

Impact of Peptide Transporter 1 on the Intestinal Absorption and Pharmacokinetics of Valacyclovir after Oral Dose Escalation in Wild-Type and *PepT1* Knockout Mice

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ABSTRACT

The primary objective of this study was to determine the *in vivo* absorption properties of valacyclovir, including the potential for saturable proton-coupled oligopeptide transporter 1 (PepT1)-mediated intestinal uptake, after escalating oral doses of prodrug within the clinical dose range. A secondary aim was to characterize the role of PepT1 on the tissue distribution of its active metabolite, acyclovir. [³H]Valacyclovir was administered to wild-type (WT) and *PepT1* knockout (KO) mice by oral gavage at doses of 10, 25, 50, and 100 nmol/g. Serial blood samples were collected over 180 minutes, and tissue distribution studies were performed 20 minutes after a 25-nmol/g oral dose of valacyclovir. We found that the C_{max} and area under the curve (AUC)₀₋₁₈₀ of acyclovir were 4- to 6-fold and 2- to 3-fold lower, respectively, in KO mice for all four oral doses of valacyclovir. The time

to peak concentration of acyclovir was 3- to 10-fold longer in KO compared with WT mice. There was dose proportionality in the C_{max} and AUC₀₋₁₈₀ of acyclovir in WT and KO mice over the valacyclovir oral dose range of 10-100 nmol/g (i.e., linear absorption kinetics). No differences were observed in the peripheral tissue distribution of acyclovir once these tissues were adjusted for differences in perfusing drug concentrations in the systemic circulation. In contrast, some differences were observed between genotypes in the concentrations of acyclovir in the distal intestine. Collectively, the findings demonstrate a critical role of intestinal PepT1 in improving the rate and extent of oral absorption for valacyclovir. Moreover, this study provides definitive evidence for the rational development of a PepT1-targeted prodrug strategy.

Introduction

Proton-coupled oligopeptide transporter 1 (PepT1, solute carrier 15A1), a member of the proton-coupled oligopeptide transporter family, is a highly conserved influx transporter that is expressed in several mammalian species, including human, rat, and mouse (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008; Smith et al., 2013). The mammalian PepT1, comprising 707-710 amino acid residues, depending on species, has 12 transmembrane domains with both the N and C termini facing the cytosolic side. PepT1 couples the influx of substrate with a proton in which the inwardly-directed proton gradient and negative membrane potential are driving forces for the uphill movement of substrate. As a high-capacity, low-affinity influx transporter, PepT1 is located principally on the brush-border membrane of epithelial cells in the small intestine and functions as the active uptake carrier of dipeptides and tripeptides generated by the digestion of dietary proteins. Since approximately 80% of digested proteins are absorbed in the form of dipeptides or tripeptides, as opposed to free amino acids, PepT1 plays an essential physiologic role in protein assimilation (Ganapathy et al., 2006).

The pharmacological relevance of PepT1 lies mainly in its ability to transport a wide spectrum of drugs from some important therapeutic classes (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008; Tsume

et al., 2008). For example, many β -lactam antibiotics (e.g., oral cephalosporin and penicillin drugs) and some angiotensin-converting enzyme inhibitors (e.g., captopril and enalapril) are known substrates of PepT1. Moreover, a number of amino acids or dipeptide-conjugated prodrugs (e.g., midodrine, valacyclovir, valganciclovir, and 5'-L-phenylalanyl-L-glycyl-floxuridine) undergo PepT1-mediated transport. Prodrugs of this type are commonly referred to as PepT1-targeted prodrugs, an area of research that is under intense investigation as a promising strategy to improve the oral availability of polar, hydrophilic compounds (Varma et al., 2010).

Valacyclovir is widely viewed as the model of PepT1-targeted prodrugs. Valacyclovir, an L-valyl ester prodrug of the potent antiviral agent acyclovir, is used for the treatment and prophylaxis of herpes, varicella zoster, and cytomegalovirus infection. In humans, the systemic availability of acyclovir, after oral administration of valacyclovir, is nearly 55%, as opposed to only 10% to 20% after oral dosing of acyclovir (Soul-Lawton et al., 1995). However, the uptake and pharmacokinetic properties of valacyclovir have shown some inconsistent or even controversial findings. On the one hand, numerous cell culture studies demonstrated the PepT1-mediated uptake of valacyclovir into cells constitutively expressing or transfected with PepT1 (Balimane et al., 1998; Ganapathy et al., 1998; Han et al., 1998; Guo et al., 1999; Balimane and Sinko, 2000). On the other hand, valacyclovir was also found to interact with other transporters such as human organic anion transporter 3 (Takeda et al., 2002), human peptide histidine transporter 1 (Bhardwaj et al., 2006), rat organic cation transporters (Sinko and Balimane, 1998), a human peptide

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ABBREVIATIONS: AUC, area under the curve; GI, gastrointestinal; HPLC, high-performance liquid chromatography; KO, *PepT1* knockout mice; PepT1, proton-coupled oligopeptide transporter 1; WT, wild-type mice.

transporter (Landowski et al., 2003), and a mouse amino acid transporter (ATB⁰⁺) (Hatanaka et al., 2004).

