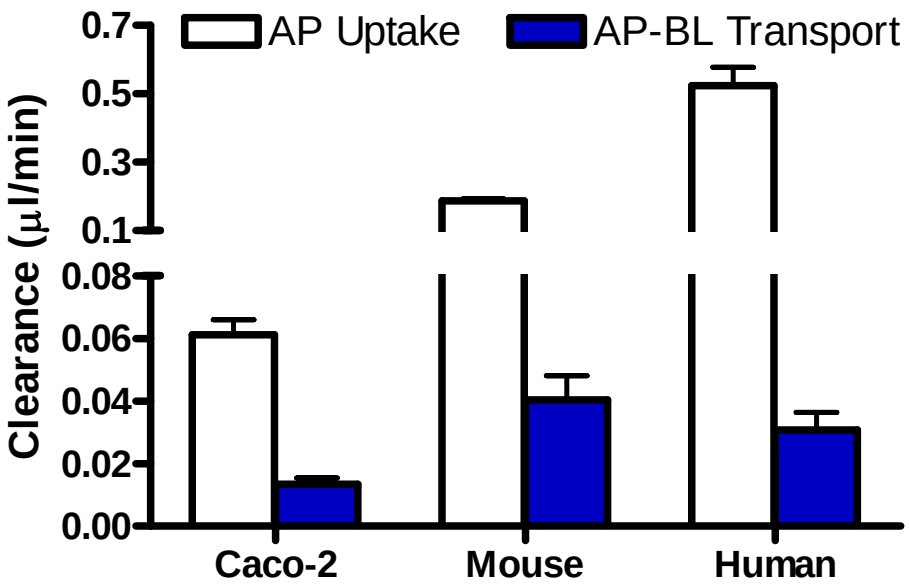


Figure 3.2. Uptake versus transport clearance of metformin in Caco-2 cell monolayers, as well as mouse and human intestinal tissue. [^{14}C]Metformin (50 µM; 0.1 µCi/ml) uptake (open bars) and transport (blue bars) was expressed as clearance values to compare the cellular uptake rate of metformin and absorptive flux. Uptake and transport clearance values were calculated by normalizing the mass of metformin accumulated intracellularly or transported into the basolateral chamber to the initial concentration of metformin in the donor chamber. Data are represented as the mean ± S.D. (n=3).

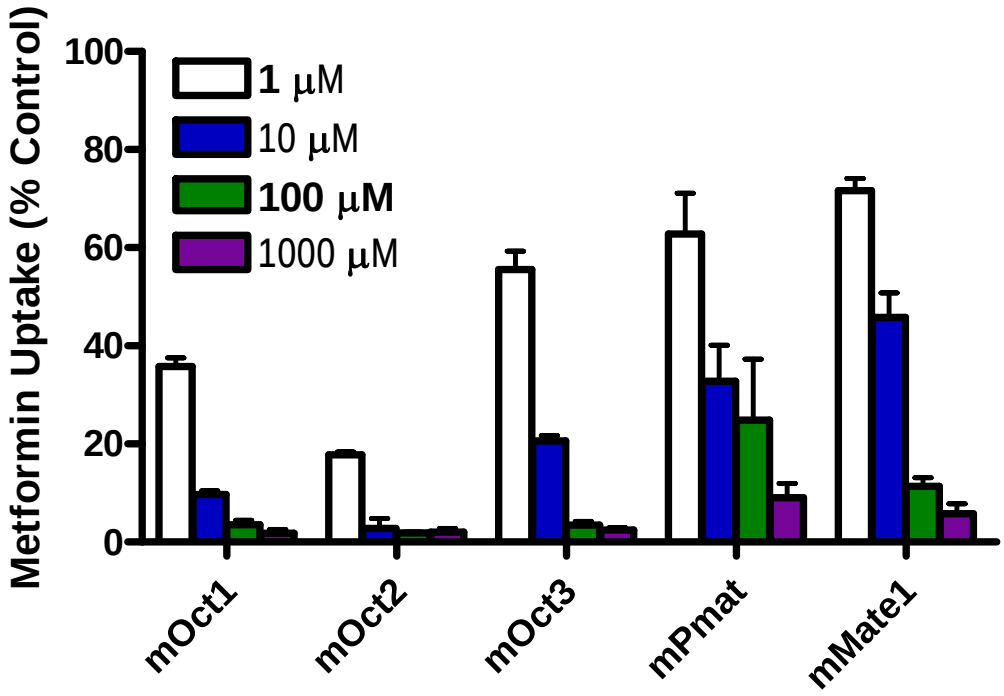


Figure 3.3. Inhibition of transporter-mediated metformin uptake by pentamidine in singly-transfected CHO cells. [^{14}C]Metformin (50 μM; 0.1 μCi/ml) uptake into the cells was measured in the presence of increasing concentrations of pentamidine (open bars = 1 μM; blue bars = 10 μM; green bars = 100 μM; purple bars = 1000 μM). Data are represented as the mean ± S.D. (n=3) and expressed as % control of metformin uptake in the absence of pentamidine.

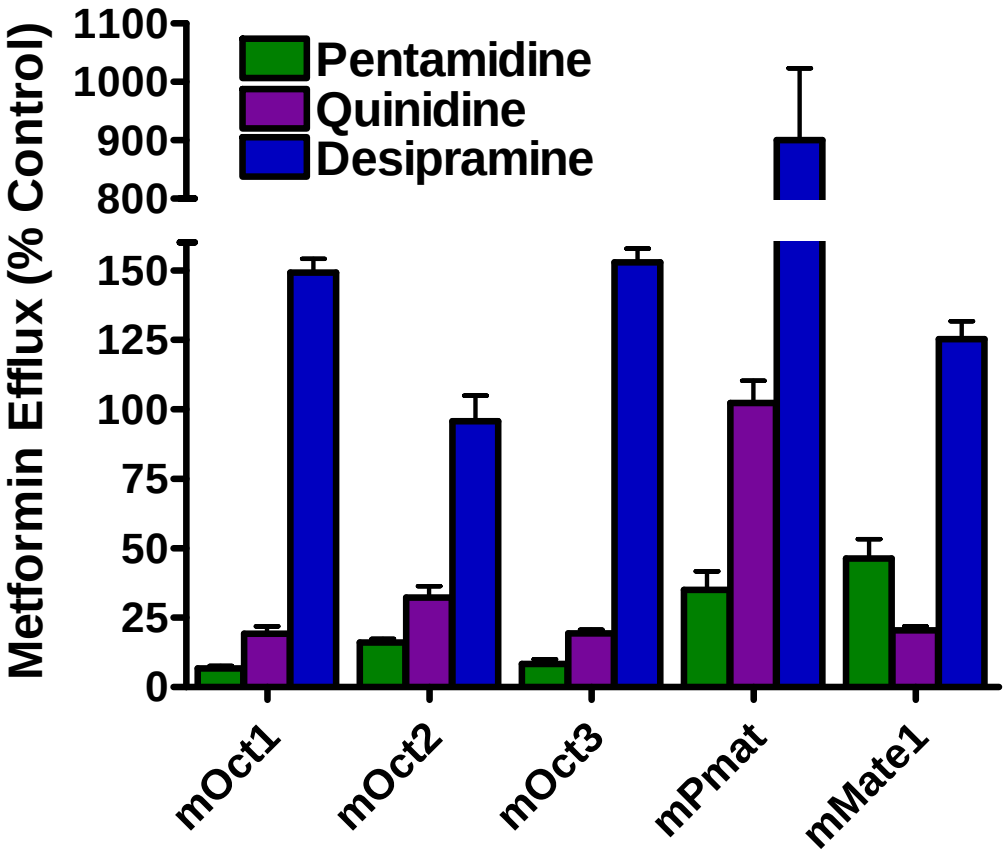


Figure 3.4. Trans-inhibition and -activation of metformin efflux from CHO cells that are singly-transfected with cation transporters by pentamidine, quinidine, and desipramine. [^{14}C]Metformin (500 μM; 0.2 μCi/ml) efflux from singly-transfected CHO cells was measured in the presence of 1 mM pentamidine (green bars), 3 mM quinidine (purple bars), or 3 mM desipramine (blue bars). Data are represented as the mean ± S.D. (n=3) and expressed as % control of metformin efflux in the absence of pentamidine.

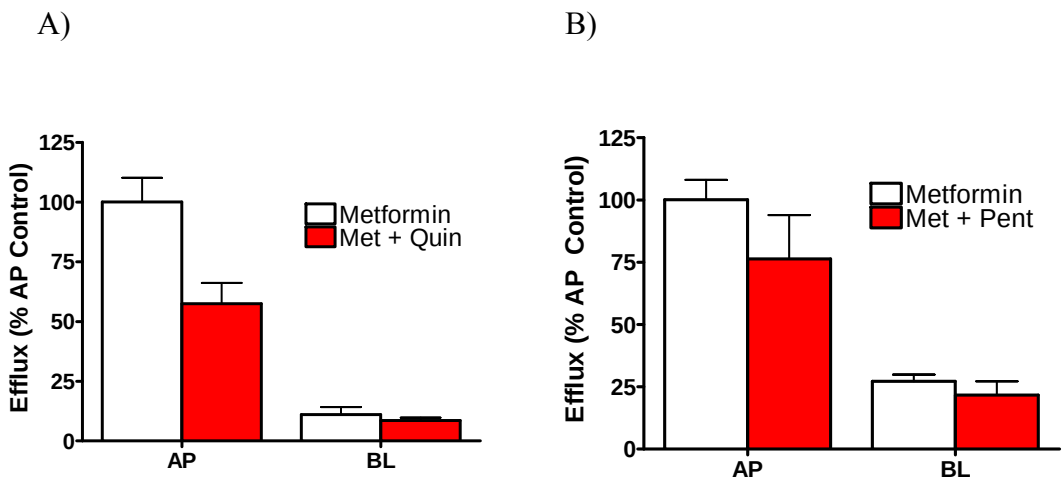


Figure 3.5. Inhibition of metformin efflux from mouse intestinal tissue by quinidine and pentamidine. Efflux from [^{14}C]metformin (500 μM; 0.3 μCi/ml) pre-loaded intestinal tissue (30 min) was determined in the absence (open bars) or presence (red bars) of transporter inhibitors, A) quinidine (200 μM), or B) pentamidine (1 mM). Data are represented as the mean ± S.D. (n=3) and are expressed as % of apical efflux in the absence of inhibitors.

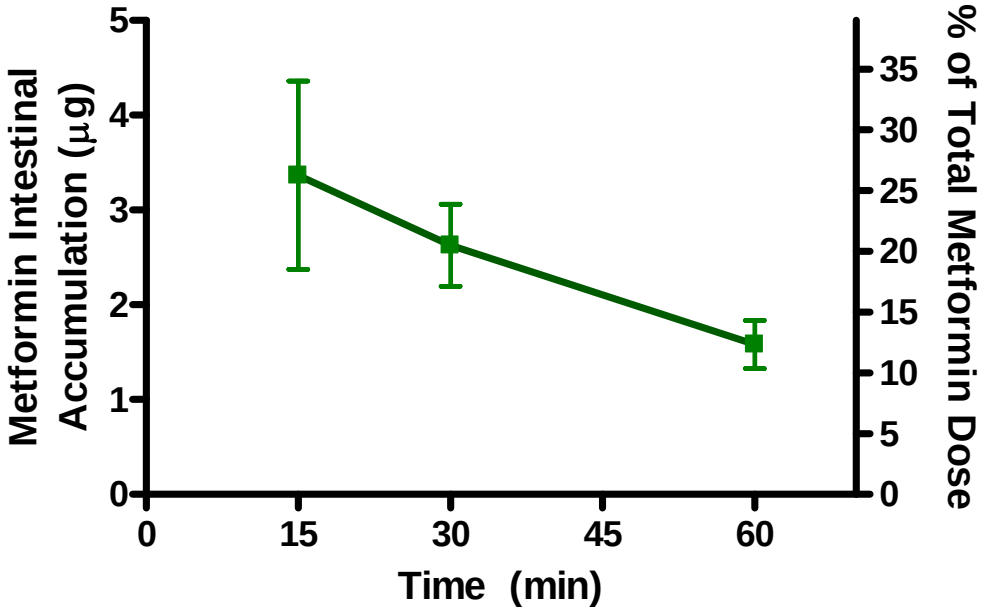


Figure 3.6. Total metformin accumulation in the small intestine of mouse. Total accumulation of [^{14}C]metformin (0.65 mg/kg; 10 ml/kg; 0.3 μCi/ml) in the small intestinal tissue of mouse following oral administration was determined at 15, 30, and 60 min. Data are expressed as the total mass of metformin associated with intestinal tissue and as a percent of the total metformin dose administered. Data are represented as the mean ± S.D. (n=3).

