Characterization of the Intestinal Permeability and Oral Absorption of Valacyclovir in Wildtype and huPepT1 Transgenic Mice

By

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DEDICATION

To my wife Erica and my son Eli

and

To my father Donovan and my mother Sue

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LIST OF ABBREVIATIONS

ACE: Angiotensin-converting enzyme

ACV: Acyclovir

$AUC_{0\text{-}180}$: Area under the plasma concentration-time curve from 0 to 180 minutes

$Ca_v1.3$: L-type calcium channel isoform 1.3

CL: Clearance

C_{max}: Maximum plasma concentration

F: Bioavailability

Gly-Sar: Glycylsarcosine

huPepT1: Humanized peptide transporter 1

Inu: Inulin

K_m: Michaelis-Menten constant

mPepT1: Mouse peptide transporter 1

NFP: Nifedipine

NHE3: Sodium proton exchanger 3

P_{eff}: Effective permeability

PepT1: Peptide transporter 1

$T_{0.5}$: Half-life

TEA: Tetraethylammonium

T_{max}: Time to reach the maximum plasma concentration

VACV: Valacyclovir

Vd_{ss}: Volume of distribution steady-state

V_{max}: Maximum velocity

ABSTRACT

PepT1 (SLC15A1) is a transporter apically expressed along the epithelial cells of the gastrointestinal tract and is responsible for the absorption of di/tripeptides, ACE inhibitors, B-lactam antibiotics and numerous prodrugs. Unfortunately, PepT1-mediated substrates that have been extensively studied were shown to exhibit species-dependent absorption and pharmacokinetics. Accordingly, in situ intestinal perfusion studies were conducted and valacyclovir uptake was shown to have a 30-fold lower K_m and 100-fold lower V_{max} in huPepT1 compared to wildtype mice. Moreover, inhibition studies demonstrated that the huPepT1 transporter alone was responsible for valacyclovir uptake, and segment-dependent studies reported significant reductions in permeability along the length of small intestine in huPepT1 mice. Subsequent oral administration studies revealed that the in vivo rate and extent of valacyclovir absorption were lower in huPepT1 mice, as indicated by 3-fold lower C_{max} and 3-fold higher T_{max} values, and an $AUC_{0\text{-}180}$ that was 80% of that observed in wildtype mice. However, no significant changes in drug disposition were observed between genotypes after intravenous bolus administration of acyclovir. Lastly, mass balance studies established that the bioavailability of acyclovir, after oral dosing of valacyclovir, was 77.5% in wildtype mice and 52.8% in huPepT1 mice, which corroborated values of 51.3% in clinical studies. Thus, it appears the huPepT1 transgenic mice may be a better model to study prodrug absorption and disposition in humans than wildtype mice.

Additional studies were conducted to determine the impact of nifedipine, a calcium channel blocker, on the PepT1-mediated uptake of valacyclovir in wildtype mice. It has been previously demonstrated in clinical studies, rat intestinal perfusion studies, and Caco-2 uptake studies that nifedipine enhanced the absorption of β-lactam antibiotics. However, co-perfusing nifedipine with valacyclovir revealed that the intestinal permeability of valacyclovir was not significantly altered in the presence of this calcium channel blocker, although a 25-30% reduction was observed. While the results obtained in these studies are difficult to reconcile, there may be a species-dependent mechanism in mice that negates the permeability enhancement of PepT1 substrates.

CHAPTER 1

Research Objectives

Mammalian members of the proton-coupled oligopeptide transporter (POT) family include PepT1 (SLC15A1), PepT2 (SLC15A2), PhT1 (SLC15A4), and PhT2 (SLC15A3). PepT1 is apically expressed mainly in the epithelial cells lining the lumen of the small intestine. It is a low-affinity, high-capacity transporter that mediates the absorption of dietary peptides along with peptidomimetic compounds with therapeutic significance such as angiotensin-converting enzyme (ACE) inhibitors, β-lactam antibiotics, and prodrugs such as valacyclovir.

Valacyclovir is the L-valyl ester prodrug of acyclovir, a nucleoside analog utilized as an antiviral agent. A multitude of *in vitro* cell culture studies have demonstrated the pH-dependent, concentration-dependent, and saturable nature of valacyclovir transport attributable to PepT1. Moreover, *in situ* perfusions in wildtype and PepT1 knockout ($Pept1^{-/-}$) mice have definitively proven that PepT1 is responsible for more than 80% of valacyclovir uptake across intestinal enterocytes.

However, a distinguishing characteristic of valacyclovir is that the prodrug exhibits species-dependent pharmacokinetics. While escalating oral doses of valacyclovir in mice have demonstrated linear pharmacokinetics, clinical administration studies revealed less than proportional, nonlinear trends. These differences are believed to be due to the intrinsic and distinctive transporter activity of the mouse and

human PepT1 orthologues. In order to investigate this premise, the intestinal and oral absorption of valacyclovir would need to be characterized in wildtype and huPepT1 transgenic mice.

Additionally, there has been a prevailing interest in investigating drug-drug interactions that impact the absorption and bioavailability of PepT1 substrates. A number of studies from the literature have shown that the calcium channel blocker nifedipine enhances the intestinal and oral absorption of various β-lactam antibiotics. However, this interaction has yet to be verified with other PepT1 substrates, such as with the prodrug valacyclovir.

The research objectives for this work are as follows:

1) To characterize and compare the intestinal permeability of valacyclovir between wildtype and huPepT1 mice
2) To characterize and compare the in vivo oral absorption and pharmacokinetics of valacyclovir in wildtype and huPepT1 mice
3) To investigate the potential drug-drug interaction between valacyclovir and nifedipine in wildtype mice via intestinal perfusions

CHAPTER 2

Background and Literature Review

2.1 Structure and Transport Properties of the Small Intestine

The human small intestine is approximately six meters long and can be subdivided into the duodenum (25 cm), jejunum (2.5 m), and ileum (3.5m). As the major absorptive site of nutrients, its surface area is maximized by the inclusion of circular folds, villi, and microvilli. Villi are 0.5-1.5 mm finger-like projections emanating from the mucosal layer and are enriched by extensive capillary beds. At the base of each villus, glands secrete mucus to combat acidic stomach chime. As a consequence, intraluminal pH will vary from 6 in the duodenum to 7.4 in the distal ileum (Fallingborg et. al, 1999). Covering each villus are hair-like projections called microvilli. These microvilli are comprised of epithelial cells: the major site for nutrient uptake and enzymatic degradation (Johnstone et. al, 2014).

Transport across epithelial cells can either be mediated by transcellular or paracellular processes. Transcellular processes include endocytosis, passive diffusion, and carrier-mediated transport. Hydrophilic drugs, metabolites, toxins, and endogenous compounds primarily need carriers to circumvent the large hydrophobic lipid bilayer (Pade and Stavchansky, 1997; Oostendorp et. al, 2009). The transporters responsible for absorption in the small intestine belong in two super-families: the ATP-binding

cassette (ABC) efflux transporters and the solute carrier (SLC) family. Important ABC efflux transporters include multidrug resistance proteins (MRPs), P-glycoproteins (P-gps), and the breast cancer resistance protein (BCRP). Major SLC transporters include organic anion transporters (OATs), organic cation transporters (OCTs), monocarboxylate transporters (MCTs), nucleoside transporters (CNTs, ENTs), and proton-coupled oligopeptide transporters (POTs) (Zakeri-Milani and Valizadeh, 2014). Conversely, the paracellular pathway involves the claudin-dependent diffusion of ions, water flux, and the passive transport of small molecules (i.e. <3.6 Å) like glucose and mannitol (Turner et. al, 2014).

2.2 Proton-Coupled Oligopeptide Transporter (POT) Family

Proton-coupled oligopeptide transporters (POTs) are a family of transport proteins that facilitate the transfer of di- and tri-peptides along with peptidomimetics across cell membranes. The four mammalian members of this family include PepT1 (SLC15A1), PepT2 (SLC15A2), PhT1 (SLC15A4), and PhT2 (SLC15A3) (Smith et. al, 2013). PepT1 was the first member successfully cloned by isolation from a rabbit intestinal cDNA library (Kanai, 1994). PepT2 shortly followed as the second member to be identified and cloned from a human renal cDNA library (Liu et al, 1995). PhT1 and PhT2 were later identified and cloned from a rat brain cDNA library (Yamashita et. al, 1997; Sakata et. al, 2001).

PepT1 and PepT2 have been the most studied POTs to date. While both transporters share similar substrate specificities, they differ in several ways. Human PepT1 and PepT2 share approximately a 50% amino acid sequence identity, with their chains being 708 and 729 residues long respectively (Daniel and Kottra, 2004). While PepT1 is a low-affinity high-capacity transporter with substrate K_m values between 200 μM to 10 mM, PepT2 is a high-affinity low-capacity transporter with substrate K_m values between 5 and 500 μM. Physiologically, PepT1 is mainly expressed in the small intestine, with lower expression in some tissues such as the kidney, bile duct, pancreas, liver, and placenta (Herrera-Ruiz et. al, 2001; Daniel and Herget, 1997). In contrast, PepT2 expression is highest in the proximal tubule of the kidneys, with appreciable levels also located in the central nervous system, lungs, and mammary glands (Döring et. al, 1998; Rubio-Aliaga and Daniel; 2002, Wang et. al, 2010).

Less information is known about the PhT1 and PhT2 transporters. While they can mediate the transport of certain di- and tri-peptides, their ability to shuttle histidine across cell membranes differentiates them from other POT members (Daniel and Kottra, 2004). In a recent study, the use of PhT1 knockout mice has demonstrated that PhT1 plays a significant role in transporting L-histidine into brain tissue, and potentially aiding in histamine homeostasis (Wang et. al, 2017). PhT1 is predominantly expressed in the brain and eye (Yamashita et. al, 1997) while PhT2 is expressed in the lymphatic system and faintly in the brain (Sakata et. al, 2001). Interestingly, it has been found that while PepT2 protein expression is present in the brain tissue of neonate mice, as they mature, PhT2 expression becomes more prominent (Hu et. al, 2014).

2.3 Proton-Coupled Oligopeptide Transporter 1 (PepT1)

2.3.1 Structure/Function of PepT1

Currently the consensus on PepT1 structure, validated by hydropathy plots and epitope-tagging, is that this transporter contains 12 transmembrane (TM) helices, has both the C and N-terminus facing in toward the cytoplasm, and exhibits a large extracellular loop between H9 and H10 (Smith, et al., 2013; Rubio-Aliaga and Daniel, 2002). PepT1 is also known to undergo extensive glycosylation, particularly at this loop (Covitz et. al, 1998). Additionally, in mice and humans, putative sites for protein kinase C-dependent phosphorylation have been identified (Liang et. al, 1995; Fei et. al, 2000). The membrane topology of PepT1 is illustrated in Figure 2.1.

An advanced structural understanding of PepT1 has largely been derived from crystalline studies on similar bacterial peptide transporters (PTRs). In 2011, Newstead et al. elucidated the crystal structure of the prokaryotic homologue $PepT_{so}$ from bacterium *Shewanella oneidensis* with a resolution of 3.6 angstroms. The structure revealed a 14 TM domain “V-shaped” protein in a ligand-bound occluded state. The binding site includes residues from eight helices: 1, 2, 4, 5, 7, 8, 10, and 11. With roughly a 30% transmembrane identity to the mammalian PepT1, the researchers found that many residues with suspected key functional activity in PepT1 are conserved in this protein as well (Newstead et. al, 2011). In addition, the crystalized structure $PepT_{ST}$ from *Streptococus thermophiles* was studied in the ligand-free open state (Solcan et. al, 2012), while GkPOT from *Geobacillus kaustophilus* and $PepT_{So2}$ from *Shewanella*

oneidensis were both studied in the ligand-bound open state (Doki et. al, 2013; Guettou et. al, 2013).

While no crystal structure has yet to be determined for mammalian PepT1, a review article has addressed the information gleaned from these crystal structure studies to propose a general mechanism for proton-coupled peptide transporters as illustrated in Figure 2.2. The main idea is that the transporter goes from an outward open state where an extracellular proton and peptide enters the binding pocket, to a peptide bound occluded state, and finally to an outward bound state where the two substrates are released inside the cell. While in the outward open state the proton is thought to bind to a conserved glutamate. This, in turn, facilitates the binding of the incoming peptide in which its specificity is modulated by conserved tyrosine residues. Movement from the outward to inward open state involves hinge-like action from select transmembrane regions coupled with salt bridge formation and disassociation (Newstead, 2015).

2.3.2 Cellular Transport Mechanism of PepT1

In 1983, researchers began to uncover the transport mechanism of dipeptide transfer. Ganapathy and Leibach (1983) found that dipeptide transfer across intestinal and renal brush-border membrane vesicles was mediated by an inward flux of protons. Further experiments involving the two-electrode voltage clamp (TEVC) technique confirmed this electrogenic process, by recording electric currents from oocytes expressing human and rabbit PepT1. Additionally, this study design revealed that

substrate transport via PepT1 follows Michaelis-Menten kinetics (Nussberger et. al, 1997; Mackenzie et. al, 1996; Amasheh et. al, 1996; Kottra et. al, 2002). Interestingly, proton to substrate transfer ratios for neutral, acidic, and basic substrates were found to be 1:1, 2:1, and 1:1, respectively (Steel et. al, 1997). In pH-dependent studies, Lister et. al (1997) discovered that anionic dipeptide transport activity increases after a corresponding drop in luminal pH, while cationic dipeptide transport activity increases under more alkaline conditions.

The mechanistic understanding of peptide and peptidomimetic transport from the lumen to the blood can be visualized on the cellular level (Figure 2.3). First, peptides and peptidomimetic compounds from the lumen of the small intestine must be transferred across the apical membrane by the action of the PepT1 transporter. Once within the enterocyte, enzymes may then metabolize these compounds. These metabolic products and/or their parent compounds can then be transported into the bloodstream through the basolateral membrane by utilizing transporters (such as amino acid or efflux) or by passive diffusion (Rubio-Aliaga and Daniel, 2002; Brandsch, 2013).

In order to have effective peptide transport, the proton gradient needs to be maintained across the cell surface. The Na^+-H^+ exchanger NHE3 located on the apical brush border membrane is thought to be a key player in this regard, with the ability to export protons out of the enterocyte. However, since proton transport is coupled to sodium its transport activity hinges on the basolateral Na^+/K^+-ATPase maintaining intracellular sodium balance (Thwaites and Anderson, 2007).

2.3.3 Substrate Specificity of PepT1

It is well established that PepT1 has wide substrate specificity. Up to 400 dipeptides and 8000 tripeptides from the 20 proteinogenic amino acids, as well as number of peptidomimetics like β-lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors, and numerous prodrugs are known to be transported by PepT1 (Rubio-Aliaga and Daniel, 2002; Brandsch et al., 2013). Undoubtedly, notable exceptions to substrate recognition and transport do exist. For example, dipeptides that consist of two basic residues and those that contain a proline at the N-terminus and a hydrophobic residue at the C-terminus demonstrate low or no transporter activity (Balvinder et al., 2006).

General guidelines have been established for ideal substrates such as: a) zwitterionic molecules are favored over those substrates with net charge; b) the peptide bond can be replaced with a ketomethylene bond (i.e. δ-aminolevulinic acid) or acid amide bond; c) a peptide bond must be in the trans-conformation; d) the NH_3^+ and carbonyl group are needed for high affinity transport; e) the COO^- group is not necessarily needed and can be switched with phosphoric acid or arylamide groups; e) there is stereoselectivity for L-isomer peptides; and f) affinity is improved with greater hydrophobicity (Brandsch et al., 2004; Brandsch et al., 2013).

2.3.4 Localization and expression of PepT1

It has been conclusively demonstrated that, in rats, humans and mice, PepT1 is

highly expressed at the apical side of enterocytes lining the small intestine. Protein and/or mRNA levels have been recorded at different segments along the gastrointestinal tract, with the duodenum, jejunum, and ileum having notable expression over the low/non-existent levels in the colon (Ogihara et al., 1996; Walker et al., 1998; Groneberg et al., 2001; Jappar et al., 2010). In particular, Japper et. al (2010) found that in wildtype mice, PepT1 transcript expression was highest in the jejunum, followed by ileum, distal colon, duodenum, and with no noticeable expression in the proximal colon. The protein expression levels, meanwhile, were highest in jejunum, intermediate in the other segments of the small intestine, and absent in both distal and proximal colon. Another study, however, conflicts with this finding by asserting that PepT1 protein levels are low but present in the distal colonic tissue of mice (Wuensch et al., 2013).

A study by Drozdzik et al., (2014) highlights the relative abundance of PepT1 in human small and large intestines. This was achieved by quantifying the protein levels of nine commonly expressed intestinal transporters derived from the tissue of Polish donors. These researchers found that 50% of the transporter protein in the small intestine was attributed to PepT1, but the same transporter was much less abundant (5%) in the colon.

PepT1 is also expressed at lower levels in the early proximal tubules of the kidneys, the apical side of epithelial cells lining the bile duct, the liver, in lysosomes of the acinar cells of the pancreas, and the placenta (Doring et al., 1998; Herrera-Ruiz et al., 2001; Knütter et al., 2002; Smith et al., 1998; Bockman et al., 1997).

2.3.5 Regulation of PepT1

Since PepT1 plays a key role in the transport of therapeutically relevant peptidomimetics, it is important to understand the various mechanisms by which this transporter is regulated and controlled. PepT1 expression and functional activity has shown to be influenced by hormones, drugs, physiological and pathological conditions, and developmental stage.

Hormones such as insulin, leptin, and thyroid hormone play an important role in PepT1 regulation as determined in Caco-2 cell lines. Insulin and leptin hormones were shown to increase the recruitment of PepT1 to the brush border membrane while mRNA levels remain constant. This effectively increases the V_{max} of PepT1 substrates, while the K_m parameter remains unchanged. Thyroid hormone, on the other hand, was shown to decrease PepT1 membrane density, thus decreasing V_{max}. (Sun et. al, 2003; Buyse et. al, 2001; Ashida et. al, 2002).

Pharmacological agents are also known to influence PepT1 regulation. Immunosuppressive compounds cyclosporine A and tacrolimus decrease PepT1 activity (Bugliani et. al, 2009), while pentazocine increases its uptake capacity (Fujita et. al, 1999). In another study, the authors found that four different Ca^{2+} channel blockers all increase the transport capacity of PepT1. They propose these blockers stimulate the NHE3 indirectly and thus create a steeper gradient for proton-coupled transfer (Wenzel et. al, 2002).

Diet has also been found to be a key regulator of PepT1. A high protein diet in rats has been found to aid the up-regulation of the transporter, thus increasing its

activity (Erickson et. al, 1995; Shiraga et. al, 1999). Conversely, fasting rats for one day also resulted in a greater transport capacity (Thamotharan et. al, 1999). Ma et. al (2012) elaborated on the topic by comparing data between fasted and fed mice. They observed that after 16 hours of fasting from a standard diet that these mice exhibited greater protein expression along with a significantly greater *in vivo* absorption of the PepT1 substrate Gly-Sar. Additionally, expression patterns were shown to exhibit a diurnal rhythm with food intake (Zucchelli et. al, 2009).

Physiological factors also contribute to PepT1 regulation. A developmental study in rats showed that PepT1 protein expression in the small intestine increased sharply to a maximal value by 3-5 days after birth, dropped, then rose to 59-88% of the maximal value at the time of weaning. By day 75, adult rats showed expression at 70% of the maximal value. Colonic expression was not detected after 5 days from birth (Shen et. al, 2001).