At present, the general consensus is that the improved systemic availability of acyclovir, after oral dosing of valacyclovir, is attributable mainly to PepT1-mediated uptake. However, convincing *in vivo* evidence in support of this contention is lacking. In this regard, some pharmacokinetic studies in humans failed to reveal the relevance of PepT1 in the intestinal absorption of valacyclovir. For instance, a significant positive correlation was observed between the area under the plasma concentration-time curve of acyclovir (after oral dosing of valacyclovir) and human peptide transporter 1 but not PepT1 mRNA expression levels in human duodenum (Landowski et al., 2003). In another study, the acyclovir area under the curve (AUC) after oral dosing of 500 mg of valacyclovir in five healthy subjects was reduced by less than 10% when coadministered orally with 500 mg of cephalexin, a human PepT1 inhibitor (Phan et al., 2003). Based on these results, the authors speculated that this minimal drug-drug interaction might reflect their belief that a higher dose of valacyclovir or cephalexin, a longer duration of therapy, or a more potent PepT1 inhibitor (i.e., cefadroxil) should have been used. Alternatively, it is also possible that valacyclovir and cephalexin are absorbed by other transporters in the intestines and, as a result, are less dependent on PepT1.

With this in mind, the primary objective of this study was to determine the *in vivo* absorption properties of valacyclovir, including the potential for saturable PepT1-mediated intestinal uptake, after escalating oral doses of prodrug within the clinical dose range. A secondary aim was to characterize the role of PepT1 on the tissue distribution of its active metabolite acyclovir. With the use of wild-type (WT) and *PepT1* knockout (KO) mice, this study provided definitive evidence for the rational development of a PepT1-targeted prodrug strategy.

Materials and Methods

Materials. Valacyclovir hydrochloride, acyclovir, and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO). Hyamine hydroxide was purchased from ICN Radiochemicals (Irvine, CA). [³H]Valacyclovir (2.1 Ci/mmol), [³H]acyclovir (12.1 Ci/mmol), and [¹⁴C]dextran-carboxyl 70,000 (1.1 mCi/g) were acquired from Moravsek Biochemicals and Radiochemicals (Brea, CA).

Animals. Gender-matched WT and *PepT1* KO mice (8–10 weeks of age) were used for all experiments (Hu et al., 2008). The mice received a standard diet and access to water *ad libitum*. All animals were maintained in a temperature-controlled environment with 12-hour light/dark cycles (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI). Mouse studies were approved by the University of Michigan Committee on Use and Care of Animals and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Pharmacokinetics of Acyclovir after Oral Valacyclovir. Wild-type and *PepT1* knockout mice were fasted overnight for ~16 hours before each experiment. Valacyclovir solutions were prepared by dissolving appropriate amounts of [³H]-labeled and unlabeled valacyclovir in normal saline. An aliquot of valacyclovir solution (200 μ l, 10 μ Ci/mouse) was administered by gastric gavage at single doses of 10, 25, 50, and 100 nmol/g body weight. Serial blood samples (20 μ l/sample) were collected by tail nicks predose and 5, 15, 30, 45, 60, 90, 120, and 180 minutes after dosing. After blood sampling, the mice were returned to their cages, where they had free access to water. Blood samples were transferred to 0.2-ml microcentrifuge tubes containing 7.5% potassium EDTA and centrifuged at 3000g for 3 minutes, ambient temperature. A 5- to 10- μ l aliquot of plasma was then transferred to a scintillation vial, and 6 ml of CytoScint scintillation fluid (MP Biomedicals, Solon, OH) was added to the sample. Radioactivity of the plasma sample was measured on a dual-channel liquid scintillation counter (Beckman LS 6000 SC; Beckman Coulter Inc., Fullerton, CA).

Pharmacokinetics of Acyclovir after Intravenous Acyclovir. Acyclovir solutions were prepared by dissolving appropriate amounts of [³H]-labeled and unlabeled acyclovir in normal saline. A 100- μ l volume of acyclovir solution (5 μ Ci/mouse) was then administered by tail vein injection at a single dose of 25 nmol/g body weight. Serial blood samples (20 μ l/sample) were collected predose and 2, 5, 15, 30, 60, 90, 120, and 180 minutes after dosing. All other experimental procedures have been described previously.

Tissue Distribution of Acyclovir after Oral Valacyclovir. Tissue distribution studies were performed in WT and *PepT1* KO mice; samples were obtained 20 minutes after oral administration of 25 nmol/g [³H]valacyclovir. To determine the tissue vascular space, 100 μ l of [¹⁴C]dextran 70,000 (0.25 μ Ci per mouse) was injected in the tail vein 5 minutes before harvesting the tissues. Following decapitation, the eye, heart, lung, liver, kidney, spleen, muscle, and gastrointestinal (GI) segments, including the stomach, duodenum, jejunum, ileum, and colon, were isolated, blotted dry, weighed, and then solubilized in 330 μ l of 1 M hyamine hydroxide and incubated overnight at 37°C (as described by the manufacturer). Three 10- μ l whole blood samples were also collected at this time. The GI segments were flushed two or three times with ice-cold saline solution to remove their contents before weighing. After the incubation period, 40 μ l of H₂O₂ (30%) was slowly added to decolorize the organs and tissues. A 6-ml volume of CytoScint scintillation fluid was added to each sample, and radioactivity was measured on a dual-channel liquid scintillation counter.

Plasma Concentration-Time Profile of Acyclovir after Oral Dosing of Unlabeled Valacyclovir. A 100 nmol/g dose of valacyclovir was administered to WT mice by oral gavage, and blood samples (50–60 μ l) were obtained via tail nicks at 5, 15, 30, 45, 60, 90, 120, and 180 minutes after dosing. The plasma was then harvested, and 20- μ l aliquots were mixed with 80 μ l of blank plasma containing the internal standard ganciclovir (30 μ M). The 100- μ l sample was mixed with acetonitrile (1:2 v/v), vortexed for 1 minute (at room temperature), and centrifuged at 15,000g for 10 minutes (4°C). The supernate was then evaporated to dryness by vacuum, reconstituted with 40 μ l of mobile phase, and a 20- μ l aliquot was introduced into the loop injector. The mobile phase consisted of 3% organic phase (0.1% v/v trifluoroacetic acid in acetonitrile) and 97% aqueous phase (0.1% v/v trifluoroacetic acid in water), pumped isocratically at 1 ml/min, ambient temperature. Acyclovir was detected by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection, as described previously (Yang and Smith, 2013). The retention times were 5.1 minutes for ganciclovir and 6.1 minutes for acyclovir.

