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***In Silico* Absorption Analysis of Valacyclovir in Wildtype and *Pept1* Knockout Mice Following Oral Dose Escalation**

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Abstract

Purpose—We developed simulation and modeling methods to predict the *in vivo* pharmacokinetic profiles of acyclovir, following escalating oral doses of valacyclovir, in wildtype and *Pept1* knockout mice. We also quantitated the contribution of specific intestinal segments in the absorption of valacyclovir in these mice.

Methods—Simulations were conducted using a mechanistic advanced compartmental absorption and transit (ACAT) model implemented in GastroPlus™. Simulations were performed for 3 hours post-dose in wildtype and *Pept1* knockout mice following single oral doses of 10, 25, 50 and 100 nmol/g valacyclovir, and compared to experimentally observed plasma concentration-time profiles of acyclovir.

Results—Good fits were obtained in wildtype and *Pept1* knockout mice. Valacyclovir was primarily absorbed from duodenum (42%) and jejunum (24%) of wildtype mice, with reduced uptake from ileum (3%) and caecum/colon (1%), for a total of 70% absorption. In contrast, the absorption of valacyclovir in *Pept1* knockout mice was slow and sustained throughout the entire intestinal tract in which duodenum (4%), jejunum (14%), ileum (10%) and caecum/colon (12%) accounted for a total of 40% absorption.

Conclusion—The ACAT model bridged the gap between *in situ* and *in vivo* experimental findings, and facilitated our understanding of the complicated intestinal absorption processes of valacyclovir.

Keywords

intestinal absorption; in situ permeability; in vivo pharmacokinetics; valacyclovir; acyclovir

INTRODUCTION

The proton-coupled oligopeptide transporter PEPT1 is a membrane-bound protein responsible for the uptake of di-/tri-peptides and a broad range of peptidomimetics in mammals. PEPT1 has been extensively studied in regard to its structural characteristics, transport mechanism, substrate specificity, tissue distribution, regulation, and physiological

and pharmaceutical relevance (1–5). In humans and mice, PEPT1 is abundantly expressed at the apical membrane of epithelial cells in small intestine, the main site of absorption for orally administered drugs. It exhibits low affinity and high capacity in facilitating the transcellular uptake of substrates across the intestinal cell barrier. Because of its favorable transport characteristics and localization, intestinal PEPT1 has long been viewed as a promising target for drug delivery, and PEPT1-targeting prodrugs have been synthesized to improve the intestinal permeation of otherwise poorly absorbable parent compounds (6–9).

Valacyclovir is an L-valyl ester prodrug of the potent antiviral agent acyclovir and is viewed as a model PEPT1-targeting prodrug. Intensive effort has been focused on delineating the quantitative importance of PEPT1 in the intestinal absorption of valacyclovir, which understandably lays a solid basis for promoting the rational design of prodrugs that selectively target intestinal PEPT1. In our laboratory, we evaluated the role and relevance of PEPT1 by performing *in situ* intestinal permeability and *in vivo* pharmacokinetic studies of valacyclovir in wildtype and *Pept1* knockout mice (10,11). The *in situ* intestinal perfusion studies demonstrated that valacyclovir was actively transported by intestinal PEPT1, which contributed approximately 90% of the permeability of valacyclovir in mouse small intestine (10). Moreover, during the *in vivo* pharmacokinetic studies of orally administered valacyclovir, 5-fold and 2-fold differences were observed between wildtype and *Pept1* knockout mice in acyclovir peak plasma concentrations (C_{max}) and area under the plasma concentration-time curves from time 0 to 180 min (AUC_{0-180}), respectively (11). Based on the *in situ* and *in vivo* results, we concluded that PEPT1 had a major role in mediating the intestinal absorption of valacyclovir. However, these methods were rather descriptive and lacked a systematic means to interpret the marked differences observed in acyclovir plasma concentration-time profiles between genotypes and the quantitative importance of each intestinal segment in valacyclovir absorption.

The intestinal absorption of valacyclovir, using *in situ* and *in vivo* experimental findings, was integrated into a mechanism-based absorption model to predict *a priori* the involvement of PEPT1 in the intestinal absorption of orally administered valacyclovir and the systemic exposure of acyclovir. The intestinal absorption of valacyclovir is influenced by numerous physical-chemical, physiological and biochemical variables such as pH, absorption surface area, gastric emptying, intestinal transit times and transporters. Therefore, modeling approaches offer a unique opportunity to assess the impact of changes in these specific variables on the rate and extent of valacyclovir intestinal absorption.

Several mechanism- or physiology-based absorption models have been published, such as the Grass (12), compartmental absorption and transit (CAT), gastrointestinal transit absorption (GITA), advanced compartmental absorption and transit (ACAT), and advanced dissolution, absorption, and metabolism (ADAM) models (13). All these examples utilize compartment models that take into account fluid movement along the digestive tract and absorption over time from each gastrointestinal segment. Simulation analyses using these models have greatly facilitated our understanding of the impact of various formulation-related, and physicochemical and physiological factors on oral drug absorption (14–17). The ACAT model, modified from the original CAT model (18,19) to include first-pass metabolism and colonic absorption, is flexible enough to account for the intestinal processes

of drug release, dissolution, precipitation, luminal degradation, active and passive uptake, and gastrointestinal transit (20). GastroPlus implements the ACAT model and has been successfully used in a number of recent simulation studies investigating the oral absorption of various drugs, including valacyclovir, via transporter and/or metabolizing enzyme pathways (21–23).