Interestingly, patients with ulcerative colitis and Crohn's disease have shown colonic tissue that aberrantly expresses PepT1, where otherwise expression is minimal in healthy patients (Zucchelli et. al, 2009; Merlin et. al, 2001). However, these findings are in stark contrast to a newer study that determined mice induced with IBD and human tissue extracted from IBD patients show reduced PepT1 expression in the ileum and colon (Wuensch et al., 2014).

2.4 Rational Prodrug Design

Many attempts have been made by scientists to improve the oral bioavailability of various drugs. This has partly been achieved by placing emphasis on improving intestinal absorption, a major barrier to systemic exposure. At first the common approach was to increase the lipophilicity of hydrophilic drugs in order to improve their passive diffusion through the lipid bilayer. This prodrug approach involved linking lipid-soluble moieties to the parent drug. However, as lipophilic xenobiotics began to demonstrate their propensity for cellular toxicity interest shifted to hydrophilic prodrugs. These compounds, although limited by passive diffusion, can enhance intestinal absorption when an attached moiety can confer carrier-mediated uptake upon the parent drug (Steffansen et. al, 2004; Varma et. al, 2010).

Other issues must also be considered when improving the bioavailability of prodrugs. The chemical and enzymatic stability of prodrugs must be favorable for transport and delivery. Many digestive enzymes are present in the gut lumen that may hydrolyze chemically labile bonds. Since these bonds link promoieties to their parent drugs, premature cleavage will undoubtedly decrease systemic exposure. However, these bonds must not be so chemically and enzymatically stable as to greatly prevent cleavage once the prodrug has traversed the epithelial barrier and entered the bloodstream. Additionally, efflux transporters located in the enterocytes may be problematic if they recognize either the prodrug or parent compound as a substrate and subsequently pumps it back out into the gut lumen (Yanni et. al, 2007).

2.5 The Prodrug Valacyclovir and Its Parent Compound Acyclovir

2.5.1 Mechanism of Action

Valacyclovir, the L-valyl ester prodrug of acyclovir, is clinically used to treat patients with genital herpes and herpes zoster (Figure 2.4). Acyclovir, as the therapeutic agent, serves as a nucleoside analog once cleaved from its promoiety. First, acyclovir enters infected cells where virally-generated thymidine kinases phosphorylate the compound to the mono-phosphate form. After a series of additional phosphorylation steps, the triphosphate form binds to viral DNA polymerase and is then incorporated into the growing DNA strand. The addition of this analog causes DNA synthesis to halt in its tracks (Baker et. al, 2002; Gnann et. al, 1983).

2.5.2 Valacyclovir as a substrate for PepT1

Numerous cell culture studies have highlighted the significance of hPepT1 in mediating the uptake of valacyclovir (Table 2). Valacyclovir transport in transfected cells (i.e. Xenopus laevis oocytes, Caco-2, CHO, and MDCK) all followed Michaelis-Menton kinetics with K_m values ranging from 0.29 to 5.94 mM. Notably, a comprehensive study by Balimane and Sinko (2000) explored the effect of pH and ionization states on the hPepT1-mediated uptake of valacyclovir in CHO cells. They found that the uptake of valacyclovir was greatest at pH 7.4. This was attributed to the

higher percentage of neutral species, which exhibited the highest affinity for transport (i.e. K_m of 1.2 mM for neutral species versus 7.4 mM for cationic species).

Consequently, valacyclovir was also found to be a substrate for other intestinal transporters as well. Gly-Sar actively inhibited valacyclovir uptake in SKPT cells expressing rat PepT2 (Ganapathy et. al, 1998). The organic anion transporter 3 (OAT3) contributed to valacyclovir uptake in S2 cells with an estimated K_m of 57.9 μM and a V_{max} of 200 pmol/mg protein/minute (Takeda et. al, 2002). The $ATB^{0,+}$ transporter, which is abundantly expressed on the luminal surface of colonic tissue, demonstrated valacyclovir uptake through inhibition in HRPE cells (Hatanaka et. al, 2004). Moreover, COS-7 cells expressing hPhT1 showed significantly greater uptake for valacyclovir over mock cells at pH 5.0 (Bhardwaj et. al, 2006). A noteworthy experiment done by Landowski et. al (2003) found that HeLa cells expressing HPT1 also transports valacyclovir. This study was conducted because Landowski and coworkers found a positive correlation between HPT expression in human duodenum and systemic exposure of acyclovir after oral dosing of valacyclovir. Interestingly, they claim that no such correlation was found to exist for the PepT1 transporter.

In response to the various *in vitro* studies attempting to attribute PepT1 or another intestinal transporter to valacyclovir uptake, Yang and Smith (2013) sought to uncover the significance and contribution of PepT1-mediated transport with mice. By measuring permeability values (via perfusions) in both wildtype and PepT1 knockout mice, the contribution of the PepT1 transporter was found to be greater than 80%. The knockout mice only had permeability values approximately 10% of that seen in wildtype. The K_m for valacyclovir was reported as 10.2 mM. Inhibition studies ruled out $ATB^{0,+}$, PhT1/2,

OATs, and OCTs as significant contributors to uptake. Subsequent oral administration studies corroborated their findings.

2.5.3 Hydrolysis of Valacyclovir

Valacyclovir is predisposed to hydrolysis due to its labile ester bond. Granero and Amidon (2006) investigated the stability of this compound in buffers and gastrointestinal fluids derived from different species. These authors made the following conclusions: 1) valacyclovir undergoes base-catalyzed pseudo-first order kinetics; 2) it has suitable stability under pH 4; and 3) the prodrug is more stable in buffer than in intestinal fluid of the same pH. Moreover, there is solid evidence to support the notion that valacyclovir hydrolysis is rapid and nearly complete *in vivo*. For example, after the oral administration of valacyclovir in humans, more than 99% of the absorbed prodrug was converted to acyclovir (Soul-Lawton et. al, 1995). Additionally, a similar study showed the valacyclovir-to-acyclovir C_{max} ratio to be minimal (i.e. 4%) following administration (Weller et. al, 1993). Rapid and complete hydrolysis has also been extended to mice, as demonstrated in studies by Yang and Smith (2013).

Esterases responsible for prodrug hydrolysis are primarily expressed in three regions: on the luminal surface, within intestinal enterocytes, and in the liver (MacDougall and Guglielmo, 2004). Many researchers have attempted to determine the specific enzyme(s) that activate valacycloivir. In 1995, Burnette and coworkers isolated and characterized an enzyme in rat that specifically hydrolyzes valacyclovir. Years later, the human valacyclovirase, biphenyl hydrolase-like protein (BPHL) was also

identified and characterized from Caco-2 cells. This enzyme was found to hydrolyze valacyclovir with a specificity constant of 420 mM^{-1}-s^{-1} (k_{cat}/K_m) (Kim et. al, 2003). However, a recent study was conducted seeking to understand the impact of BPHL on the hydrolysis of valacyclovir in mice. After the oral administration of valacyclovir in wildtype and BPHL knockout mice, it was determined that the AUC ratio of valacyclovir to acyclovir was 5.5% and 9.1% for wildtype and BPHL knockout mice respectively. Finding only a marginal reduction in the hydrolase activity of valacyclovir in BPHL KO mice, the authors concluded that this enzyme is not essential for prodrug activation (Hu et. al, 2018).

2.5.4 Pharmacokinetics of Valacyclovir and Its Parent Compound Acyclovir

The bioavailability of acyclovir in humans was reported as 54.2% after the oral administration of 1000 mg valacyclovir (Soul-Lawton et. al, 1995). In contrast, bioavailability was observed to only be 15-30% when acyclovir was administered instead (Laskin, 1983). This is commonly attributed to its poor intestinal permeability. PepT1 does not transport acyclovir (Han et. al, 1998), but instead undergoes passive absorption (Meadows and Dressman, 1990). Acyclovir distributes well into tissues including the CSF (~50% of plasma levels), with protein binding found to be 15% in patients (MacDougall and Guglielmo, 2004). An *in vitro* study showed acyclovir to bind in mouse plasma at 14% and in human plasma at 22-33% (de Miranda et. al, 1981).

In clinical trials acyclovir was administered intravenously with doses that ranged from 2.5-15 mg/kg. Elimination of acyclovir followed a two-compartment model with a

terminal half-life of 2-3 hours. Dose proportionally was observed in respect to C_{max} and AUC values. Renal contribution to clearance was approximately 77% of total clearance with tubular secretion and filtration both contributing to renal drug excretion (Whitley et. al, 1982; Laskin et. al, 1982). In terms of metabolism 9-carboxymethoxymethylguanine (CMMG) comprised 8.5-14.1% of the total administered dose in humans, with >.5% being 8-OHACV (Soul-Lawton et. al, 1995). Metabolites excreted from mice constituted 4% of the dose (de Miranda et. al, 1981).

In a pivotal study from Yang et. al (2013), the pharmacokinetic features of acyclovir were determined after the oral administration of valacyclovir in wildtype and PepT1 knockout mice. $AUC_{0\text{-}180}$ and C_{max} decreased substantially in the PepT1 knockout mice across four doses: 10, 25, 50, and 100 nmol/g. Oral dose escalation revealed that both C_{max} and AUC increase linearly with dose in wildtype and knockout mice. Intravenous acyclovir concentration-time profiles were similar across genotypes, indicating absorption is the main driver for differences in valacyclovir pharmacokinetics. Tissue to blood ratios were not significantly different between genotypes.

In contrast to the aforementioned studies in mice, some clinical research has showcased non-linear pharmacokinetics for acyclovir following orally administered valacyclovir. Weller et. al (1993) found less than proportional increases in the C_{max} and AUC of acyclovir in single-dose studies ranging from 100-1000 mg, as well as from multiple-dose (i.e. QID for 11 days) studies ranging from 250-2000 mg. In particular, they determined from their single-dose escalation studies that C_{max} increased from 0.83 to 5.65 mg /L, while AUC increased from 2.28 to 19.52 mg/L*hr. A similar clinical study reported by Jacobson (1993) discovered only a non-linear trend for AUC values, while a

third clinical study by Ormrod et. al (2000) found linear trends for both C_{max} and AUC over the dose range of 100-1000 mg.

2.6 Issues with Predicting Clinical Pharmacokinetics: In Vitro Scaling and Interspecies Differences

Currently, two approaches attempt to predict the clinical pharmacokinetic parameters from ADME processes in animals. One approach involves the use of physiological modeling with *in vitro* data (Kang and Lee, 2011). In a particular case, Hallifax et. al (2010) compiled data with this method and found intrinsic clearance in cryogenic hepatocytes consistently under-predicts intrinsic clearance *in vivo*. While this situation can be corrected empirically, the scenario illustrates a lack of knowledge in the mechanistic details attributing to the miscorrelation. The authors suggest differences may be due to reduced enzymatic activity or lower permeability in these hepatocytes.

The second approach, which is more commonly utilized, involves allometric scaling. This method often involves the use of three animals models in which pharmacokinetic and physiological parameters are acquired for each species. After a regression analysis is conducted, human pharmacokinetic parameters are extrapolated from the data (Polekhina et. al, 2014). Although success can sometimes be achieved, at other times animal models can fail to properly predict clinical values. Thus, commonly used measures of allometric scaling success (e.g., correlation coefficient and allometric exponent) failed to discriminate between successful and failed allometric predictions (Ward and Smith, 2004).

The challenges faced by allometric scaling can certainly be due to species differences in the functional activity of intestinal transporters. As previously mentioned, clinically administered valacyclovir has demonstrated less than proportional increases in AUC and C_{max} with increasing oral dose. Yet, Yang and Smith (2013) found that

comparable doses in mice yielded linear pharmacokinetics. These differences are thought to be chiefly due to the fact hPepT1-mediated intestinal transport is saturated at high doses. Thus, it is quite possible to over-predict clinical values if wildtype mice are utilized in interspecies scaling. However, what could be the mechanistic reason behind these differences? Yang and Smith (2013) believed these differences were due to the mouse PepT1 and human PepT1 transporters having different affinities for the substrate. This assertion was well founded. In a novel study, human, mouse, and rat PepT1 isoforms expressed in yeast *P. Pastoris* demonstrated distinct transport kinetics for the model dipeptide Gly-Sar. Specifically, the substrate affinity (K_m) was found to be the following: 0.86 mM for human, 0.3 mM for mouse, and 0.16 mM for rat (Hu et. al, 2012).

PepT1 protein expression levels could also be different between species. Although no definitive study has been done to address protein levels, one research group found mRNA levels encoding transporters in the intestine to be quite different among mice, rats, and humans. In particular, PepT1 mRNA transcripts in clinical tissue were approximately 2-fold and 6-fold lower than what was found in mice and rats, respectively (Kim et. al, 2007). As for expression in the kidney, mRNA levels were moderate in the rat but low in the mouse (Lu and Klaassen, 2006).

2.7 Humanized Pept1 Mice

In an attempt to mitigate the interspecies differences observed following valacyclovir and cefadroxil administration in mice and humans (Yang et. al, 2013; Weller et. al, 1993; Posada and Smith, 2013; Garrigues et. al, 1991), Hu et. al (2014) generated the humanized PepT1 (huPepT1) transgenic mouse model. The development of these mice involved the insertion of the huPepT1 gene (with both coding and regulatory elements) into a one-celled embryo with a $PepT1^{-/-}$ background.

Once generated, these mice needed to be fully validated. A full phenotypic analysis was conducted and the results were found to be normal. mRNA expression studies demonstrated that huPepT1 mice had measureable levels in every segment of the small intestine but lower expression in the large intestine. Similarly, protein expression studies revealed high levels in the small intestine but trace levels in colonic tissue. No measurable PepT1 was found in the kidney. Additionally, other transporter genes were monitored to check for potential compensatory changes in huPepT1 mice, with wildtype acting as a baseline. Most notably, *mPat1* transcripts in the small intestine and *mOat* transcripts in the kidney increased by approximately 2-fold in huPepT1 mice. Lastly, functional activity for huPepT1-meditated transport was assessed in these mice by conducting intestinal perfusion studies with Gly-Sar. The uptake of this substrate exhibited Michaelis-Menton kinetics in wildtype and huPepT1 mice, with V_{max} values of 3.75 and 0.5 nmol/cm^2/sec, respectively, and K_m values of 13.2 and 3.3 mM, respectively (Hu et. al, 2014).

More recently, the intestinal permeability and oral absorption of cefadroxil was evaluated in both wildtype and huPepT1 transgenic mice. Following segment-dependent studies, it was determined that small intestinal permeability in huPepT1 mice was only 50-60% of wildtype values, and that colonic permeability was 14-fold higher in the humanized transgenic mice. Furthermore, concentration-dependent studies revealed that the K_m and V_{max} of transport in huPepT1 mice (as compared to wildtype) were approximately 2-fold and 7-fold lower, respectively. Subsequently, IV bolus administration studies determined that there were no differences in drug disposition for cefadroxil in wildtype and huPepT1 mice. However, oral dose escalation studies in huPepT1 mice revealed less than proportional increases in C_{max} and AUC, while only linear trends were observed in wildtype. These differences became compelling after huPepT1 mice were found to better match the trends and values derived from comparable clinical doses (Hu and Smith, 2016; Garrigues et. al, 1991).

2.8 The Ca_v1.3 Calcium Channel and Inhibition by the Dihydropyridine Nifedipine

2.8.1 Introduction of Cav1.3 and Nifedipine

In total, there are 10 calcium channel isoforms. Each isoform is differentiated by a unique α1 subunit, which is the pore-forming structure of the channel. Specifically, the Ca_v1.3 isoform belongs to the subclass of long lasting voltage-gated (L-type) calcium channels. These Ca_v1.3 channels are expressed in many different tissues of the body. They are prominently found in sinoatrial node cells where they help mediate pacemaking activity of the heart. Additionally, these channels contribute to auditory transduction in the ear and endocrine secretion (Striessnig et. al, 2015; Catterall, 2011). Ca_v1.3 is also expressed in intestinal enterocytes. A protein expression study in rats found these channels along the gastrointestinal tract with peak levels in the distal jejunum and proximal ileum (Morgan et. al, 2003). Moreover, gene expression studies done with clinical and mouse intestinal tissue revealed the presence of Ca_v1.3 transcripts in all segments of the small and large intestine. It is important to note that in mice, transcript levels were found to be much greater in the colon than in the small intestine (Radhakrishnan et. al, 2016; Reyes-Fernandez and Fleet, 2015).

As depicted in Figure 2.5, there are primarily three routes that can initiate calcium transport from the lumen to the blood. Uptake can occur through the tight junctions, the vitamin D dependent calcium channel TRPV6, or the calcium channel Ca_v1.3. The relative contribution of the paracellular and transcellular routes for calcium

flux is seemingly contentious (Kellett, 2011; Radhakrishnan et. al, 2016). On the other hand, TRPV6 and $Ca_v1.3$ transport are proposed to work in complementary ways. Under depolarizing conditions the $Ca_v1.3$ channel should be more active than the TRPV6 channel, while under hyperpolarizing conditions the inverse should be true. Depolarization across the intestinal enterocyte occurs via nutrient uptake, like when glucose and Na^+ ions are transported by SGLT1 (Kellett, 2011). In fact, the co-perfusion of 10 μM nifedipine and 20 mM glucose resulted in the inhibition of 78% of total calcium uptake, while the perfusion of nifedipine alone dropped total inhibition to 52%.

The dihydropyridine nifedipine is commonly administered to patients with angina or hypertension. The compound behaves as an arterial vasodilator by binding to L-type calcium channels and preventing calcium influx into smooth muscle cells (Snider et. al, 2008). Furthermore, nifedipine is a highly potent compound that was shown to block 50% of the $Ca_v1.3$ channels at a concentration of 20.1 nM in tsa201 cells (Sinnegger-Brauns et. al, 2009). However, the perfusion of 1 μM nifedipine though a segment of rat jejunum was determined to be insufficient at blocking calcium influx. This changed when the concentration was elevated to 10 μM, as calcium influx was then blocked by 73% (Morgan et. al, 2003). In contrast, another study found that nifedipine did not appear to have an effect on calcium absorption in rat duodenum, unlike verapamil at concentrations higher than 1 mM (Fox and Green, 1986). The rat duodenum, though, is known to express relatively low levels of $Ca_v1.3$ (Morgan et. al, 2003). This fact may perhaps diminish the value of this study.

2.8.2 Proposed mechanism of nifedipine and its effect on PepT1 activity

A proposed mechanism that links nifedipine administration to the increased functional activity of PepT1, and for which is largely supported in the work by Wenzel et. al (2002), is illustrated in Figure 2.6. First, nifedipine binds to the $Ca_v1.3$ channel and blocks calcium influx into the enterocyte. This would then result in the decreased activity of other transporters reliant on calcium like the basolateral Na^+/Ca^+ (Barboza et. al, 2015). An imbalance in sodium may then enhance the activity of the NHE3 transporter. In turn, the increase in proton efflux would effectively provide a larger gradient for PepT1-mediated transport. However, there is some doubt about the role of the NHE3 transporter and its ability to modulate PepT1-mediated transport. In a clinical study involving the oral administration of cefadroxil and tenapanor (a NHE3 inhibitor), the researchers observed that cefadroxil pharmacokinetics remain unchanged in the presence of this other drug (Johansson et. al, 2017).

Another mechanism was also proposed for the drug-drug interaction involving nifedipine and PepT1 substrates. It was suggested that the calcium-blocking effects of nifedipine would modulate the activity of the enteric nervous system, and thus affect the activity of PepT1. A series of rat intestinal perfusion studies investigated this premise (Harcouet et. al, 1997; Berlioz et. al, 1999). However, the enhancement of cefixime uptake in CaCo2 cells, while in the presence of nifedipine and other blockers, refutes this neuron-centric mechanism (Wenzel et. al, 2002). Additionally, no studies on the impact of the enteric nervous system were ever carried out *in vivo,* as noted by Wenzel et. al (2002). In a more positive light, some interesting insights have come out

of these aforementioned intestinal perfusion studies as related to the impact of nifedipine, including: 1) the enhancement of PepT1 absorption was not due to any hemodynamic effect; and 2) there were no changes to gastric motility (Harcouet et. al, 1997).