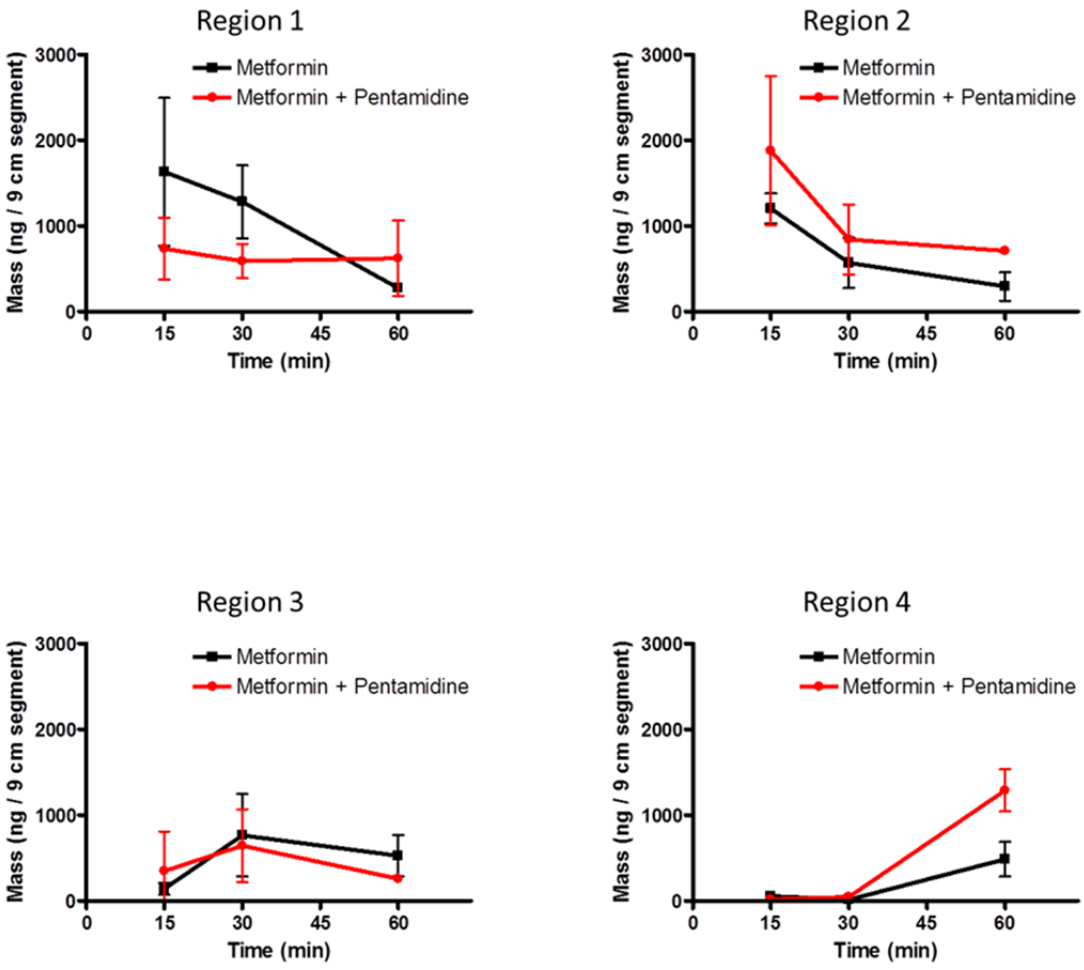


Figure 3.7. Intestinal accumulation of metformin with and without orally co-administered pentamidine. Small intestinal accumulation of [^{14}C]metformin (0.65 mg/kg; 10 ml/kg; 0.3 µCi/ml) with and without orally co-administered pentamidine (3.4 mg/kg) was evaluated in mouse. Intestinal tissue was harvested at 15, 30, and 60 min. Each region represents a 9 cm segment of the small intestine, with region 1 being the most proximal segment and region 4 the most distal segment. Data are represented as the mean ± S.D. (n=3).

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Chapter 4

APICAL UPTAKE AND EFFLUX TRANSPORTERS ENHANCE THE ORAL ABSORPTION OF METFORMIN VIA THE PARACELLULAR ROUTE: PROOF OF CONCEPT FOR THE NOVEL ORAL ABSORPTION MECHANISM OF METFORMIN

4.A. OVERVIEW

The oral absorption of metformin is believed to occur through an intestinal transporter-mediated process. Based on studies conducted with Caco-2 cell monolayers, Proctor *et al.* (2008) put forward a "sponge" mechanism for the intestinal absorption of metformin which proposes that intestinal transporters mediate cellular uptake and efflux of metformin across the apical but not the basolateral membrane, and indirectly augment the oral absorption of metformin via the paracellular route through increased exposure of metformin to the paracellular space. The goal of the current study is to test this hypothesis in an *in vivo* mouse model. Transporter-selective chemical inhibitors, namely desipramine and pentamidine, when dosed orally, decreased the area under the plasma concentration-time curve ($AUC_{0\text{-}6\ hrs}$) of orally dosed metformin by ~35%. Orally co-administered pentamidine did not affect the pharmacokinetic concentration-time profile of intravenously administered metformin. In portal vein cannulated mice, the portal and systemic exposures of orally dosed metformin were reduced by ~3.6-fold, with a corresponding 3.7-fold decrease in portal bioavailability (F_g) when pentamidine was co-administered. Further, pentamidine significantly reduced metformin plasma AUC when

its oral administration was delayed by 15 minutes after metformin oral dosing. This result provided circumstantial evidence that not only the transporter-mediated uptake of metformin into the enterocytes, but also its transporter-mediated efflux contributed to the oral absorption of metformin. Parallel studies with ranitidine, which has been shown to have a significant transcellular component in its intestinal absorption, showed no change in its pharmacokinetic profile with delayed administration of pentamidine. The oral absorption of mannitol, a paracellular probe, was also unaffected by pentamidine. These results, taken together, provide strong evidence for the "sponge" mechanism of the oral absorption of metformin, in which the oral dose of metformin is taken up into the intestinal epithelium, with a small percentage of the dose absorbed via the paracellular route. Subsequently, the metformin dose that is trapped in the enterocytes egresses into the lumen. This allows some of the dose to be absorbed paracellularly, some of the dose to transit forward in the lumen due to intestinal motility, and some to be taken up again into the enterocytes. The experimental design employed in this work provides a novel strategy to study the contributions of paracellular versus transcellular route for drug absorption *in vivo*.

4.B. INTRODUCTION

The widely prescribed antidiabetic drug metformin is a highly hydrophilic compound. As described in previous chapters, physicochemical properties of metformin suggest that passive diffusion through biological membranes such as the intestinal epithelium is an inefficient process. However, metformin is a well-absorbed drug with an oral bioavailability as high as ~60%, and its dose-normalized absorption is reduced at higher oral doses (Pentikainen et al., 1979), suggesting that transporters may play a role in its oral absorption.

Metformin shows substrate activity for a number of solute carrier (SLC) transporters that are classisfied as polyspecific organic cation transporters (Kimura et al., 2005a; Kimura et al., 2005b; Masuda et al., 2006; Koepsell et al., 2007; Tanihara et al., 2007; Zhou et al., 2007; Nies et al., 2009). Included in this group of known metformin transporters are the organic cation transporters 1, 2, and 3 (OCT1-3; *SLC22A1-3*), the plasma membrane monoamine transporter (PMAT; *SLC29A4*), and the multidrug and toxin extrusion transporters 1 and 2 (MATE1-2; *SLC47A1-2*). Recent results have identified the high affinity choline transporter (CHT; *SLC5A7*) and the serotonin reuptake transporter (SERT; *SLC6A4*) as additional metformin transporters (Han et al., 2012). The mOcts in the sinusoidal membrane of hepatocytes mediate uptake of metformin from the systemic circulation into the liver cells, where metformin is known to exert its major pharmacologic effect of reducing gluconeogenesis. Similarly, in renal proximal tubules, mOcts in the basolateral membrane mediate renal excretion, the major route of elimination of metformin (Wang et al., 2002). On the apical membrane of mouse hepatocytes and renal cells, the proton antiporters mMate1 and mMate2 are the major

transporters responsible metformin efflux into the bile and urine, respectively (Ito et al., 2010; Kusuhara et al., 2011; Ito et al., 2012). However, the role of transporters in the intestinal absorption of metformin is predominantly limited to studies conducted in a cellular model of intestinal absorption, Caco-2 cell monolayers, which have identified OCT1, PMAT, SERT, and HCT as the major contributors to the luminal uptake of metformin (Han et al., 2012).

In 2008, Proctor *et al.* proposed a novel absorption mechanism of metformin based on detailed kinetic studies of this drug across the apical and basolateral membranes of Caco-2 cell monolayers, as well as its overall transport across this cellular intestinal model. In these studies, efficient apical uptake and efflux of metformin was observed with poor rate-limiting basolateral efflux via the transcellular route. A three-compartment kinetic model utilized to describe metformin permeability in the Caco-2 Transwell® model revealed that >90% of metformin was transported across the epithelial monolayer via the paracellular route (Proctor et al., 2008). These results are consistent with previous estimates of an 88% paracellular flux of metformin in Caco-2 cell monolayers (Saitoh et al., 2004), where a more simple methodology was implemented in which the apparent passive permeability determined in a parallel artificial membrane permeability assay (PAMPA) was subtracted from apparent permeability (P_{app}) values determined in Caco-2 cells. The findings in these studies by Proctor and Saitoh are interesting given that paracellular absorption is generally considered inefficient, due to the narrow space between adjacent epithelial cells and the presence of the protein complex known as tight junctions. The high oral absorption of metformin is in stark contrast to the 16% bioavailability of mannitol (Artursson and Karlsson, 1991), which is

a classic paracellular probe. To explain the discrepancy in data between the efficient oral absorption of metformin in humans and its predominantly paracellular transport *in vitro*, the "sponge" mechanism of the intestinal absorption of metformin was proposed by Proctor *et al* (2008) that was consistent with the observed transport data in the Caco-2 cell model as well as the clinical observations showing dose-dependent absorption, "flip-flop" kinetics, and relatively flat plasma concentration *versus* time profile after oral dosing of metformin (Proctor et al., 2008). This hypothesis stated that cation-selective transporters facilitate the uptake of metformin across the luminal membrane of enterocytes, and due to a lack of efficient basolateral efflux, metformin accumulates in the enterocytes. As the dose of metformin in the intestinal lumen travels distally through the gastrointestinal tract, the concentration gradient across the apical membrane reverses, and efflux of metformin occurs through electrogenic cation transporters. This cycling of metformin between the gut lumen and enterocytes increases the access of metformin to the paracellular space. Therefore, the paracellular absorption of metformin is indirectly augmented by apical transporter-mediated uptake and accumulation of metformin and its subsequent transporter-mediated efflux.