There were two major objectives in performing this analysis. The first was to develop simulation and modeling methods for predicting the *in vivo* pharmacokinetic profiles of acyclovir, following escalating oral doses of valacyclovir, in wildtype and *Pept1* knockout mice. This was accomplished by integrating experimental findings regarding the *in situ* intestinal permeability and degradation of valacyclovir, expression levels of PEPT1 in the intestines, and *in vivo* disposition of acyclovir into the ACAT model. The second major objective was to quantitate the contribution of specific intestinal segments in the absorption of valacyclovir in PEPT1-expressing mice and during PEPT1 ablation.

MATERIALS AND METHODS

Computer Hardware and Software

Simulations were conducted on a standard Dell desktop computer using GastroPlus™ (version 7, Simulations Plus, Lancaster, CA, USA).

ACAT Model

The ACAT model implemented in GastroPlus was used to simulate *in silico* the pharmacokinetics of valacyclovir following escalating oral administration in wildtype and *Pept1* knockout mice (11). The intestinal fate of orally dosed valacyclovir solution was governed by three simultaneous kinetic processes in the gastrointestinal (GI) tract including GI transit, intestinal absorption and luminal degradation, based on findings from the previous *in situ* perfusion studies (10). Simulations were then performed by coupling the three intestinal processes of valacyclovir (i.e., GI transit, absorption and degradation) to a pharmacokinetic compartment model for intravenously administered acyclovir.

Using GastroPlus, the GI transit of dissolved valacyclovir was modeled as a first-order process across a sequence of 9 consecutive compartments corresponding to the stomach and 8 different regions of the small and large intestines. The luminal degradation of valacyclovir was extensive in mouse (24) and modeled as a first-order pH dependent process. Valacyclovir GI transit and luminal degradation processes were modeled the same way for wildtype and *Pept1* knockout mice based on the assumption that these two processes were not affected by *Pept1* ablation. The key difference in the intestinal handling of valacyclovir between genotypes resides in the absorption of drug-related species across the apical membrane of enterocytes. As reported in our perfusion studies (10), permeability of valacyclovir in the small intestine of wildtype mice was quite high and attributable primarily to PEPT1-mediated and, to a lesser extent, passive absorption. Moreover, permeability of the luminal degradation product acyclovir was found to be insignificant in the small intestine. Therefore, only the absorption of intact valacyclovir, via active and passive pathways, was modeled in wildtype mice. In contrast, intestinal permeability of valacyclovir was similarly

low to that of acyclovir during PEPT1 ablation. As a result, the intestinal absorption of both valacyclovir and acyclovir were modeled in *Pept1* knockout mice. Mathematically, the PEPT1-mediated absorption of valacyclovir was modeled in wildtype mice using a Michaelis-Menten equation while absorption, via passive diffusion, was modeled as a first-order process in both wildtype and *Pept1* knockout mice. Once absorbed into epithelial cells, valacyclovir was assumed to undergo instantaneous hydrolysis via biphenyl hydrolase-like protein to generate its active species so that only acyclovir was available to the systemic circulation. This assumption was reasonable based on experimental evidence that only acyclovir was measurable in the portal vein of blood samples obtained from both genotypes (10). All the kinetic processes described above were expressed by a system of coupled linear and non-linear differential equations that can be integrated numerically in GastroPlus.

Input Parameters

Model-dependent input parameters had to be specified in order to perform the pharmacokinetic simulations. Thus, GastroPlus included parameters associated with dose, the physical-chemical properties of valacyclovir and acyclovir, mouse GI physiology including intestinal PEPT1 protein expression, valacyclovir luminal degradation, valacyclovir and acyclovir permeability, and the *in vivo* pharmacokinetics of intravenous bolus acyclovir.

Physical-Chemical, Formulation and Dose Information

The physical-chemical properties, formulation and dose information for valacyclovir and acyclovir are summarized in Table I. Default values in GastroPlus were used when no other information was available.

Mouse GI Physiology

Information regarding mouse GI transit times, segmental volumes, length (L), radius (R), and luminal pH profiles along the GI tract were provided by GastroPlus as the default values in fasted mice. These physiological parameters were used internally by GastroPlus for the calculation of first-order transit rate constants governing the movement of dissolved valacyclovir along consecutive GI compartments. Luminal pH profiles were also used in the estimation of first-order degradation rate constants characterizing the luminal hydrolysis of valacyclovir.

Absorption scale factors (ASF) were needed to convert intestinal permeability (denoted as P_{eff}) of each segment to a corresponding absorption rate constant (denoted as k_a), based on the relationship between P_{eff} and k_a :

$$k_a = \frac{2 * P_{eff}}{R}, \text{ ASF} = \frac{2}{R} \quad (1)$$

Note that other models for the calculation of ASF were also available in GastroPlus. However, the above relationship was chosen because it offered the best model fit.

Intestinal expression of PEPT1 in mice was extracted from a study reported by Jappar et al (25). In this study, it was found that PEPT1 protein levels were highest in the jejunum, followed closely by levels in the duodenum and ileum. PEPT1 was not detected in the colon of mice. Thus, in our simulations PEPT1 was assumed to be uniformly distributed along the small intestine but absent from the large intestine of wildtype mice. Not surprising, PEPT1 protein was not expressed in the small or large intestine of *Pept1* knockout mice.

Valacyclovir Luminal Degradation

The luminal degradation of valacyclovir was modeled as a first-order process characterized by the degradation rate constant k_{de} , which was estimated by reanalyzing *in situ* intestinal perfusion results obtained in the jejunum of wildtype mice at pH 5.5, 6, 6.5, 7 and 7.5 (26), as well as those perfusion results obtained in different intestinal segments of wildtype mice at pH 6.5 (10). The derivation of k_{de} is described below.

