

# Gene Expression in the Human Intestine and Correlation with Oral Valacyclovir Pharmacokinetic Parameters

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## ABSTRACT

The transport of valacyclovir, the L-valyl ester of acyclovir, has been suggested to be mediated by several carrier-mediated pathways in cell culture and animal models. The role and importance of these transporters in modulating valacyclovir absorption in humans has not been determined, however. Recent advances in genomic technology have facilitated the rapid and simultaneous determination of global mRNA expression profiles for thousands of genes in tissue biopsies directly associated with the absorption process, thereby dramatically increasing the value of studies in humans. In this article, we describe correlations of pharmacokinetic parameters following oral valacyclovir or acyclovir administration with expression levels of intestinal genes in humans. Highly positive and significant correlations were observed with 4F2hc, an activator of cation-preferring amino acid transport systems, and human oligopep-

tide transporter (HPT1), an oligopeptide transporter expressed at higher levels in the human intestine compared with oligopeptide transporter (PEPT1). The validation of HPT1 microarray data with reverse transcription-polymerase chain reaction and the enhanced valacyclovir uptake in HeLa/HPT1 cells suggest that the role of HPT1 in transport of peptides and peptidomimetics drugs needs to be examined in more detail. The interrelation of 4F2hc and HPT1 in transport may be of interest. No significant correlations of valacyclovir pharmacokinetic parameters with PEPT1 and with organic cation or anion transporter expression levels were observed. The highly negative correlations observed with known efflux pumps such as MDR1 (P-glycoprotein) and MRP2 (cMOAT), as well as with the CYP450 IIIA subfamily may indicate that these proteins may regulate the cellular accumulation and metabolism of acyclovir.

The enhancement in oral valacyclovir bioavailability, the L-valyl ester of the antiviral agent acyclovir, has been attributed to its enhanced permeation across the intestine compared with acyclovir. The early acyclovir disposition studies following oral administration of the L- and D-valyl stereoisomers to rats clearly indicated stereoselective absorption, thus suggesting a carrier-mediated mechanism (Beauchamp et al., 1992; Beauchamp and Krenitsky, 1993; Purifoy et al., 1993). The L-valacyclovir transport mechanism has since been extensively examined in animal models and in cell culture systems (Smith et al., 1993; Balimane et al., 1998; Han et al.,

1998a,b; Sinko and Balimane, 1998). These studies revealed that valacyclovir absorption may be facilitated by several carrier-mediated transporters in the intestine. Thus, in addition to the intestinal proton-dependent oligopeptide transporter PEPT1, it has been suggested that organic anion (OATs) and organic cation (OCT) transporters may also play a role in intestinal valacyclovir uptake (Sinko and Balimane, 1998). The relative contribution of oligopeptide and other transporters in overall valacyclovir uptake in humans has not yet been reported, however.

Over the past few years the dramatic increase in prodrug strategies to improve both oral absorption as well as efficacy and safety considerations can be directly attributed to a growing emphasis to better understand the role and importance of carrier-mediated transport in the human intestine (Shin et al., 2003). The wide use of cell culture systems and/or animal models as surrogates for the human intestine has

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**ABBREVIATIONS:** OAT, organic anion transporter; OCT, organic cation transporter; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; PEPT1, oligopeptide transporter; HPT1, human oligopeptide transporter; MES, 4-morphoethanesulfonic acid; AUC, area under the curve; CNT2, concentrative purine nucleoside transporter.

contributed enormously to this endeavor. Nevertheless, such reliance on model systems may also lead to the implication of multiple transporters contributing simultaneously to overall intestinal transport. Furthermore, such model systems cannot address the important issues of relevancy to the role of human intestinal transporters *in vivo*, and when relevant, the relative contributions of these transporters *in vivo*. Thus, it is essential to investigate the role and importance of multiple transporters in the *in vivo* transport of prodrugs across the human intestine. With the emergence of genomics and advances in microarray technology, thousands of genes from tissues or cells can be simultaneously analyzed to acquire the mRNA expression levels. This technology is of particular importance to *in vivo* studies with humans since it not only obviates the need to assay gene expression levels one at a time but also facilitates the global assessment of the role of several thousands of genes in the modulation of biopharmaceutical processes and parameters of interest. Thus, it would be possible to generate a global database of gene expression in intestinal tissues from a group of healthy human volunteers. If the absorption of an orally administered drug is also determined in the same group of volunteers, the global database of gene expression can then be examined to identify plausible putative or novel mediators of drug transport and oral drug absorption in the intestinal tissues.

In this article, we describe the results of a multiphase study in humans designed specifically to identify genes that mediate the oral absorption of valacyclovir and acyclovir in healthy humans. Thus, duodenal tissue biopsies were first obtained from all subjects in phase 1 of the study. Subsequently, in phases 2 and 3 of the study, pharmacokinetic studies were conducted in the same subjects to monitor acyclovir absorption following valacyclovir and acyclovir oral administration, respectively. The gene expression profiles determined from duodenal tissue biopsies were then compared with acyclovir pharmacokinetic parameters obtained for each individual to elicit correlations. Gene expression was analyzed using microarray expression technology (Affymetrix GeneChip, Affymetrix, Inc., Santa Clara, CA) that contains 12,559 gene transcripts. The possible involvement of relevant transporters in determining overall valacyclovir absorption, as indicated by significant positive correlations with expression levels, was then examined with functionality tests in cell culture constructs *in vitro*. These studies are expected not only to provide a more thorough understanding of valacyclovir transport in humans *in vivo* but also reveal general indications regarding the importance of various transporters in the intestinal transport of amino acid ester prodrugs and peptidomimetics.

## Materials and Methods

**Materials.** Acyclovir (Zovirax, 400 mg; GlaxoSmithKline, Uxbridge, Middlesex, UK) and valacyclovir hydrochloride (Valtrex, 500 mg; GlaxoSmithKline) were obtained from the Hospital Pharmacy, University of Michigan Hospital System (Ann Arbor, MI). TRIzol reagent and SuperScript Choice System for cDNA synthesis kit were purchased from Invitrogen (Carlsbad, CA). BioArray high-yield RNA transcript labeling kit was purchased from Enzo Biochem (New York, NY). GeneChips were purchased from Affymetrix. The GeneChip hybridization and scanning was performed at the

Genomic Information Support Facility at Michigan State University (East Lansing, MI). Tissue culture plates were purchased from BD Biosciences (San Jose, CA). All cell culture medium and reagents were from Invitrogen. [<sup>3</sup>H]Valacyclovir (specific activity, 5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). All solvents used were high-performance liquid chromatography (HPLC) grade, and all chemicals used were analytical grade.