This novel intestinal absorption mechanism of metformin was based on studies in Caco-2 cell monolayers, which is a static system. Gastrointestinal transit of metformin is undoubtedly a prerequisite for the enhanced oral absorption of this drug, which could not be tested in the Caco-2 cell model or other cell-based static models. Therefore, the goal of the current study is to test the key components of the proposed novel absorption mechanism of metformin in an *in vivo* mouse model, such as its transporter-mediated luminal uptake and efflux in the small intestine, and the contribution of each to the oral

absorption of the drug. The transporter-mediated apical uptake and efflux of metformin, as well as its poor efflux across the basolateral membrane observed in the Caco-2 cell model were also demonstrated in *ex vivo* mouse intestinal tissue in diffusion chamber studies (Chapter 3). Therefore, the mouse was considered an appropriate model for studies designed to test the proposed absorption mechanism for metformin. The results show that (1) apical uptake transporters mediate the *in vivo* intestinal absorption of metformin, and (2) transporter-mediated apical efflux contributes to the enhanced absorption of metformin, thus providing support for the "sponge hypothesis" explaining the absorption mechanism of metformin.

4.C. METHODS

Materials

Metformin, desipramine, pentamidine, and ranitidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{14}C]Metformin (110 mCi/mmol) and [^{3}H]pentamidine (500 mCi/mmol) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). Sterile saline (0.9% NaCl) was purchased from Med-Vet International (Mettawa, IL, USA).

Animals

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were 9-12 weeks of age at time of experimentation. The animals were housed according to requirements and approved protocols of the Association for Assessment and Accreditation of Laboratory Animal Care and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. The animal housing facility was under the supervision, care, and husbandry of the University of North Carolina at Chapel Hill's Division of Laboratory Animal Medicine. All animals were maintained under a normal 12-hour day/night schedule.

Pharmacokinetic Studies in Conscious Mouse

Mice were fasted overnight prior to the initiation of each study. [^{14}C]Metformin (0.65 mg/kg; 15 μCi/ml), [^{3}H]pentamidine (3.4 mg/kg; 20 μCi/ml), or [^{3}H]mannitol (0.1 mg/kg; 5 μCi/ml) dissolved in 0.9% NaCl was administered by oral gavage in a volume of 10 ml/kg. For metformin and mannitol studies, serial blood samples (~20 μl/sample) were collected from the saphenous vein at 5, 15, 30, 60, 120, 240, and 360 minutes post dose administration utilizing heparinized microhematocrit capillary tubes (Fisher

Scientific, Pittsburgh, PA, USA). A [^{3}H]pentamidine oral pharmacokinetic profile was determined by serial sampling at 5, 15, 30, 45, 60, 120, and 180 minutes. For chemical inhibition studies, orally dosed metformin was co-administered with desipramine (8.0 mg/kg) or pentamidine (3.4 mg/kg) to achieve intestinal concentrations of 3 mM and 1 mM, respectively. Pentamidine doses used in delayed administration were similar to those that were co-administered with metformin. However, in experiments involving delayed administration, metformin or ranitidine and pentamidine were given as two separate oral doses (*i.e.*, 0.65 mg/kg; 5 ml/kg for metformin or 1.57 mg/kg; 5 ml/kg for ranitidine, and 3.4 mg/kg; 5 ml/kg pentamidine 15 or 30 min post metformin administration). Blood samples were collected at 5, 15, 30, 45, 60, 90, and 120 minutes. To determine systemic plasma concentrations of [^{14}C]metformin (0.65 mg/kg; 5 ml/kg) following intravenous dosing, serial blood samples were collected from the saphenous vein at 5, 15, 30, 45, 60, 90, and 120 min post dose administration. For intravenous studies using the transporter inhibitor pentamidine (3.4 mg/kg; 10 ml/kg), mice were orally dosed with inhibitor followed by an intravenous metformin dose administered at the estimated T_{max} of pentamidine. Plasma concentrations of [^{14}C]metformin, [^{3}H]mannitol, and [^{3}H]pentamidine (10 μl) were quantified by liquid scintillation spectrometry.

Metformin Pharmacokinetics in Portal Vein Cannulated Mouse

Male C57BL/6J mice were anesthetized by an intraperitoneal injection of urethane (1.5 g/kg). The depth of anesthesia was monitored throughout the surgical procedure and experiment by the toe pinch reflex. The portal vein was cannulated as previously described by Dufek *et al.* (2013). Briefly, an abdominal midline incision was

made and the small intestine was gently pushed aside to expose the portal vein, which was cannulated by implanting a saline-filled silastic catheter (0.025" OD x 0.012" ID) (Braintree Scientific, Braintree, MA, USA) with a 26 gauge needle tip (Becton Dickinson, Franklin Lakes, NJ, USA) attached to the end into the portal vein. The cannula was secured to the surrounding tissue with a micro-serrefine vascular clamp (FST, Foster City, CA, USA). Following the oral administration of [^{14}C]metformin (0.32 mg/kg; 5 ml/kg) in the absence and presence of pentamidine (1.7 mg/kg; 5 ml/kg), portal blood samples were collected at 5, 15, 30, 60, and 120 minutes. Matched systemic samples via the tail vein were collected from the same animal and [^{14}C]metformin plasma concentrations were determined by liquid scintillation spectrometry.

The portal bioavailability was calculated as previously described by Dufek *et al.* (2013). Briefly, the mass of metformin absorbed ($M_{Absorbed}$) following oral administration was calculated according to equation 1,

Equation 1:

$$M_{Absorbed} = M_{Portal} - M_{Systemic}$$

where M_{Portal} is the mass of drug in the portal circulation and $M_{Systemic}$ is the mass of drug in the portal circulation returned from the systemic circulation. The M_{portal} and $M_{Systemic}$ were calculated using the portal blood flow (Q_{portal}), the area under the portal plasma concentration-time curve (AUC_{portal}), and the area under the systemic plasma concentration-time curve ($AUC_{systemic}$) as described below.

Equation 2:

$$M_{portal} = R_B * Q_{Portal} * AUC_{portal}$$

Equation 3:

$$M_{systemic} = R_B * Q_{Portal} * AUC_{systemic}$$

Plasma concentrations of metformin in the portal and systemic circulations were converted to blood concentrations using the blood-to-plasma ratio (R_B) of metformin, which was calculated by spiking [^{14}C]metformin into fresh mouse plasma or whole blood to obtain a final concentration of 5 μM. Blood samples were incubated at 37°C with shaking for 30 minutes and then centrifuged for 10 minutes at 9000 x g at 4ºC for 10 minutes. [^{14}C]metformin concentrations in the plasma samples were measured by liquid scintillation spectrometry. F_g was calculated using equation 4,

Equation 4:

$$F_g = \frac{M_{Absorbed}}{Dose}$$

Ranitidine Assay

Ranitidine was extracted from 10 μl of mouse plasma by protein precipitation with 100 μl of acetonitrile and 10 μl of 100 nM famotidine as an internal standard. Samples were vortexed for 1 minute, centrifuged at 9000 x g at 4°C for 10 minutes, and the supernatant was transferred to a clean tube. Ranitidine and famotidine were quantified by liquid chromatography-mass spectrometry (LC-MS/MS). The LC/MS/MS system comprised LC10-ADVP quaternary pumps (Shimadzu, Kyoto, Japan) that were fitted with a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC, USA) and a Sciex API-4000 triplequadropole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The chromatographic separation of analytes was performed with a linear gradient of 0–80% B at a flow rate of 0.75 mL/min over four min and a sample injection volume of 4 μl. The analytical column was a Zorbax SB-C18 2.1x50

mm, with a 5 μm particle size (Agilent, Santa Clara, CA, USA). The samples were ionized using TuroboIonSpray ion source and the positive ions were monitored at the following Q1/Q3 transitions (m/z): 315.15→176.2 for ranitidine and 338.40→189.0 famotidine (internal standard). Calibration standard curves that ranged from 1–5000 nM were linear ($R^2 > 0.99$) with accuracy ± 15% nominal and were analyzed at the beginning and end of the run. The data were acquired and processed using Analyst 1.4.1 (Applied Biosystems, Foster City, CA, USA).

Data Analysis

Pharmacokinetic parameters of orally and intravenously administered metformin were obtained by fitting a two-compartment model to metformin plasma concentration versus time data after oral and intravenous administration in mice using WinNonlin Version 5.3 (Pharsight, Mountain View, CA, USA). Significance was determined using a Student's T-test for two treatment groups or One-Way analysis of variance (ANOVA) followed by a Tukey's post-test for three groups using GraphPad Prism® version 4.03 for Windows (GraphPad Software Inc., La Jolla, CA, USA). A minimum of three mice were used for each treatment group. All data are reported as mean ± S.D. The criterion for a significant difference in values was $p<0.05$.

4.D. RESULTS

Effect of Cation-selective Transporter Inhibitors, Desipramine and Pentamidine, on the Oral and Intravenous Pharmacokinetics of Metformin in Conscious Mouse

Metformin (0.65 mg/kg) was administered via oral gavage in the presence and absence of the pan cation-selective transporter inhibitors desipramine (8.0 mg/kg) or pentamidine (3.4 mg/kg) to C57BL/6J mice (Figure 4.1A). The inhibitor doses used in this study were selected with the goal of achieving intestinal concentrations previously shown (Chapters 2 and 3) to inhibit metformin uptake by the putative mouse transporters of metformin (*i.e.*, mOct1-3, mPmat, and mMate1). Systemic plasma $AUC_{0\text{-}6hrs}$ of metformin was reduced by 37% and 33% ($p<0.01$) in the presence of desipramine and pentamidine, respectively, compared to control mice (Figure 4.1B). Desipramine and pentamidine decreased the estimated maximal concentration (C_{max}) of metformin by 55% and 52% ($p<0.01$), respectively.

To minimize the possibility of confounding systemic effects of inhibitors, pentamidine, a compound with a poor oral bioavailability, was chosen for subsequent *in vivo* studies. To determine whether orally dosed pentamidine affects metformin disposition at the systemic level, metformin pharmacokinetics was evaluated after intravenous dosing of the drug with or without concurrent oral administration of the inhibitor (Figure 4.2A). Following an oral dose of pentamidine (3.4 mg/kg; 10 ml/kg), metformin was administered intravenously 30 minutes later, which is the estimated T_{max} of pentamidine (Figure 4.3). There was no statistically significant difference in metformin $AUC_{0\text{-}120\ min}$, steady state volume of distribution (V_{ss}), or clearance (CL) between the pentamidine-treated and the control animals (Table 1).

Effect of Pentamidine on Metformin Pharmacokinetics in Portal Vein Cannulated Mouse

Because oral and intravenous pharmacokinetic studies in conscious mice suggested transporter-mediated intestinal absorption of metformin, the effect of pentamidine on the intestinal absorption of metformin was further investigated using a portal vein cannulated mouse model. This method allows for direct measurement of the drug in the portal circulation as the drug traverses the intestinal epithelium so that involvement of other organs in the pharmacokinetics of metformin could be minimized. . Both the portal and systemic $AUC_{0\text{-}120\ min}$ of metformin decreased by ~3.6-fold ($p<0.05$) in the presence of 1 mM pentamidine (Figure 4.4A/B; Table 4.2). The calculated F_g in control and pentamidine-treated mice also decreased by 3.7-fold from 1.04 ± 0.51 to 0.28 ± 0.25, respectively. These calculations were based on the experimentally determined blood-to-plasma ratio of 0.70 ± 0.04 and a previously reported portal blood flow rate in mouse of 1.45 ml/min (Davies and Morris, 1993). The results from the study in conscious animals and in portal vein cannulated mice showed that cation-selective uptake transporters contributed to increased metformin absorption after oral dosing.