During the intestinal perfusion studies, absorption of lumenally-formed acyclovir was assumed negligible when estimating the *in situ* permeability of valacyclovir. This assumption was valid considering the marked difference in permeability between valacyclovir and acyclovir in wildtype mice. Based on this assumption, the following mass balance equations hold for each intestinal segment under steady-state perfusion conditions:

$$\text{Rate of valacyclovir degradation} = \text{Rate of acyclovir formation} \quad (2)$$

$$\text{Rate of valacyclovir degradation} = k_{de} * V * C_{ave} \quad (3)$$

$$\text{Rate of acyclovir formation} = Q * C_{out,acyclovir} \quad (4)$$

where V is the volume of a given intestinal segment, C_{ave} is the logarithmic mean of valacyclovir luminal concentration within the intestinal segment, Q is the perfusion flow rate (0.1 mL/min) and $C_{out,acyclovir}$ is the acyclovir concentration exiting the perfused segment. C_{ave} was calculated using the measured inlet ($C_{in,valacyclovir}$) and outlet ($C_{out,valacyclovir}$) concentrations of valacyclovir:

$$C_{ave} = \frac{(C_{in,valacyclovir} - C_{out,valacyclovir})}{\ln(C_{in,valacyclovir}/C_{out,valacyclovir})} \quad (5)$$

By combining equations 2–4, k_{de} can be calculated as:

$$k_{de} = \frac{Q * C_{out,acyclovir}}{V * C_{ave}} \quad (6)$$

Using equation 6, k_{de} was estimated in jejunum at different pH values. Subsequently, the relationship between k_{de} or log-transformed k_{de} ($\log-k_{de}$) and pH was modeled using a linear function, and goodness of fit was assessed by the coefficient of determination r^2 . The better-fitting function was then used to estimate k_{de} at pH values found in the GI tract of fasted mice. k_{de} was also estimated in different intestinal segments at pH 6.5 to assess whether or not regional differences existed at the same pH value.

***In Situ* Intestinal Permeability of Valacyclovir in Wildtype Mice**

Total permeability of valacyclovir in the small intestine of wildtype mice was comprised of a major PEPT1-mediated component (denoted as $P_{eff,PEPT1}$) and a minor passive component (denoted as $P_{eff,passive}$). $P_{eff,passive}$ alone accounted for the total permeability of valacyclovir in large intestine due to the absence of PEPT1 in this segment. For the pharmacokinetic simulations in wildtype mice, $P_{eff,passive}$ was assumed to be equal in both the small and large intestines, and initially set at 0.27×10^{-4} cm/sec, the *in situ* P_{eff} of valacyclovir measured in the colon of wildtype mice during previous perfusion studies (10).

$P_{eff,PEPT1}$ was described by the Michaelis-Menten equation:

$$P_{eff,PEPT1} = \frac{V_{max} * C_{luminal}}{K_m + C_{luminal}} \quad (7)$$

where the maximum transport velocity V_{max} was estimated to be 1.4 nmol/cm²/sec and the apparent Michaelis constant K_m estimated to be 10 mM in single-pass concentration-dependent perfusion studies performed in the jejunum of wildtype mice (10). Note that the *in situ* V_{max} estimate was reported for completeness; however, this *in situ* value could not be directly converted to the *in vivo* V_{max} needed in the simulations. Instead, the *in vivo* V_{max} was obtained through an optimization step as described later in the “Parameter Optimization” section. $C_{luminal}$ was the time-varying luminal concentration of valacyclovir generated by GastroPlus. In the simulations, V_{max} and K_m were assumed to remain constant over the entire small intestine. $P_{eff,PEPT1}$ was calculated internally by GastroPlus using equation 7 after considering the regional expression levels of PEPT1 in the small intestine of wildtype mice.

***In Situ* Intestinal Permeability of Valacyclovir and Acyclovir in *Pept1* Knockout Mice**

As described previously in the ACAT Model section above, the absorption of both valacyclovir and acyclovir needed to be simulated in *Pept1* knockout mice because of extensive luminal hydrolysis of valacyclovir to acyclovir, and the similarly low intestinal permeability of both species. However, the version of GastroPlus used for the present pharmacokinetic simulations did not have the capability of modeling simultaneously the intestinal absorption of two compounds. A solution to circumvent this limitation was to assume the same passive permeability of valacyclovir and acyclovir so that these two compounds were treated equally in terms of their intestinal absorption process. This assumption was reasonable based on previous perfusion studies demonstrating the similar and low intestinal permeabilities of valacyclovir and acyclovir in *Pept1* knockout mice (10).

In the simulations, initial input values of valacyclovir (and acyclovir) permeability in *Pept1* knockout mice were based on four approaches using results obtained from previous *in situ* perfusion studies (10): Method #1, corresponding to the mean P_{eff} of valacyclovir in small intestine; Method #2, corresponding to the mean P_{eff} of valacyclovir in both small and large intestines; Method #3, corresponding to the mean P_{eff} of valacyclovir in duodenum; and Method #4, corresponding to the mean P_{eff} of acyclovir in jejunum. In doing so, the four methods could be evaluated separately to assess which P_{eff} estimate resulted in the best model predictions. The four P_{eff} values, along with their 90% confidence intervals, are summarized in Table II. Although the intestinal P_{eff} estimates of valacyclovir and acyclovir were numerically different in *Pept1* knockout mice, the differences were not statistically significant.