**Human Study Protocol.** Eleven healthy subjects (seven males and four females) gave written informed consent to participate in the study. This investigation complied with tenets of the Declaration of Helsinki promulgated in 1964 and was approved by the University of Michigan Institutional Review Board. The subjects were 21 to 36 years of age ( $29.0 \pm 5.8$  years) and were within 20% of their ideal body weight ( $75.7 \pm 15.7$  kg). Subjects were deemed healthy based on medical history, physical examination, and complete blood count and serum chemistries. Persons with a history of renal, hepatic, gastrointestinal, cardiovascular, or psychiatric disease were excluded from the study, as were subjects with a history of clinical illness within 2 weeks of the start of their participation in the study. In addition, all subjects were medication free, including over-the-counter agents, for at least 3 days before the study (hormonal contraceptive medications were permitted). This crossover study consisted of three phases, and each subject participated in all three phases. Phase I was always conducted first with each subject, and the sequence of phase II and phase III studies were conducted in a randomized manner. Female subjects had to test negative in pregnancy tests before participation in each phase of the study. A washout period of at least 5 days was allowed between each phase of the study.

**Phase I.** The duodenal biopsy samples for the measurement of mRNA expression levels for subsequent gene correlations were obtained in this phase. The studies in phase I also involved the estimation of jejunal valacyclovir and acyclovir permeability using a regional perfusion technique, the results of which will be reported elsewhere. Briefly, following a 10-h overnight fast, subjects were admitted to the General Clinic Research Center at the University of Michigan Medical Center at 7 AM on the day of the study and fed a standard breakfast over the next half-hour. The subjects remained fasted for the duration of the study, approximately 14 h. The intubation and placement of the perfusion tube in the upper jejunum was performed according to the procedure described previously (Takamatsu et al., 1997). Briefly, esophago-gastroduodenoscopy was performed to facilitate the passage of a fiberoptic endoscope to the upper duodenum of the small intestine. Ten biopsy samples of approximately 5 mg each were then obtained from the duodenal mucosa using the forceps at the tip of the endoscope. The biopsy specimens were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA was processed for microarray analysis.

**Phases II and III.** Phases II and III of the study involved the estimation of acyclovir pharmacokinetics following oral valacyclovir and acyclovir administration, respectively. Briefly, following a 10-h overnight fast, subjects were admitted to the General Clinic Research Center at the University of Michigan Medical Center on the day of the study at 7 am. Subsequently, a single dose of either 500 mg of Valtrex (phase II) or 400 mg of Zovirax (phase III) was orally administered with 180 ml of water. Blood samples for measurement

of acyclovir and valacyclovir plasma concentrations were obtained at specified times. The subjects were fed a standard meal 4 and 10 h following drug administration.

**Collection of Blood Samples and Drug Analysis.** Blood samples were obtained through a forearm venous catheter for multiple blood draws and placed in heparinized Vacutainer vials (BD Biosciences). In phase II studies, 10-ml samples were withdrawn at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, and 4 h, followed by 5-ml samples at 6, 8, 10, and 12 h. In phase III studies, 5-ml samples were obtained at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 4, 6, 8, 10, and 12 h. The blood samples were immediately centrifuged at 3000 rpm for 5 to 10 min at 4°C. Plasma was removed, snap frozen in liquid nitrogen, and immediately stored at -80°C until further analysis.

The acyclovir and valacyclovir concentrations in plasma samples were simultaneously assayed by HPLC. The HPLC system consisted of a Waters interface module system, a Waters WISP 712 Autosampler, a Waters 996 photodiode array detector, and a Waters HPLC 515 pump (Waters, Milford, MA). The reversed-phase column used was an Ultrasphere ODS-1 (5  $\mu\text{m}$ , 250  $\times$  4.6 mm; Beckman Coulter, Inc., Fullerton, CA) column equipped with a guard column. The mobile phase used was 25 mM sodium acetate buffer, pH 3.5, containing 4.5% (v/v) acetonitrile. The flow rate used was 1 ml/min, and the UV detection wavelength was set at 254 nm. The HPLC system was controlled with Waters Millennium software (Version 3.0.1; Waters). Assays of plasma samples were carried out as follows. In a typical assay, plasma samples were thawed at room temperature and 0.5 ml of 20% (v/v) trifluoroacetic acid in water was added to 1 ml of plasma in an Eppendorf tube. The mixture was vortexed for 1 min and centrifuged at 12,500 rpm and 4°C for 15 min. The supernatant was filtered using a 0.45- $\mu\text{m}$  filter cartridge, and 100  $\mu\text{l}$  of the filtered supernatant was injected directly onto the column for HPLC analyses. The retention times were  $\sim$ 5 and  $\sim$ 10 min for acyclovir and valacyclovir, respectively. Standard curves using solutions of acyclovir and valacyclovir in distilled water were constructed over the concentration range of 0.3 to 300  $\mu\text{M}$  and were found to be linear ( $r^2 > 0.999$ ). Additionally, plasma blanks spiked with known acyclovir and valacyclovir standards were subjected to the extraction procedure described above and assayed to determine extraction efficiency. The recovery was greater than 98% over the concentration range of 0.3 to 80  $\mu\text{M}$  for both acyclovir and valacyclovir. All samples were assayed in triplicate. The limit of quantitation was set at the lowest concentration of 0.3  $\mu\text{M}$  ( $\sim$ 0.07  $\mu\text{g/ml}$ ) used in the standard curve. The limit of detection was  $\sim$  0.1  $\mu\text{M}$  (0.02  $\mu\text{g/ml}$ ).