Data Analysis. The plasma concentration-time profiles of acyclovir were analyzed by a noncompartmental approach using WinNonlin version 5.3 (Pharsight Inc., Mountainview, CA). After oral dosing of valacyclovir, peak plasma concentrations of acyclovir (C_{max}) and the time to reach a peak concentration (T_{max}) were taken from the observed results. Area under the acyclovir plasma concentration-time curve (AUC) was calculated by linear trapezoidal rule. After intravenous administration of acyclovir, the AUC and total clearance of drug were determined.

For tissue distribution studies, acyclovir concentrations were corrected for vascular space using the following equation (Ocheltree et al., 2005; Shen et al., 2007): $C_{tiss,corr} = C_{tiss} - DS \cdot C_b$, where $C_{tiss,corr}$ and C_{tiss} are the respective corrected and uncorrected tissue concentrations of acyclovir (nmol/g), DS is the dextran space (blood vascular space) in the tissue (ml/g), and C_b is the blood concentration of acyclovir (nmol/ml).

All results were expressed as mean \pm standard error (S.E.) unless otherwise indicated. Unpaired two-tail *t* tests were used to compare differences between WT and *PepT1* KO mice. A *P* value \leq 0.05 was considered statistically significant.

Results

Pharmacokinetics of Acyclovir after Oral Valacyclovir. As shown in Fig. 1, the plasma concentration-time profiles of acyclovir after oral administration of valacyclovir were substantially different between the two genotypes for all four doses. In WT mice, acyclovir plasma concentrations increased quickly to reach C_{max} values within 30 minutes of dosing. In contrast, the accumulation of acyclovir

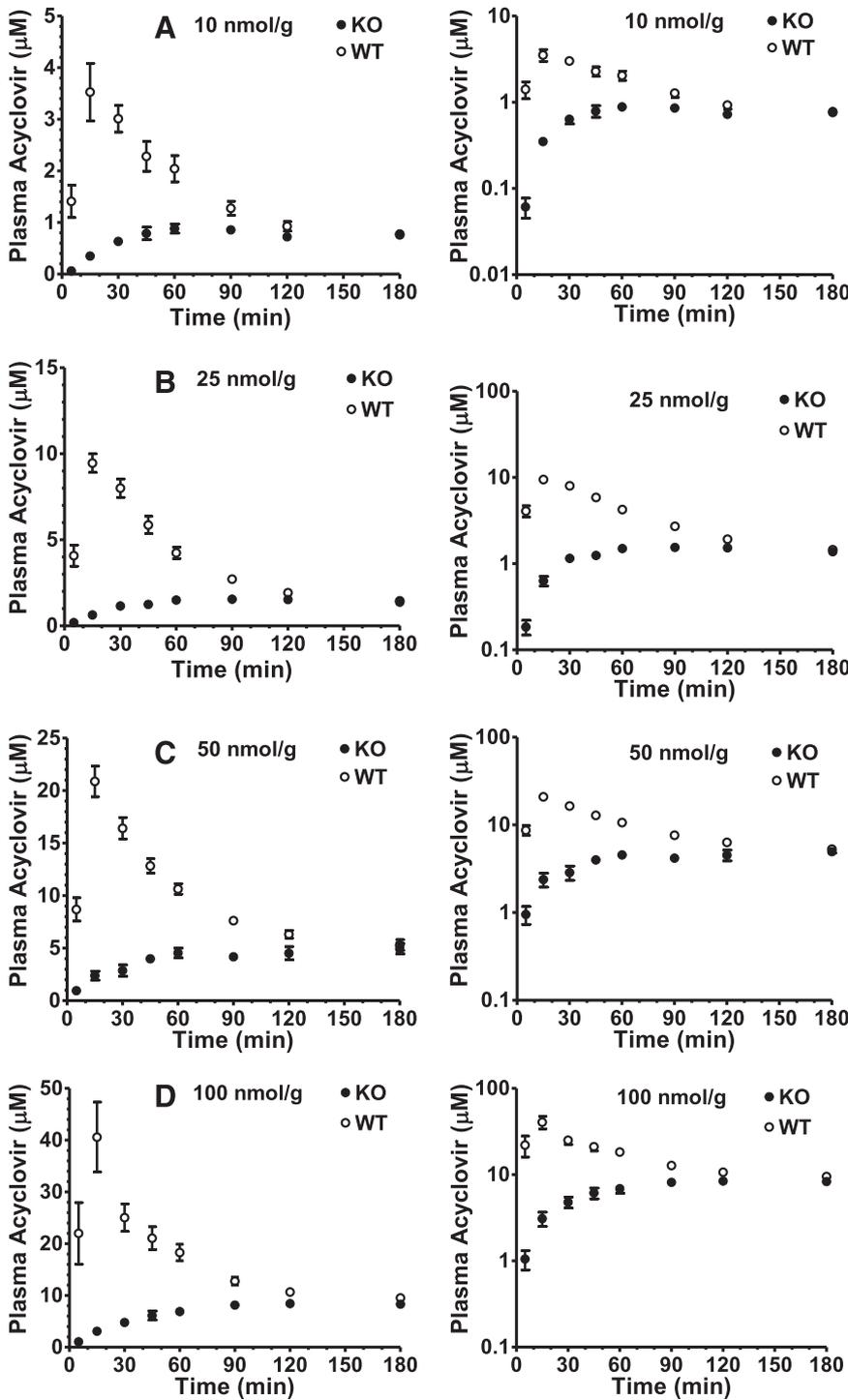


Fig. 1. Plasma concentration-time profiles of acyclovir in WT and *PepT1* KO mice after 10 nmol/g (A), 25 nmol/g (B), 50 nmol/g (C), and 100 nmol/g (D) oral doses of [³H]valacyclovir. Data are expressed as mean ± S.E. (*n* = 4–7) in which the y-axis is displayed on linear (left panel) and logarithmic (right panel) scales.