***In Vivo* Pharmacokinetics of Intravenous Acyclovir**

Individual plasma concentration-time curves of acyclovir in wildtype and *Pept1* knockout mice, following a 25 nmol/g intravenous bolus dose of acyclovir, were fitted to one-, two- and three-compartment models using the PKPlus™ module of GastroPlus. Akaike's Information Criteria (AIC) and Schwarz's Bayesian Criteria (SBC) were used to select the most appropriate model. Since these acyclovir plasma concentrations were virtually superimposable over time, as reported previously in wildtype and *Pept1* knockout mice (11), the estimated values from both genotypes were combined and then used to calculate mean pharmacokinetic parameters for initial input in the simulations. The unbound fraction of acyclovir in plasma was set at 0.87, which was obtained from the literature (27).

Modeling and Simulation Procedure

The plasma concentration-time profiles of acyclovir were simulated in wildtype and *Pept1* knockout mice following single oral doses of 10, 25, 50 and 100 nmol/g valacyclovir. The simulations followed three sequential steps, including parameter sensitivity analysis (PSA), parameter optimization, and comparison of the simulated pharmacokinetic profiles of acyclovir with previously reported experimental data (11). Acyclovir results from the 25 nmol/g oral dose of valacyclovir in wildtype mice served as a "training set" in evaluating the PSA and optimization procedures, while the remaining results (i.e., other three oral dose groups) were used as external validation datasets in the final comparison step.

Parameter Sensitivity Analysis

Inaccurate input parameters could impair the predictive performance of the specified ACAT model. Therefore, PSA was performed to evaluate which input parameters most influenced the simulated plasma concentrations of acyclovir, as well as C_{max} and AUC_{0-180} . Parameters evaluated in the PSA step were associated with the *in situ* PEPT1-mediated and passive intestinal permeability of valacyclovir, as well as the *in vivo* pharmacokinetics of intravenous acyclovir. The initial input values were varied by multiplying them with 10 scaling factors in the range of 0.1–10 to allow an order of magnitude increase or decrease. Acyclovir C_{max} and AUC_{0-180} were then evaluated at each of the 10 scaling factors for each of the parameters studied.

Parameter Optimization

Following PSA, parameter optimization was performed to fine-tune the most influential input parameters for improving the model fit of the training set. In particular, the *in vivo* value for PEPT1 V_{max} had to be obtained through optimization since the *in situ* V_{max} value cannot be directly scaled to the *in vivo* value needed in GastroPlus. Parameter optimization was, therefore, performed using a built-in optimization module in GastroPlus.

Comparison of Simulated and Experimental Acyclovir Pharmacokinetic Profiles

Once parameter optimization (if performed) was completed, the parameter values were fixed and then used for predicting the plasma concentration-time profiles of acyclovir after the escalating oral doses of valacyclovir in wildtype and *Pept1* knockout mice. Note that when performing the simulations in *Pept1* knockout mice, PEPT1-mediated permeability was turned off and four initial passive P_{eff} values were used separately. The quality of simulations was evaluated by the following three criteria: 1) visual inspection of the simulated and observed plasma concentration-time curves of acyclovir, 2) comparison of the predicted and observed C_{max} and AUC_{0-180} values, and 3) inspection of the coefficient of determination r^2 .

Model Application

Once the predictive performance of the specified ACAT model was fully validated, it was used to quantify the contribution of each intestinal segment to the overall oral absorption of valacyclovir in wildtype and *Pept1* knockout mice. The fraction of oral dose absorbed in each segment was calculated as the ratio of amount of prodrug disappearing from that segment to the administered dose. These fractional absorption values were computed directly and reported by GastroPlus after the simulations. The ACAT model was also used to evaluate the influence of luminal degradation on extent of valacyclovir absorption by comparing the predicted total fraction absorbed in the presence and absence of luminal degradation.

RESULTS

Valacyclovir Luminal Degradation

Figure 1 depicts the estimated k_{de} (panel A) or $\log-k_{de}$ (panel B) of valacyclovir as a function of pH, both of which are overlaid with regression lines from linear models. The plots clearly demonstrate an increase in the luminal hydrolysis of valacyclovir with increasing pH values. Given the better fit of the log-transformed k_{de} relationship, this equation was used to predict the k_{de} of valacyclovir at physiological pH along the entire length of intestine in fasted mice. The predicted k_{de} , degradation half-life (calculated as $0.693/k_{de}$), and intestinal pH values in fasted mice are listed in Table III. These k_{de} predictions were used as input values in the pharmacokinetic simulations. In addition, analysis of the estimated valacyclovir k_{de} in different intestinal segments of wildtype mice at pH 6.5 suggested there were no regional differences in the luminal degradation of valacyclovir (data not shown).

***In Vivo* Pharmacokinetics of Intravenous Acyclovir**

A three-compartment model achieved the smallest AIC and SBC values when fitted to the plasma concentration-time profiles of acyclovir following an intravenous bolus injection of 25 nmol/g acyclovir in wildtype and *Pept1* knockout mice. The pharmacokinetic parameters needed to fully characterize this model included volume of central compartment V_L , clearance CL , first-order transfer rate-constants between the central and shallow peripheral compartment k_{12} and k_{21} , and first-order transfer rate-constants between the central and deep peripheral compartment k_{13} and k_{31} . Mean pharmacokinetic parameter estimates along with standard deviation (SD) for this three-compartment model were used as initial input values in the simulations and are summarized in Table IV.

PSA Results

As shown in Figure 2 for wildtype mice, PSA suggested that the predicted AUC_{0-180} of acyclovir was most sensitive to changes in CL , followed by changes in V_L , k_{13} , k_{31} , V_{max} and K_m (panel A). The predicted C_{max} of acyclovir was most sensitive to changes in V_L , followed by changes in V_{max} and K_m (panel B). In contrast, the predicted AUC_{0-180} and C_{max} values were insensitive to changes in $P_{eff,passive}$, showing less than 30% and 60% changes, respectively, when $P_{eff,passive}$ was varied over a 100-fold range. Since the predicted C_{max} was similarly sensitive to changes in V_{max} and K_m , and since V_{max} had to be obtained through optimization, only V_{max} was selected for subsequent optimization. Initial *in situ*-derived values for K_m were used directly without further refinement. The pharmacokinetic parameters V_L , CL , k_{13} and k_{31} were also optimized based on the PSA results.