**GeneChip Analysis.** The human duodenal samples were prepared as described earlier (Sun et al., 2002). Briefly, the tissue samples were homogenized in TRIzol, and total RNA was isolated. From the total RNA, cDNA was made and then converted back to biotin labeled cRNA. The biotin-labeled cRNA was fragmented and hybridized along with controls (Bio B, C, D, and Cre) to the U95A GeneChip (Affymetrix). The GeneChip<sup>®</sup> was then washed and stained with streptavidin phycoerythrin solution. After washing, the GeneChip was scanned with a laser scanner (Affymetrix). The gene expression profiles were analyzed by Affymetrix Microarray Suite and Data Mining Tool software.

**Semiquantitative RT-PCR Analysis.** For RT-PCR, total RNA from the tissue and Caco-2 cell samples was purified

using TRIzol reagent. One microgram of total RNA from each sample was subjected to RT-PCR (PCR access system; Promega, Madison, WI) using PEPT1- and HPT1-specific primers. The PEPT1 RT-PCR assay was performed as described previously (Sun et al., 2002). The HPT1 assay was done using the forward primer (CATAGAAGTGAAGGACA) and the reverse primer (GATGGGGATCTGATCATTG). The first-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase at 48°C for 45 min. This was followed by a 2-min cycle at 94°C to inactivate avian myeloblastosis virus reverse transcriptase and to denature the primers and cDNA. The PCR was performed for 25 cycles of 94°C for 30 s, primer annealing for 1 min at 55°C, extension at 68°C for 1 min, and a final extension at 68°C for 7 min. The conditions were established to obtain linear amplification of PCR product. The expected HPT1 PCR fragment was  $\sim$ 1 kilobase. The reaction mixture was separated on a 4 to 20% Tris borate-EDTA-polyacrylamide gel (Invitrogen) and visualized with SYBR Green nucleic acid gel stain (Molecular Probes, Eugene, OR).

**Transfection of HPT1 into HeLa Cells.** HeLa cells were cultured in Dulbecco's modified Eagle's medium with high glucose supplemented with 1% nonessential amino acid, 1% L-glutamine, 1% sodium pyruvate, and 10% fetal bovine serum. Cells were plated onto a 12-well plate (Falcon, Cowley, UK) for 24 h before transfection. Transfection was performed after the cells reached 50 to 70% confluence. The HPT1/pcDNA3.0 construct (a gift of Eli Lilly and Company, Indianapolis, IN) was transfected into the cells using Fugene reagent (Roche, Indianapolis, IN) after incubating the cells with Fugene/DNA complex (3:1) in Dulbecco modified Eagle medium with 10% fetal bovine serum for 48 h before functional assay.

**[<sup>3</sup>H]Valacyclovir Uptake Studies in HPT1 Transfected HeLa Cells.** After a 48-h transfection, cells were washed twice with transport buffer (pH 6, 1 mM CaCl<sub>2</sub>, 1 mol/l MgCl<sub>2</sub>, 150 mM NaCl, 3 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM D-Glucose, 5 mM MES) and incubated with 10  $\mu\text{M}$  valacyclovir (9.80  $\mu\text{M}$  valacyclovir and 0.20  $\mu\text{M}$  [<sup>3</sup>H]valacyclovir) in 1 ml transport buffer for 30 min at room temperature. After 30 min, the uptake was stopped by the addition of 0.5 ml of ice-cold transport buffer. Cells were washed 3 times with ice-cold transporter buffer, collected in 0.5 ml of 1.5% Triton X-100, and sonicated 3 times for 10 s. Sonicated cell suspension (200  $\mu\text{l}$ ) was used for scintillation counting, and the remaining sample was saved for protein assay.

## Results

**Acyclovir Pharmacokinetic Parameters after Oral Administration of Acyclovir and Valacyclovir.** The relevant pharmacokinetic parameters calculated using noncompartmental analyses of plasma-time curves following oral acyclovir and valacyclovir administration are listed in Tables 1 and 2, respectively. The parameters shown in Tables 1 and 2 were not dose-normalized. Maximum plasma concentrations ( $C_{\text{max}}$ ) were obtained from the observed plasma concentration-time profiles. The finite and infinite areas under the acyclovir plasma concentration-time curves,  $\text{AUC}_{0-\text{last}}$  and  $\text{AUC}_{0-\text{inf}}$ , respectively, were determined by a trapezoidal method with extrapolation. The valacyclovir levels in all plasma samples were below the detection limit (0.1  $\mu\text{M}$ ) at all

TABLE 1

Acyclovir pharmacokinetic parameters following oral administration of 400 mg acyclovir to humans ( $n = 11$ )

| Subject | $C_{\max}$              | $AUC_{0-\text{last}}$                  | $AUC_{0-\text{inf}}$ |
|---------|-------------------------|--|----------------------|
|         | $\mu\text{g}/\text{ml}$ | $\mu\text{g} \cdot \text{h}/\text{ml}$ |                      |
| 5100    | 0.38                    | 2.19                                   | 2.57                 |
| 5102    | 0.51                    | 2.65                                   | 3.59                 |
| 5104    | 0.29                    | 1.57                                   | 2.26                 |
| 5106    | 0.49                    | 2.22                                   | 2.66                 |
| 5107    | 0.51                    | 2.35                                   | 2.40                 |
| 5108    | 0.76                    | 2.89                                   | 3.02                 |
| 5109    | 0.26                    | 1.22                                   | 1.52                 |
| 5110    | 0.41                    | 2.09                                   | 2.21                 |
| 5111    | 0.58                    | 2.25                                   | 2.35                 |
| 5112    | 0.43                    | 1.96                                   | 2.21                 |
| 5115    | 0.53                    | 1.00                                   | 1.09                 |
| Mean    | 0.47                    | 2.04                                   | 2.35                 |
| % CV    | 30.00                   | 28.00                                  | 28.00                |