2.9 Interaction Studies Involving Nifedipine and Pept1 Substrates

Many studies have investigated and found significant drug-drug interactions (DDIs) involving nifedipine and various PepT1 substrates (Table 2.2). Notably, two of these studies were clinically focused. The first study involved the oral administration of 1 gram amoxicillin either alone or 30 minutes after the oral administration of 20 mg nifedipine. It was determined that in the presence of nifedipine the following pharmacokinetic parameters changed for amoxicillin: a 22% increase in AUC, a 21% increase in bioavailability, a 15% increase in C_{max}, and a 21% decrease in T_{max}. However, there were no changes in the volume of distribution or clearance for this compound. Thus, the authors concluded that nifedipine enhanced the oral absorption of amoxicillin (Westphal et. al, 1990). In a similar clinical study 200 mg of cefixime was orally administered alone or 30 minutes after the oral administration of 20 mg nifedipine. Again, the following pharmacokinetic parameters changed for cefixime while in the presence of nifedipine: a 46% increase in C_{max}, a 69% increase in AUC_{ext}, and a 71% increase in bioavailability. In addition, a deconvolution analysis revealed that 50% of the total dose was absorbed in the presence of nifedipine, but was markedly lower, namely 30%, when nifedipine was absent. Coupling these results with the knowledge that no changes to drug disposition occurred, the authors concluded that nifedipine enhanced the oral absorption of cefixime (Duverne et. al, 1992).

Three DDI studies were conducted in rats. In the first study, rats were orally administered 22.4 mg/kg cephalexin either alone or 30 minutes after the oral administration of 10 mg/kg nifedipine. In the presence of nifedipine, cephalexin

bioavailability increased 23%, the C_{max} increased 57%, the AUC increased 34%, and the $T_{0.5}$ and T_{max} remained unchanged. The authors concluded, as with the clinical studies, that nifedipine enhanced the oral absorption of this PepT1 substrate (Berlioz, et. al, 2000). As for the other two studies, these involved *in situ* intestinal perfusions of cephalexin or cefixime ± nifedipine through the small intestine. Specifically, nifedipine or a drug-free solution was perfused for 15 minutes. This was then followed by the perfusion of 1 mM of cephalexin or cefixime for 60 minutes. Upon completion of the intestinal perfusions, the plasma concentrations of both cephalexin and cefixime were both at least 2-fold greater in the presence of nifedipine. These increases were again attributed to the enhancement of intestinal absorption (Berlioz et. al, 1999; Harcouet et. al, 1997). However, these same researchers claim that nifedipine did not enhance cefixime uptake in previously conducted *in vitro* studies. This is in contrast to the comprehensive study by Wenzel et. al (2002), where they observed a nearly 2-fold increase in the Caco-2 cell permeability of cefixime in the presence of nifedipine.

2.10 Figures and Tables

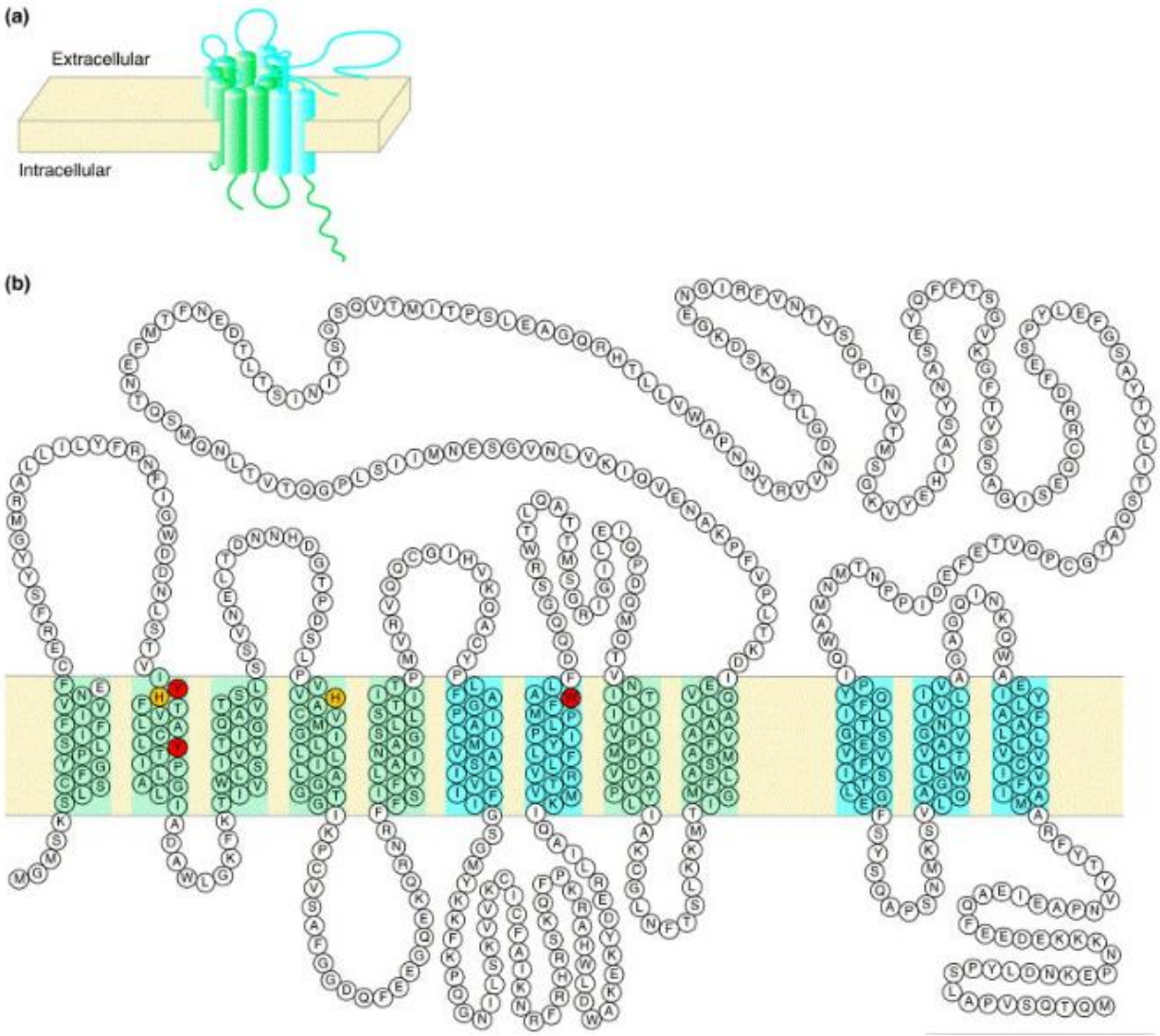

Figure 2.1 The membrane topology of PepT1. (Adopted from Rubio-Aliaga and Daniel, 2002)

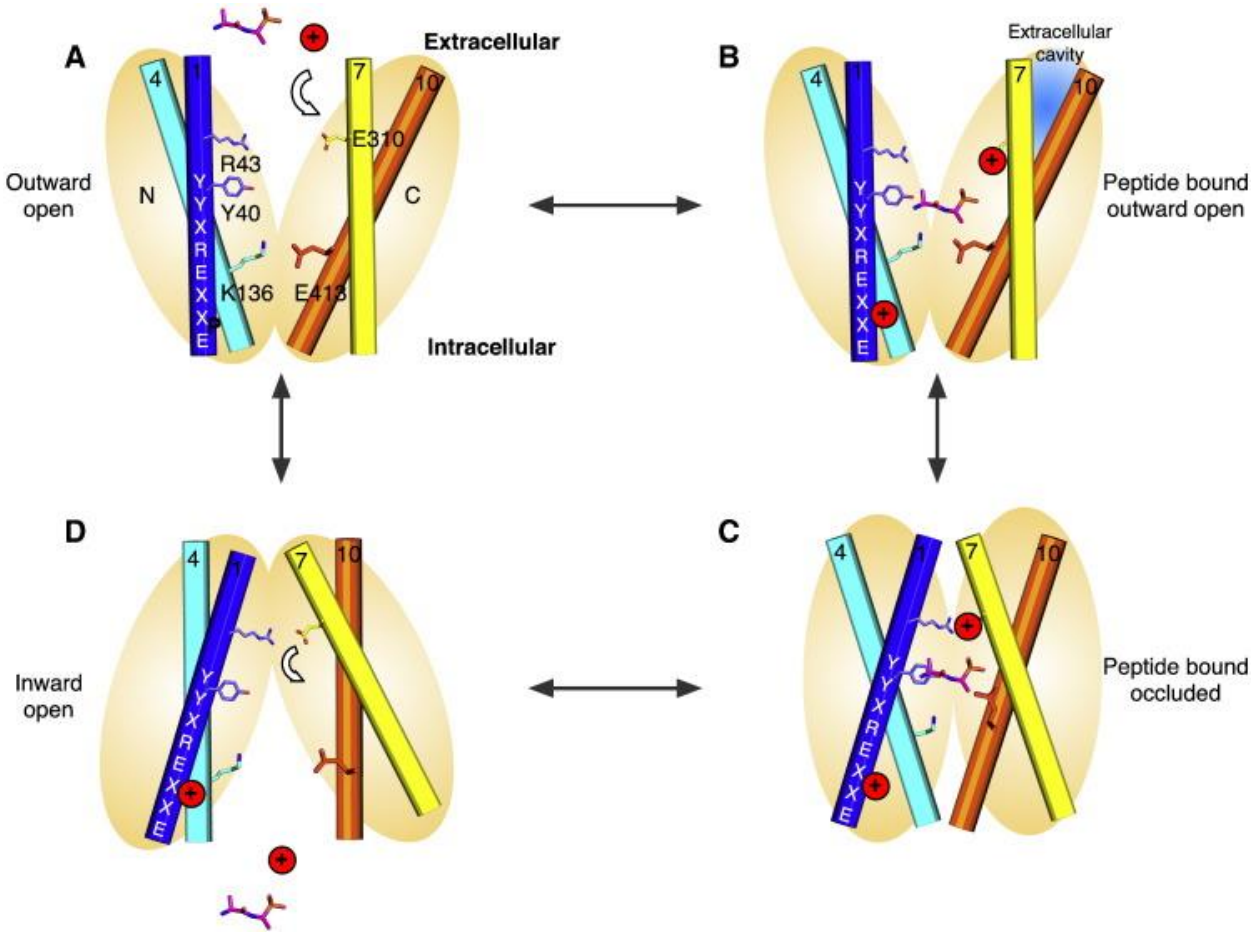

Figure 2.2 A general mechanism for peptide transport. (Adopted from Newstead, 2015)

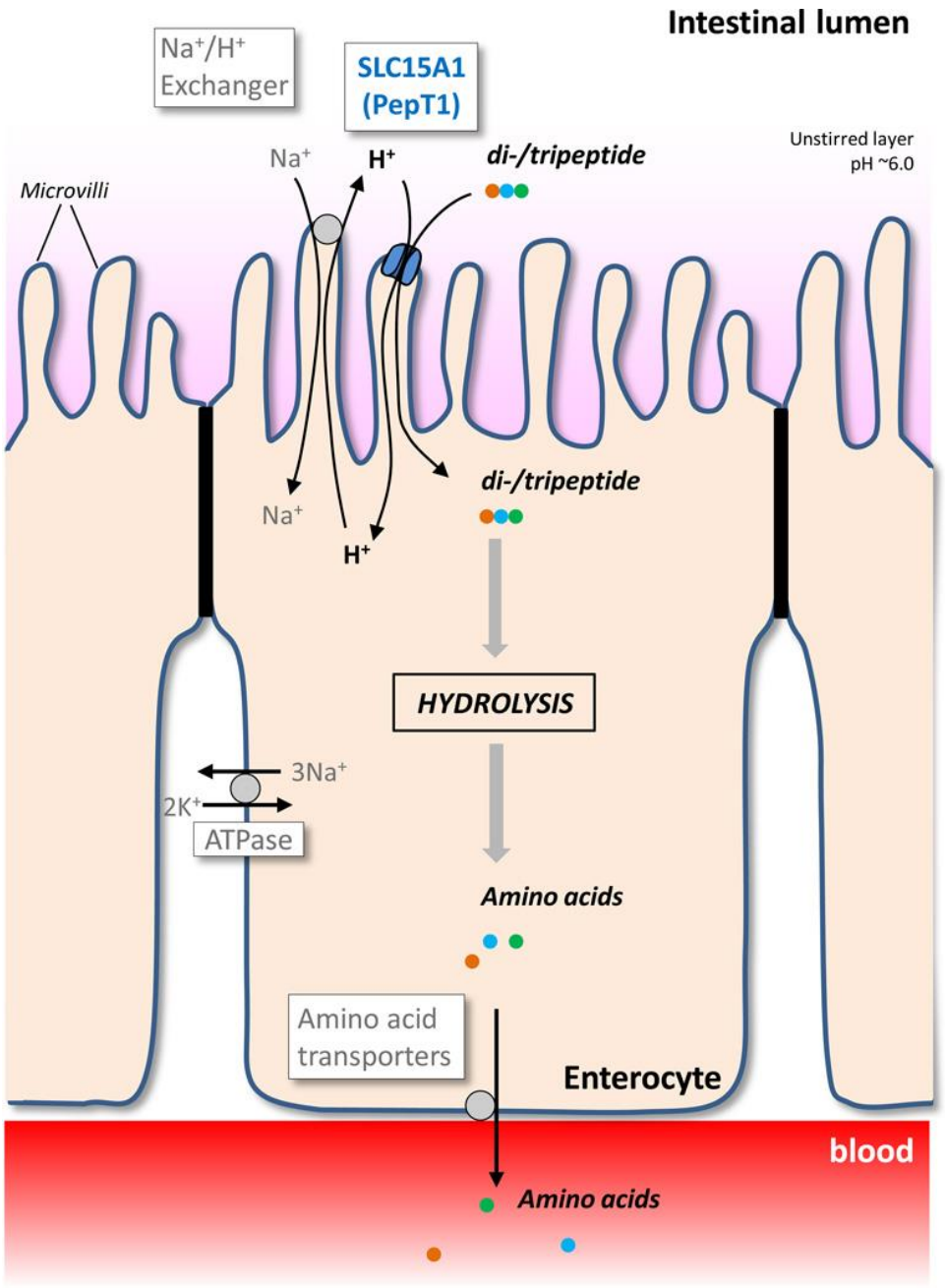

Figure 2.3 An illustrative model showing PepT1-meditated uptake of dietary peptides in intestinal enterocytes. (Adopted from Smith et. al, 2013)

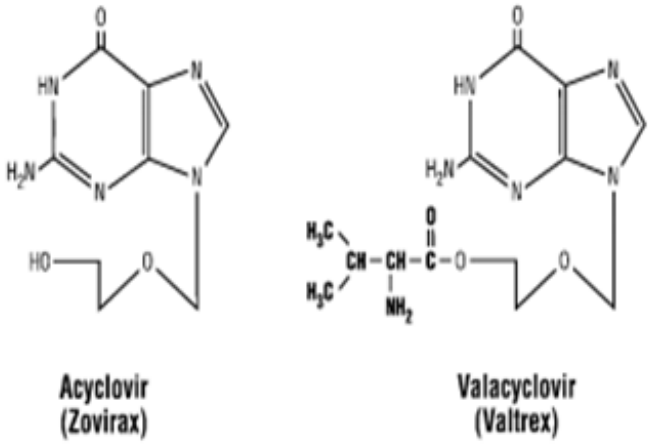

Figure 2.4 The chemical structures of acyclovir and valacyclovir.

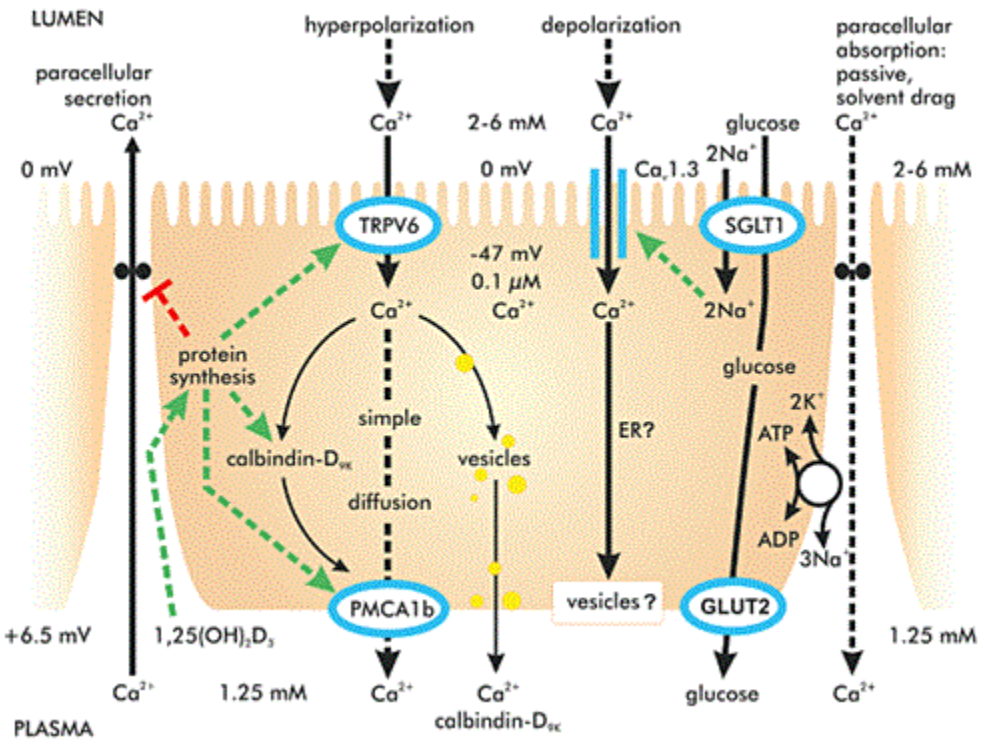

Figure 2.5 Possible routes for calcium uptake in the intestinal enterocyte. (Adopted from Kellett, 2011)

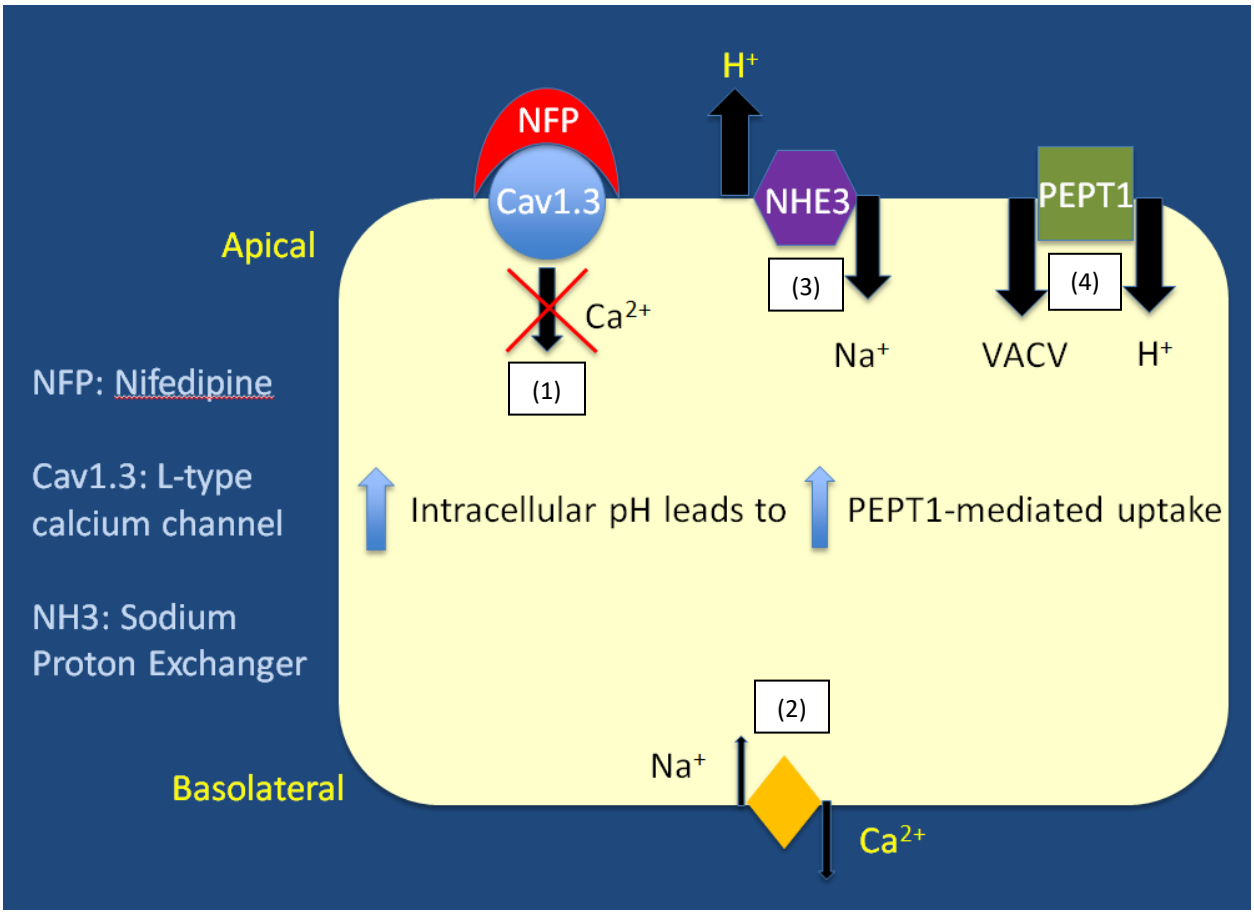

Figure 2.6 A proposed mechanism that links nifedipine administration with an enhancement of PepT1-mediated transport for valacyclovir (VACV). The steps for the mechanism are as follows: (1) NFP inhibits $Ca_v1.3$ at the apical membrane; (2) reduced intracellular Ca^{2+} attenuates Ca^{2+}/Na^+ exchange at the basolateral membrane; (3) reduced intracellular Na^+ stimulates NHE3 at the apical membrane; and (4) PepT1 activity is stimulated by the increased proton gradient at the apical membrane.