Parameter Optimization

The maximum rate of PEPT1-mediated transport, V_{max} , was optimized at 0.000726 mg/sec for a best fit of simulations to the observed data. In addition, the parameters V_L , CL , k_{13} and k_{31} were also fine-tuned using the selected training set. These optimized pharmacokinetic parameters are summarized in Table IV. Most optimized parameters showed <1.5-fold change relative to the initial input values. The optimized value for k_{31} had an acceptable 2.1-fold change compared with its initial estimate.

Comparison of Simulated and Experimental Acyclovir Pharmacokinetic Profiles in Wildtype Mice

Figure 3 depicts the simulated and experimental plasma concentration-time profiles of acyclovir in wildtype mice following escalating oral doses of valacyclovir. Figure 3A demonstrates that the simulated curves are contained within the range of individually observed data for the four dose groups studies, while Figure 3B demonstrates good agreement between the simulated and observed mean data with slight under-prediction at the terminal time points for the two higher dose groups (i.e., 50 and 100 nmol/g). As shown in Table V, adequate model predictions were further confirmed in wildtype mice by the high r^2 values (> 0.875) across four doses and the close agreement between simulated and observed C_{max} (< 12% difference) and AUC_{0-180} (< 19% difference) values of acyclovir.

Comparison of Simulated and Experimental Acyclovir Pharmacokinetic Profiles in *Pept1* Knockout Mice

Figure 4 depicts the simulated and mean observed pharmacokinetic profiles of acyclovir in *Pept1* knockout mice. Using a passive P_{eff} of 0.074×10^{-4} cm/sec (method #4) in the simulations, a pronounced under-prediction was observed at the early time points (i.e., < 45 min) but a reasonable fit at later time points (i.e., 45 to 180 min). Model predictions obtained using the other P_{eff} values listed in Table II (i.e., methods #1, #2 and #3) all over-predicted the observed pharmacokinetic profiles of the four dose groups (data not shown). Simulations were also performed using the upper and lower limits corresponding to the 90% CI of the mean P_{eff} value of valacyclovir estimated in the small intestine of *Pept1* knockout mice (method #5). As shown in Figure 5, at a typical dose of 25 nmol/g oral valacyclovir, the observations fell within the 90% CI enclosed by the two simulations. However, it was still challenging to satisfactorily match the observed observations using this method. Similar results were observed for the other dose groups (data not shown).

In a subsequent *ad hoc* parameter optimization procedure, P_{eff} was optimized separately for each dose level (method #6) in order to improve the model fit. The optimized P_{eff} values were 0.096×10^{-4} cm/sec, 0.070×10^{-4} cm/sec, 0.101×10^{-4} cm/sec and 0.086×10^{-4} cm/sec for the 10, 25, 50 and 100 nmol/g dose groups, respectively. These optimal values were similar to the jejunal P_{eff} estimate of acyclovir in *Pept1* knockout mice (method #4) and fell within the 90% CI of mean P_{eff} estimates listed in Table II. As shown in Figure 6, the model simulations using method #6 significantly improved the prediction of experimental data, although some degree of model misfit was still visible during the early absorption phase. The need to use optimized P_{eff} values for obtaining reasonable model fits suggests a high sensitivity to the P_{eff} input values in the simulation of poorly permeable compounds. Even statistically insignificant differences in P_{eff} estimates may lead to poor *in silico* pharmacokinetic predictions. Table V summarizes the predicted and observed C_{max} (< 12% difference) and AUC_{0-180} (< 8% difference) values of acyclovir in *Pept1* knockout mice using optimized method #6. The r^2 values (0.814) further support a reasonable fit between the simulated and observed plasma concentration-time profiles of acyclovir in *Pept1* knockout mice using this final method.

Model application

Figure 7 displays the contribution of specific regions of the small and large intestines toward the oral absorption of 25 nmol/g valacyclovir in wildtype and *Pept1* knockout mice. Similar results were observed in the other three dose groups (data not shown). In wildtype mice, the duodenum was found to be the most important absorption site, accounting for 42% of the dose absorbed (Figure 7A). The two jejunal segments accounted for 24% of dose absorbed, and the ileum, caecum and colon combined for the remaining 4%, resulting in a total dose absorbed of 70%. In contrast, in *Pept1* knockout mice, the duodenum accounting for only 4% of the dose absorbed (Figure 7B). However, the two jejunal segments accounted for 14% of dose absorbed, the ileum 10%, and the caecum and colon combined for 12%, resulting in a total dose absorbed of 40%. Finally, when the simulations were performed for wildtype mice in the absence of luminal hydrolysis (i.e., valacyclovir $k_{\text{de}} = 0$), nearly 100% of the prodrug was absorbed within 3 hr of oral dosing (Figure 7C). Thus, it appears that the

incomplete oral absorption of valacyclovir was due to competing luminal degradation rather than to insufficient permeability or intestinal residence times.