TABLE 2

Acyclovir pharmacokinetic parameters following oral administration of 500 mg valacyclovir to humans ( $n = 10$ )

| Subject | $C_{\max}$              | $AUC_{0-\text{last}}$                  | $AUC_{0-\text{inf}}$ |
|---------|-------------------------|--|----------------------|
|         | $\mu\text{g}/\text{ml}$ | $\mu\text{g} \cdot \text{h}/\text{ml}$ |                      |
| 5100    | 1.79                    | 6.98                                   | 7.33                 |
| 5102    | 2.37                    | 8.62                                   | 9.12                 |
| 5104    | 2.31                    | 8.95                                   | 9.70                 |
| 5106    | 2.80                    | 7.13                                   | 7.68                 |
| 5107    | 3.68                    | 12.00                                  | 12.20                |
| 5108    | 3.86                    | 11.70                                  | 12.10                |
| 5109    | — <sup>a</sup>          | — <sup>a</sup>                         | — <sup>a</sup>       |
| 5110    | 2.37                    | 9.15                                   | 9.96                 |
| 5111    | 3.17                    | 8.72                                   | 9.98                 |
| 5112    | 1.72                    | 10.00                                  | 10.40                |
| 5115    | 3.47                    | 11.10                                  | 12.40                |
| Mean    | 2.75                    | 9.44                                   | 10.09                |
| % CV    | 28.00                   | 19.00                                  | 18.00                |

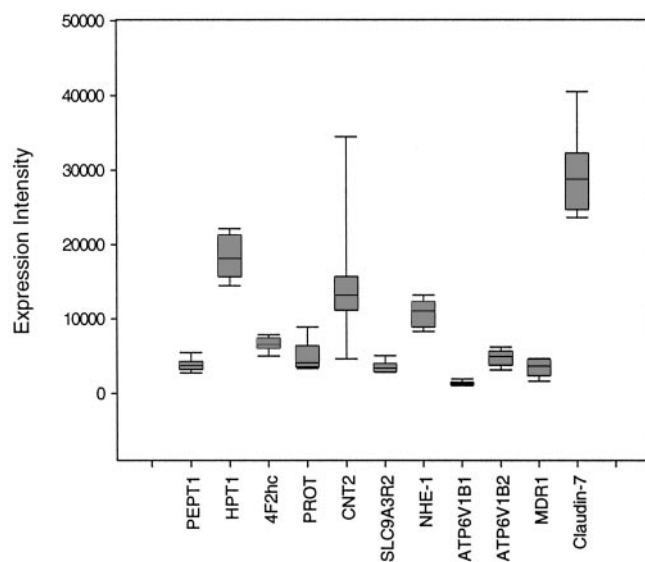
<sup>a</sup> Subject did not participate in this Phase.

time points. Peak plasma acyclovir levels were 4- to 6-fold higher following oral valacyclovir administration. AUC values were about 4-fold higher following oral valacyclovir administration compared with that obtained after acyclovir administration. It is also seen from Tables 1 and 2 that the variability associated with the pharmacokinetic parameters is slightly lower after valacyclovir oral administration compared with acyclovir administration. A detailed evaluation of the single-dose pharmacokinetics of acyclovir following oral acyclovir and valacyclovir administration will be described elsewhere (S. S. Menon C. Ramachandran, D. R. Foster, L. S. Welage, J. L. Barnett, and L. Amidon, submitted for publication).

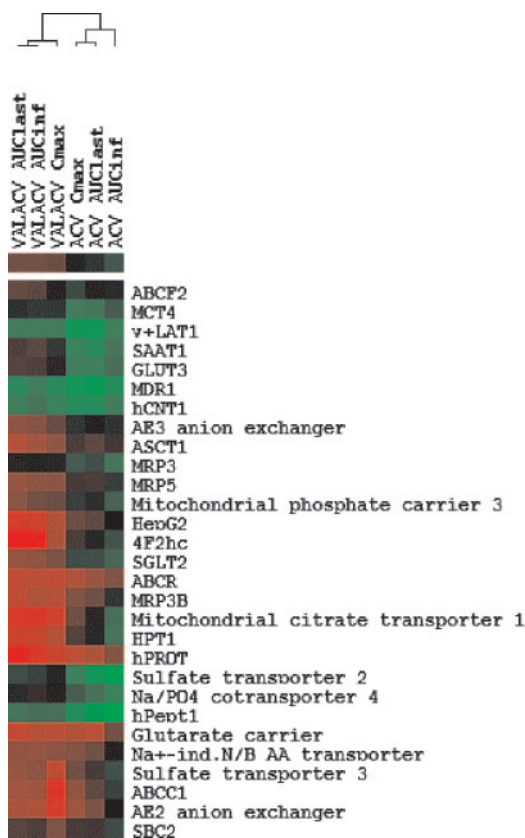
**Intestinal mRNA Expression and Variability of Selected Genes.** The mRNA expression data for 12,559 gene sequences from biopsy samples of the 10 subjects (excluding subject 5,115), assayed using GeneChip expression analysis and reported previously (Sun et al., 2002), were used in this correlation study. A narrower list of 281 transporters, channels, and metabolizing enzymes was selected based on the expression levels in the tissues. The variability in expression levels with the 10 subjects observed with the 281 genes was in the range of 5 to 148%, with an average of 37%. The average variability in expression levels of various classes of genes were as follows: transporters only, 33%; channels/exchangers only, 33%; and enzymes only, 38%. The mRNA expression intensities and variabilities of selected transport-

ers (peptide, amino acid, and nucleoside), ion exchangers, and intestinally related genes are shown in Fig. 1. Of the intestinal peptide transporters, the average HPT1 expression level in human duodenum was 4.5-fold higher than the average PEPT1 expression level. Furthermore, the variability in PEPT1 expression levels (25%) was lower than the average variability in expression levels of all transporters in the set (33%), whereas the variability in HPT1 expression levels was even lower (14%). The highest variability in expression levels of solute transporters expressed in the duodenum was found with the purine nucleoside transporter CNT2 (54%), whereas the sodium/glucose cotransporter SGLT1 exhibited the lowest variability (11%).