Pharmacokinetic Behavior of Metformin and Ranitidine Following Delayed Administration of Pentamidine

To assess the role of transporter-mediated intestinal apical efflux of metformin in the oral absorption of the drug, metformin was administered via oral gavage to allow accumulation in the small intestinal tissue. At 15 or 30 minutes post-metformin dose, pentamidine or saline was orally administered (Figure 4.6A/B). A statistically significant decrease of 27% ($p<0.05$) in metformin $AUC_{0\text{-}120\ min}$ was observed with the 15 minute

delayed inhibitor dose while no change was associated with the 30 minute delayed dose (Figure 4.6C). The C_{max} of metformin was reduced from 133 to 89 ng/ml (33% decrease) after delayed pentamidine administration compared to control, although this did not reach statistical significance. Additionally, a flattened metformin oral plasma concentration-time profile was observed after administration of pentamidine (15 and 30 min delay), indicating a decreased but sustained rate of absorption of metformin.

The effect of a delayed pentamidine dose on the oral absorption of ranitidine was also evaluated. Both paracellular and transcellular routes of absorption appear to contribute nearly equally to transport of ranitidine across Caco-2 cell monolayers at concentrations ranging from 0.1–2 mM (Bourdet et al., 2006). Because the basolateral egress of ranitidine occurs via passive diffusion it was hypothesized that blocking the efflux (if any) of ranitidine by delayed administration of pentamidine would have no effect on ranitidine absorption. As expected, ranitidine pharmacokinetic profile was unaltered with no statistically significant changes in rantidine $AUC_{0\text{-}120\ min}$ (Figure 4.7) upon delayed oral dosing of pentamidine.

Effect of Pentamidine on Paracellular Absorption of Mannitol

The oral pharmacokinetics of mannitol (0.1 mg/kg), a paracellular probe, was evaluated with or without co-administration of pentamidine to investigate effects of the inhibitor on paracellular transport. Co-administration of pentamidine showed no statistically significant change in mannitol $AUC_{0\text{-}60\ min}$ or plasma concentration at any of the individual time points (Figure 4.8).

4.E. DISCUSSION

The oral absorption of metformin is assumed to be transporter-mediated, yet evidence for such an absorption mechanism has been lacking. In Chapter 3, it was demonstrated that the pan cation-selective transporter inhibitor, pentamidine, reduced the *in vivo* intestinal accumulation of metformin in mice when orally co-administered with metformin. Here it is shown for the first time that inhibitors of cation-selective transporters reduce the intestinal absorption of orally co-administered metformin (Figure 4.1). Collectively, these data clearly suggest that apical intestinal cation-selective transporters increase the oral absorption of metformin through a process that involves intestinal accumulation.

Oral pharmacokinetic studies conducted with mMate1 knockout mice and mOct1/2 double knockout mice have shown an ~4-fold increase in metformin systemic AUC compared to control animals (Tsuda et al., 2009; Higgins et al., 2012). The significant increase in V_{ss} of metformin in mOct1/2 double knockout mice and not in mMate1 knockout animals was presumably due to differences in transporter localization, with mOct1 and mOct2 mediating basolateral uptake of metformin from blood, and mMate1 mediating its efflux on the apical membrane of both kidney and liver cells. Since these studies demonstrate the importance of transporters on the pharmacokinetics and tissue accumulation of metformin, the potential effects of pentamidine on intravenously administered metformin disposition was investigated. In the current study, intravenously administered metformin in the presence of pentamidine showed no significant change in AUC or V_{ss} of metformin, indicating that cation-selective transporters in the liver or kidney were not inhibited after an oral dose of pentamidine.

Furthermore, an oral pharmacokinetic study of pentamidine showed (Figure 4.2) that systemic concentrations of this inhibitor were lower than the levels required to substantially inhibit mouse metformin transporters, with the exception of mOct2 (Chapter 3). To explicitly show that the transporter inhibitory effects of pentamidine occur at the intestinal level, a portal vein cannulated mouse model was employed. This method clearly demonstrated a significant decrease in the portal and systemic exposure of metformin upon co-administration of pentamidine (Figure 4.4), with a corresponding 3.7-fold reduction in the calculated F_g, suggesting the involvement of intestinal transporters in the oral absorption of metformin.

In the absence of co-administered pentamidine, the F_g of metformin in portal vein cannulated mice was 1.04 ± 0.51, which was comparable to a systemic bioavailability (F) of 1.04 that was calculated from metformin AUC values derived from a two compartment model fit to intravenous and oral metformin pharmacokinetic data. The absolute oral F was ~2-fold higher than the reported F in humans (~40–60%), suggesting possible enterohepatic recirculation of metformin.

Orally administered metformin is known to accumulate within the enterocytes of the small intestine in both human and mouse (Wilcock and Bailey, 1994; Bailey et al., 2008) as was seen in the studies described in Chapter 3. As per the "sponge hypothesis", the accumulated metformin would efflux across the apical membrane and then get absorbed via the paracellular route rather than getting absorbed transcellularly by passage across the basolateral membrane. To test this component of the hypothesis, a study was designed where orally administered metformin was allowed to accumulate within the enterocytes for 15 min before oral administration of the transporter inhibitor, pentamidine

(15 min is the time point at which highest intestinal accumulation of metformin was observed; Chapter 3). It is important to note that the basolateral egress of metformin was not affected by pentamidine in metformin preloaded mouse intestinal tissue (Chapter 3). Several different hypothetical pharmacokinetic profiles of metformin can be conceived for this dosing regimen of metformin and pentamidine, which would reveal the mechanism of absorption of intestinally accumulated metformin (Figure 4.5). (1) If accumulated metformin is not apically effluxed, and therefore crosses the basolateral membrane by a pentamidine-insensitive process, no change in the oral profile of metformin the metformin plus pentamidine group is expected (Figure 4.5A). (2) Even if metformin is effluxed via apical transporters, it could still be absorbed transcellularly. If apical efflux of metformin is followed by subsequent re-uptake into the enterocytes, an increase in its rate of absorption is expected when pentamidine is co-administered (*i.e.*, shorter T_{max}) (Figure 4.5B). (3) Additionally, if metformin efflux is extensive and the apical transporters attenuate metformin absorption in the absence of inhibitor, pentamidine would cause an increase in systemic AUC (Figure 4.5C). (4) Finally, accumulated metformin could be apically effluxed and absorbed paracellularly as its major route of absorption. In this case, the co-administration of an apical efflux inhibitor would slow the absorption rate (Figure 4.5D).

In the present study, the results provide strong evidence for the apical efflux of metformin followed by its paracellular absorption in mouse intestine. A 15 minute delayed administration of pentamidine decreased the C_{max} of metformin by 33% and generated a flattened and virtually indistinguishable T_{max} (Figure 4.6A). These data suggest that pentamidine reduces the apical efflux and therefore absorption rate of

metformin across mouse intestinal tissue. It is possible that there is a small degree of mixing of pentamidine with unabsorbed metformin in the intestinal lumen, which could also reduce the absorption of metformin via inhibition of its apical uptake. Administration of pentamidine 30 minutes after metformin oral dosing also resulted in a flattening of the oral profile of metformin, although there was no change in systemic plasma AUC by the inhibitor treatment (Figure 4.6B/C). This finding is consistent with the inhibition of apical efflux of metformin and not its apical uptake, since all of the accumulated metformin in the small intestine would be absorbed, although at a slower rate according to this model.

Compartmental analysis of transport and accumulation of ranitidine shows that this drug traverses Caco-2 cell monolayers by paracellular and transcellular mechanisms (Bourdet et al., 2006). Bourdet *et al.* reported that ranitidine is a substrate of OCT1 as well as the efflux transporter P-glycoprotein (P-gp) at the apical membrane of Caco-2 cells (Bourdet et al., 2006; Bourdet and Thakker, 2006). Inhibition of mOct1 by pentamidine had no effect on ranitidine pharmacokinetics as this drug is capable of crossing the basolateral membrane either by passive diffusion ($pK_a = 8.2$) or an unknown efflux mechanism, independent of cation-selective transporters (Figure 4.7).

Because these data support at least partial absorption of metformin via the paracellular route, the effect of pentamidine on the oral absorption of a classic paracellular marker, mannitol, was also evaluated. A significant change in the systemic plasma $AUC_{0\text{-}60\ min}$ of mannitol in the presence of pentamidine was not observed over the first hour. Interestingly, a dramatic increase in plasma concentrations of mannitol was seen at later time points (≤ 90 minutes) in animals treated with pentamidine (data not

shown). This high rate of mannitol absorption corresponds with the small intestinal transit time of approximately1 hr, and the time at which the oral doses would reach the large intestine in mouse (Hamada et al., 1999). Krugliak *et al.* reported varying rates of mannitol absorption along the gastrointestinal tract of rat, with >5-fold higher mannitol permeability in the colon compared to jejunum (Krugliak et al., 1994). This was attributed to the large amount of water re-absorption in the colon, leading to paracellular transport of mannitol by convective forces. Because metformin absorption occurs predominantly within the small intestine, the potential effects of pentamidine on increased colonic absorption of the paracellular marker, mannitol, should not influence the paracellular absorption of metformin.

The use of pentamidine as an inhibitor of polyspecific organic cation transporters allowed us to evaluate the role of efflux transporters in the oral absorption of metformin. However, the experiments conducted in this study were not designed to evaluate the contributions of individual transporters. Given that both mOct1 and mMate1 are highly expressed in the mouse small intestine (see Chapter 2), they are the primary candidates that need to be investigated in future studies related to the intestinal absorption of metformin. As with ranitidine, the efflux of organic cations via electrogenic transporters, such as the Octs is energetically unfavorable since they require a large concentration gradient to overcome the negative resting potential of the enterocytes (Koepsell et al., 2007). However, based on the findings of this study and previous reports demonstrating high intestinal accumulation of metformin in human and mouse intestine (Chapter 3; (Wilcock and Bailey, 1994; Bailey et al., 2008), a large concentration gradient very likely exists. Maximum theoretical concentrations of metformin in the small intestinal lumen

following clinically relevant doses can range from ~15–25 mM (500–850 mg metformin / 250 ml of water). As the metformin dose in the lumen travels along the gastrointestinal tract, the luminal concentration would decrease while the intracellular metformin concentration in the enterocytes would remain high. In addition to mOct1, mMate1 is a very likely candidate to facilitate egress of metformin across the apical membrane, based on gene expression data (Chapter 2). The precise membrane localization of mMate1 in the small intestine has not been elucidated but future studies with transporter specific inhibitors or gene knockout/knockdown studies could confirm this. If mMate1 is indeed on the apical membrane of enterocytes, the presence of a proton gradient, particularly in the upper small intestine would help facilitate metformin efflux, as MATE transporters are proton antiporters (Otsuka et al., 2005). While these transporters are plausible mediators of metformin efflux in the mouse intestine, known species differences in transporter expression suggest that the relative roles of individual transporters in the intestinal absorption of metformin are likely to be dissimilar between mouse and humans. Bourdet reported high levels of OCT3 mRNA in the human small intestine compared to OCT1 and OCT2 (Bourdet, 2005), which may also play a role in metformin absorption. It is not within the scope of the current set of experiments to show evidence of any specific efflux transporter that may play a role in metformin intestinal absorption in humans. Rather, inhibition of all putative transporters of metformin in mouse intestine via a pan inhibitor provides proof-of-concept that apical efflux of metformin enhances its paracellular absorption.