DISCUSSION

In the present study, we used an *in silico* analysis to investigate the role and relevance of intestinal PEPT1 on the oral absorption of the model prodrug valacyclovir, and the systemic exposure of the active species acyclovir in wildtype and *Pept1* knockout mice. This was accomplished by optimizing the input parameters (e.g., *in situ* intestinal permeabilities of valacyclovir and acyclovir, luminal degradation of valacyclovir, PEPT1 intestinal expression levels, *in vivo* disposition kinetics of acyclovir) when simulating the plasma concentration-time profiles of active drug. Upon integrating these simulations into an ACAT model, and comparing them with the experimental data, we achieved excellent model predictions for both wildtype and *Pept1* knockout mice over a 10-fold range of oral doses. From this analysis, three major observations were made: 1) in wildtype mice, the primary absorption sites of valacyclovir were the duodenum (42%) and jejunum (24%) in which 66% of the total 70% absorption of prodrug occurred; 2) in *Pept1* knockout mice, the absorption of valacyclovir was slow but sustained across the entire intestinal tract in which the duodenum (4%), jejunum (14%), ileum (10%) and caecum/colon (12%) accounted for the total 40% absorption of prodrug; and 3) in the absence of luminal degradation, the intestinal uptake of valacyclovir was essentially complete (99%), thereby, revealing that permeability was not a rate-limiting factor in the reduced *in vivo* oral absorption of prodrug. Collectively, these findings are novel in reporting for the first time the quantitative contribution of specific intestinal segments on the *in vivo* absorption of valacyclovir in the presence and absence of PEPT1. Moreover, the findings demonstrate conclusively that the reduced *in vivo* oral absorption of valacyclovir is solely the result of luminal degradation.

A mechanistic model that adequately captures the *in vivo* intestinal processes of valacyclovir is a key determinant in successfully characterizing the plasma concentration-time profiles of the active drug acyclovir. Provided that the ACAT structural model is correct, the quality of the *in silico* simulations depends mainly upon the accuracy of input parameters. Thus, parameters pertaining to the intestinal permeability and luminal degradation of valacyclovir, and the *in vivo* disposition of acyclovir, had to be reliably estimated and when necessary optimized. The luminal degradation of valacyclovir was examined by re-analyzing the results from a previous intestinal perfusion study in mice (10). These results represented our best estimate of valacyclovir degradation *in vivo* since gastrointestinal physiology remains intact presumably under *in situ* conditions. In this analysis, the luminal degradation of valacyclovir was found to increase with increasing pH, a finding consistent with another study showing that valacyclovir was stable under acidic conditions but metabolically labile under basic conditions in rats and humans (28). However, valacyclovir degradation rate constants determined *in situ* in mice were much higher than values in buffer solutions of the same pH, suggesting that membrane-bound enzymes accelerated the hydrolysis of valacyclovir in the intestinal lumen. The best-fitting function between log-transformed degradation rate constants and pH was then used to predict degradation rate constants of valacyclovir in mouse intestinal lumen under physiological pH conditions (Figure 1 and Table III), and applied to the current simulations.

Accurate parameter estimates were also needed for the active and passive permeability of valacyclovir, along with the *in vivo* pharmacokinetics of acyclovir. However, substantial inter-subject variability was observed for some of these parameters. Since we used parameter values collected from one group of mice to predict the pharmacokinetics in other group of mice (i.e., animals were not crossed-over), parameter sensitivity analysis was performed to evaluate the influence of population variability on the prediction results. Given the dominance of PEPT1-mediated intestinal uptake of valacyclovir in wildtype mice, it is conceivable that predictions were sensitive to changes in parameters characterizing this active transport process (i.e., $V_{\max}$ and K_m). Conversely, the predicted AUC_{0-180} and $C_{\max}$ values of acyclovir were almost invariant to 100-fold changes in valacyclovir passive permeability (Figure 2), suggesting an insignificant role of passive diffusion during the intestinal absorption of prodrug in wildtype mice. The AUC_{0-180} and $C_{\max}$ values of acyclovir were highly sensitive to variability in the *in vivo* kinetics of acyclovir (e.g., CL and V_1), suggesting that minor inaccuracies in acyclovir disposition would significantly bias the results. As a result, optimized values were used for these parameters (Table IV). Most parameters were not optimized for a variety of reasons. For example, the default physiological parameters in the ACAT model were not optimized since we assumed these values were correct in the absence of our own experimental findings. In addition, degradation rate constants were not optimized because GastroPlus did not allow this option. It should be noted, however, that parameter optimization is only feasible during retrospective studies where experimental pharmacokinetic data are available as the training dataset. In the absence of such data, approaches taking into account population variability in input parameters would be more suitable to generate the simulations.

Using the ACAT model, *in silico* plasma concentration-time profiles of acyclovir were simulated following a 10-fold oral dose range of valacyclovir (i.e., 10–100 nmol/g). For wildtype mice, there was good agreement between the simulations and experimental observations, with slight under-prediction of acyclovir plasma concentrations being observed at two terminal time points for the 50 and 100 nmol/g dose groups (Figure 3). One possible explanation for the slight lack of fit is that some input parameters are still not accurately parameterized for these two cohorts of mice. It is also possible, that at the two higher doses, our assumption of linear disposition of acyclovir is invalid because of the involvement of organic anion and cation transporters limiting the renal secretion of drug (29). For *Pept1* knockout mice, the *in silico* simulations showed high sensitivity to the low intestinal permeability values of valacyclovir and acyclovir in the absence of PEPT1. By inspecting the optimized permeability values for the four dose groups, it was obvious that slight numerical changes in passive permeability could significantly impact the model predictions of *Pept1* knockout mice. As a result, ad hoc optimization of passive permeability had to be performed at each dose level (i.e., method #6) to obtain a reasonable model fit (Figure 6). This finding, along with previous PSA results showing that wildtype mice were highly sensitive to K_m and $V_{\max}$, suggests the importance of intestinal permeability in affecting the pharmacokinetic profiles of drugs with either high or low permeability.