**Correlations of Gene Expression Levels in Duodenal Biopsies with Acyclovir Pharmacokinetic Parameters following Oral Administration of Valacyclovir and Acyclovir.** To identify transporters that may potentially contribute to valacyclovir or acyclovir absorption, linear correlations between the microarray expression profiles determined from duodenal biopsy samples and the corresponding pharmacokinetic parameters from the same individual were determined. The correlation parameters are summarized in a cluster diagram (Fig. 2). The areas in red denote the existence of positive correlations, whereas those in green represent negative correlations. Areas in black in the cluster diagram indicate lack of any correlation between the two parameters of interest. Positive correlations (red areas) between valacyclovir-associated pharmacokinetic parameters and several solute transporters are evident (Fig. 2). Curiously, the PEPT1 expression levels correlated poorly and negatively with valacyclovir pharmacokinetic parameters ( $r = -0.147$ ,  $p = 0.710$  with  $AUC_{0-\text{last}}$ ;  $r = -0.132$ ,  $p = 0.740$  with  $AUC_{0-\text{inf}}$ ;  $r = -0.589$ ,  $p = 0.095$  with  $C_{\max}$ ). On the



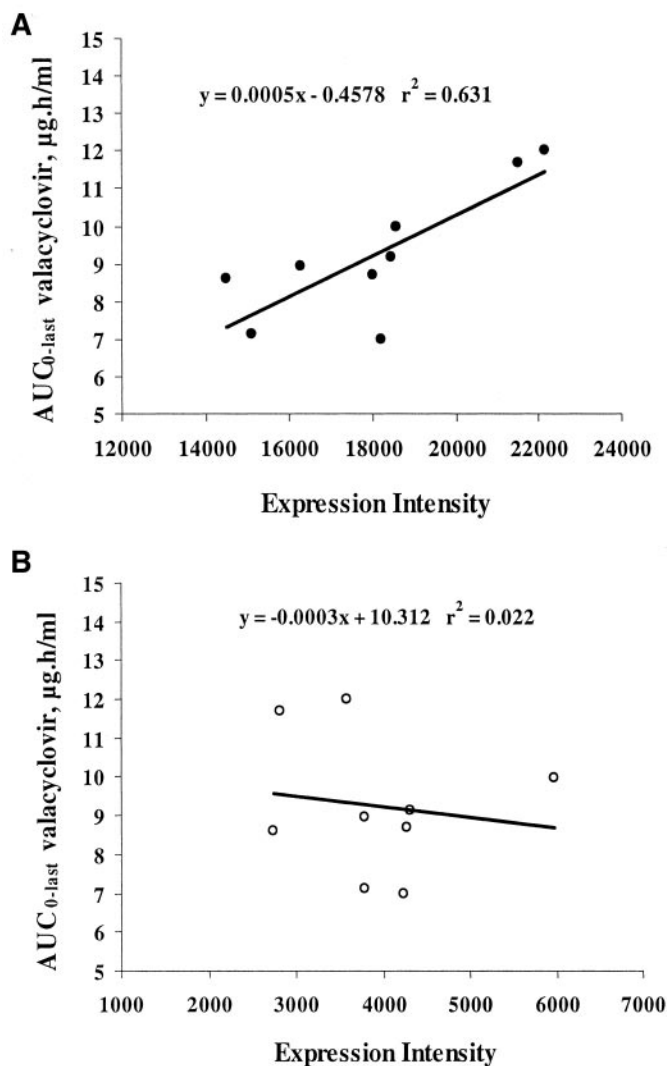
**Fig. 1.** Variability and expression of selected transporters, ion exchange proteins, ATPases, and intestinal protein genes in human duodenal biopsies ( $n = 10$ ). Shaded box indicates 25 to 75% of expression range, the line within the box marks the median, and error bars indicate 10 to 90% of expression range. PEPT1 (di-/tripeptide transporter); HPT1 (LI-cadherin; peptide transporter); 4F2hc (activator of dibasic and neutral amino acid transport); PROT (L-proline transporter); CNT2 (concentrative purine nucleoside transporter); SLC9A3R2 ( $\text{Na}^+/\text{H}^+$  exchanger protein); NHE-1 (SLC9A1; sodium/hydrogen exchanger); ATP6V1B1 and ATP6V1B2 (proton transporting ATPases); MDR1 (P-gp); Claudin-7 (tight junction protein).



**Fig. 2.** Cluster diagram of correlation of transporter expression levels with AUC values and  $C_{\max}$  following oral administration of valacyclovir ( $n = 9$ ) or of acyclovir ( $n = 10$ ). Areas in red denote positive correlations; green colored areas indicate negative correlations and black areas indicate absence of any correlation.

other hand, positive and significant correlations were observed between AUC values following valacyclovir oral administration and the expression levels of HPT1 peptide transporter ( $r = 0.794$ ,  $p = 0.011$  with  $AUC_{0-1\text{last}}$ ;  $r = 0.766$ ,  $p = 0.016$  with  $AUC_{0-\text{inf}}$ ). The linear correlations of HPT1 and PEPT1 expression levels with  $AUC_{0-1\text{last}}$  following oral valacyclovir administration are shown in Fig. 3. The highest positive linear correlations of valacyclovir parameters were observed with the expression levels of 4F2hc, a membrane glycoprotein ( $r = 0.875$ ,  $p = 0.002$  with  $AUC_{0-\text{inf}}$ ), and with proline transporter, an amino acid transporter ( $r = 0.857$ ,  $p = 0.003$  with  $AUC_{0-1\text{last}}$ ). A linear correlation plot of 4F2hc expression levels with  $AUC_{0-1\text{last}}$  after valacyclovir oral administration is shown in Fig. 4. No positive correlations were found involving the valacyclovir-associated pharmacokinetic parameters and organic cation transporter (OCT1 and OCT2) expression levels or with a variety of organic anion transporters.

Positive correlations were observed between valacyclovir-related pharmacokinetic parameters and ion channel and exchanger expression levels. Although such genes are not expected to be involved in direct valacyclovir transport, the ion gradients generated could potentially influence ion-coupled transporters. Figure 5 shows the linear correlations in a cluster diagram. It was found that the expression levels of the  $\text{Na}^+/\text{H}^+$  exchanger gene, NHE-1, exhibited a better positive correlation with  $AUC_{0-1\text{last}}$  following valacyclovir administration ( $r = 0.680$ ,  $p = 0.044$ ) (Fig. 4) compared with



**Fig. 3.** A, correlation of expression intensities of HPT1 in human duodenal biopsies with  $AUC_{0-1\text{last}}$  following oral administration of valacyclovir ( $n = 9$ ). B, correlation of expression intensities of PEPT1 in human duodenal biopsies with  $AUC_{0-1\text{last}}$  following oral administration of valacyclovir ( $n = 9$ ).