In summary, the current studies show that (1) cation-selective transporters at the apical membrane of the mouse intestine mediate both the uptake and efflux of metformin,

which together facilitate the intestinal accumulation of this orally administered drug, and (2) apical efflux of metformin that is accumulated in the enterocytes enhances its paracellular absorption across the intestinal epithelium. These studies also provide a relevant strategy to address paracellular versus transcellular absorption of intestinally accumulated drugs in an *in vivo* system. This strategy can be applied to the study of metformin in a clinical setting or to understand the mechanism of intestinal absorption of other drugs.

TABLES AND FIGURES

Table 4.1. Oral and intravenous pharmacokinetic parameters of metformin

A) Oral pharmacokinetic parameters of metformin

Parameter	Metformin	Metformin + Pentamidine	Metformin + Desipramine
PO Dose (mg/kg)	0.65	0.65	0.65
AUC (min*ng/ml)	16149	10789	10108
CL/F (ml/min)	0.77	1.04	1.18
V/F (ml)	34.6	68.6	108.9
T_{max}	28.2	36.2	32.7
C_{max}	152.0	77.7	74.8

B) Intravenous pharmacokinetic parameters of metformin

Parameter	Metformin	Metformin + Pentamidine
IV Dose (mg/kg)	0.65	0.65
AUC (min*ng/ml)	15477	13232
C_o (ng/ml)	1178	986
CL (ml/min)	0.84	0.98
V_{ss} (ml)	21.3	20.1

Table 4.2. Portal and systemic exposure of metformin with or without co-administered pentamidine

	$AUC_{0\text{-}120\ min}$ (min*ng/ml)			
	Metformin		Metformin + Pentamidine	
	Average	S.D.	Average	S.D.
Portal Exposure	16464	6916	4496	2742
Systemic Exposure	8560	3980	2372	903

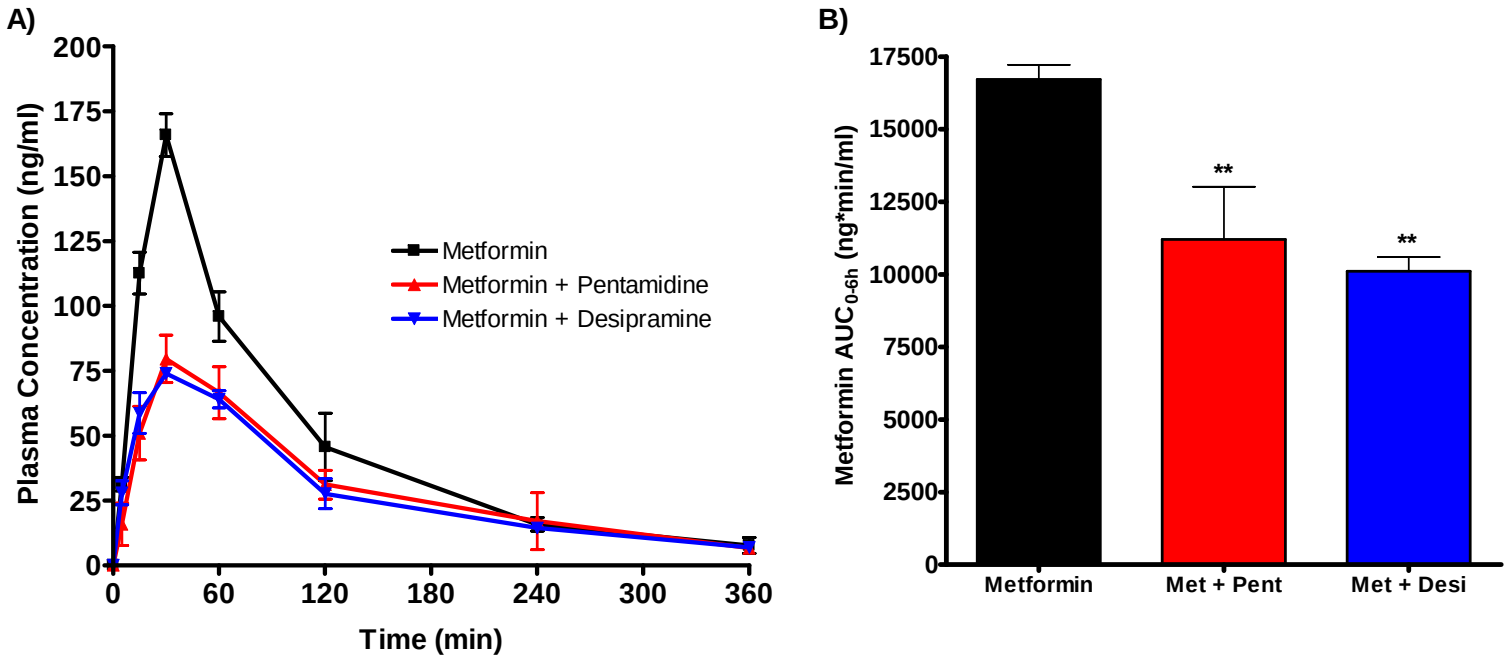

Figure 4.1. Metformin AUC with and without co-administered transporter inhibitors. A) Plasma concentration-time profile of [^{14}C]metformin (0.65 mg/kg; 15 μCi/ml), with co-administered pentamidine (3.4 mg/kg) or desipramine (8.0 mg/kg) versus time. B) $AUC_{0\text{-}6hrs}$ of metformin, metformin + pentamidine, and metformin + desipramine. Serial saphenous vein blood samples were collected at 5, 15, 30, 60, 120, 240, and 360 min. Data are represented as the mean ± S.D. with a minimum of 3 mice/group. **p<0.01

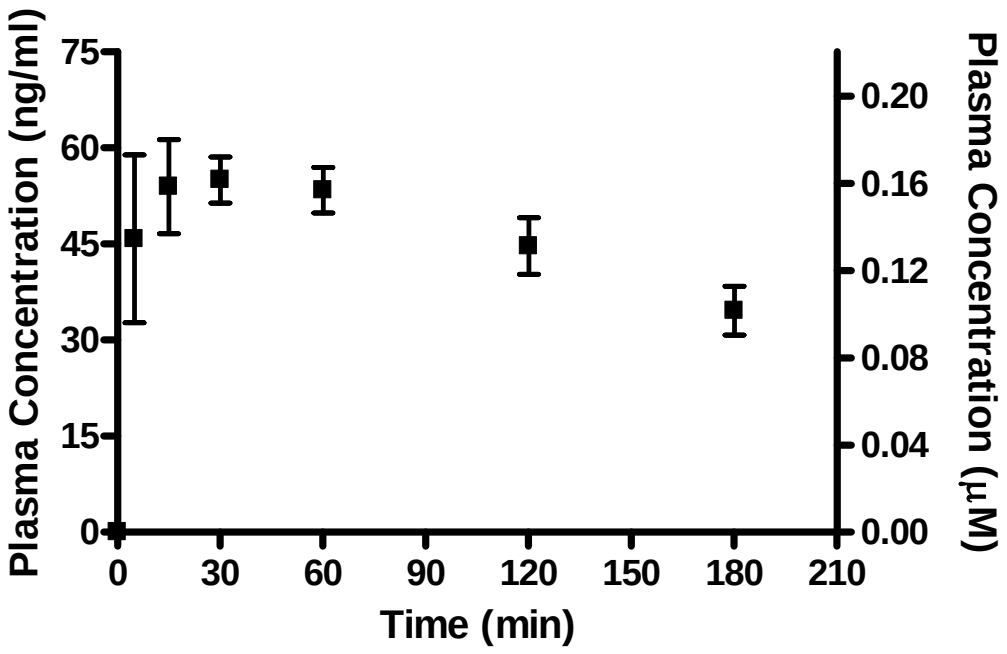

Figure 4.2. Plasma concentration *versus* time profile for pentamidine after oral administration. Pharmacokinetic profile of [^{3}H]pentamidine (3.4 mg/kg; 20 μCi/ml administered to mice via oral gavage. Blood samples were collected at 5, 15, 30, 60, 120, and 180 min from the saphenous vein. Plasma concentrations of pentamidine were determined by liquid scintillation spectrometry. Data are represented as the mean ± S.D. of 3 mice.

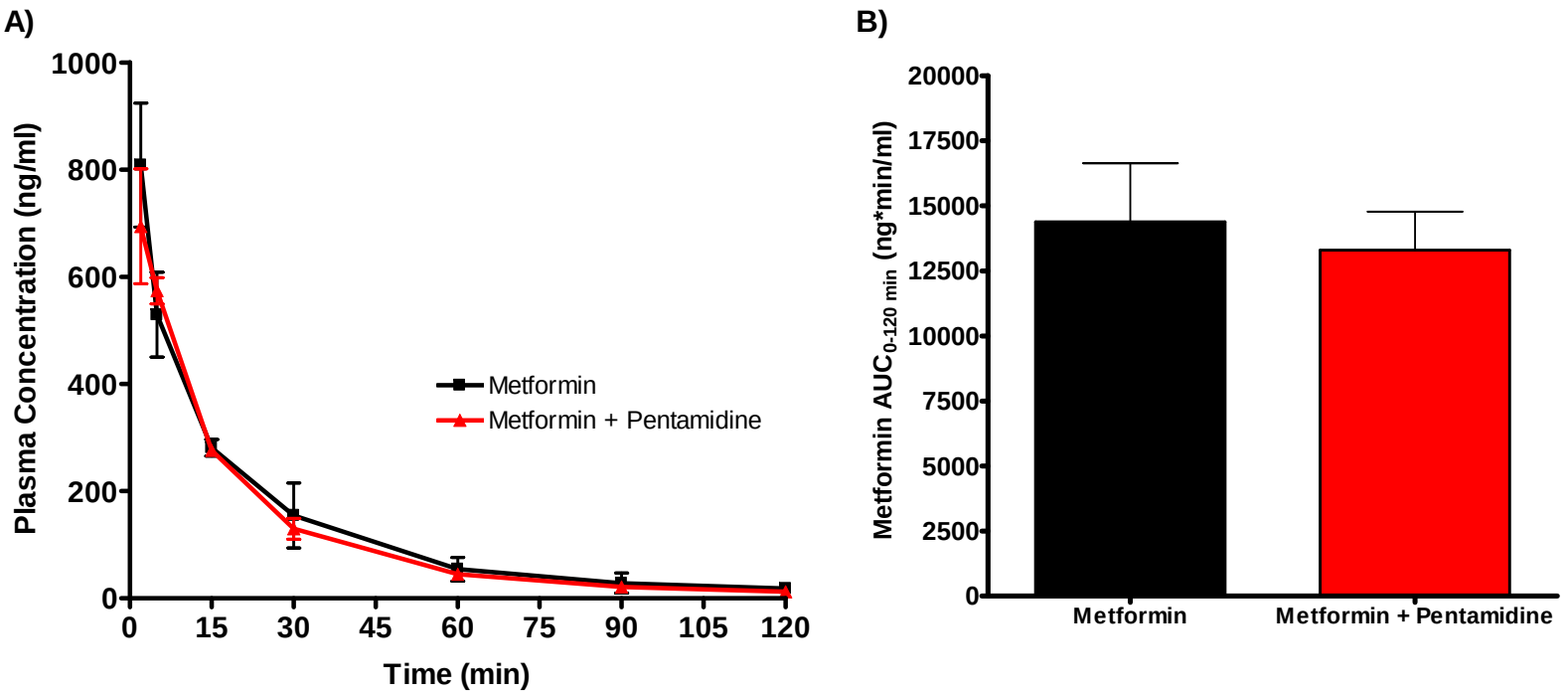

Figure 4.3. Systemic disposition of metformin with and without co-administered pentamidine. A) Plasma concentration-time profile following [^{14}C]metformin (0.65 mg/kg; 15 μCi/ml) intravenous bolus dose 30 min after a pentamidine (3.4 mg/kg) or saline oral dose. B) $AUC_{0\text{-}120\ min}$ of metformin with or without co-administration of pentamidine. Serial saphenous vein blood samples were collected at 2, 5, 15, 30, 60, 90, and 120 min. Data are represented as the mean ± S.D. with a minimum of 3 mice/group.