Table 2.1 Various uptake studies performed with valacyclovir in cells transfected with hPepT1.

Cell line	pH of uptake buffer	K_m (mM)	V_{max} (Various Units)	Reference
Xenopus laevis oocytes	5.5	5.94	1.68 nmol/hr/oocyte	Balimane et. al, 1998
CaCo2	7.4	0.292	15.7 nmol/h*cm^2	Han et. al, 1998
CHO	7.5	1.64	23.34 nmol/mg protein/5 min	Guo et. al, 1999
CHO	7.5	2.69	N/A	Balimane and Sinko, 2000
MDCK	6.0	5.05	5.88 nmol/mg protein	Bhardwaj et. al ,2005
CaCo2	6.0	1.55	2.93 nmol/mg protein	Bhardwaj et. al, 2005

Table 2.2 Various drug-drug interaction studies involving nifedipine and a PepT1 substrate.

Species/Cell culture	Study Type	Nifedipine Dose/conc.	PepT1 Substrate	Buffer Solution	DDI Result on PepT1 substrate	Reference
Clinical	Oral administration	20 mg	200 mg cefixime	Water	71% increase in bioavailability	Duverne et. al, 1992
Clinical	Oral administration	20 mg	1 gram amoxicillin	Water	21% increase in bioavailability	Westphal et. al, 1990
CaCo2	Cell uptake	100 µM	1 mM cefixime	Modified Krebs, pH 5.0, 10 mM glucose	Nearly 50% increase in uptake	Wenzel et. al, 2002
Rat	Oral administration	10 mg/kg	22.4 mg/kg cephalexin	Krebs-ringer, pH 7.5	23% increase in bioavailability	Berlioz et. al, 2000
Rat	Jejunal perfusion	100 µM	1 mM cephalexin	Krebs-ringer, pH 7.5	50% increase in plasma concentration	Berlioz et. al, 1998
Rat	Duodenojejum perfusion	100 µM	1.1 mM cefixime	Krebs-ringer, pH 5.5, 9.2 mM citric acid	76% increase in portal vein blood concentration	Harcouet et. al, 1996

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CHAPTER 3

Evaluating the Intestinal and Oral Absorption of the Prodrug Valacyclovir in Wildtype and Hupept1 Transgenic Mice

3.1 Abstract

The purpose of this work was to evaluate the intestinal permeability, oral absorption and disposition of the ester prodrug valacyclovir in wildtype mice and a huPepT1 transgenic mouse model. PepT1 (SLC15A1) is a transporter apically expressed along the epithelial cells of the gastrointestinal tract and is responsible for the absorption of di-/tripeptides, ACE inhibitors, β-lactam antibiotics and numerous prodrugs. Unfortunately, PepT1-mediated substrates that have been extensively studied were shown to exhibit species-dependent absorption and pharmacokinetics. Accordingly, in situ intestinal perfusion studies were conducted and valacyclovir uptake was shown to have a 30-fold lower K_m and 100-fold lower V_{max} in huPepT1 compared to wildtype mice. Moreover, inhibition studies demonstrated that the huPepT1 transporter alone was responsible for valacyclovir uptake, and segment-dependent studies reported significant reductions in permeability along the length of small intestine in huPepT1 mice. Subsequent oral administration studies revealed that the in vivo rate and extent of valacyclovir absorption were lower in huPepT1 mice, as indicated by 3-fold lower C_{max} and 3-fold higher T_{max} values, and an $AUC_{0\text{-}180}$ that was 80% of that observed in

wildtype mice. However, no significant changes in drug disposition were observed between genotypes after intravenous bolus administration of acyclovir. Lastly, mass balance studies established that the bioavailability of acyclovir, after oral dosing of valacyclovir, was 77.5% in wildtype mice and 52.8% in huPepT1 mice, which corroborated values of 51.3% in clinical studies. Thus, it appears the huPepT1 transgenic mice may be a better model to study prodrug absorption and disposition in humans than wildtype mice.

3.2 Introduction

The proton-coupled oligopeptide transporter PepT1 (SLC15A1), which is expressed along the apical side of intestinal enterocytes, has become a prime target for prodrug binding and uptake. A prodrug is similar in structure to a pharmacologically active parent compound but contains an added chemical promoiety that, in this case, confers PepT1-mediated transport upon the entire molecule, thus enhancing intestinal absorption (Rubio-Aliaga and Daniel, 2002; Dahan et. al, 2014). One reason PepT1 has become a preferential target is because of this transporter's abundant expression along the gastrointestinal tract. This fact is substantiated in a study by Drozdzik et. al (2014), showing that relative PepT1 protein expression in the human small intestine constitutes half of all tested transporter proteins. Secondly, PepT1 is targeted due to its high capacity transport and broad substrate specificity. Known substrates to be transported by PepT1 include di/tripeptides, peptidomimetic compounds such as β-lactam antibiotics and ACE inhibitors, and a growing number of prodrugs (e.g. valacyclovir, valganciclovir, L-dopa-L-Phe, and zanamivir) (Rubio-Aliaga and Daniel, 2002; Dahan et. al, 2014; Brandsch et. al, 2013; Sugawara et. al, 2000).

Valacyclovir is the L-valine prodrug of acyclovir, a nucleoside antiviral that is used for the treatment of genital herpes and herpes zoster (Baker et. al, 2002). Importantly, this compound has been studied extensively as a substrate for PepT1-mediated transport. In a novel study by Yang and Smith (2013), the authors found that PepT1 contributed >80% of the *in situ* intestinal permeability of valacyclovir in wildtype mice and, in a subsequent study by Yang et. al (2013), they observed a substantially greater

oral absorption of valacyclovir as compared to PepT1 knockout mice. Many *in vitro* studies have also demonstrated that cells transfected with human PepT1 exhibit functional activity for valacyclovir transport (Balimane et. al, 1998; Han et. al, 1998; Guo et. al, 1999; Balimane and Sinko, 2000; Bhardwaj et. al, 2005).

Although studies investigating PepT1-mediated uptake are numerous, a definitive species difference in PepT1 transporter kinetics was first reported in Pichia pastoris expressing the mouse, rat, and human orthologs (Hu et. al, 2012). In light of this finding, Hu et. al (2014) developed a novel humanized transgenic mouse line (huPepT1) that was proven to be viable, express human PepT1 protein along the intestinal tract, and demonstrate functional activity with the model dipeptide glycylsarcosine. More recently, these same researchers validated huPepT1 with the β-lactam antibiotic cefadroxil (Hu and Smith 2016). The huPepT1 mice clearly showed a higher affinity (i.e., lower K_m) and lower capacity for transport (i.e. lower V_{max}) as compared to wildtype mice. Equally important, oral dose escalation studies revealed that AUC and C_{max} profiles in huPepT1 mice over a large dose range exhibited non-linear pharmacokinetics, as seen in human clinical trials (Weller et. al, 1993), but unlike the linear pharmacokinetics observed in wildtype mice (Posada and Smith, 2013). The huPepT1 mouse line, therefore, demonstrated its use as a potential model for predicting human pharmacokinetics involving compounds that are mainly absorbed by the action of PepT1. Nonetheless, huPepT1 mice have not been tested with respect to their *in situ* intestinal permeability, and *in vivo* absorption and disposition kinetics of prodrugs.

With this in mind, the purpose of the current study was to evaluate the intestinal permeability, oral absorption and disposition of the ester prodrug valacyclovir in a

huPepT1 transgenic mouse model. We hypothesized that valacyclovir would exhibit a higher affinity for intestinal transport in huPepT1 mice, and that the rate of absorption and extent of systemic exposure would be lower than that observed in wildtype mice. In particular, initial studies evaluated the *in situ* intestinal permeability and kinetics of the transport of valacyclovir, the specificity of prodrug transport, and the intestinal segment-dependent permeability of valacyclovir in both wildtype and huPepT1 mice. Subsequent studies evaluated the *in vivo* oral and intravenous pharmacokinetics of valacyclovir (and acyclovir) as related to oral absorption, systemic exposure, and bioavailability.

3.3 Materials and Methods

3.3.1 Chemicals

Valacyclovir, acyclovir, glycylsarcosine (Gly-Sar), cephalexin, tetraethylammonium (TEA), L-histidine, L-lactate and L-valine were purchased from Sigma-Aldrich (St. Louis, MO). CytoScint^{TM} scintillation solution were purchased from MP Biomedicals (Solon, OH). [^{3}H]Valacyclovir (1.1 Ci/mmol) and [^{3}H]acyclovir (10.6 Ci/mmol) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). [^{14}C]Inulin 5000 (1.1 mCi/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

3.3.2 Animals

Gender-matched and age-matched (8-12 weeks) mice utilized in these studies have a C57BL/6 background and consisted of the mPepT1$^{+/+}$ (wildtype) and mPepT1$^{-/-}$ /huPepT1$^{+/-}$ (humanized PepT1) genotypes. The viability and functional activity of the humanized PepT1 mouse line were previously established in our laboratory (Hu et. al 2014). Inheritance of the huPepT1 gene was confirmed by genotyping. All mice were bred and housed in a temperature-controlled environment with 12 hour light and dark cycles, and a standard diet and water a*d libitum* (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

3.3.3 In Situ Single-Pass Intestinal Perfusion Studies

As reported previously (Yang and Smith, 2013; Jappar et. al, 2010), wildtype and huPepT1 mice were fasted overnight (14-18 hours) before experimentation. Mice were then given 40-60 mg/kg of pentobarbital as an intraperitoneal injection for anesthesia. The abdominal area was sanitized with alcohol before a 1.5 cm incision was made along the midline section. An 8-cm segment of jejunum (~2 cm distal to the Ligament of Trietz) was then isolated, cannulated (2.0 mm outer diameter), and tied off with silk sutures. The mouse was then placed in a temperature-controlled chamber (31°C) and saline-wetted gauze, along with Parafilm, was laid out across the abdominal section to prevent dehydration. A syringe containing pH 6.5 perfusion buffer (10 mM MES/Tris, 135 mM NaCl, 5 mM KCl), and 50 µM valacyclovir, was housed in a syringe pump (Harvard Apparatus PHD Ultra, South Natick, MA). Inlet tubing connected the syringe to the perfused segment and outlet tubing connected the perfused segment to the collection vials. Buffer solution was perfused through the cannulated segment at a programmed flow rate of 0.1 mL/min for 90 minutes. Perfusate samples were collected every 10 minutes for the last 60 minutes of the perfusion. No samples were collected during the first 30 minutes in order to pass over the pre-equilibration period. Upon completion, the perfused intestinal segments were removed, and the length was measured and recorded.

Concentration-dependent studies were conducted by perfusing valacyclovir through the jejunum at concentrations ranging from 0.05-30 mM in wildtype mice and from 0.025-1 mM in huPEPT1 mice. Additionally, inhibition studies conducted in both

genotypes evaluated the specificity of valacyclovir transport from the jejunum. Valacyclovir was perfused at 50 μM either alone (control) or with inhibitors at 25 mM. Potential inhibitors included glycylsarcosine (Gly-Sar), cephalexin, tetraethylammonium (TEA), L-histidine, L-lactate, and L-valine. All perfusion buffers containing inhibitors had their pH readjusted to 6.5, as needed.

For segment-dependent studies, 50 μM valacyclovir was simultaneously perfused through a 2 cm segment of duodenum (~0.25 cm distal to the pyloric sphincter), the jejunum (as described before), a 6 cm segment of the ileum (~1 cm proximal to the cecum), and a 3 cm segment of the colon (~0.5 cm distal to the cecum).

3.3.4 UPLC Analytical Method

All perfusate samples were analyzed by a UPLC Waters Acquity H-class system. Peaks corresponding to valacyclovir and acyclovir were separated by a gradient method through a 100-cm Acquity HSS T3 column. The method starts with 100% water in 0.1% TFA, and changes linearly over the next five minutes to 90% water/10% acetonitrile. This ratio holds for 2 minutes before reverting back to 100% aqueous content almost instantly. Each sample run lasts 12 minutes to ensure column pressure equilibration. Column temperature was maintained at 40°C and the flow rate remained constant at 0.5 mL/min. Compounds were detected by UV detection at 254 nm and retention times were approximately 2.4 and 4.9 minutes for acyclovir and valacyclovir, respectively. All perfusate samples were centrifuged at 15000 rpm for 10 minutes and 25 μL aliquots of supernatant were injected into the column by automation.

Method validation over three consecutive days showed accuracy within 6% of specified values and precision within 2% (relative standard deviation). Linearity was established from 0.5-50 μM for acyclovir and from 2-200 μM for valacyclovir. The limits of quantitation for acyclovir and valacyclovir in perfusion buffer were 50 and 10 nM respectively. Additionally, the examination of blank perfusate samples showed that endogenous compound noise was essentially absent within the analyte retention time windows.

3.3.5 Intravenous and Oral Administration Studies

IV bolus and oral administration studies were conducted in a similar manner to that reported previously (Sugawara et. al, 2000). For IV bolus studies mice were administered, by tail vein, 100 μL of saline solution containing 24 nmol/g of acyclovir and [^{3}H] acyclovir (5 μCi/mouse). Blood samples (~20 μL) were collected by tail nick at 1, 5, 10, 20, 30, 45, 60, 90, 120 and 180 minutes after administration. Prior to the oral studies, mice were fasted overnight (16-18 hours) before being administered 200 μL of saline solution containing 24 nmol/g of valacyclovir and [^{3}H] valacyclovir (10 μCi/mouse) by oral gavage. Blood samples were collected similarly as described for IV bolus, with the exception that the first collection time was at 2 minutes. Blood samples from both studies were placed in microcentrifuge tubes with K3-EDTA and centrifuged for 3 minutes at 3000 rpm. Aliquots (5 μL) of plasma were placed in scintillation vials along with 6 mL of CytoScint scintillation fluid (MP Biomedicals, Solon, OH). The plasma

samples were placed in a dual-channel liquid scintillation counter (Beckman LS 6000 SC, Beckman Coulter Inc., Brea, CA) and radioactivity was measured.

3.3.6 Mass Balance Studies

Mice were fasted overnight (16-18 hours) and then 100 µL [^{14}C]inulin (5 µCi/mouse) was injected by IV bolus in the tail vein. Immediately after, these mice were administered 24 nmol/g valacyclovir with [^{3}H]valacyclovir (10 µCi/mouse) by oral gavage. Mice were then placed in a metabolic cage for 24 hours for the purpose of separately collecting the urine and feces. Food and water were provided to the mice while in the cage. After several cage washes, 5-10 µL aliquots of the diluted urine and feces were placed in separate vials with 6 mL of scintillation fluid, and radioactivity was measured by the scintillation counter.

3.3.7 Data Analysis

For in situ perfusion studies, the calculation of effective permeability (P_{eff}) assumed a complete radial mixing (parallel tube) model (Komiya et. al, 1980; Kou et. al, 1991):

$$Peff = \frac{-Qin \times \ln(\frac{C'out}{Cin})}{2\pi RL} \quad (1)$$

where Q_{in} is the inlet perfusion flow rate (0.1 mL/min), C'_{out} is the sum of valacyclovir and acyclovir concentrations in the outlet perfusate corrected for water flux, Cin is the

concentration of valacyclovir in the inlet perfusate, R is the intestinal radius (0.1 cm for small intestine and 0.2 cm for colon), and L is the intestinal segment length. The equation used to determine C'_{out} was calculated as:

$$C'out = (Cout, VACV + Cout, ACV - Cin, ACV) \times (\frac{Qout}{Qin})$$

(2)

where Q_{out} is determined gravimetrically (Sutton et. al, 2001). Concentration-dependent studies in both wildtype and huPepT1 mice were modeled according to Michaelis-Menten kinetics:

$$V = Peff \times Cin = \frac{Vmax \times Cin}{Km + Cin}$$

(3)

where V_{max} is the maximal velocity of transport and K_m is the transporter affinity in terms of the inlet concentration of valacyclovir. The kinetic data was modeled by nonlinear regression and assessed for goodness of fit using Prism version 7 software (GraphPad Software, Inc., La Jolla, CA).

Data were reported as mean ± SD, unless noted differently. Comparisons between two groups were assessed by the unpaired t–test. Comparisons between multiple groups were analyzed by one-way ANOVA followed by either Dunnett's or Tukey's test using Prism software. Statistical significance was attributed when $p \leq 0.05$. For oral and IV bolus studies, pharmacokinetic parameters were determined by noncompartmental analysis using Phoenix WinNonlin 8.0 software (Certara, St Louis).