that with  $AUC_{0-1\text{last}}$  ( $r = 0.230$ ,  $p = 0.050$ ) following acyclovir oral administration. A relatively high positive correlation was also observed between expression levels of SLC4A2, an ion exchange protein, and valacyclovir related pharmacokinetic parameters ( $r = 0.785$ ,  $p = 0.012$ , with  $C_{\max}$  and  $r = 0.600$ ,  $p = 0.088$  with  $AUC_{0-\text{inf}}$ ). There were a few other  $\text{Na}^+/\text{H}^+$  exchanger protein genes such as SLC9A3R2 that also exhibited similar correlations. It was also found that  $\text{Na}^+/\text{K}^+$ -ATPase proteins on the basolateral membrane may potentially be involved as well. Thus, the  $\text{Na}^+/\text{K}^+$ -ATPase $\beta$ 1 (ATP1 $\beta$ 1) and  $\beta$ 2 (ATP1 $\beta$ 2) subunit expression levels, especially the  $\beta$ 1 subunit, exhibited a positive correlation with valacyclovir pharmacokinetic parameters. The highest significant positive correlation observed was between the ATP6V1 $\beta$ 1 proton transporting ATPase expression levels and  $AUC_{0-1\text{last}}$  following valacyclovir oral administration ( $r = 0.780$ ,  $p = 0.013$ ) and is shown in Fig. 4.

The linear correlations of pharmacokinetic parameters following valacyclovir and acyclovir oral administration with expression levels of select metabolizing enzymes are shown

as a cluster diagram in Fig. 6. High negative correlations were obtained with expression levels of efflux proteins such as MDR1 and MRP2 (cMOAT) with pharmacokinetic param-

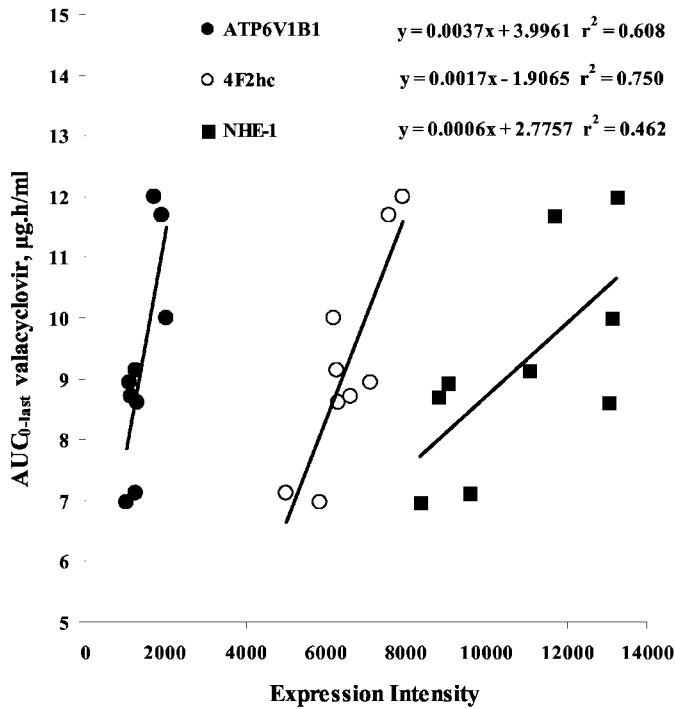


Fig. 4. Positive linear correlations of expression levels of select genes in human duodenum with  $AUC_{0-last}$  following oral valacyclovir administration ( $n = 9$ ).

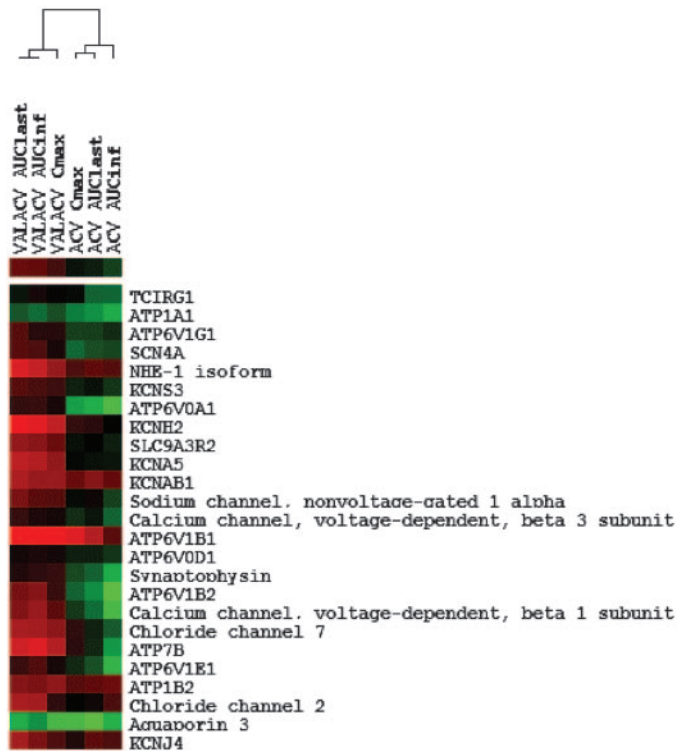


Fig. 5. Cluster diagram of correlations of expression levels of ion channels, exchanger proteins, and ATPases with AUC values and  $C_{max}$  following oral administration of valacyclovir ( $n = 9$ ), or of acyclovir ( $n = 10$ ). Color notation same as in Fig. 2.