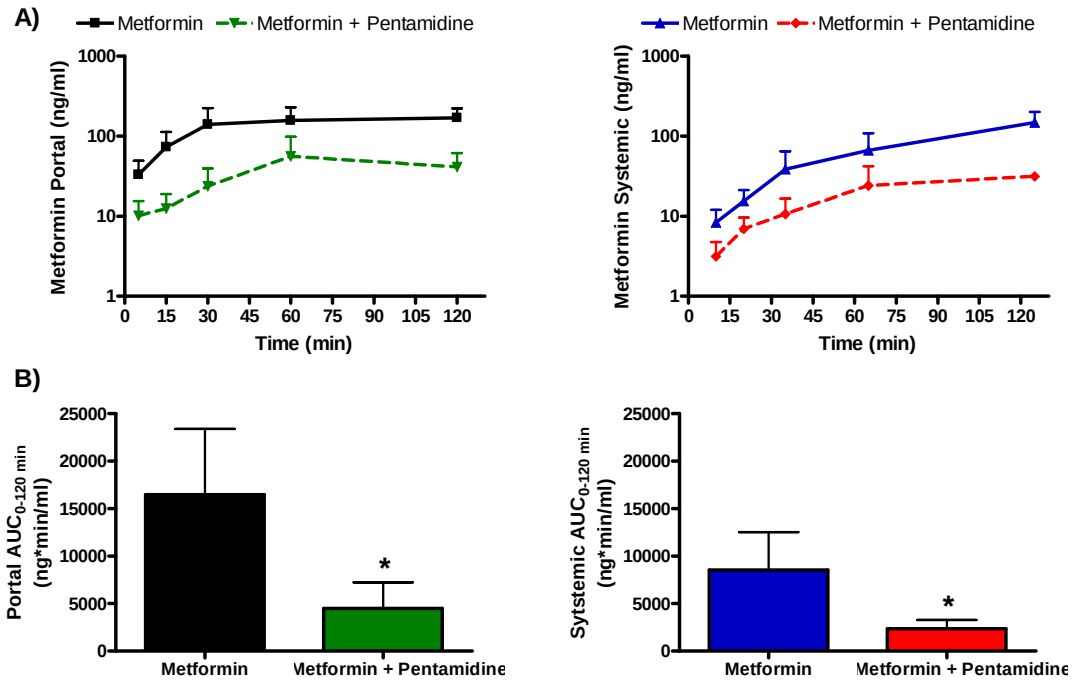

Figure 4.4. Effect of pentamidine on metformin absorption into the portal and systemic circulation of portal vein cannulated mice. A) Metformin portal and systemic plasma concentration-time profiles in urethane-anesthetized mice following [^{14}C]metformin (0.32 mg/kg; 30 μCi/ml) with and without co-administered pentamidine (1.7 mg/kg). B) Portal and systemic $AUC_{0\text{-}120min}$ of metformin with and without pentamidine. Data are represented as the mean ± S.D. with a minimum of 3 mice/group. *$p<0.05$.

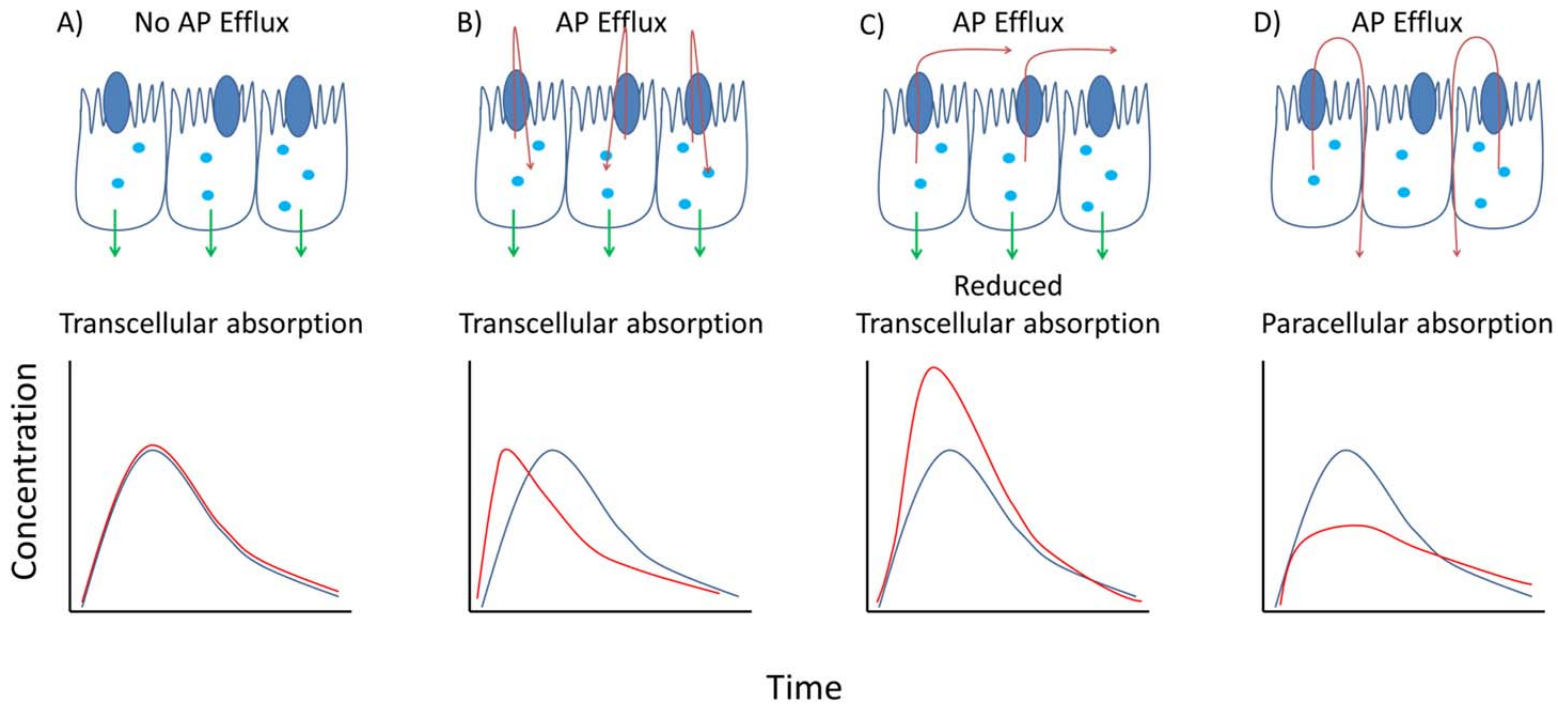

Figure 4.5. Hypothetical changes in metformin pharmacokinetics after delayed administration of pentamidine. Expected profile changes with (Red) and without (Blue) delayed pentamidine administration when A) apical efflux transporters are not involved in intestinal absorption of metformin, B) apical transporters efflux metformin followed by its apical uptake and transcellular absorption, C) apical efflux transporters reduce metformin absorption through the transcellular route, and D) apical efflux transporters enhance metformin absorption through the paracellular route.

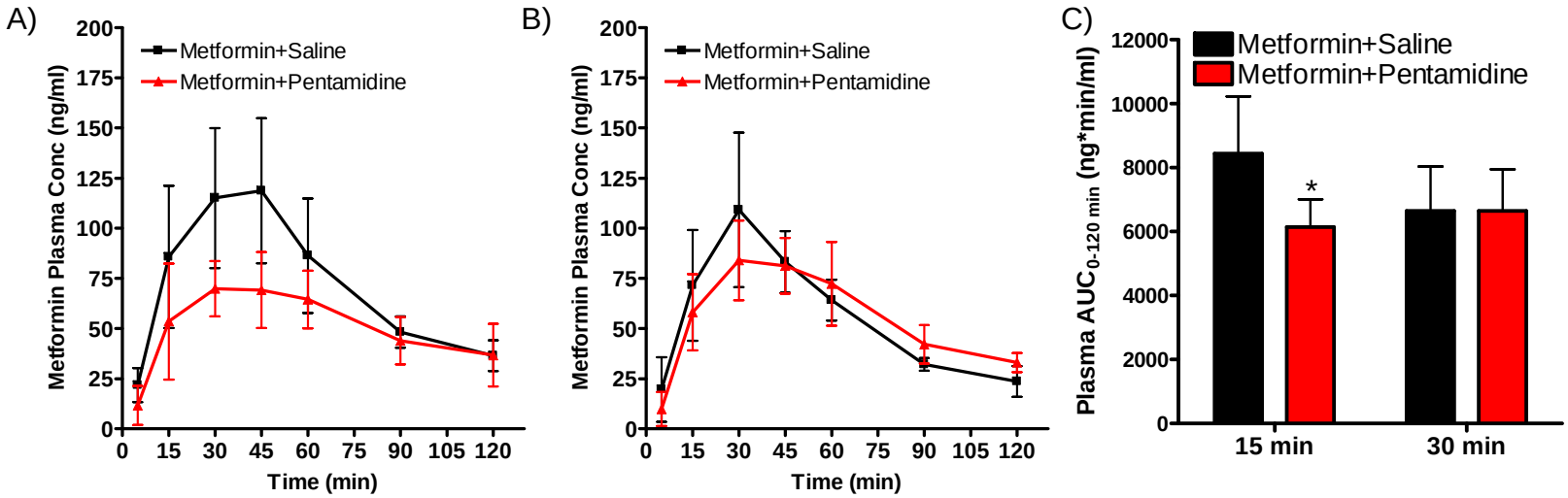

Figure 4.6. Metformin plasma concentration versus time profile after oral dosing of metformin with or without delayed administration of pentamidine. Plasma concentration-time profile following a [^{14}C]metformin (0.65 mg/kg; 15 μCi/ml; 5 ml/kg) dose and subsequent oral pentamidine (3.4 mg/kg; 5 ml/kg) administration at A) 15 minutes post-metformin dose or B) 30 minutes post-metformin dose. C) Metformin $AUC_{0\text{-}120\ min}$ after delayed pentamidine or saline administration. Serial saphenous vein blood samples were collected at 5, 15, 30, 45, 60, 90, and 120 min. Data are represented as the mean ± S.D. with a minimum of 3 mice/group. *$p<0.05$.