3.4 Results

3.4.1 Valacyclovir Transport Kinetics During In Situ Jejunal Perfusions

Both wildtype and huPepT1 mice exhibited distinctive Michaelis-Menten kinetics for valacyclovir uptake with characteristic transport saturability (Figures 3.1, 3.2 and 3.3). The kinetic parameters for wildtype mice (Figure 3.1) were V_{max} = 782 ± 53 pmol/cm^2/sec and K_m = 7.7 ± 1.0 mM (r^2=0.992), and for huPepT1 mice (Figure 3.2) were V_{max} = 7.83 ± 1.37 pmol/cm^2/sec and K_m = 0.25 ± 0.10 mM (r^2=0.898). The V_{max} value was approximately 100-fold greater in wildtype mice than in huPepT1 mice, while the K_m value was approximately 30-fold less (i.e. affinity was 30-fold greater). The inclusion of a nonsaturable term did not improve the goodness of fit, so only a saturable term was considered in the final model.

3.4.2 mPepT1 and huPepT1 Specificity for Valacyclovir Transport During In Situ Jejunal Perfusions

The co-perfusion of various potential inhibitors with valacyclovir in wildtype mice (Figure 3.4) demonstrated that glycylsarcosine and cephalexin significantly reduced the permeability of valacyclovir by approximately 2-fold from the control value. In contrast, the substrates tetraethylammonium, L-histidine, L-lactate, and L-valine did not significantly change valacyclovir permeability. These same inhibitor studies in huPepT1 mice (Figure 3.5) showed a significant 16-fold reduction in the permeability of

valacyclovir by glycylsarcosine, whereas cephalexin did not induce a significant reduction. Moreover, co-perfusion of valacyclovir with other potential inhibitors did not significantly change the permeability from control values, as similarly observed in the wildtype results.

3.4.3 Regional Valacyclovir Permeability During In Situ Intestinal Perfusions

The permeability of valacyclovir, obtained from segments of the small intestine, were shown to be significantly different between, but not within, the two genotypes **(**Figure 3.6). The huPepT1 mice had permeability values that were substantially smaller than wildtype mice and were approximately 12-fold lower in the duodenum, 10-fold lower in the jejunum, and 7-fold lower in the ileum. On the other hand, permeability values from the colon were not different between wildtype and huPepT1 mice and, in huPepT1 animals, were not different from other segments of the small intestine.

3.4.4 Pharmacokinetic Parameters In Vivo from IV Bolus and Oral Administrations

The IV bolus administration of acyclovir in wildtype and huPepT1 mice yielded pharmacokinetic profiles that were nearly superimposable between the two genotypes (Figure 3.7). This observation is supported by the pharmacokinetic analysis in which all key parameters showed no significant differences between the wildtype and huPepT1 mice (Table 3.1). In contrast, the oral administration of valacyclovir produced plasma concentration-time curves of acyclovir that were visually different between the two

genotypes (Figure 3.8). This difference was noted by the 3-fold higher C_{max} in wildtype mice, the 3-fold larger T_{max} in huPepT1 mice, and the AUC_{0-180} of huPepT1 mice that was approximately 80% of the value seen in wildtype animals (Table 3.2). Despite these trends, only the C_{max} values were statistically different from each other.

3.4.5 Acyclovir Bioavailability In Vivo from Mass Balance Studies

As shown in Table 3.3, the bioavailability of acyclovir, after oral administration of valacyclovir, was 77.5% and 52.8% for wildtype and huPepT1 mice, respectively. This significant difference corresponds to a relative bioavailability in huPept1 mice that was 68% of the value seen in wildtype animals. Although the urine from huPepT1 mice contained 20% less of the administered dose, their feces contained approximately 20% more of the dose, as compared to wildtype mice. Inulin, a marker for urine collection efficiency, revealed adequate recoveries of drug-related radiolabel in these studies (~90%). Additionally, trace amounts of inulin in the feces (~1%) confirmed that there was a minimal level of urine-to-feces cross-contamination.

3.5 Discussion

Applicability of the huPepT1 transgenic mouse line for predicting clinical pharmacokinetic parameters has been validated recently through studies performed with cefadroxil (Hu and Smith, 2016). However, these findings were limited in scope because the substrates that PepT1 can transport encompass more than just β-lactam antibiotics (Rubio-Aliaga and Daniel, 2002; Dahan et. al, 2014; Brandsch et. al, 2013). The current study has sought to broaden the utility of these transgenic mice by further validating the model with the ester prodrug valacyclovir. Specifically, several major findings were revealed: 1) huPepT1 mice had a greater affinity (30-fold lower K_m) and reduced capacity (100-fold lower V_{max}) for valacyclovir during jejunal perfusions than wildtype mice; 2) the jejunal permeability of valacyclovir was specific for PepT1; 3) the permeability of valacyclovir was significantly lower in all segments of huPepT1 mouse small intestine as compared to wildtype; 4) the disposition kinetics of intravenously administered valacyclovir did not differ between the two genotypes; and 5) the relative bioavailability of orally dosed valacyclovir was about 30% lower in huPepT1 mice than wildtype animals, and its absolute bioavailability in huPepT1 mice was better correlated with clinical studies.

In a previous perfusion study, Yang and Smith (2013) reported the jejunal transport kinetics of valacyclovir as V_{max} = 1.4 nmol/cm^2/s and K_m = 10.2 mM in wildtype mice. These values were comparable to those obtained in the current study (i.e., V_{max} = 0.78 nmol/cm^2/s and K_m = 7.71 mM). In regard to hPepT1-mediated transport, previous cell culture studies revealed K_m values ranging from 0.29 - 5.94 mM (Balimane et. al, 1998;

Han et al, 1998; Guo et. al, 1999; Balimane and Sinko, 2000; Bhardwaj et. al, 2005). In the current study, huPepT1 mice had a K_m of 0.25 mM, a value approaching the lower bounds of the literature range. The reasons for variation between our in situ perfusion studies and other in vitro studies may be attributed to the use of different cell expression systems, a lack of blood flow during in vitro studies, and differences in buffer pH that may alter valacyclovir affinity for PepT1 (Balimane and Sinko, 2000).

An understanding of PepT1 specificity for valacyclovir in the huPepT1 mouse model is paramount for model validation. Inhibition studies, conducted in both wildtype and huPepT1 mice, indicated that L-valine, L-histidine, L-lactate and tetraethylammonium had little to no effect on valacyclovir-mediated uptake, thereby, ruling out an influence of $ATB^{0,+}$, PhT1/2, MCT1 and OCT transporters. Conversely, the reduction in valacyclovir uptake during co-perfusion of glycylsarcosine and cephalexin indicated that valacyclovir was transported substantially by PepT1 in both mouse models. This finding is in agreement with a similar study in wildtype mice by Yang and Smith (2013), with the novelty of also testing MCT1 given its known expression in mouse small intestine (Iwanaga et. al, 2006). It is noteworthy to mention that cephalexin did not significantly reduce valacyclovir uptake in huPepT1 mice, although a trend was observed. This may have to do with differences in inhibitor affinity of cephalexin for this transporter. Two previous studies in Caco-2 cells (pH 6.0 uptake buffer) revealed that the K_m values for glycylsarcosine and cephalexin were approximately 4-fold different (i.e., 2 and 8 mM respectively) (Irie et. al, 2005; Watanabe et. al, 2006).

With respect to segment-dependency, wildtype mice showed uptake trends that were roughly similar to the relative levels of protein expressed in each segment (Jappar

et. al, 2010). However, segment-dependent uptake in huPepT1 mice did not correspond well with protein levels. While uptake was low and statistically similar across all segments, our previous study reported higher protein levels in the small intestine and only trace amounts in the colon (Hu and Smith, 2016). This "apparent" discrepancy is hard to reconcile. However, a similar study with cefadroxil revealed non-significant differences in uptake between the small and large intestines of huPepT1 mice, although greater differences were observed (Hu and Smith, 2016).

In a clinical study, where 10 μM of valacyclovir was perfused through the subject's jejunum using the Loc-I-Gut® method (pH 6.5 sodium phosphate buffer), the effective permeability was reported as 1.66 x 10^{-4} cm/s (Cao et. al, 2006). In the current study, the jejunal permeability of 50 μM valacyclovir was 0.92 x 10^{-4} in wildtype mice and 0.12 x 10^{-4} cm/s in huPepT1 mice. Using these permeability values, it was possible to estimate the absorption rate constant (Ka) of valacyclovir by the equation:

$$Ka = (2 \times Peff)/R \tag{4}$$

where R is the intestinal radius of the jejunum (1.75 cm for humans and 0.1 cm for mice) (Yu et. al, 1999). Accordingly, the K_a values were 0.68 hr^{-1} in humans, 6.62 hr^{-1} in wildtype and 0.86 hr^{-1} in huPepT1 mice (Table 3.4), indicating a better correlation in the absorption rate constant between humans and huPepT1 mice. Thus, it appears that huPepT1 transgenic mice may predict the absorption of valacyclovir in humans with improved accuracy.

The 24 nmol/g valacyclovir dose chosen for all *in vivo* experiments in the current study was selected from the 10-100 nmol/g dosing range established previously (Yang

et. al, 2013). These authors justified the dosing range by relating the C_{max} values of acyclovir obtained from their wildtype mice with the C_{max} values determined from clinical studies in which 250, 500, 1000 and 2000 mg oral doses of valacyclovir were administered (Weller et. al, 1993). Apart from establishing dosing rationale, pharmacokinetic trends can be elucidated from these studies as well. For example, escalating oral doses of valacyclovir in wildtype mice revealed linear increases in acyclovir AUC and C_{max}, but clinical studies showed less than proportional increases in these parameters. Investigators (Yang et. al, 2013; Weller et. al, 1993) have attributed these clinical trends to saturation of transporter-mediated intestinal absorption, which we believe is substantiated by comparing the oral absorption results in the current study between wildtype and huPepT1 mice.

The *in vivo* oral absorption and disposition of 25 nmol/g valacyclovir has been reported previously in wildtype and PepT1 knockout mice (Yang et. al, 2013). In these studies, PepT1 knockout mice had a lower C_{max} (5- to 6-fold), a higher T_{max} (5-fold), and an $AUC_{0\text{-}180}$ for acyclovir that was 35% of that observed in wildtype mice. While PepT1 ablation clearly demonstrated a deep reduction in the rate and extent of oral valacyclovir absorption, more modest differences were observed between wildtype and huPepT1 mice (Table 3.2). Nonetheless, as clearly shown by our mass balance studies (Table 3.3), the bioavailability of orally administered valacyclovir was 52.8% in huPepT1 mice, as compared to 77.5% for wildtype animals. Notably, a similar bioavailability was observed in the clinical setting where a value of 51.3% was observed after the oral administration of 1000 mg valacyclovir (Soul-Lawton et. al, 1995). It is worth mentioning that the bioavailability of acyclovir as determined from the $AUC_{0\text{-}180}$ values, derived from

the oral and IV bolus studies (Tables 3.2 and 3.3), were 106% and 76.4% for wildtype and huPepT1 mice, respectively. While a similar reduction in bioavailability is evident in huPepT1 mice, the results from the mass balance studies are believed to be superior for the following reasons: 1) only a single population of mice is needed to determine bioavailability in these mass balance studies and 2) only partial AUC values are utilized in the IV bolus and oral administration studies.

As shown previously by our group, jejunal permeabilities that differed by 10-fold between wildtype and PepT1 knockout mice resulted in systemic availabilities that differed by only 2-fold for glycylsarcosine (Jappar et. al, 2010; Jappar et. al, 2011) and by 2- to 3-fold for valacyclovir (Yang and Smith, 2013; Yang et. al, 2013) after oral dosing. In these reports, we advanced the concept that PepT1 knockout mice may take advantage of the residual length of small and large intestines, resulting in greater than expected passive absorption and, thereby, minimizing the reduced systemic availability of substrate expected during PepT1 ablation. This concept was subsequently validated during an in silico analysis by our group (Yang and Smith, 2017), demonstrating that whereas wildtype mice primarily absorb valacyclovir in the duodenal and jejunal segments of the small intestine (i.e., 66% of a total 70% absorbed), the absorption of valacyclovir in PepT1 knockout mice was slow but sustained throughout the entire intestinal tract (i.e. 4% duodenum, 14% jejunum, 10% ileum and 12% cecum/colon for a total 40% absorbed). Although a similar analysis was not performed for valacyclovir in huPepT1 mice, this transgenic group appears to fall in the middle of the results observed between wildtype and PepT1 knockout mice, namely, having an absorption of 53%.

An underlying assumption throughout these studies was that valacyclovir was rapidly degraded to its parent compound *in vivo* and that the radiolabel being measured was essentially all acyclovir. We believe, for several reasons, that this was a reasonable assumption. First, Yang and Smith (2013) found that after a 90-min intestinal perfusion of valacyclovir, only acyclovir was observed in the portal venous blood of mice. Second, these investigators reported very similar plasma concentration-time profiles of acyclovir in mice following oral administration of either radiolabeled or unlabeled valacyclovir, as measured by liquid scintillation counting or HPLC, respectively. Finally, studies in humans, monkeys and rats also confirmed the rapid and efficient metabolic conversion of prodrug (Weller et. al, 1993; Burnette and de Miranda, 1994; de Miranda and Burnette, 1994).

In conclusion, the *in situ* mechanistic studies provided here demonstrate that valacyclovir has a greater affinity (and lower capacity) for PepT1-medidated transport in huPepT1 than wildtype mice. The *in vivo* pharmacokinetic and mass balance studies reflect the saturability of intestinal uptake in huPepT1 mice, as judged by decreases in valacyclovir's rate and extent of absorption or bioavailability. Taken as a whole, the discrete differences in wildtype and huPepT1 mice, when held against clinical data, further rationalize the usefulness of this transgenic model for studying other prodrugs. The huPepT1 mice, along with other transgenic mouse models, are currently being used to evaluate the feasibility of developing oral prodrugs for parenteral anti-cancer therapeutics.

3.7 Figures and Tables

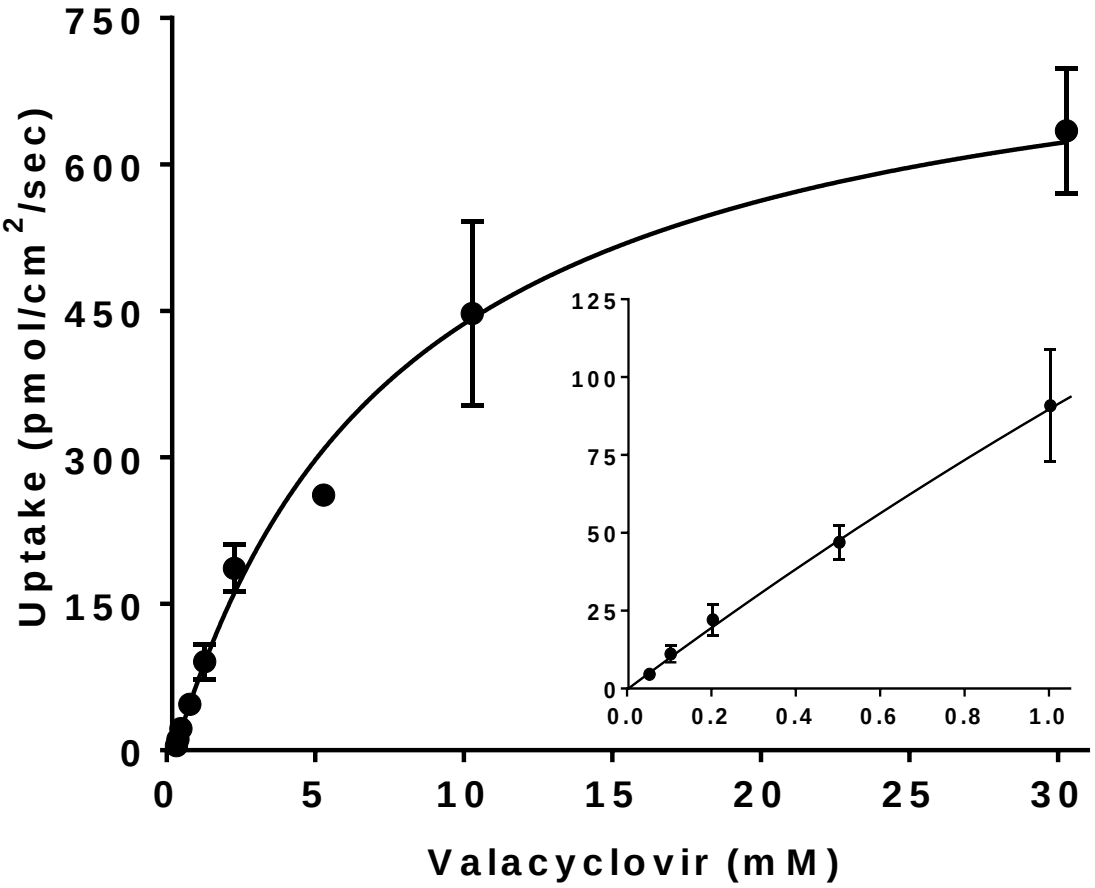

Figure 3.1 Uptake of valacyclovir after in situ perfusion of wildtype mice at given perfusate concentrations. Data are expressed as mean±S.E. (n=3-4). Mean data was fit into the model. Inset shows the data points at low valacyclovir concentrations.

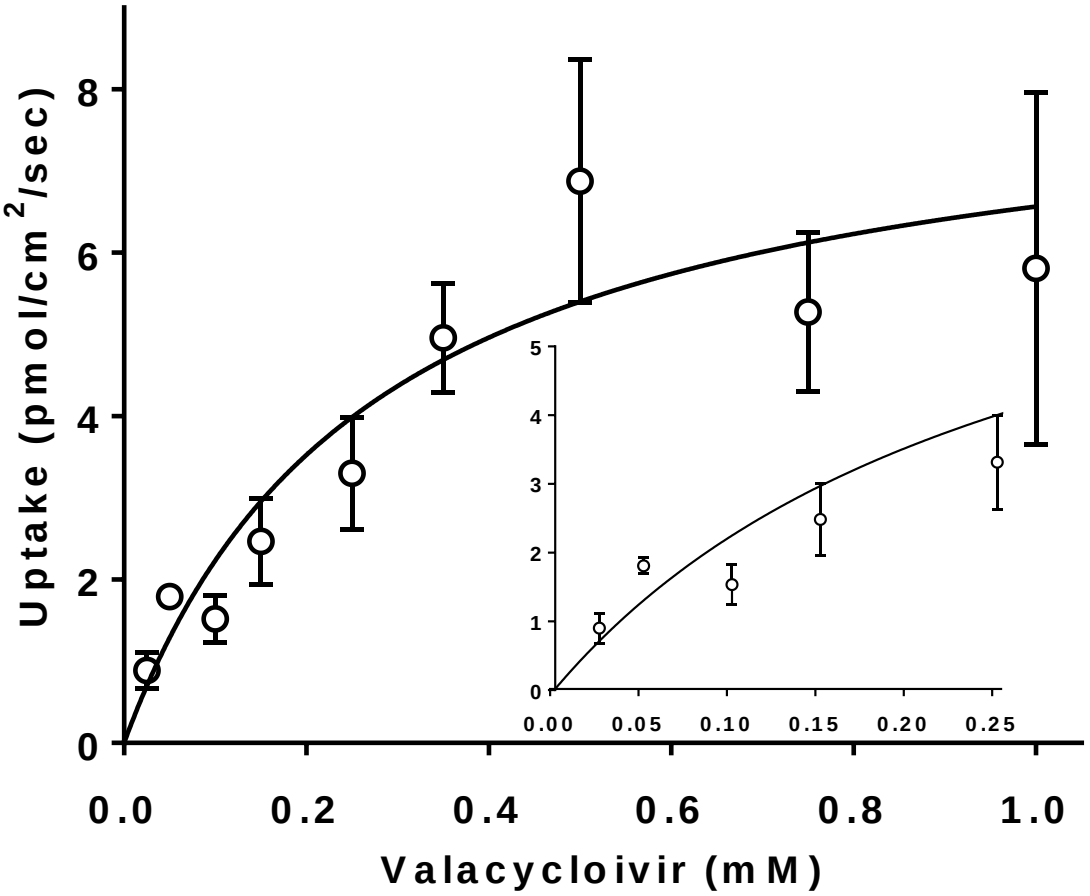

Figure 3.2 Uptake of valacyclovir after in situ perfusion of huPepT1 mice at given perfusate concentrations. Data are expressed as mean±S.E. (n=3-7). Mean data was fit into the model. Inset shows the data points at low valacyclovir concentrations.