plasma concentrations was much slower in *PepT1* KO mice, where C_{max} values were substantially lower and reached peak values in about 1.0–2.5 hours. More important, the systemic exposure of acyclovir in *PepT1* knockout mice, as judged by AUC values over 180 minutes (AUC_{0-180}), was only 35% to 46% of that observed in wild-type animals. These results are summarized in Table 1 and demonstrate the significant differences between genotypes, at all four doses, for T_{max} (3.0- to 10.4-fold longer in *PepT1* knockout mice), C_{max} (4.2- to 5.6-fold lower in *PepT1* knockout mice), and AUC_{0-180} (2.2- to 2.8-fold lower in *PepT1* knockout mice).

The contribution of intestinal *PepT1* to the rate of valacyclovir absorption was further probed by determining the partial cumulative AUC of acyclovir as a function of time. As illustrated in Fig. 2, the slopes of the curves were much steeper for wild-type than for *PepT1* knockout mice during the early time periods (i.e., 5–60 minutes). However, the slopes of the curves were almost parallel between the two genotypes during the later time periods (i.e., 90–180 minutes). A quantitative assessment of these differences is shown in Table 2; early on, the slopes are about 5- to 6-fold lower in *PepT1* knockout mice. At later times, there is only a 20% difference in slopes between the two genotypes.

TABLE 1

Pharmacokinetics of acyclovir after escalating oral doses of [^3H]valacyclovir in wild-type (WT) and *PepT1* knockout (KO) mice

Data are expressed as mean \pm S.E. (n = 4–7).

Parameter	Dose (nmol/g)			
	10	25	50	100
WT				
T_{\max} (min)	24 \pm 4	19 \pm 3	15 \pm 0	15 \pm 0
C_{\max} (μM)	3.8 \pm 0.5	9.6 \pm 0.5	20.9 \pm 1.5	40.6 \pm 6.8
AUC_{0-180} (min \times μM)	278 \pm 13	663 \pm 34	1675 \pm 66	2923 \pm 190
KO				
T_{\max} (min)	71 \pm 11*	96 \pm 16**	156 \pm 24**	150 \pm 19**
C_{\max} (μM)	0.9 \pm 0.1**	1.7 \pm 0.1***	5.0 \pm 0.5***	8.8 \pm 0.5*
AUC_{0-180} (min \times μM)	128 \pm 8***	235 \pm 11***	743 \pm 78***	1233 \pm 79**

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with WT mice.

The potential for nonlinear kinetics (i.e., saturable PepT1-mediated absorption) was determined by evaluating the dose proportionality of acyclovir C_{\max} and AUC_{0-180} after 10–100 nmol/g oral doses of valacyclovir. As shown in Fig. 3, A and B, values for the C_{\max} and AUC_{0-180} of acyclovir were linearly correlated with dose in both wild-type and *PepT1* knockout mice ($P < 0.001$), having nonzero regression slopes. Moreover, the dose-normalized values for C_{\max} and AUC_{0-180} of acyclovir were independent of dose, and the slopes of the linear regression lines were not different from zero (Fig. 3, C and D).

Pharmacokinetics of Acyclovir after Intravenous Acyclovir. Since

$$\text{AUC}_{\text{oral}} = \text{Dose}_{\text{oral}} * (F/CL),$$

differences in AUC after oral dosing can reflect changes in intestinal absorption (F) or drug clearance (CL). For this reason, and to rule out any non-PepT1 dispositional effects, acyclovir was studied after intravenous administration in wild-type and *PepT1* knockout mice. As shown in Fig. 4, the plasma concentration-time profiles of acyclovir after intravenous dosing of 25 nmol/g acyclovir were virtually superimposable between genotypes. In fact, the clearance of acyclovir in *PepT1* knockout mice (1.17 ± 0.15 l/hr/kg) was only 12% lower than that observed in wild-type

animals (1.33 ± 0.12 l/hr/kg) ($P = 0.459$). Thus, it appears that PepT1 ablation does not affect the systemic disposition of acyclovir and, as a result, differences in acyclovir AUC after oral valacyclovir dosing are reflective of intestinal PepT1 expression and functional activity.

Tissue Distribution of Acyclovir after Oral Valacyclovir. Since differences in acyclovir systemic exposure after oral administration of valacyclovir were essentially the result of intestinal PepT1, tissue distribution studies were performed at 20 minutes, the T_{\max} after 25 nmol/g of prodrug in wild-type mice. As shown in Fig. 5A, the concentrations of acyclovir in peripheral tissues were all significantly higher in wild-type mice than in *PepT1* knockout animals. A 5-fold difference between the two genotypes was also observed in whole-blood concentrations of acyclovir ($P < 0.001$). Therefore, to rule out differences in tissue distribution being the consequence of differences in systemic exposure, tissue-to-blood ratios were determined for acyclovir. As shown in Fig. 5B, no significant differences were observed between the two genotypes once these peripheral tissues were adjusted for differences in perfusing concentrations of acyclovir. With respect to tissue distribution along the GI tract (Fig. 5C), no differences were observed between genotypes in proximal regions such as the