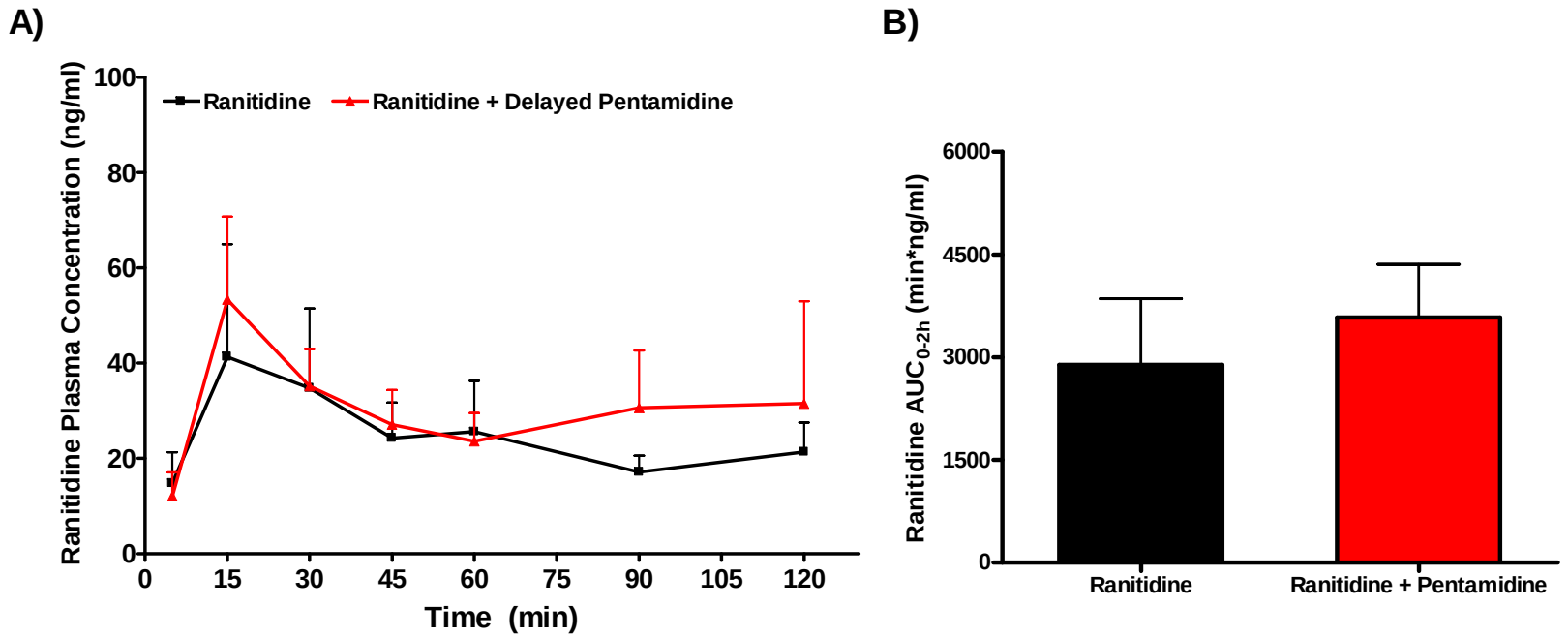

Figure 4.7. Oral ranitidine pharmacokinetic profile after delayed pentamidine administration. A) Plasma concentration-time profile following a ranitidine (1.57 mg/kg; 5 ml/kg) dose and subsequent oral pentamidine (3.4 mg/kg; 5 ml/kg) administration at 15 min post-ranitidine dose. B) Ranitidine $AUC_{0\text{-}120\ min}$ after delayed pentamidine or saline administration. Serial saphenous blood samples were collected at 5, 15, 30, 45, 60, 90, and 120 min. Data are represented as the mean ± S.D. with a minimum of 3 mice/group.

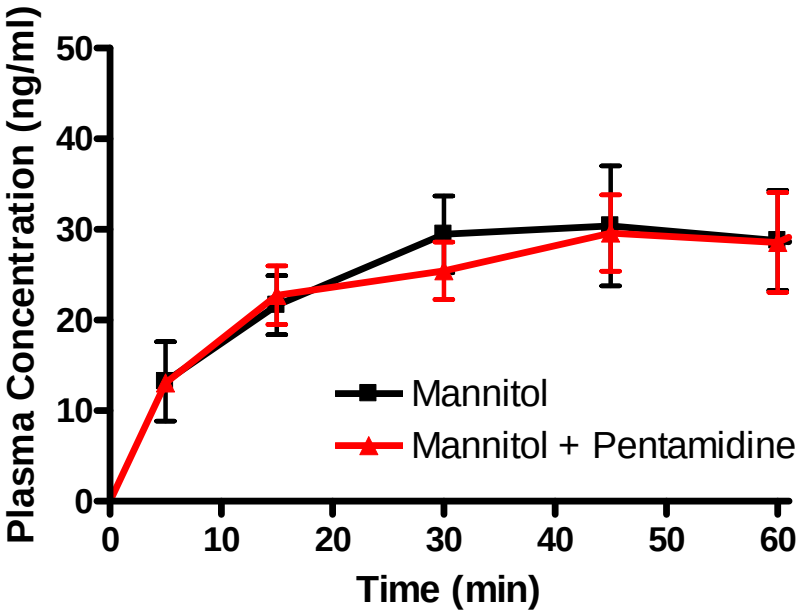

Figure 4.8. Oral mannitol pharmacokinetic profile with and without co-administered pentamidine. Plasma concentration-time profile following a [^{3}H]mannitol (0.1 mg/kg; 5 μCi/ml) oral dose with and without pentamidine (3.4 mg/kg). Serial saphenous blood samples were collected at 5, 15,30, 45, and 60 min. Data are represented as the mean ± S.D. with a minimum of 3 mice/group.

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Chapter 5

CONCLUSIONS

The overarching goal of the research conducted in this dissertation project was to further our understanding on the intestinal absorption mechanisms of the anti-hyperglycemic drug, metformin, in an *in vivo* system. Metformin is the most widely prescribed drug for the treatment of type 2 diabetes, a disease that has reached global epidemic levels. Despite the fact that this orally administered drug is well-absorbed and used by millions of patients, the fact remains that the mechanism by which the drug is so well-absorbed is not known. The unique chemical structure of the drug, with two guanidine groups on a very small carbon skeleton (the drug has only four carbons and five nitrogens in it structure), with permanent charge and $\log D_{pH6.0}$ at –6.13, has led to a unique transport behavior of the compound. It is taken up into the enterocytes or Caco-2 cells across the apical membrane efficiently by transporters, but it is not able to cross the basolateral membrane due to the lack of appropriate efflux transporters in this membrane. When a high concentration builds up inside the cell, the drug is able to egress across the apical membrane via some of the bidirectional SLC transporters. Because of a very inefficient transport of metformin across the basolateral membrane, over 90% of the absorptive transport of the drug is via the paracellular route. These findings were synthesized to propose a novel mechanism of intestinal absorption for metformin by Proctor et al. (2008), which is coined as the “sponge hypothesis”. This hypothesis

proposed that efficient apical uptake of metformin via transporters and poor basolateral efflux leads to accumulation of metformin in the enterocytes with a small percent of the drug being absorbed via the paracellular route. The accumulated metformin is effluxed across the apical membrane by bidirectional transporters, and the drug in the lumen goes through the same cycle again. This leads to overall improved efficiency of paracellular absorption of metformin, thus making it possible to have >60% oral bioavailability despite over 90% of its absorptive transport being paracellular.

The specific studies conducted in this body of work were designed with the major objective of testing a novel absorption mechanism as proposed above by the Thakker laboratory (Proctor et al., 2008). Although this absorption hypothesis was supported by human data from numerous clinical studies reported in the literature, the mechanism was conceptualized based on the transport properties of metformin in the Caco-2 Transwell® model, which is a static system. However, key components of the proposed mechanism of the intestinal absorption of metformin clearly involve dynamic changes in drug concentration within the lumen of the small intestine due to drug uptake into enterocytes as well as gastrointestinal transit. Therefore, the critical step in ultimately testing this hypothesis was to employ an *in vivo* model which would enable the evaluation of intestinal absorption of metformin and the role of drug transporters in this process.

The mouse model was chosen for *in vivo* pharmacokinetic studies to evaluate the contribution of apical drug transporters in the oral absorption of metformin. The use of this rodent model provides several advantages, including the availability of transporter knockout animals, several diabetic mouse models, as well as previous pharmacokinetic studies in mice reported in the literature to serve as a guide for the current research.

Because the mechanism of absorption of orally administered metformin includes the critical role of cation-selective transporters to facilitate metformin movement across the apical membrane of enterocytes, the first major step was to characterize the transporters found in mouse intestine. This was necessary, not so much because the role of each transporter in the intestinal absorption of metformin was to be determined (this is a subject of a separate dissertation project being pursued by Kevin Han in the Thakker Laboratory), but to enable the identification of pan-transporter inhibitors of metformin apical transport and the design of critical experiments that would test the role of transporter-mediated apical uptake and efflux in enhancing the paracellular absorption of metformin. The transporters of metformin in humans, which have been uncovered over the course of more than a decade of research in the field of organic cation transporters (OCTs) are the OCTs, multidrug and toxin extrusion (MATE) proteins, and plasma membrane monoamine transporter (PMAT). The kinetic behavior of metformin toward these transporters that are capable of translocating metformin across cellular membranes has been extensively studied (Kimura et al., 2005a; Kimura et al., 2005b; Masuda et al., 2006; Tanihara et al., 2007; Zhou et al., 2007). However, at the outset of this project, the kinetic behavior of metformin towards the mouse orthologs of the putative human metformin transporters was not reported in the literature.

To address this gap in knowledge, stable cell lines singly-expressing mOct1–3, mMate1, or mPmat transporters were generated (Chapter 2). These cells were used to characterize metformin uptake kinetics *in vitro* and identify chemical inhibitors of metformin transporters that can be employed as pharmacologic tools in subsequent experiments in this dissertation project, with the purpose of implicating transporters in

the intestinal absorption of metformin. Through this set of *in vitro* experiments, several chemical inhibitors of the mouse metformin transporters, mOct1–3, mMate1, or mPmat were investigated, which led to the identification of the pan inhibitors desipramine, quinidine, and pentamidine.

The second series of experiments were conducted to characterize metformin transport across the intestinal epithelium of mouse (Chapter 3). Specific features of metformin transport in Caco-2 cell monolayers that were incorporated into the sponge hypothesis, such as transporter-mediated metformin intestinal accumulation and efflux, were evaluated in an *ex vivo* model. Metformin accumulation in the enterocytes that line the small intestine has been shown in human, mouse, and in Caco-2 cell monolayers (Wilcock and Bailey, 1994; Bailey et al., 2008; Proctor et al., 2008). Drug accumulation is expected during oral absorption when rate-limiting processes at the basolateral membrane of the intestinal epithelium slow the egress of drug into the portal blood circulation. Studies conducted in Caco-2 cell monolayers identified a saturable transporter-mediated uptake process at the apical membrane, which was inhibited by classic organic cation substrates and inhibitors such as quinidine and 1-methyl-4-phenylpyridinium (MPP^+) (Proctor et al., 2008). Implementing Ussing-type diffusion chambers allowed the evaluation of transporter-mediated processes at the apical membrane of mouse intestinal tissue. *Ex vivo* studies using mouse intestinal tissues showed similar transport properties of metformin to those seen in Caco-2 cell monolayer studies. The choice of appropriate concentrations of both metformin and transporter inhibitors in these studies was only possible due to the *in vitro* characterization of metformin uptake kinetics and transporter inhibitors in Chapter 2. The data obtained

from diffusion chamber studies were supported by results from *in vivo* intestinal accumulation experiments involving co-administration of metformin and pentamidine. Decreased accumulation of metformin in the upper small intestine in the presence of the pan-transporter inhibitor is consistent with the absorption kinetics of pentamidine. After establishing a role for the apical uptake transporters of mouse in the intestinal accumulation of metformin, the role of apical efflux of accumulated metformin from intestinal tissue was assessed in the diffusion chamber system. Again, by first employing the previously generated transporter-expressing cell lines, the ability of various organic cationic compounds to trans-inhibit the apical efflux of metformin that had been pre-loaded into cells was evaluated. This strategy not only enabled the identification of potent inhibitors of metformin apical efflux, but also provided insight into the mechanism of inhibition by different compounds. For instance, desipramine, which was identified as a pan uptake inhibitor, showed the ability to trans-stimulate metformin efflux via 4 of the 5 transporters evaluated (mOct1, mOct3, mPmat, and mMate1, but not mOct2) suggesting substrate activity of desipramine for those transporters, and therefore a competitive mechanism of inhibition. Utilizing desipramine as an efflux inhibitor was therefore ruled out, as desipramine-mediated inhibition of metformin efflux would only occur after achieving sufficient intracellular accumulation of desipramine, a process that would first increase metformin efflux through a trans-stimulatory effect.