Overall, the quality of simulations was somewhat worse for *Pept1* knockout mice (r^2 0.814) than for wildtype animals (r^2 0.875) even when using optimized permeability values (Table 5). Although speculative, this may be due to several factors. For example, the

assumption of equal intestinal permeabilities in different intestinal regions of *Pept1* knockout mice may not be sufficiently accurate since differences in passive permeability, although not statistically significant (10), were observed in the *in situ* studies (Table II). While it is possible that the use of optimized P_{eff} values for different intestinal segments may help to better understand the source of minor misfits in the simulated results, we decided not to pursue this course since it is unlikely that the approach would significantly enhance the predictive ability of the ACAT model. It is also possible, as noted before for wildtype animals, that population variability in input factors can confound the quality of simulations as compared to the observed results.

Our final ACAT model was used to examine the effect of hydrolysis as well as the contribution of specific intestinal regions on the absorption of valacyclovir. We found that luminal hydrolysis caused an incomplete oral availability of valacyclovir in wildtype mice even though its small intestinal permeability was sufficiently high (Figure 7C). The results also indicated that the duodenum played a major role in the absorption of valacyclovir in wildtype mice but that more distal intestinal segments were important for valacyclovir absorption in the absence of PEPT1 (Figure 7A–B). Similar findings were reported by Hironaka et al (17) where they found in rats that PEPT1 accounted for about 50% of the total oral absorption of cephalexin (another PEPT1 substrate). However, their simulation study indicated an 83% bioavailability of cephalexin in the absence of PEPT1, which they attributed to greater absorption of drug in the lower intestinal segments.

The simulation analysis provided a pivotal mechanistic explanation for our previous findings in which mouse genotypes exhibited a greater magnitude of change in the *in situ* intestinal permeability of PEPT1 substrates than in their *in vivo* oral absorption. For example, even though glycylsarcosine (25,30) and valacyclovir (10,11) had jejunal permeabilities that were 10-fold lower during PEPT1 ablation, their systemic availabilities were only 2-fold and 2- to 3-fold lower, respectively, after oral dosing. At that time, we proposed that in *Pept1* knockout mice glycylsarcosine and valacyclovir may take advantage of the residual length and prolonged residence times in more distal regions of the small and large intestines, resulting in a greater than expected passive absorption to partially compensate for the loss of PEPT1. The quantitative findings from the present simulation study supports this contention in demonstrating that even in the absence of intestinal PEPT1, passive absorption provides a consistent 5–10% absorption of valacyclovir along the entire length of the intestines.

The oral dose range of valacyclovir (i.e., 10 to 100 nmol/g) was chosen so that plasma concentration of acyclovir in mice would be similar to that produced in humans during commonly prescribed oral doses. Thus, peak plasma concentrations of acyclovir ranged from 3.8 – 40.4 μM over this 10-fold dose range (11), whereas in adult human subjects, acyclovir had peak plasma concentrations of 10 – 37 μM following 250 to 2000 mg oral doses of valacyclovir (31,32). It was also estimated that, at the highest oral dose given, the initial concentration of valacyclovir in the gastrointestinal tract would be 10 mM, which approximated the value observed in mice during *in situ* jejunal perfusions (10).

A more complete plasma concentration-time profile would have been preferred for acyclovir in wildtype and, even more so, in *Pept1* knockout mice. However, the simulations were

based on results from our previous paper (11) and, as a result, sampling was limited to 0–3 hr for both genotypes. In this previous paper, no attempt was made to measure plasma concentrations of valacyclovir in the systemic circulation. This was reasonable since *in situ* intestinal perfusions of valacyclovir had indicated that only acyclovir was present in the portal vein of samples obtained from wildtype and *Pept1* knockout mice (10). Moreover, 90% of valacyclovir was converted to acyclovir by presystemic metabolism in rats after a 90 nmol/g dose (33), which was similar to the 100 nmol/g oral dose of valacyclovir given to mice in the present study.

CONCLUSIONS

In concluding, these simulations integrated the physical-chemical and pharmacokinetic properties of valacyclovir and acyclovir, along with mouse physiology and biological determinants, in predicting the prodrug's intestinal absorption (and subsequent systemic availability of acyclovir) in wildtype and *Pept1* knockout mice. Results from this study were particularly noteworthy in delineating the quantitative *in vivo* contribution of PEPT1 in distinct regions of the small and large intestines, and the significance of luminal hydrolysis in reducing the oral absorption of valacyclovir. Moving forward, a similar analysis should be performed with valacyclovir in other transgenic models such as humanized *PEPT1* mice (34) and biphenyl hydrolase-like (*Bphl*) knockout mice. Ultimately, future studies should be aimed at translating these animal models to human in order to better predict *a priori* oral drug performance, drug-drug interactions, and the development of PEPT1 targeted drugs or prodrugs.

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DISCLOSURES

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ABBREVIATIONS

ACAT	advanced compartmental absorption and transit
AUC_{0–180}	area under the plasma concentration-time curve from time 0 to 180 min
C_{max}	peak plasma concentration
GI	gastrointestinal
P_{eff}	intestinal permeability
PEPT1	peptide transporter 1
PSA	parameter sensitivity analysis

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Figure 1. Relationship between the luminal degradation rate-constant k_{de} of valacyclovir and pH (A) or log-transformed k_{de} and pH (B) when fitted by linear regression.

Figure 2. Sensitivity of predicted acyclovir AUC₀₋₁₈₀ (A) and C_{max} (B) to input parameters after oral administration of 25 nmol/g valacyclovir in wildtype mice. Parameters were changed by multiplying the initial input values with scaling factors in the range of 0.1–10.