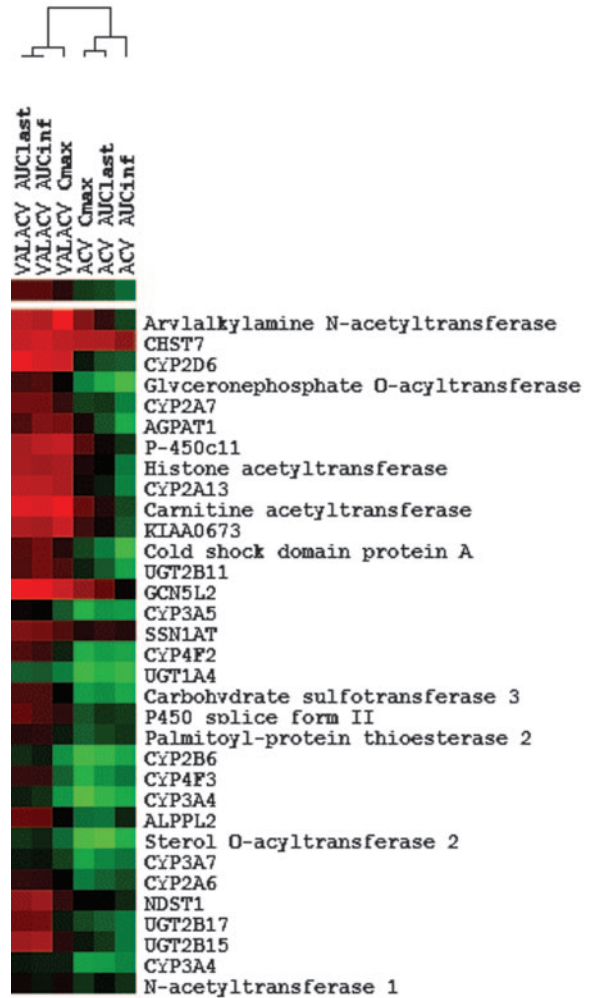
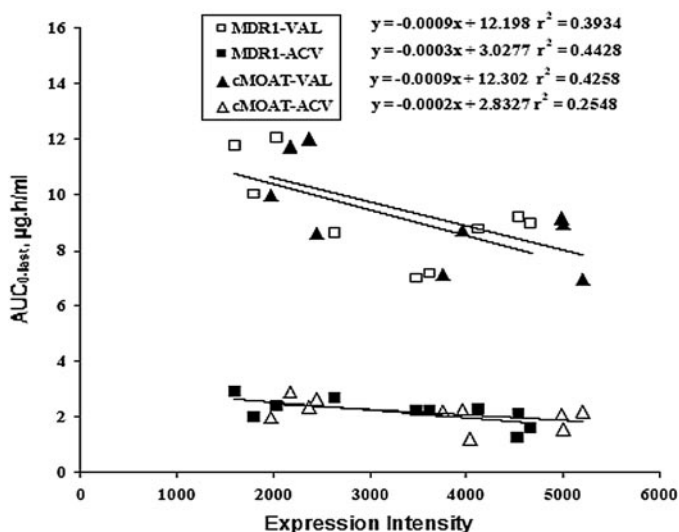


Fig. 6. Cluster diagram of correlations of expression levels of metabolizing enzymes with AUC values and  $C_{max}$  following oral administration of valacyclovir ( $n = 9$ ) or of acyclovir ( $n = 10$ ). Color notation same as in Fig. 2.

eters following either acyclovir or valacyclovir oral administration (Fig. 7). Similar high negative correlations were also observed with the cytochrome P450 IIIA subfamily metabolism enzymes ( $r$  values ranging from  $-0.6$  to  $-0.8$ ).

Linear correlations of pharmacokinetic parameters with expression levels of junction proteins and other intestinal proteins were also determined. The best positive correlations were between expression levels of the tight junction protein claudin-7, with  $AUC_{0-last}$  ( $r = 0.788$ ,  $p = 0.012$ ),  $AUC_{0-inf}$  ( $r = 0.708$ ,  $p = 0.033$ ), and  $C_{max}$  ( $r = 0.544$ ,  $p = 0.130$ ) following valacyclovir oral administration. There also appears to be a weak relationship of these valacyclovir-related pharmacokinetic parameters with the mucin protein secreted in the intestine.

The prominent positive correlation coefficients of gene expression levels with acyclovir pharmacokinetic parameters  $AUC_{0-last}$ ,  $AUC_{0-inf}$ , and  $C_{max}$  following oral valacyclovir administration are summarized in Table 3. There were few significant positive correlations between the acyclovir pharmacokinetic parameters and transporter expression. The moderate positive correlation of CNT2 (purine transporter) expression levels with  $AUC_{0-inf}$  ( $r = 0.602$ ,  $p = 0.065$ ) may be of interest.



**Fig. 7.** Negative linear correlations of expression intensities of select genes in human duodenum with  $AUC_{0-last}$  following oral acyclovir ( $n = 10$ ) or oral valacyclovir administration ( $n = 9$ ).

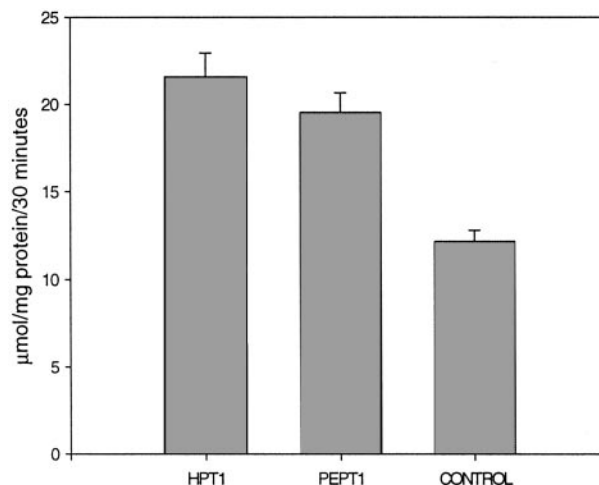
TABLE 3

Summary of correlation coefficients of expression levels of select genes and pharmacokinetic parameters following oral administration of valacyclovir to humans ( $n = 9$ )

| GI No.  | Gene      | $AUC_{0-last}$ | $AUC_{0-inf}$ | $C_{max}$ | $r$ or $p$ |
|---------|-----------|----------------|---------------|-----------|------------|
| 854174  | HPT1      | 0.794          | 0.766         | 0.555     | $r$ value  |
|         |           | 0.011          | 0.016         | 0.121     | $p$ value  |
| 1839269 | PROT      | 0.857          | 0.791         | 0.365     | $r$ value  |
|         |           | 0.003          | 0.011         | 0.334     | $p$ value  |
| 182864  | 4F2hc     | 0.866          | 0.875         | 0.613     | $r$ value  |
|         |           | 0.003          | 0.002         | 0.079     | $p$ value  |
| 1809029 | SLC4A2    | 0.572          | 0.600         | 0.785     | $r$ value  |
|         |           | 0.151          | 0.088         | 0.012     | $p$ value  |
| 1770309 | SLC25A1   | 0.584          | 0.618         | 0.845     | $r$ value  |
|         |           | 0.098          | 0.076         | 0.004     | $p$ value  |
| 544775  | NHE-1     | 0.680          | 0.610         | 0.179     | $r$ value  |
|         |           | 0.044          | 0.081         | 0.645     | $p$ value  |
| 190459  | ATP6V1B1  | 0.780          | 0.716         | 0.304     | $r$ value  |
|         |           | 0.013          | 0.300         | 0.427     | $p$ value  |
| 4128014 | Claudin-7 | 0.788          | 0.708         | 0.544     | $r$ value  |
|         |           | 0.012          | 0.033         | 0.130     | $p$ value  |