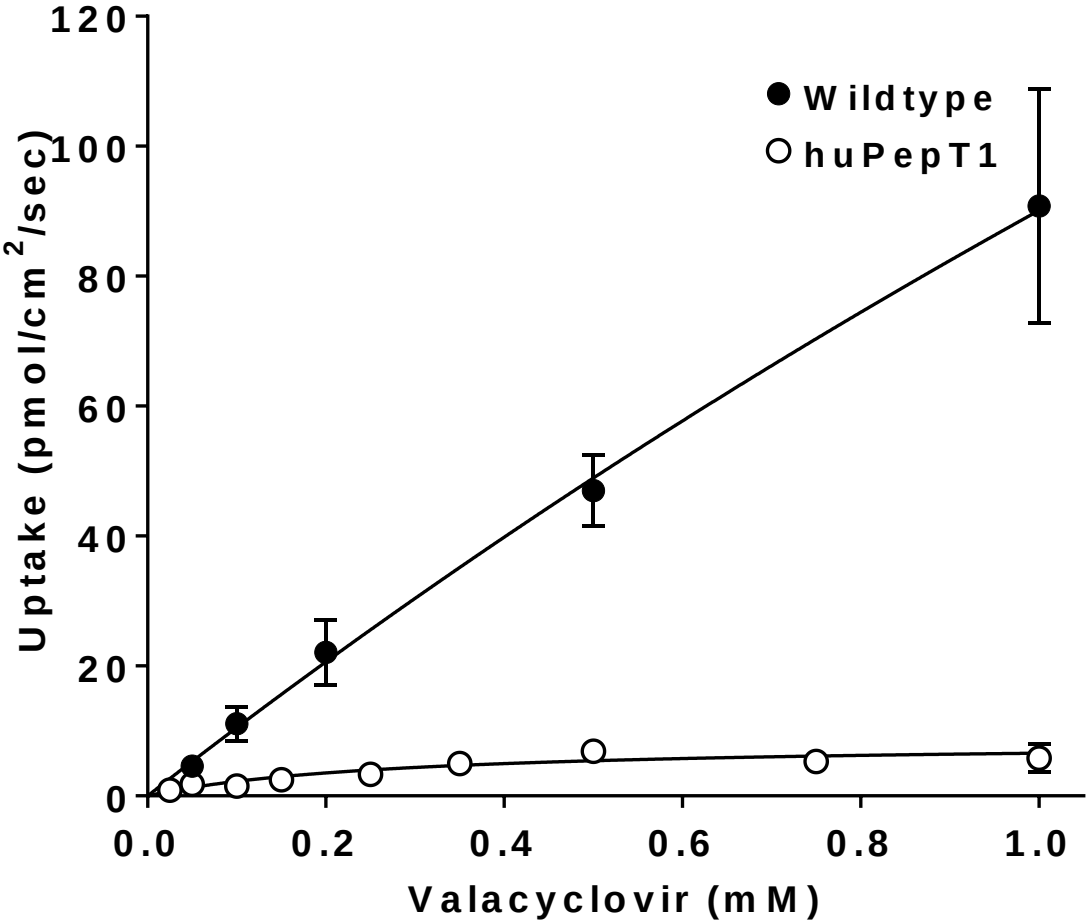

Figure 3.3 Comparison of in situ perfusions of valacyclovir in wildtype and huPepT1 mice. All data are expressed as mean±S.E. (n=3-7). Mean data was fit into model.

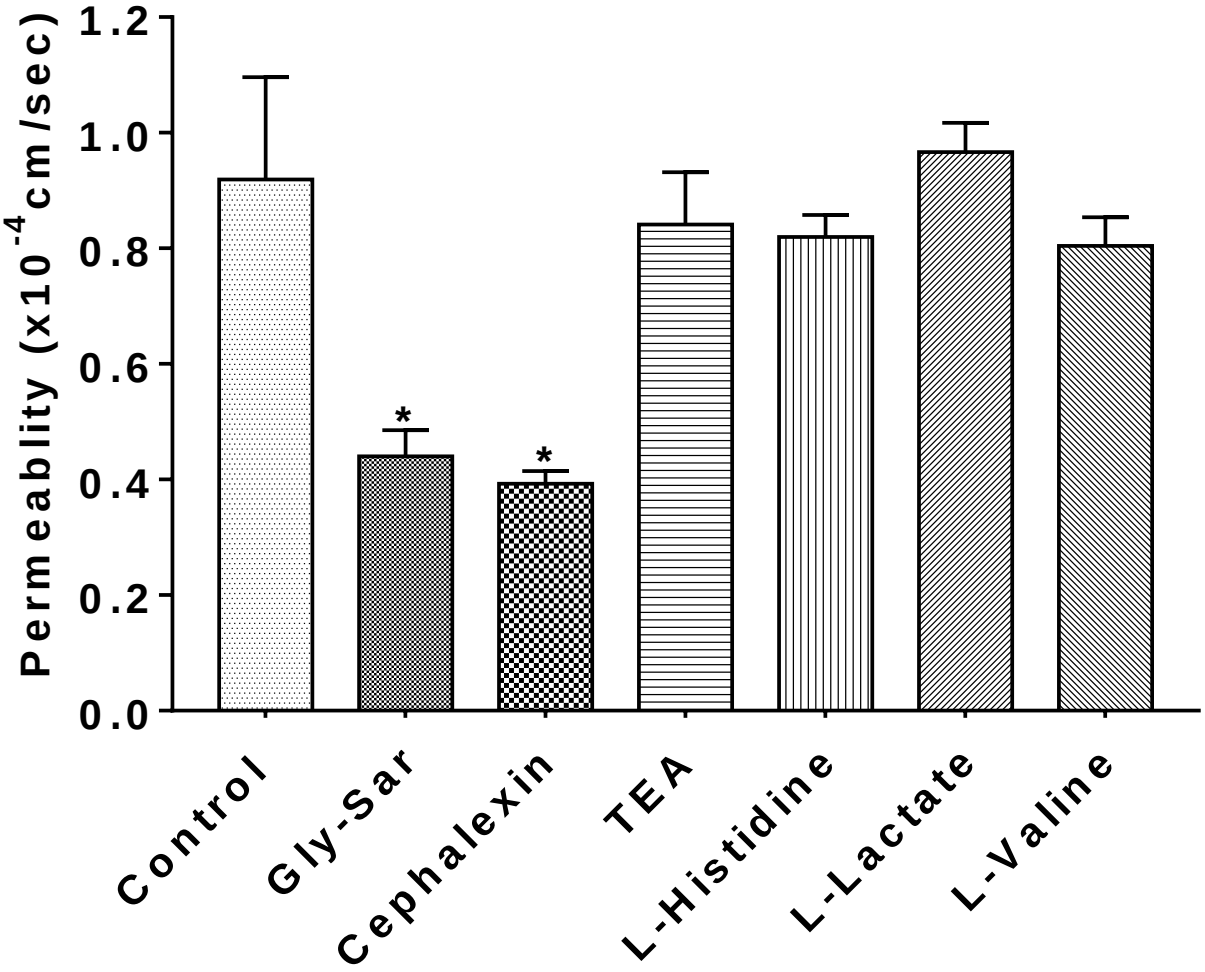

Figure 3.4 Valacyclovir at 50 µM was co-perfused (pH 6.5) with various potential inhibitors at 25 mM in wildtype mice (n=3-7). The control column represents the perfusion of valacyclovir alone. *p ≤ 0.05 compared to control, as determined by ANOVA and Dunnett's' test.

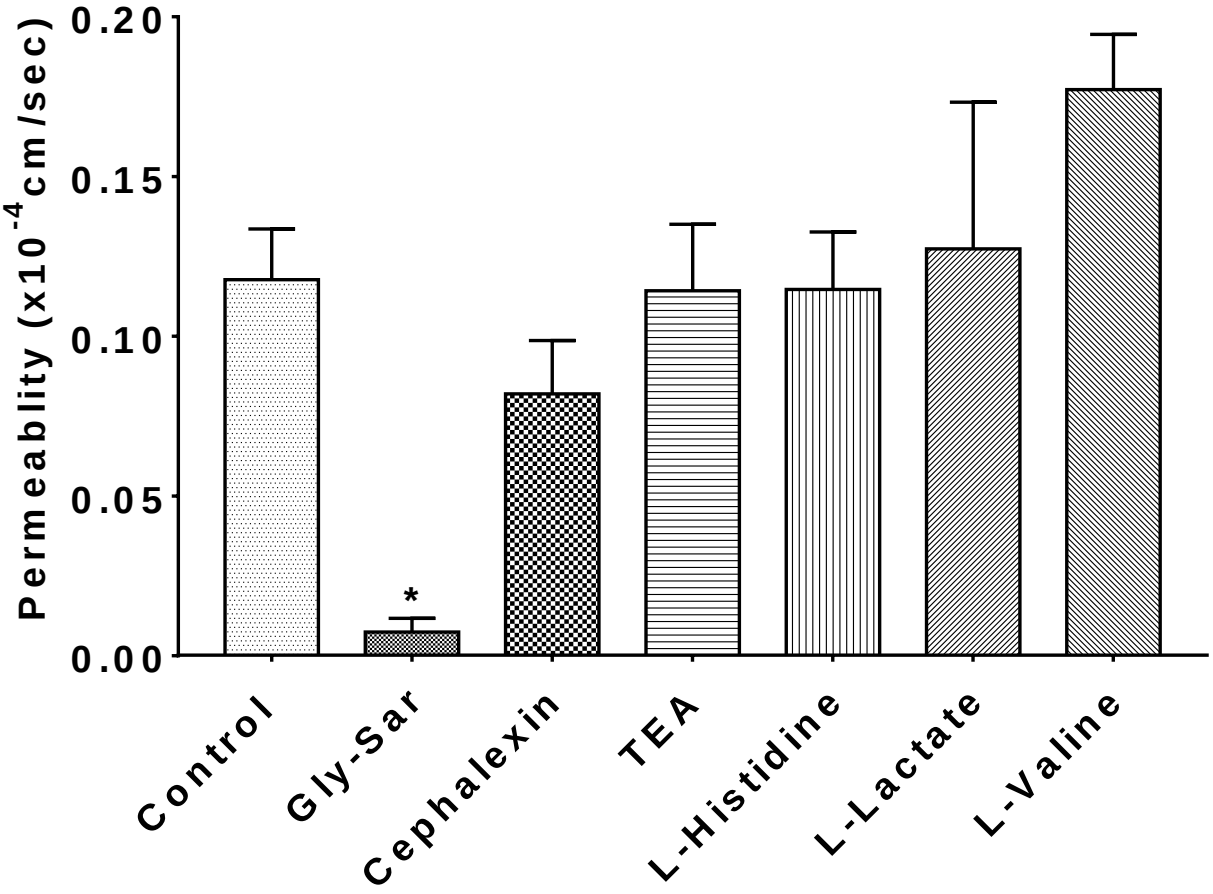

Figure 3.5 Valacyclovir at 50 µM was co-perfused (pH 6.5) with various potential inhibitors at 25 mM in huPepT1 mice (n=5-8). The control column represents the perfusion of valacyclovir alone. *$p \leq 0.05$ compared to control, as determined by ANOVA and Dunnett's' test.

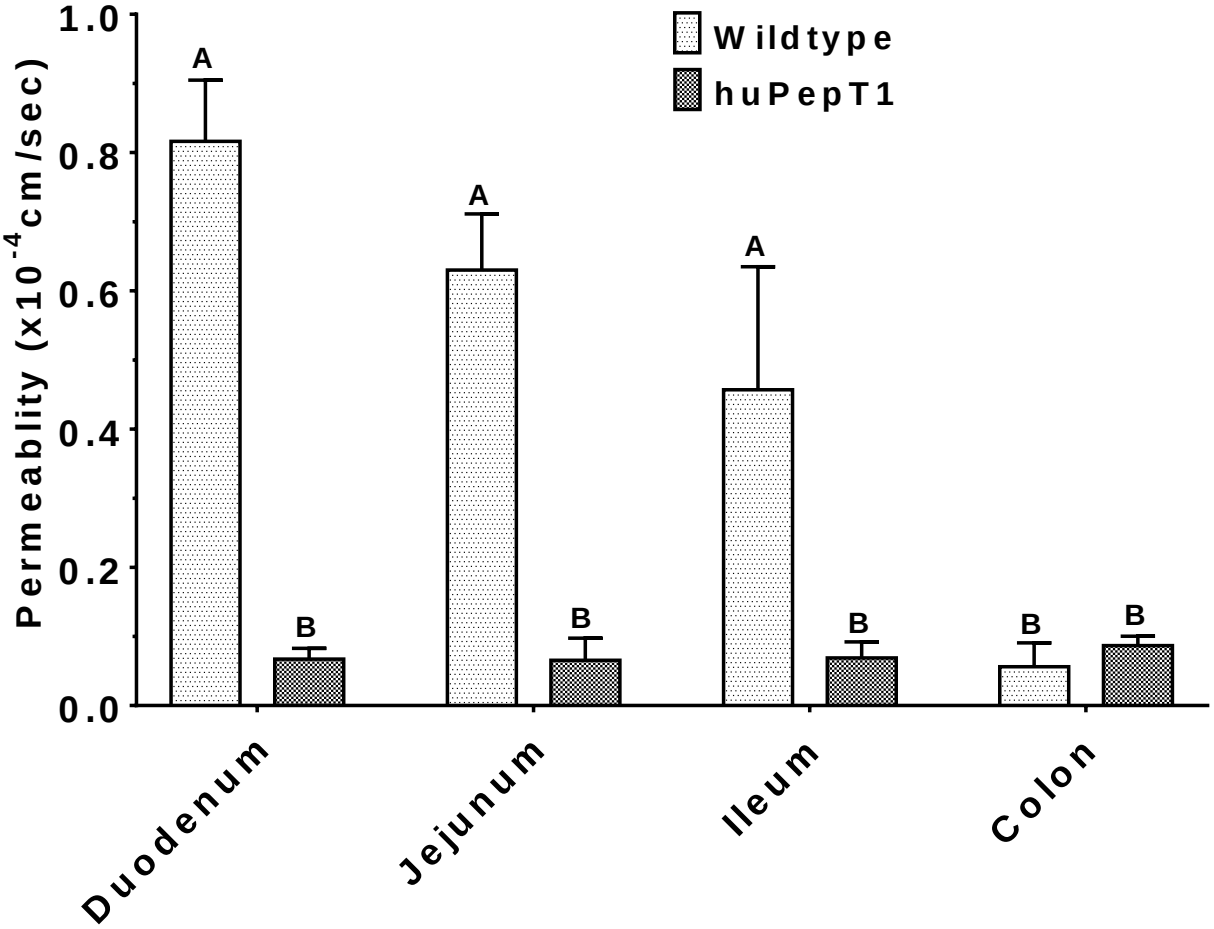

Figure 3.6 All four intestinal segments were perfused simultaneously with valacyclovir in both wildtype and huPepT1 mice. Data are expressed as mean ± SE (n=4). Segments with the same letter were not significantly different, as determined by ANOVA and Tukey's test.

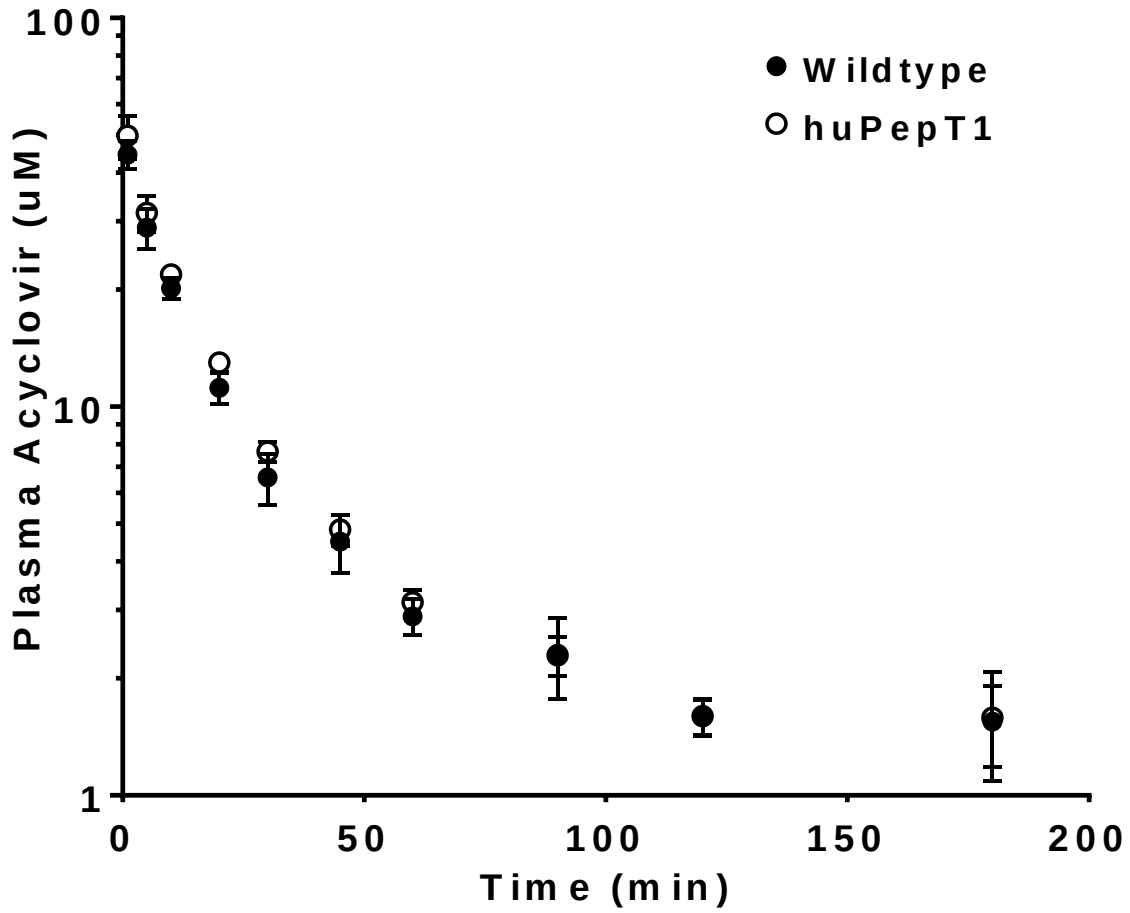

Figure 3.7 Acyclovir plasma concentration-time profile after IV bolus administration of 24 nmol/g of acyclovir in wildtype and huPept1 mice. Data are expressed as mean ± SE (n=4).