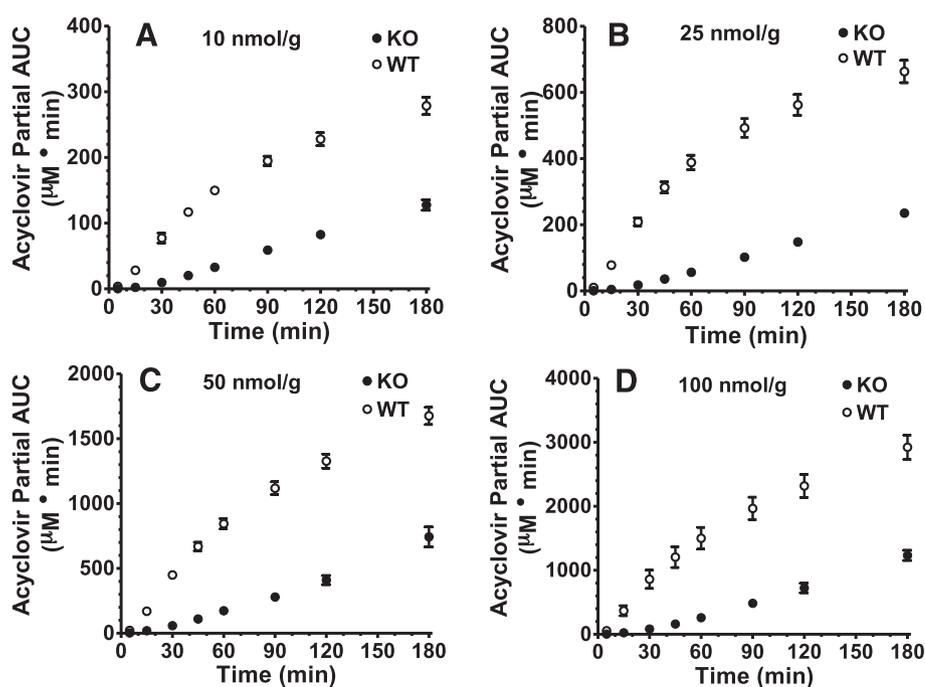


Fig. 2. Partial cumulative area under the plasma concentration-time curve (AUC) of acyclovir as a function of time in WT and *PepT1* KO after 10 nmol/g (A), 25 nmol/g (B), 50 nmol/g (C), and 100 nmol/g (D) oral doses of [^3H]valacyclovir. Data are expressed mean \pm S.E. (n = 4–7).

TABLE 2

Dose-corrected slopes of the partial cumulative AUC of acyclovir versus time after escalating oral doses of [³H]valacyclovir in wild-type (WT) and *PepT1* knockout (KO) mice

Dose (nmol/g)	Slope (5–60 min)			Slope (90–180 min)		
	WT	KO	Ratio (KO/WT)	WT	KO	Ratio (KO/WT)
10	0.27	0.06	0.22	0.09	0.08	0.83
25	0.28	0.04	0.15	0.08	0.06	0.79
50	0.31	0.06	0.20	0.12	0.10	0.83
100	0.27	0.05	0.17	0.11	0.08	0.76

stomach, duodenum, and jejunum. In contrast, acyclovir concentrations were significantly lower in the distal regions of *PepT1* knockout mice, such as in the ileum and colon.

Discussion

In situ perfusion studies were performed previously by our laboratory in wild-type and *PepT1* knockout mice (Yang and Smith, 2013), demonstrating that intestinal PepT1 was the predominant route of valacyclovir absorption in the small intestine, accounting for about 90% of prodrug permeability. This finding was the first quantitative measure of the relative significance of intestinal PepT1 in enhancing the intestinal absorption of valacyclovir, a result that was corroborated by our in vivo absorption studies (Yang and Smith, 2013). However, the in vivo absorption studies were limited in that valacyclovir was given as a single oral dose of 25 nmol/g; as a result, we were not able to evaluate the potential for capacity-limited uptake by intestinal PepT1. Moreover, tissue distribution studies were not performed to evaluate the effect of this transporter on drug distribution to peripheral tissues. Because of the preliminary nature of these in vivo findings (Yang and Smith, 2013), and because of reports indicating less than proportional increases in C_{max} and AUC during oral dose ranging experiments in humans (Weller et al., 1993; Ormrod and Goa, 2000), the current study was designed to determine whether valacyclovir

can exhibit nonlinear oral absorption kinetics because of intestinal PepT1 saturation.

In studying acyclovir, after escalating oral doses of valacyclovir in wild-type and *PepT1* knockout mice, several important findings were made, including the following: 1) PepT1 ablation had a profound effect on the rate and extent of valacyclovir absorption for all doses in which the T_{max} of acyclovir was increased 3- to 10-fold, and the C_{max} and AUC_{0-180} of acyclovir were reduced 4- to 6-fold and 2- to 3-fold, respectively; 2) the C_{max} and AUC_{0-180} of acyclovir were dose-proportional after 10–100 nmol/g of oral valacyclovir, indicating that intestinal PepT1 was not saturated over this dose range; 3) the systemic exposure of acyclovir after 25 nmol/g intravenous administration of acyclovir was not significantly different between genotypes, indicating that PepT1 ablation did not influence the systemic disposition of acyclovir once formed; and 4) PepT1 ablation had no significant effect on the distribution of acyclovir to peripheral tissues (i.e., outside the GI tract) once adjustment was made for differences in systemic concentrations of drug.