The last and most critical phase of this research project was to understand the role of intestinal transporters of metformin in its *in vivo* biopharmaceutic and pharmacokinetic behavior in a mouse model (Chapter 4). First, the cation-selective transporter inhibitors, desipramine and pentamidine, were evaluated for their ability to decrease the intestinal

absorption of metformin. Both inhibitors, although likely acting through different mechanisms, showed similar abilities to decrease the intestinal absorption of metformin when orally co-administered. This result provided evidence that intestinal transporters play a critical role in enhancing metformin absorption after oral dosing. Because the oral absorption of desipramine is extensive compared to pentamidine, it was hypothesized that its intestinal concentration would decline rapidly and therefore decrease its utility as an effective intestinal transporter inhibitor. Additionally, desipramine showed a clear trans-stimulatory effect in metformin pre-loaded transporter-transfected cell lines, again suggesting that it would be a poor inhibitor *in vivo*. In contrast to desipramine, pentamidine was a potent inhibitor of metformin uptake and efflux in *in vitro* and *ex vivo* studies. To rule out the possible effects of transporter inhibitors on the systemic disposition of metformin, including alterations in its distribution or elimination, the effect of an orally dosed transporter inhibitor on intravenously administered metformin was evaluated. Results from this study showed no effect of the inhibitor on the pharmacokinetic profile of metformin. To definitively demonstrate that a transporter-mediated process in the intestine was responsible for the observed increased systemic exposure of metformin, a portal vein cannulated mouse was used. Data from this set of experiments showing a ~75% reduction in portal bioavailability of metformin by co-administered pentamidine provided clear evidence of the involvement of intestinal transporters in metformin disposition. Lastly, the contribution of metformin efflux transporters in the apical membrane of the enterocytes to the intestinal absorption of the drug was investigated by using a novel approach. Pentamidine was previously shown to trans-inhibit metformin efflux *in vitro* in non-polarized transporter-transfected CHO cells,

while inhibition of basolateral efflux of metformin was not observed in *ex vivo* studies using mouse intestinal tissues (Chapter 3). Hence, an experimental approach was designed to inhibit apical efflux of metformin that was accumulated in the intestine, and then investigate the effect of this inhibition on the oral absorption of metformin. In this approach, metformin was dosed orally to mice, and then pentamidine was dosed at defined time intervals after the metformin dosing. Results from delayed pentamidine dosing studies showed a reduction in the rate of metformin absorption, evidenced by a decreased metformin AUC and a flattened C_{max} after pentamidine administration. Delayed pentamidine experiments evaluating ranitidine absorption, which is known to have a substantial transcellular absorption component, showed no difference between delayed saline and pentamidine treated animals. Because these results suggest an apical efflux and subsequent paracellular absorption mechanism for metformin, the oral absorption of mannitol, a molecule used to assess paracellular transport across epithelial monolayers, was also assessed with and without pentamidine. Mannitol absorption in the small intestine was not affected by co-administered pentamidine. Collectively, these results provided strong circumstantial evidence that not only do metformin efflux transporters enhance the intestinal absorption of metformin *in vivo,* but they do so by augmenting paracellular absorption of metformin across the intestinal epithelium.

The series of *in vitro, ex vivo, and in vivo* studies reported in Chapters 2, 3, and 4, provided proof of concept that metformin uptake and efflux leading to metformin intestinal accumulation plays a critical role in the efficient absorption of metformin by enhancing access to the paracellular space. The strategy of addressing the various components of the "sponge" mechanism was necessary because the direct inhibition of

paracellular transport is not experimentally feasible. The development of these studies led to the design of a novel experimental method to indirectly assess the contribution of paracellular absorption through the inhibition of apical drug efflux. This method may be applied to *in vivo* studies aimed at understanding the absorption mechanism of other compounds involving apical efflux. While the goal of this work was to test the overall mechanism of oral absorption of metformin, future *in vivo* studies using selective transporter inhibitors or knockout animals will identify major transporters of metformin in the intestine. These proposed studies will clarify the importance of individual transporters in the absorption of metformin and highlight the risk of potential drug-drug interactions.

Elucidation of the intestinal absorption mechanism of metformin and its transporter-mediated accumulation in enterocytes impacts our understanding of the proposed metformin pharmacology in the intestine and observed metformin pharmacology in the liver. The intestine has been directly implicated in the glucose lowering effect mediated by metformin by reducing the oral absorption of glucose (Bailey et al., 2008). Seemingly contradictory reports demonstrate increased glucose uptake via the rapid translocation of the glucose transporter 2 (GLUT2) to the apical membrane of the small intestine in the presence of metformin (Walker et al., 2005). However, anaerobic glucose metabolism is higher in the enterocytes in the presence of metformin and may offset the increase in cellular glucose uptake (Wilcock and Bailey, 1990; Bailey et al., 1992; Cuber et al., 1994). Thus, understanding the contribution of drug transporters to metformin accumulation may also help in elucidating the role of the intestine in metformin's most serious side effect, lactic acidosis. Additionally, a first-

pass pharmacodynamic effect has been demonstrated in rats dosed with metformin via various routes of administration (Stepensky et al., 2002). The greatest pharmacodynamic effect was observed when a steady input of metformin was delivered to the liver through the portal vein, suggesting the slow rate of intestinal absorption enhances the efficacy of this anti-hyperglycemic. Furthermore, recent studies in the Thakker laboratory have shown that adverse gastrointestinal side-effects, such as diarrhea and nausea commonly associated with the initiation of metformin therapy, are likely related to the inhibition of the serotonin transporter, elevated serotonin in the gut lumen, and consequently increased gastrointestinal motility (Han, 2013). Because these side-effects has been reported to occur in ~50% of patients, and causes ~6% to eventually discontinue therapy (BMS, 2009) and nearly 60 million metformin prescriptions are filled per year in the United States alone, the new knowledge gained from this dissertation work has the potential to positively affect the tolerability of metformin in a substantial number of individuals.

In addition to the direct pharmacologic effects mediated by the intestine, potential drug-drug interactions may occur within the small intestine. While interactions in the systemic circulation are generally associated with increased drug exposure and toxicity, intestinal drug interactions may lead to decreased drug efficacy. Alternatively, reduced intestinal absorption of a drug could increase its intestinal exposure which may also have negative local effects. The most well-known interaction of metformin occurs with cimetidine (Somogyi et al., 1987), although there are conflicting interpretations of the mechanism of this interaction. In particular, OCT2 is generally considered the transporter protein involved in the metformin-cimetidine interaction (Giacomini et al., 2010; FDA, 2012), yet new evidence strongly suggests that this interaction likely

involves the renal transporters MATE1 and/or MATE2-K that mediate the apical efflux of metformin into urine (Matsushima et al., 2009; Tsuda et al., 2009; Ito et al., 2012). Although no intestinal drug interactions with cimetidine have been reported, it is possible that cimetidine-mediated decreased absorption of metformin is masked by the wide therapeutic window of metformin that results in increased dosing of metformin when its desired pharmacodynamic effects are not attained. Additionally, a reduction in the metformin oral absorption due to interactions with cimetidine may not be observed because of decreased metformin distribution and/or clearance rates resulting in increased metformin plasma concentration. Future studies conducted with and without co-administered cimetidine to determine metformin bioavailability (measure total metformin elimination in urine), or studies conducted in portal vein cannulated animals would allow more direct assessment of absorbed metformin and would clearly address the possible masking of the effects of co-administered cimetidine. While this example of a drug interaction involving metformin transporters demonstrates the effects of decreased transporter function, polymorphisms that code for reduced protein expression or function may also lead a decrease in the oral absorption of metformin. In fact, a growing body of work has identified several OCT1 and MATE polymorphisms that affect the disposition and pharmacology of metformin (Shu et al., 2007; Wang et al., 2008; Becker et al., 2009a; Becker et al., 2009b).

Using this dissertation work as a guide, clinical studies can be designed to confirm the intestinal absorption of metformin in humans. *In vivo* pharmacokinetic experiments in this dissertation work showed that transporter inhibitors decreased the intestinal absorption of a low dose (0.65 mg/kg) of metformin that generates a luminal

concentration of 0.5 mM (Chapter 4). However, no decrease in oral absorption of this drug was observed at a higher dose (3.5 mg/kg) that resulted in ~8 mM intestinal concentrations (data not reported). This was likely due to the inability of the transporter inhibitors to decrease metformin uptake that would occur at maximal velocity (*i.e.*, transporter saturation) at the higher dose (K_m values for mouse transporters range from 0.3–4.3 mM; Chapter 2). Although an inhibitory effect was not observed under these conditions, transporters would nonetheless aid in the accumulation of metformin into enterocytes. To address whether transporters play a role in the intestinal absorption of metformin at clinically relevant doses, identifying inhibitors such as non-competitive or mechanism-based inhibitors is necessary, as either of these two inhibitor types would decrease the maximal velocity of metformin transporters. Alternatively, taking advantage of known transporter polymorphisms and their associated reduced function may prove to be a useful approach. However, unless the major intestinal transporter(s) of metformin is inactivated, changes in the pharmacokinetic behavior of this drug are not likely to be observed due to compensation by secondary transporters. Nevertheless, clinical studies analogous to the mouse *in vivo* studies conducted in Chapter 4 could be designed with microdosing strategies, similar to those conducted with pyrimethamine to implicate MATE1 and MATE2-K in metformin renal efflux (Kusuhara et al., 2011). Although the possible effect of pyrimethamine on the oral absorption of metformin was not discussed by the authors, a statistically significant decrease in the fraction of dose excreted in urine from 77.7 to 64.5% over 24 hours at a 100 μg metformin dose was observed, whereas at a therapeutic dose of 250 mg, no difference was seen in the presence and absence of inhibitor. While the pyrimethamine dose was selective for renal

MATE tranporters, the intestinal concentrations achieved in the Kusuhara study was above those required for metformin transporter inhibition in the intestine (Ito et al., 2012). In addition to the effects of other drugs on metformin disposition and pharmacology, the effect of metformin on the absorption of other cationic drugs cannot be ignored. The high dose leading to the high concentration achieved in the small intestinal lumen is well above the K_m of metformin for cation-selective transporters. This has the obvious potential to decrease intestinal absorption of OCT, PMAT, and MATE transporter substrates. Alternatively, drugs that may be effluxed across the apical membrane by any of the metformin transporters during intestinal absorption are at risk of higher than expected drug absorption when metformin is co-administered due to the high intestinal accumulation of metformin. Clinical studies can easily test for this possible interaction by administering a high dose of metformin prior to the oral administration of the victim drug while monitoring for increased plasma concentrations.

In summary, the studies conducted in this dissertation research have significantly enhanced our current understanding of the oral absorption of metformin. The findings presented here provide evidence that the intestinal absorption of metformin is mediated by apical transporters with limited transport across the basolateral membrane due to the cationic nature of metformin and the lack of an efficient basolateral transporter. Additionally, through *in vivo* inhibition studies in mouse, strong circumstantial evidence is provided to support the sponge mechanism of metformin intestinal absorption which suggests high drug accumulation followed by the efflux of metformin across the apical membrane that enhances its paracellular absorption. Future clinical studies utilizing the approaches developed within this project will confirm the intestinal absorption

mechanism of metformin in humans which will help in understanding the oral absorption mechanisms of other hydrophilic drugs and serve as a guide in the generation of future metformin-based therapies.

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