Figure 3. Model predicted (solid lines) and observed (open diamonds) plasma concentration-time profiles of acyclovir after escalating oral doses of valacyclovir in wildtype mice using individual (A) and mean (B) results. The observed data in panel B were expressed as mean $\pm$ SD (n=4–7).

Figure 4.

Model predicted (solid lines) and observed (open diamonds) plasma concentration-time profiles of acyclovir after escalating oral doses of valacyclovir in *Pept1* knockout mice. Model simulations were obtained using a P_{eff} input value of 0.074×10^{-4} cm/sec for valacyclovir (method #4). The observed data were expressed as mean $\pm$ SD (n=4–7).

Figure 5.

Comparison of mean observations (open diamonds) with simulations (solid lines) using 0.04×10^{-4} cm/sec (lower line) and 0.32×10^{-4} cm/sec (upper line) as the valacyclovir P_{eff} input values following oral administration of 25 nmol/g valacyclovir in *Pept1* knockout mice.

These two P_{eff} values represent the upper and lower limits of the 90% confidence interval for the estimated P_{eff} of valacyclovir in the small intestine of *Pept1* knockout mice (method #5).

The observed data were expressed as mean $\pm$ SD (n=7).

Figure 6.

Model predicted (solid lines) and observed (open diamonds) plasma concentration-time profiles of acyclovir after escalating oral doses of valacyclovir in *Pept1* knockout mice using individual (A) and mean (B) results. Model simulations were obtained using separately optimized P_{eff} values for each dose level (method #6). The observed data in panel B were expressed as mean $\pm$ SD (n=4–7).

Figure 7.

Contribution of specific intestinal regions in the oral absorption of 25 nmol/g valacyclovir in wildtype (A) and *Pept1* knockout (B) mice, and in wildtype mice in the absence of luminal degradation (C).

Table I

Physical-Chemical, Formulation and Dose Information of Valacyclovir and Acyclovir

Property	Valacyclovir	Acyclovir	Source
Molecular formula	C ₁₃ H ₂₀ N ₆ O ₄	C ₈ H ₁₁ N ₅ O ₃	
Molecular weight	324.3	225.2	
Predicted log P (neutral)	-0.95		Bolger et al (21)
pKa1	1.9		Balimane and Sinko (23)
pKa2	7.47		Balimane and Sinko (23)
pKa3	9.43		Balimane and Sinko (23)
Aq. solubility (pH 7)	174 mg/mL		Bolger et al (21)
Dosage form	Valacyclovir solution		
Doses (nmol/g)	10.0	6.9	
	25.0	17.4	
	50.0	34.7	
	100.0	69.4	
Dose volume	0.2 mL		
Mean precipitation time	900 sec		GastroPlus default value
Diffusion coefficient	7.50×10 ⁻⁶ cm/sec		GastroPlus default value
Drug particle density	1.2 g/mL		GastroPlus default value
Particle size	25 µm		GastroPlus default value

Table II*In Situ* Intestinal Permeability (P_{eff}) of Valacyclovir or Acyclovir in *Pept1* Knockout Mice

P_{eff} (estimation method)	Mean value ($\times 10^{-4}$ cm/sec)	90% CI ($\times 10^{-4}$ cm/sec)
Valacyclovir P_{eff} in small intestine (#1)	0.18	(0.04, 0.32)
Valacyclovir P_{eff} in entire intestine (#2)	0.23	(0.11, 0.35)
Valacyclovir P_{eff} in duodenum (#3)	0.27	(−0.02, 0.57)
Acyclovir P_{eff} in jejunum (#4)	0.074	(−0.023, 0.72)

Table III

In Situ Degradation Rate Constants of Valacyclovir at Physiological pH Values in the Intestinal Lumen of Fasted Mice

Segment	pH ^a	k _{de} (min ⁻¹) ^b	Half-life (min)
Duodenum	4.74	0.031	22
Jejunum	5.01	0.038	18
Ileum	5.24	0.045	15
Caecum	4.63	0.028	24
Colon	5.02	0.038	18

^aGastroPlus default values.

^bDetermined using the equation: $\log k_{deg} = 0.771 \times \text{pH} - 7.61$.

Table IV

Pharmacokinetic Parameters of Intravenous Acyclovir Derived From a Three- Compartment Model

Parameter (unit)	Fitted (Mean $\pm$ SD)	Optimized	Optimized/Fitted
V_1 (L/kg)	0.38 ± 0.08	0.545	1.4
$k_{12}(\text{hr}^{-1})$	6.54 ± 3.25	–	–
$k_{21}(\text{hr}^{-1})$	13.2 ± 7.4	–	–
$k_{13}(\text{hr}^{-1})$	2.91 ± 0.68	2.221	0.8
$k_{31}(\text{hr}^{-1})$	0.44 ± 0.36	0.908	2.1
CL (L/hr/kg)	1.0 ± 0.5	1.078	1.1

Table V
Simulation Results for Acyclovir Following Escalating Oral Doses of Valacyclovir in Wildtype and *Pept1* Knockout Mice

Genotype	Dose (mmol/g)	C _{max} (μM)		AUC ₀₋₁₈₀ (min·μmol/L)		r ²
		Observed	Predicted	Observed	Predicted	
Wildtype	10	3.8	3.9	278	279	0.898
	25	9.6	9.7	663	691	0.969
	50	20.9	19.0	1675	1364	0.902
	100	40.6	35.9	2923	2636	0.875
<i>Pept1</i> Knockout	10	0.9	1.0	128	131	0.897
	25	1.7	1.8	235	238	0.877
	50	5.0	5.2	743	687	0.814
	100	8.8	8.8	1233	1176	0.885