After oral administration of valacyclovir, marked differences were observed for all doses in the plasma concentration-time profiles of acyclovir between wild-type and *PepT1* knockout mice. Thus, several metrics were used to assess the differences between genotypes in the extent and rate of valacyclovir absorption. The key finding from this part of the study was that PepT1 deletion led to 2- to 3-fold differences in AUC (a well-accepted indicator of systemic exposure) and 4- to 6-fold differences in C_{max} (a much-criticized indicator of absorption rate), supporting the quantitative significance of PepT1 in the extent of intestinal absorption for valacyclovir. Partial cumulative AUC, especially during early time periods after drug administration, was also proposed as a measure of absorption rate since the method is more discriminating than C_{max} or T_{max} for this type of evaluation (Chen, 1992; Chen et al., 2001). In our study, the slopes of acyclovir partial AUC versus time in *PepT1* knockout mice, when evaluated from 5–60 minutes, were reduced by 80% compared with wild-type animals. In contrast, the slopes, when evaluated from 90–180 minutes, were very similar and differed between genotypes by only 20%. Similar findings were

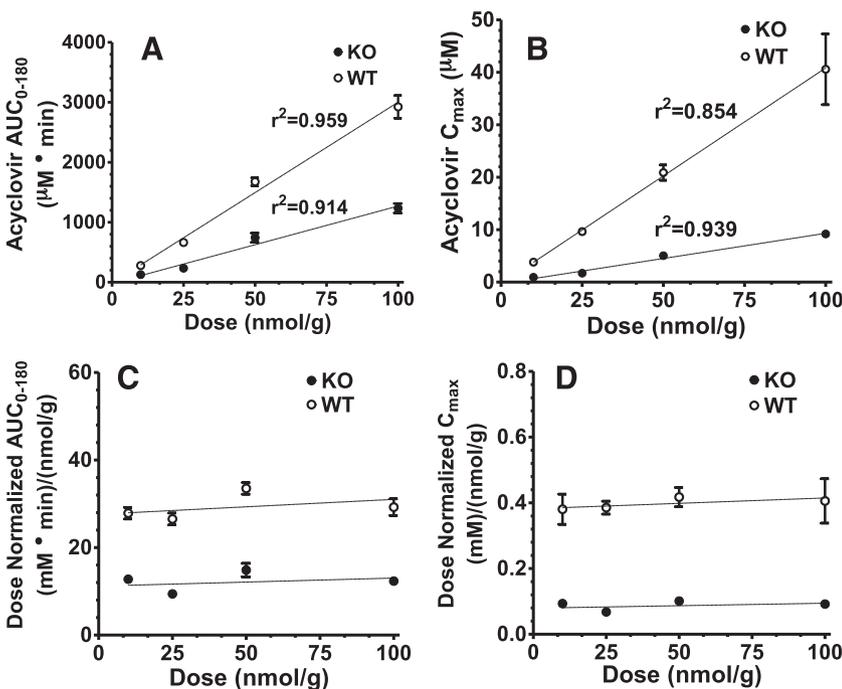


Fig. 3. Relationship between acyclovir AUC_{0-180} vs. dose (A), acyclovir C_{max} vs. dose (B), acyclovir AUC_{0-180}/dose vs. dose (C), and acyclovir C_{max}/dose vs. dose (D) in WT and *PepT1* KO after 10 nmol/g, 25 nmol/g, 50 nmol/g, and 100 nmol/g oral doses of [³H]valacyclovir. Data are expressed as mean \pm S.E. ($n = 4-7$).

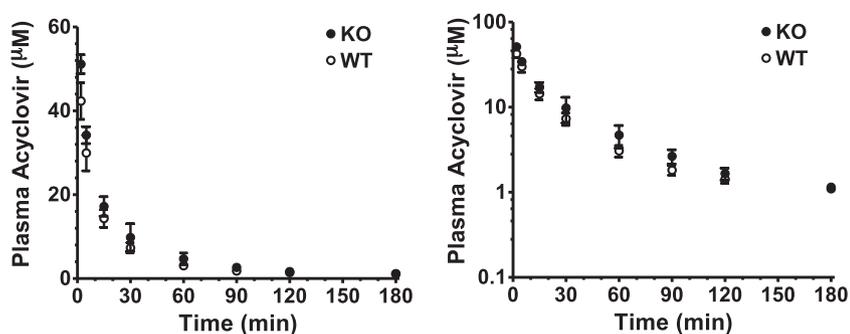


Fig. 4. Plasma concentration-time profiles of acyclovir in WT and *PepT1* KO mice after a 25 nmol/g intravenous bolus dose of [3 H]acyclovir. Data are expressed as mean \pm S.E. ($n = 3$) in which the y-axis is displayed on linear (left panel) and logarithmic (right panel) scales.

observed between wild-type and *PepT1* knockout mice in the rate and extent of cefadroxil absorption after escalating oral doses of 44.5–356 nmol/g (Posada and Smith, 2013a,b).

In these analyses, we assumed that all the radioactivity was represented by acyclovir, with little or no prodrug being present. We believe this is a

reasonable assumption based on several lines of evidence. First, only acyclovir was observed in portal venous blood during in situ perfusions of valacyclovir in mice (Yang and Smith, 2013), indicating that the prodrug was rapidly and completely degraded to acyclovir during its passage through the enterocytes. Second, valacyclovir was nearly completely converted to acyclovir ($\geq 90\%$) by first-pass intestinal and hepatic metabolism in rats after a 90-nmol/g dose (Anand et al., 2004), similar to the highest dose of 100 nmol/g during the present study in mice. Finally, an exploratory study by our group found that after 100 nmol/g of oral valacyclovir, there was not much difference in the systemic exposure of acyclovir in mice, whether administered as labeled or unlabeled drug (Fig. 6). In fact, the AUC_{0-180} was 12% higher when acyclovir was measured by HPLC (i.e., 3281 min \times μ M) as opposed to liquid scintillation counting (i.e., 2923 min \times μ M), a finding in the opposite direction of that expected if prodrug was present in the sample. Thus, it is unlikely that our analyses of plasma acyclovir were confounded by nonspecific measurements of valacyclovir. Although it is possible that a decreased appearance of acyclovir in the plasma of *PepT1* knockout mice could be partially due to an increased conversion rate of luminal valacyclovir to active drug, we do not believe this to be the case. As estimated in jejunum during in situ perfusions (Yang and Smith, 2013), and using the equation $K_{\text{degradation}} = \text{formation rate of acyclovir divided by the average amount of valacyclovir in lumen}$ for the first-order degradation rate constant, $K_{\text{deg}} = 0.086 \pm 0.011 \text{ minute}^{-1}$ for wild-type mice and $K_{\text{deg}} = 0.050 \pm 0.005 \text{ minute}^{-1}$ for *PepT1* knockout mice.