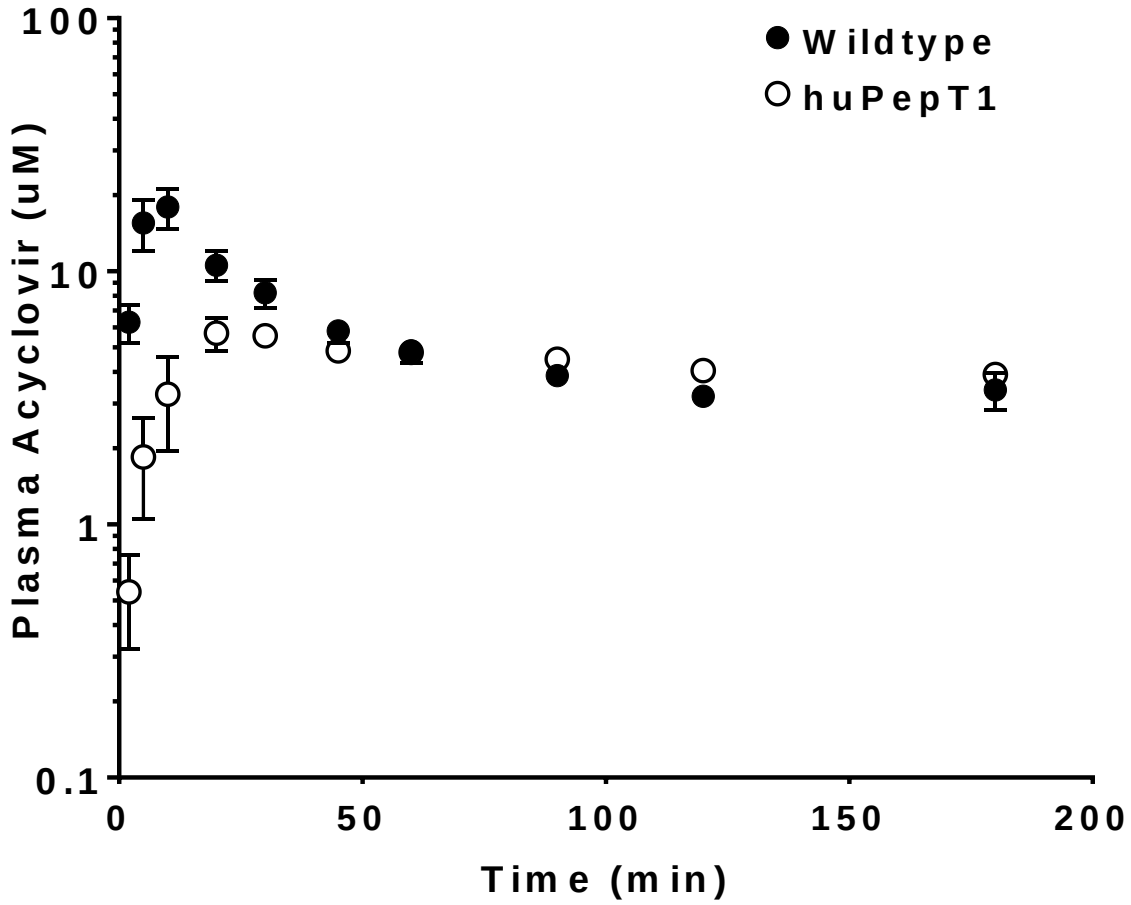

Figure 3.8 Acyclovir plasma concentration-time profiles after oral administration of 24 nmol/g of valacyclovir in wildtype and huPept1 mice. Data are expressed as mean ± SE (n=4).

Table 3.1 Pharmacokinetic parameters of acyclovir after IV bolus administration of acyclovir (24 nmol/g) in wildtype and huPeptT1 mice

Parameter (unit)	Wildtype	huPepT1	Ratio (huPepT1/WT)
Vd_{ss} (L/kg)	2.1 ± 0.8	1.4 ± 0.2	0.67
CL (L/hr/kg)	1.3 ± 0.2	1.3 ± 0.2	1.0
$T_{0.5}$ (min)	106 ± 46	63.4 ± 4.0	0.60
$AUC_{0\text{-}180\ min}$ (μM*min)	929 ± 126	1015 ± 92	1.1

Data are shown as mean ± SD (n=4). No significant difference was observed for any of the key parameters, as determined by unpaired t-test.

Table 3.2 Pharmacokinetic parameters of acyclovir after oral administration of valacyclovir (24 nmol/g) in wildtype and huPept1 mice

Parameter (unit)	Wildtype	huPepT1	Ratio (huPepT1/WT)
T_{max} (min)	10 ± 0	33 ± 19	3.3
C_{max} (μM)	18.0 ± 6.5	6.1 ± 1.4*	0.34
$AUC_{0\text{-}180\ min}$ (μM*min)	982 ± 230	775 ± 86	0.79

Data are shown as mean ± SD (n=4). *p ≤ 0.05 compared to wildtype mice, as determined by unpaired t-test.

Table 3.3 Bioavailability of acyclovir after the oral administration of valacyclovir (24 nmol/g) in wildtype and huPepT1 as determined from mass balance

	Wildtype			huPepT1		
	Urine	Feces	F	Urine	Feces	F
% Acv	68.1	11.6	77.5	47.4	31.6	52.8*
% Inu	87.9	1.4	89.3	89.1	0.5	89.6

Data are expressed as mean values and represent three mice per group. Each animal was injected with [^{14}C]inulin (Inu) by IV bolus, and then dosed with [^{3}H]valacyclovir [VACV] by oral gavage. Animals were then placed in a metabolic cage, and the urine and feces collected over 24 hours. Bioavailability (F) of acyclovir (ACV) was calculated as: %ACV recovered in the urine divided by %Inu recovered in the urine. Bioavailability (F) of Inu was calculated as: %Inu recovered in the urine plus %Inu recovered in the feces. *$p \leq 0.05$ compared to wildtype mice, as determined by unpaired t-test.

Table 3.4 The effective permeability and calculated K_a of the prodrug valacyclovir as determined from the jejunum in humans, wildtype and huPepT1 transgenic mice

Model	Peff (cm/s) x 10^{-4}	K_a (hr^{-1})
Clinical	1.66*	0.68
HuPepT1	0.12	0.86
Wildtype	0.92	6.62

* Value taken from Cao et. al., 2006.

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CHAPTER 4

The Effect of Nifedipine on the Intestinal Permeability of Valacyclovir in Wildtype Mice

4.1 Abstract

There have been many drug interaction studies conducted in the past that have investigated the influence of the calcium channel blocker nifedipine on the absorption and pharmacokinetics of various PepT1 substrates belonging to a single class of β-lactam antibiotics. The results from the clinical and rat administration studies found that nifedipine significantly enhanced the absorption of these compounds. Therefore, the focus of the current investigation was to ascertain if the absorption-enhancing effects of nifedipine could be extended to other PepT1 substrates, specifically to the prodrug valacyclovir. Wildtype mice were co-perfused with valacyclovir and nifedipine and the results revealed that the intestinal permeability of the prodrug was non-statistically diminished (i.e. 26-28%) in the presence of this calcium channel blocker. While the outcomes from these experiments have been difficult to reconcile in light of previous studies, perhaps the valacyclovir-nifedipine interaction involves a species-dependent mechanism that denies absorption-enhancing effects in mice.

4.2 Introduction

Drug-drug interactions have become an emerging concern for government regulators and drug developers alike over the last couple decades, with guidance for the industry first established in 1997. This concern was so evident that from 1999-2003 half of all drugs pulled from US markets were due to undesirable drug interactions. These interactions occur when a “perpetrator” compound affects the pharmacokinetic profile of a “victim” compound by altering its absorption, distribution, metabolism, or excretion. At first, much effort was placed on trying to understand interactions that were mediated by changes in metabolic activity, particularly with cytochrome P450 enzymes. However, this field has expanded recently with drug transporters now considered important mediators in drug interactions as well (Prueksaritanont et. al, 2013; Huang et. al, 2013).

There are many drug-drug interaction studies from the literature that suggest the calcium channel blocker nifedipine can enhance the oral absorption of β-lactam antibiotics, a class of drugs absorbed by the action of PepT1 (Rubio-Aliaga and Daniel, 2002). Specifically, two clinical interaction studies with amoxicillin and cefixime (Westphal et. al, 1990; Duverne et. al, 1992), and one oral administration study with cephalexin in rats (Berlioz et. al, 2000) have supported this premise. Furthermore, nifedipine was found to enhance the intestinal absorption of cephalexin and cefixime after a series of intestinal perfusions conducted in rats (Harcouet et. al, 1997; Berlioz et. al, 1999). Interestingly, the same trend was

observed for cefixime in Caco-2 cells (Wenzel et. al, 2002), where the authors attributed an increase in uptake to a concomitant increase in PepT1-mediated activity (due to an elevated proton gradient).

Despite the insight provided from these previous drug interaction studies, it remains to be seen if other PepT1 substrates that are not β-lactam antibiotics will act similarly in the presence of nifedipine. Thus, the purpose of this current study is to examine and assess the potential interaction between the prodrug valacyclovir (a PepT1 substrate) and nifedipine in wildtype mice. The underlying hypothesis is that the co-administration of nifedipine will enhance the intestinal absorption of valacyclovir. Therefore, intestinal perfusions in wildtype mice have been carried out to determine the impact of nifedipine on the permeability of this prodrug.

4.3 Materials and Methods

3.3.1 Chemicals

Valacyclovir and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO). CytoScint™ scintillation solution was purchased from MP Biomedicals (Solon, OH). [^{3}H]Valacyclovir (1.1 Ci/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). [^{14}C]Inulin 5000 (1.1 mCi/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other buffer components were purchased from standard sources.

3.3.2 Animals

Gender-matched and age-matched (8-12 weeks) wildtype mice are utilized in these studies and have a C57BL/6 background. All mice were bred and housed in a temperature-controlled environment with 12 hour light and dark cycles, and a standard diet and water a*d libitum* (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

3.3.3 Perfusate Buffer Composition

The buffer solution used in the following intestinal perfusion studies was

partially adopted from the drug interaction studies conducted by Wenzel et. al (2002). It is a modified Krebs buffer with 10 mM HEPES, 0.3 mM KH_2PO_4, 0.3 mM NaH_2PO_4, 1.0 mM $MgSO_4$, 2.8 mM $CaCl_2$, 5.4 mM KCl, 137 mM NaCl, and 10 mM β-D-glucose. Tris was added to the solution to achieve pH 6.5. The non-absorbable marker [^{14}C]inulin 5000 (0.18 µCi/mouse) was added to solution to account for changes in water flux, as done before (Hu and Smith, 2016). Valacyclovir was perfused at 50 µM with [^{3}H]valacyclovir (1.8 µCi/mouse), while 90-100 µM nifedipine was added during co-perfusions.

3.3.4 In Situ Single-Pass Intestinal Perfusion Studies

As reported previously (Yang and Smith, 2013; Jappar et. al, 2010), wildtype mice were fasted overnight (16-18 hours) before experimentation. Mice were then given 40-60 mg/kg of pentobarbital as an intraperitoneal injection for anesthesia. The abdominal area was sanitized with alcohol before a 1.5 cm incision was made along the midline section. An 8-cm segment of jejunum (~2 cm distal to the Ligament of Trietz) was isolated, cannulated (2.0 mm outer diameter), and tied off with silk sutures. The mouse was then placed in a temperature-controlled chamber (31°C) and saline-wetted gauze, along with Parafilm, was laid out across the abdominal section to prevent dehydration. Inlet tubing connected the syringe (which contained the perfusion buffer) to the intestinal segment and outlet tubing connected the intestinal segment to the collection vials.

Buffer solution was perfused through the cannulated segment at a programmed flow rate of 0.1 mL/min for 90 minutes by the action of a Harvard Apparatus PHD

syringe pump (South Natick, MA). Two different perfusion schedules were utilized in these studies. In the first study, valacyclovir and nifedipine were co-perfused for the first 10 minutes while valacyclovir was perfused alone for the remaining 80 minutes. In the second study, valacyclovir and nifedipine were co-perfused for the first 30 minutes while valacyclovir was perfused alone for the remaining 60 minutes. Perfusate samples were collected every 10 minutes for the last 60 minutes for each schedule. Upon completion, the perfused intestinal segments were removed, and the length was measured and recorded. Aliquots (100 µL) of inlet and outlet perfusate were placed in scintillation vials along with 6 mL of CytoScint scintillation fluid (MP Biomedicals, Solon, OH). These samples were placed in a dual-channel liquid scintillation counter (Beckman LS 6000 SC, Beckman Coulter Inc., Brea, CA) and radioactivity was measured.

3.3.5 Data Analysis

For in situ perfusion studies, the calculation of effective permeability (P_{eff}) assumed a complete radial mixing (parallel tube) model (Komiya et. al, 1980; Kou et. al, 1991):

$$Peff = \frac{-Qin \times \ln(\frac{C'out}{Cin})}{2\pi RL} \quad (1)$$

where Q_{in} is the inlet perfusion flow rate (0.1 mL/min), C'_{out} is the sum of valacyclovir and acyclovir concentrations in the outlet perfusate corrected for water flux, Cin is the concentration of valacyclovir in the inlet perfusate, R is the intestinal radius (0.1 cm for

small intestine and 0.2 cm for colon), and L is the intestinal segment length. The equation used to determine C'_{out} was calculated as:

$$C'out = Cout \times (INUin/INUout) \quad (2)$$

where INU_{in} and INU_{out} are the inlet and outlet counts of [^{14}C]Inulin 5000 contained within the perfusate (Sutton et. al, 2001).

Data were graphed on Prism software (Irvine, CA) and reported as mean ± SE. Comparisons between two groups were assessed by the unpaired t–test.

4.4 Results

4.4.1 First study: Co-perfusion of valacyclovir and nifedipine for 10 minutes

The permeability of valacyclovir in the control group was 0.83×10^{-4} cm/sec, while the permeability of valacyclovir after a 10-minute co-perfusion with 100 μM nifedipine was 0.61×10^{-4} cm/sec (Figure 4.1). The permeability of valacyclovir in the presence of nifedipine was reduced by 26.5%, but this difference was not statistically significant.

4.4.2 Second study: Co-perfusion of valacyclovir and nifedipine for 30 minutes

The permeability of valacyclovir in the control group was 0.75×10^{-4} cm/sec, while the permeability of valacyclovir after a 30-minute co-perfusion with 90 μM nifedipine was 0.54×10^{-4} cm/sec (Figure 4.2). The permeability of valacyclovir in the presence of nifedipine was reduced by 28.0%, but this difference was not statistically significant. These results were not markedly different from the first perfusion schedule (Table 4.1).

4.5 Discussion

There are several drug interaction studies found in the literature that have effectively demonstrated the absorption-enhancing effects of nifedipine on various PepT1 substrates (Westphal et. al, 1990; Duverne et. al, 1992; Harcouet et. al, 1997; Berlioz et. al, 199; Berlioz et. al, 2000; Wenzel et. al, 2002).However, these studies have only focused on a single class of drugs: the β-lactam antibiotics. Therefore, the current studies have sought to understand if the prodrug valacyclovir can be affected in a similar fashion while in the presence of this calcium channel blocker. After a series of intestinal perfusion studies in wildtype mice it became evident that nifedipine modestly reduced the permeability of valacyclovir (i.e. 26-28%) in a non-significant manner. These results were surprising as they directly clashed with the conclusions drawn from other oral administration and intestinal perfusion studies, but also ran counter to the mechanism proposed by Wenzel et. al (2002). Therefore, upon further reflection, additional studies were not performed.

A possible explanation for the non-significant interaction between valacyclovir and nifedipine may be due to the prevailing ionization state of valacyclovir in the perfusate buffer. Valacyclovir is known to have three pKa values: 1.90, 7.47, and 9.43. The neutral form of the compound, which has the greatest affinity and functional activity toward PepT1-mediated transport, is the predominant form between pH 7.47 to 9.43. However, the pH 6.5 perfusate buffer utilized in these studies, coupled with the nifedipine-induced acidification of the intestinal lumen (Wenzel et.al, 2002), would leave much of the prodrug in the less active, cationic form. The presence of this cationic form

results in an approximate 40% reduction in uptake when the pH is reduced from 7.4 to 6.5 (Balimane and Sinko, 2000). On the other hand, β-lactam antibiotics are commonly present in the zwitterionic form at physiological pH. For example, amoxicillin is primarily zwitterionic between pH 2.4 and 7.4 (Elmolla and Chaudhuri, 2010), while cephalexin is primarily zwitterionic between pH 2.56 and 6.88 (Legnoverde et. al, 2014). Moreover, the zwitterionic form of cefixime dramatically increases under pH 6.0 (Wenzel et. al,1996), which explains why the Caco-2 and rat perfusion studies were conducted in buffers with pH ranging from 5.0 to 5.5 (Wenzel et. al, 2002; Harcouet et. al, 1996). Since these zwitterionic forms would be expected to enhance PepT1 functional activity (Brandsch et. al, 2004), this may help explain why these β-lactam antibiotics (and not valacyclovir) are affected by nifedipine.

In light of these drug interaction studies, however, it would certainly be rational to question the efficacy of nifedipine in these intestinal perfusions. Indeed, efficacy was a legitimate concern after observing a non-significant decrease in permeability following a 10-minute co-perfusion with nifedipine. This was the very reason the co-perfusion period was increased to 30 minutes. Nevertheless, efficacy is not likely an issue in these studies. In previous intestinal perfusion experiments nifedipine was perfused at 100 μM (a comparable concentration) for 15 minutes, followed by the 60-minute perfusion of cefixime or cephalexin (Harcouet et. al, 1997; Berlioz et. al, 1999). Despite this time differential, nifedipine still had a notable effect on the antibiotics even an hour after nifedipine ceased to be perfused through the intestinal segment.

Since nifedipine is intrinsically linked to the activity of the $Ca_V1.3$ channel (Striessnig et. al, 2015; Snider et. al, 2008), it would also be prudent to address a

study that has questioned the role of this channel as a mediator for calcium absorption in mice (Reyes-Fernandez and Fleet, 2015). In summary, these researchers found that serum calcium levels were not significantly different between mice that were co-administered calcium with either 25 mM fructose or 25 mM glucose. Based on these results they asserted that $Ca_v1.3$ does not have an essential role in intestinal calcium transport since the more depolarizing conditions induced by glucose did not impact total calcium influx. This assertion is primarily based on the notion that $Ca_v1.3$ channels are fully activated (i.e., the most influential) in the presence of at least 20 mM glucose, as indicated in a previous intestinal study conducted in rats (Mace et. al, 2007). However, the conclusions Reyes-Fernandez and Fleet have drawn from their study are not convincing. If these researchers wanted to study the impact of $Ca_v1.3$, they should have used a calcium channel blocker to monitor the contribution of this channel under different conditions. As indicated in the review by Kellett (2011), the TRPV6 channel operates under more hyperpolarizing conditions, and thus can compensate for the lack of Cav1.3 activity under glucose-deficient conditions.

Although depolarizing nutrients like glucose can increase the activity of the $Ca_v1.3$ channel, it is interesting to observe substantial interactions between nifedipine and various PepT1 substrates under relatively hyperpolarized conditions. Namely, the clinical and rat oral administration studies, and the rat intestinal perfusion studies were performed under the fasted state with little depolarizing nutrient aside from the PepT1 substrates themselves (Westphal et. al, 1990; Duverne et. al, 1992; Harcouet et. al, 1997; Berlioz et. al, 1999; Berlioz et. al, 2000). The Caco-2 cell studies in fact utilized the highest concentration of depolarizing agent: 10 mM glucose (Wenzel et. al, 2002).

This concentration was adopted for the current studies, and was believed to be sufficient to facilitate an interaction under fasted conditions in mice.

In conclusion, the co-perfusion of valacyclovir and nifedipine in wildtype mice resulted in a non-significant decrease in the permeability of this prodrug. It has been suggested that these results may deviate from past studies involving β-lactam antibiotics because of this prodrug's unfavorable ionization state at physiological pH. Therefore, conducting interaction studies between β-lactam antibiotics and nifedipine in mice would be a logical next step to determine if positive interactions are due to differences in compound ionization or to unattributed species differences.