The oral doses selected for this study (i.e., 10–100 nmol/g) were scaled so that plasma concentrations produced in mice would be equivalent to those obtained after commonly prescribed doses in humans. Thus, the C_{max} values of acyclovir in wild-type mice, after oral valacyclovir doses of 10, 25, 50, and 100 nmol/g, were 3.8, 9.6, 20.9, and 40.6 μ M, respectively. By comparison, the C_{max} values of acyclovir in adult human subjects, after oral valacyclovir doses of 250, 500, 1000, and 2000 mg, were about 10, 15, 25, and 37 μ M, respectively (Weller et al., 1993; Ommrod and Goa, 2000). In our study, the oral absorption kinetics of valacyclovir were linear over a 10-fold dose range, for both genotypes, as demonstrated by the dose-proportional increases in C_{max} and AUC_{0-180} of acyclovir. Given a water content of 0.2 ml in the mouse stomach (McConnell et al., 2008), the initial concentration of valacyclovir at the highest oral dose was estimated at 10 mM, a value approximating the K_m of 10.2 mM as determined by in situ jejunal perfusions in mice (Yang and Smith, 2013). However, it is likely, as a result of the proximal absorption of prodrug with no change in water content, that the luminal concentration of valacyclovir will decrease as it travels down the small intestine (i.e., from proximal to distal regions). Consequently, the concentrations of valacyclovir in much of the small intestine will be well below the value needed for saturation of *PepT1*-mediated absorption.

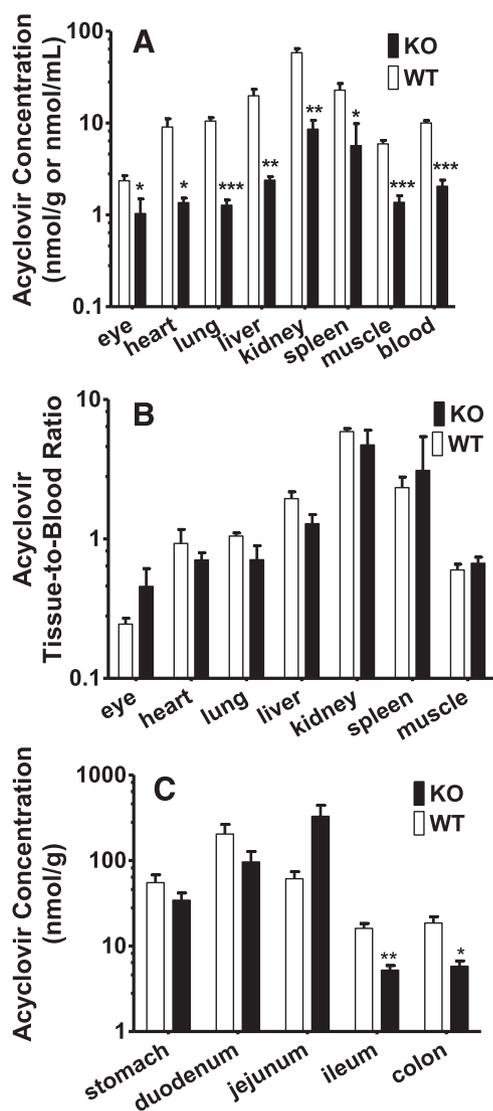


Fig. 5. Tissue concentrations of acyclovir in peripheral tissues (A), tissue-to-blood concentration ratios of acyclovir in peripheral tissues (B), and tissue concentrations of acyclovir in the gastrointestinal tract (C) of WT and *PepT1* KO mice 20 minutes after oral administration of 25 nmol/g [3 H]valacyclovir. Data are expressed as mean \pm S.E. ($n = 4-6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with WT mice.

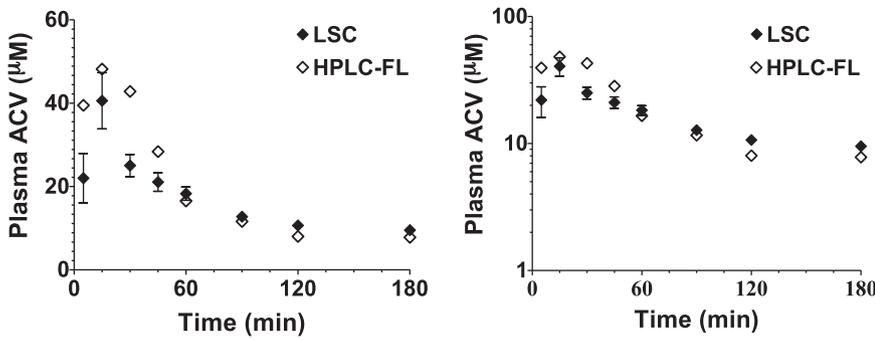


Fig. 6. Plasma concentration-time profiles of acyclovir in wild-type mice after a 100 nmol/g oral dose of radiolabeled and unlabeled valacyclovir. Plasma concentrations were determined by liquid scintillation counting (LSC) and HPLC-fluorescence (HPLC-FL) detection, respectively. Data are expressed as mean ± S.E. for the radiolabeled dose (*n* = 4) and as one or two animals per sample for the unlabeled dose. The y-axis is displayed on linear (left panel) and logarithmic (right panel) scales.

Whereas our results in mice showed linearity between the systemic exposure of acyclovir and valacyclovir oral dose, the relationship was nonlinear in human subjects (Weller et al., 1993; Ormrod and Goa, 2000). We believe this “apparent” discrepancy may be due to a species difference in the *K_m* of valacyclovir. As shown in Table 3, the affinity estimates of PepT1 for valacyclovir were quite variable, differing by as much as 34-fold in the three animal species listed (albeit under different experimental conditions) and 5.3-fold when studied using the same experimental system (i.e., Caco-2 cells). Notwithstanding this uncertainty, the *K_m* of valacyclovir in the mouse was consistent with the low-affinity transport properties of PepT1, as well as being similar to values reported in the literature. Still, the *K_m* estimate in mouse was at the high end of values observed in other experimental systems using human PepT1 and, given the critical dependence of intestinal PepT1 on luminal drug concentrations, might account for differences between mice and humans in the oral absorption kinetics of valacyclovir.

Results from the current *in vivo* oral absorption study confirm our findings from a previous *in situ* intestinal perfusion study in mice (Yang and Smith 2013), indicating a major role for PepT1 in the uptake of valacyclovir. However, the contribution made by intestinal PepT1 toward valacyclovir absorption was less pronounced in the *in vivo* experiments (i.e., a 60% reduction of oral absorption in *PepT1* knockout mice) compared with *in situ* perfusions (i.e., 90% reduction of permeability in *PepT1* knockout mice). The likely reason for this finding is that, in wild-type mice, valacyclovir is absorbed rapidly and efficiently because of the abundant expression of PepT1 along the duodenal, jejunal, and ileal regions of the mouse small intestine (Jappari et al., 2010). In *PepT1* knockout mice, valacyclovir takes advantage of the residual length of intestine, the prolonged residence times of drug in both small

and large intestines, and the larger concentration gradients of drug in intestinal segments so that a greater than expected passive absorption occurs, thereby compensating in part for the absence of PepT1.

When evaluating peripheral tissues, 20 minutes after oral dosing of 25 nmol/g valacyclovir, significant differences were seen between wild-type and *PepT1* knockout mice in the distribution of acyclovir in the eyes, heart, lung, liver, kidney, spleen, and muscle. However, when adjusted for differences in perfusing drug concentrations, the tissue-to-blood ratios of acyclovir were similar between the two genotypes. These results support our acyclovir intravenous dosing experiments, in which we found that PepT1 does not play an important role in affecting the *in vivo* systemic disposition of drug (i.e., drug clearance was unchanged). GI segments were also evaluated 20 minutes after the 25 nmol/g oral dose of valacyclovir. Although no differences were observed between genotypes in the distribution of acyclovir in the stomach, duodenum, and jejunum, concentrations of drug were significantly lower in the ileum and colon of *PepT1* knockout mice. This finding is difficult to reconcile since we would have expected to see differences of acyclovir distribution in the proximal regions of small intestine, not the distal region and colon. Still, it must be appreciated that although systemic plasma and tissue concentrations represent acyclovir, GI tissue samples may represent a mixture of both acyclovir and valacyclovir before prodrug conversion and drug efflux from the enterocyte. As a result, it may be more difficult to interpret the results from regional tissue samples.

In conclusion, our results in mice have provided definitive evidence that PepT1 deletion significantly reduced the *in vivo* oral absorption of valacyclovir using doses that reflected clinically relevant doses in humans. Intestinal PepT1 exhibited a high capacity for valacyclovir uptake in which dose-linear plasma concentrations of acyclovir were observed over

TABLE 3
Species-dependent affinity of PepT1 for valacyclovir

Animal Species	VACV <i>K_m</i> (mM)	Experimental Conditions ^a	Reference
Human	0.3	Caco-2, pH 7.4, HPLC-UV	Han et al., 1998
Human	5.9	cRNA-injected XLO, pH 5.5, [³ H]label-LSC	Balimane et al., 1998
Human	1.6	cDNA-transfected CHO, pH 7.5, [³ H]label-LSC	Guo et al., 1999
Human	2.2-7.4	cDNA-transfected CHO, pH 7.9-5.5, [³ H]label-LSC	Balimane and Sinko, 2000
Human	1.6	Caco-2, pH 6.0, [³ H]label-LSC	Bhardwaj et al., 2005
Human	3.8-5.0	cDNA-transfected MDCK, pH 6.0, [³ H]label-LSC	Bhardwaj et al., 2005
Rat	1.2 ^b	Single-pass jejunal perfusion, pH 6.5, HPLC-UV	Sinko and Balimane, 1998
Mouse	10.2	Single pass jejunal perfusion, pH 6.5, HPLC-FL	Yang and Smith, 2013a

^aCaco-2 refers to human epithelial colorectal adenocarcinoma cells. CHO, Chinese hamster ovary cells; FL, fluorescent detection; LSC, liquid scintillation counter; MDCK, Mardin-Darby canine kidney epithelial cells; UV, ultraviolet; VACV, [³H]label to the radioisotope of valacyclovir; XLO, *Xenopus laevis* oocytes.
^bIntrinsic *K_m* calculated using “estimated” concentrations at the membrane wall.

10–100 nmol/g oral doses of prodrug. These findings strongly support a PepT1-targeting strategy to improve the oral performance of poorly permeable drugs (i.e., Biopharmaceutics Classification System 3 and 4).

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Authorship Contributions

Participated in research design: Yang, Smith.

Conducted experiments: Yang.

Performed data analysis: Yang, Hu.

Wrote or contributed to the writing of the manuscript: Yang, Smith.

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