4.6. Figures and Tables

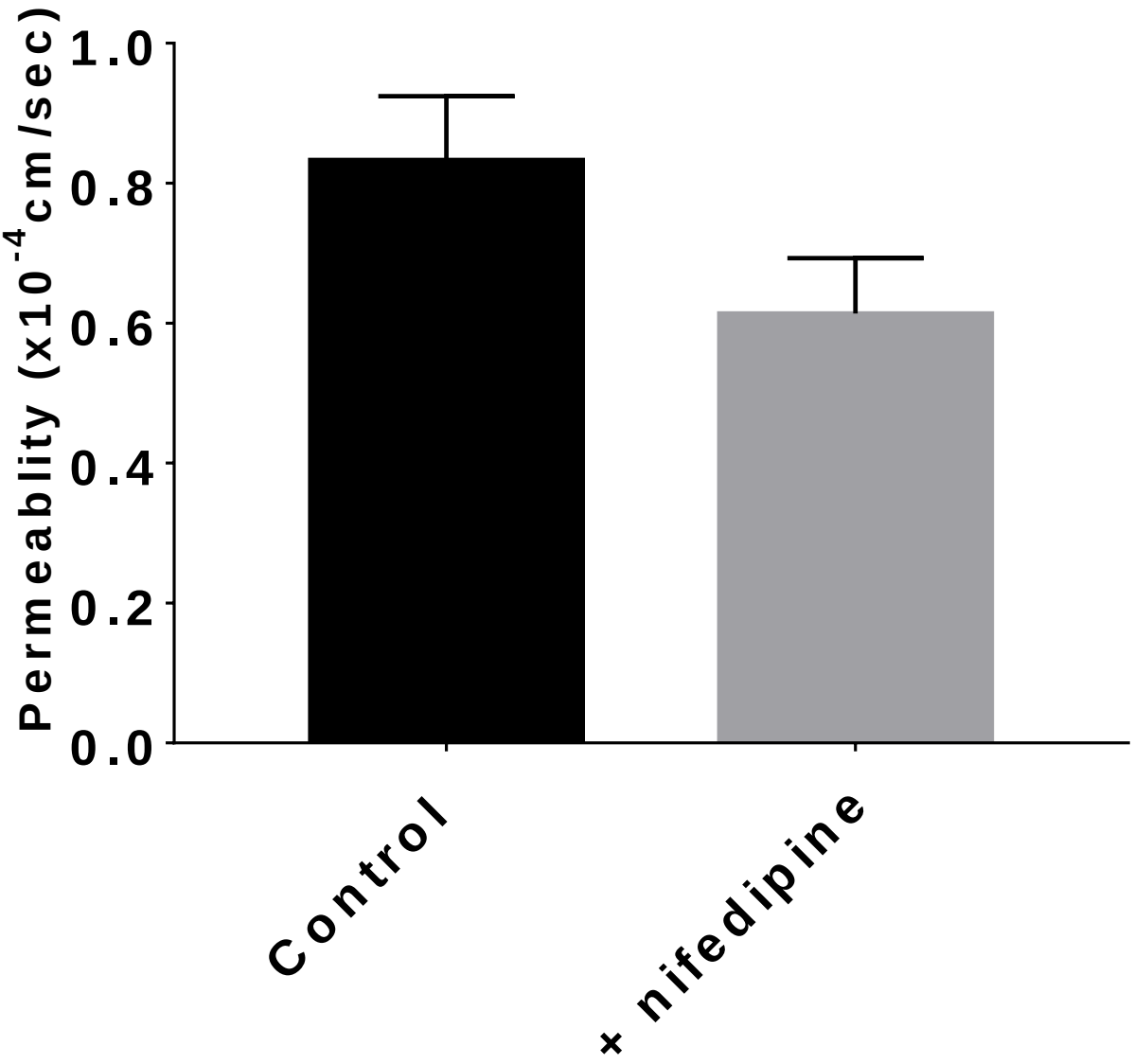

Figure 4.1 Wildtype mice were perfused with 50 μM valacyclovir alone (control) or co-perfused with 100 μM nifedipine for the first 10 minutes (n=4) of the intestinal perfusion. There were no significant differences as determined by unpaired t test.

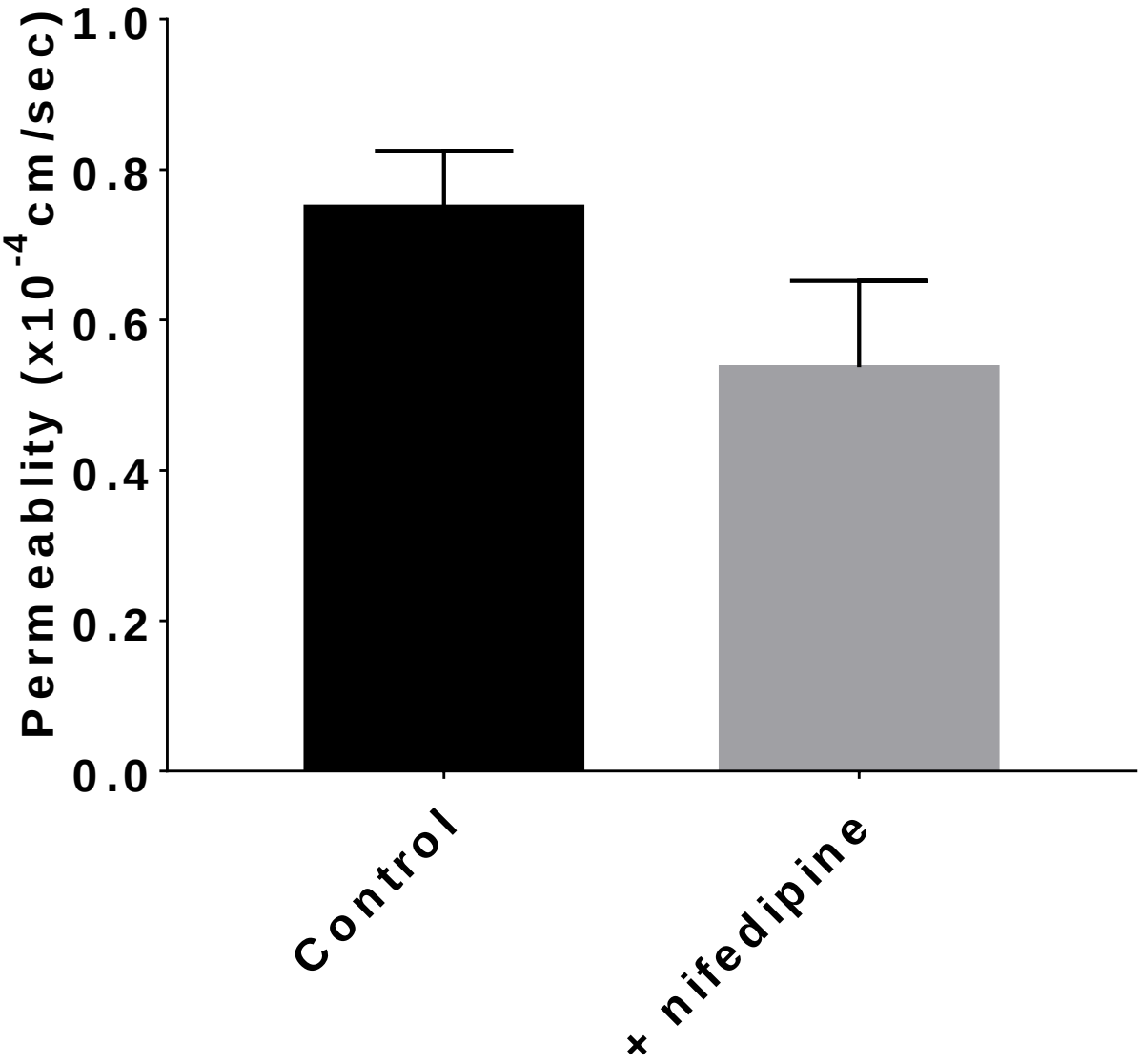

Figure 4.2 Wildtype mice were perfused with 50 μM valacyclovir alone (control) or co-perfused with 90 μM nifedipine for the first 30 minutes (n=5) of the intestinal perfusion. There were no significant differences as determined by unpaired t test.

Table 4.1 Comparison of the results from the two different perfusion schedules involving valacyclovir and nifedipine

Study	Group	Permeability (cm/sec $x10^{-4}$)	Percent Change
1st Perfusion Study (10-minute co-perfusion)	Control + Nifedipine	0.83 0.61	-26.5%
2nd Perfusion Study (30-minute co-perfusion)	Control + Nifedipine	0.75 0.53	-28.0%

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CHAPTER 5

Future Directions

The goal of the studies presented in Chapter 4 was to determine if the intestinal absorption of valacyclovir would be enhanced in the presence of nifedipine. This was the prevailing hypothesis because previous administration studies involving β-lactam antibiotics and nifedipine asserted that there was an enhancement in the intestinal and oral absorption of these PepT1 substrates in both humans and rats. However, this trend was not observed with the current studies conducted in mice. Instead, the intestinal permeability of valacyclovir was modestly and insignificantly reduced by 26-28% in the presence of nifedipine. This seemingly contradictory result opens two new lines of inquiry that deserve further exploration.

First, the efficacy of nifedipine on the intestinal inhibition of calcium and its reliance with nutrient uptake should be investigated in these wildtype mice. It was demonstrated in rat jejunum that calcium uptake was blocked by 52% with 10 μM nifedipine, and that the addition of 20 mM glucose increased calcium inhibition to 78%. Currently, no such studies have been conducted in mice, but understanding the effect nifedipine has on calcium uptake is integral when interpreting interaction studies. Upon satisfactorily completing this investigation, conducting interaction studies with higher glucose concentrations may prove to be an interesting prospect. This is because the $Ca_V1.3$ channel is known to be more highly activated under increasingly depolarized

conditions.

Secondly, it is quite possible that the unexpected results obtained from these mice are due to the reduction in PepT1 functional activity as dictated by the unfavorable ionization state of valacyclovir at pH 6.5. To determine if this is the case, other compounds should be tested in mice that are primarily zwitterionic under experimental conditions. Good candidate compounds would be amoxicillin and cephalexin. These β-lactam antibiotics have already been co-administered with nifedipine in humans and rats, so observing results that deviate from these previous studies may suggest an unknown species-dependent mechanism instead .

APPENDIX : UPLC Method Validation (Figures and Tables)

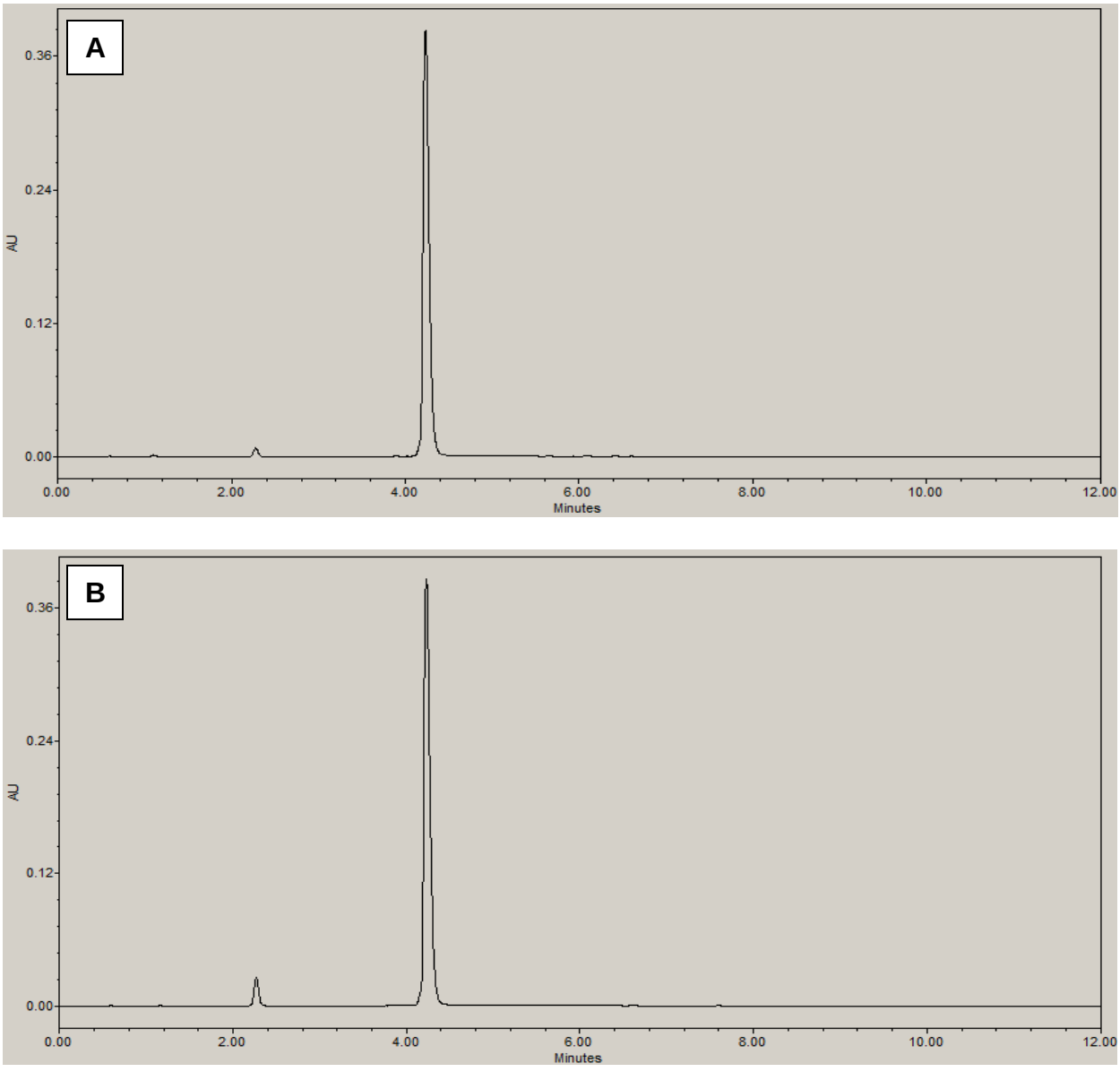

Figure A.1 Chromatograms of A) inlet and B) outlet perfusate following the *in situ* intestinal perfusion of the prodrug valacyclovir. Acyclovir has a retention time of 2.3 minutes and valacyclovir has a retention time of 4.2 minutes.

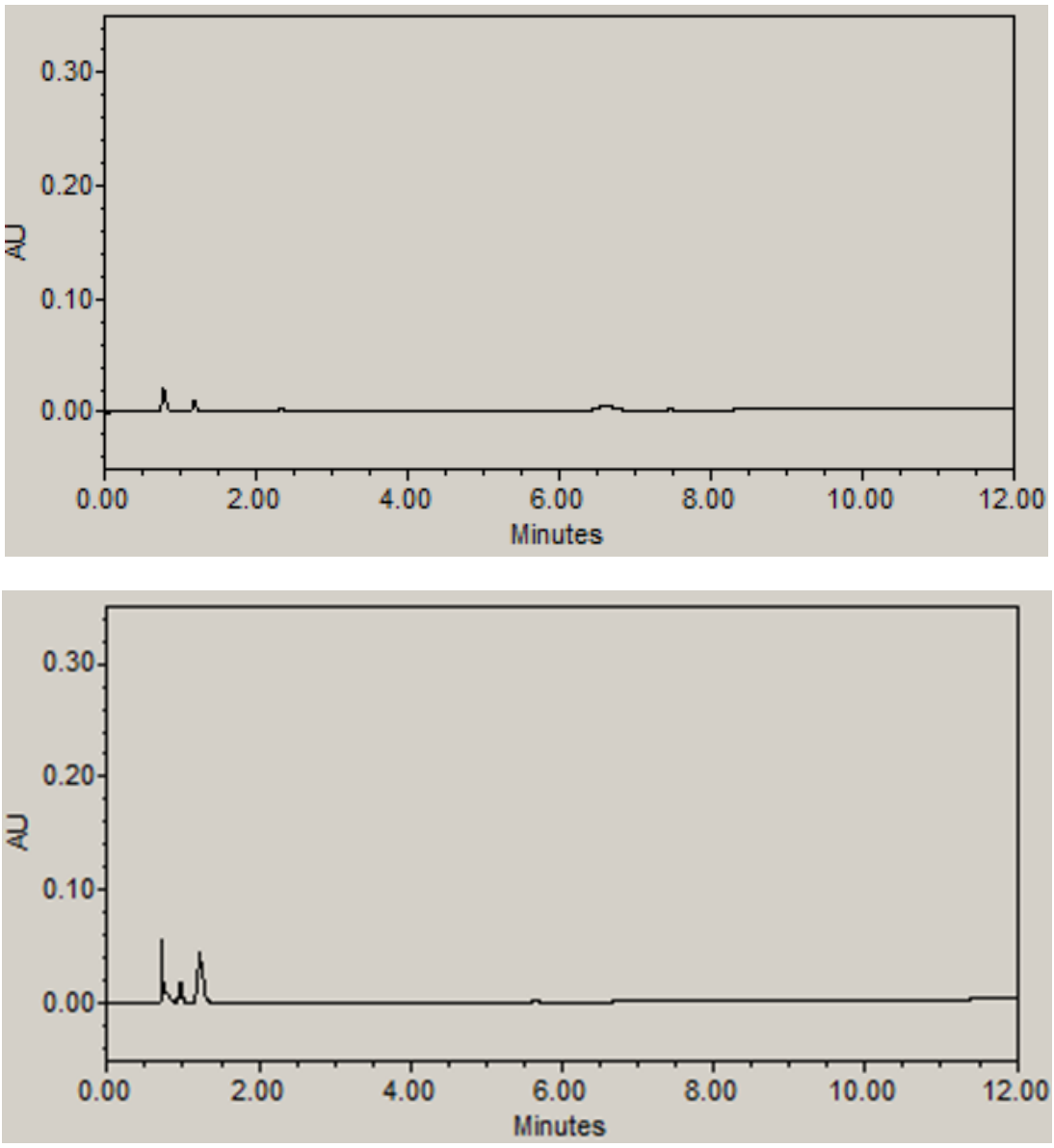

Figure A.2 An assessment of method specificity: chromatograms of blank perfusate. Each chromatogram is derived from separate mice.

Table A.1 The UPLC gradient method utilized for the separation of acyclovir and valacyclovir

Time (min)	Flow Rate (mL/min)	Water + 0.1% TFA	Acetonitrile +0.1% TFA
Initial	0.5	100%	0%
5	0.5	90%	10%
7	0.5	90%	10%
7.1	0.5	100%	0%

Table A.2 The intraday and interday accuracy of acyclovir and valacyclovir as assessed by quality control samples

Compound	Quality Control Sample (μM)	Intraday Accuracy (% of true value)	Interday Accuracy (% of true value)
Acyclovir	50	96.4 - 100.1	98.7
	10	99.9 - 102.8	101.6
	2.5	103.7 - 105.5	104.5
Valacyclovir	200	101.4 - 103.1	102.1
	50	101.6 - 101.8	101.7
	10	100.0 - 102.3	101.3

Intraday and interday accuracy were assessed over three consecutive days. Intraday values were recorded as a range.

Table A.3 The intraday and interday precision of acyclovir and valacyclovir as assessed by quality control samples

Compound	Quality Control Sample (μM)	Intraday Precision RSD (%)	Interday Precision RSD (%)
Acyclovir	50	0.03 - 0.25	2.03
	10	0.14 - 0.26	1.67
	2.5	0.12 - 0.45	0.55
Valacyclovir	200	0.22 - 0.71	1.17
	50	0.25 - 0.34	0.99
	10	0.31 - 0.34	1.59

Intraday and interday precision were assessed over three consecutive days. Intraday values were recorded as a range.