**RT-PCR Analysis.** The duodenal mRNA expression profiles obtained for PEPT1 and HPT1 from microarray data analyses were validated using semiquantitative RT-PCR. PEPT1 mRNA expression in the individual biopsies determined by RT-PCR exhibited a pattern similar to that observed with the microarray data ( $r^2 = 0.89$ ). HPT1 mRNA expression determined by RT-PCR was also found to parallel the expression pattern in the microarray data ( $r^2 = 0.80$ ).

**[ $^3H$ ]Valacyclovir Uptake by HPT1.** The [ $^3H$ ]valacyclovir uptake in transiently HPT1-expressing HeLa cells was compared with uptake in normal HeLa cells. The HPT1 mRNA expression in the transfected cells was enhanced compared with control HeLa cells (data not shown). The uptake experiment results are shown in Fig. 8. It is seen from Fig. 8 that the uptake of [ $^3H$ ]valacyclovir after a 30-min incubation period was  $\sim 1.8$ -fold higher ( $p < 0.05$ ) than that obtained with control HeLa cells. Figure 8 also shows the [ $^3H$ ]valacyclovir uptake results obtained with HeLa cells overexpressing PEPT1. The valacyclovir uptake was found to be  $\sim 1.6$ -fold ( $p < 0.05$ ) greater in HeLa cells overexpressing PEPT1 compared with that obtained in normal HeLa cells. The va-



**Fig. 8.** Direct uptake of [ $^3H$ ]valacyclovir in HeLa/HPT1 and HeLa/PEPT1 cells (uptake in HeLa cells overexpressing HPT1 or PEPT1 significantly higher compared with control HeLa cells;  $p < 0.05$ ).

lacyclovir uptake in HeLa/HPT1 cells and in HeLa/PEPT1 cells was not statistically different ( $p = 0.296$ ).

## Discussion

The immense potential of recent advances in genomic technologies to determine global intestinal expression of genes was used in this study to identify the contributions from various transporters, exchangers, and metabolizing enzymes to in vivo intestinal valacyclovir absorption in humans. The 3- to 5-fold enhanced acyclovir absorption following oral valacyclovir compared with the parent compound acyclovir observed in this study with healthy humans was consistent with previous studies (Weller et al., 1993; Soul-Lawton et al., 1995). The absence of detectable amounts of valacyclovir in plasma also suggests rapid conversion of valacyclovir to acyclovir following transport.

The significant positive linear correlations of absorption parameters following valacyclovir oral administration with expression levels of 4F2hc, a membrane glycoprotein, PROT, a proline transporter, and HPT1, a less widely examined peptide transporter that has been reported to be present in the human intestine (Dantzig et al., 1994; Yang, 1998; Yang et al., 1999), suggests their possible involvement in valacyclovir transport. The lack of positive linear correlations between valacyclovir pharmacokinetic parameters and PEPT1 expression levels (Figs. 2 and 3B) is rather surprising in light of previous studies that demonstrated dipeptide and valacyclovir transport by this oligopeptide transporter (Han et al., 1998a,b; Oh et al., 1999; Chu et al., 2001; Shin et al., 2003). The absence of significant positive correlations of valacyclovir absorption parameters with organic cation, organic anion, and nucleoside transporters strongly suggests that conclusions based on rat perfusion studies may not be tenable in humans. Although no direct evidence of valacyclovir transport by organic cation, organic anion, and nucleoside transporters has been reported, it appears that the contribution of these transporters and PEPT1 to valacyclovir transport and subsequent absorption may be negligible in vivo compared with that from HPT1.

HPT1 is an intestinal peptide transporter that was identified from Caco-2 membrane proteins and reported almost

simultaneously with the discovery of rabbit PEPT1 (Dantzig et al., 1994; Fei et al., 1994). HPT1, containing 832 amino acids with a reported mass  $\sim 120 \pm 10$  kDa, is apically expressed in Caco-2 cells and may contain one to six transmembrane domains (Hoffman and Stoffel, 1993; Dantzig et al., 1994). HPT1 and PEPT1 exhibit only 16% identity and 41% similarity in their amino acid sequences (Liang et al., 1995). The PEPT1 transporter has been extensively studied for its role in transporting a variety of peptides and peptidomimetic compounds (Oh and Amidon, 1999; Oh et al., 1999). This 708 amino acid transporter has been functionally expressed in a variety of cell systems including Chinese hamster ovary cells and HeLa cells (Covitz et al., 1996; Han et al., 1999; Surendran et al., 1999; Chu et al., 2001; Sun et al., 2001). It was demonstrated that PEPT1 in overexpressed cells transported several di- and tripeptides as well as a few peptidomimetic compounds but was not capable of transporting amino acids. These studies have established that PEPT1 is a proton-coupled, low-affinity, high-capacity transporter, with substrate  $K_m$  values in the millimolar range.

The expression of several oligopeptide transporters in human and rat gastrointestinal tracts and in Caco-2 cells obtained using RT-PCR and Southern blot analysis has recently been reported (Herrera-Ruiz et al., 2001). The authors found that PEPT1 was predominantly expressed in the human duodenum, with minimal expression in the jejunum and ileum. HPT1 expression, however, was significant in all regions of the gastrointestinal tract. In contrast, the authors found that the rat isoforms of PEPT1 and HPT1 were widely expressed throughout the rat gastrointestinal tract. The results reported by Herrera-Ruiz et al. (2001) are consistent with an earlier report of the discovery of rPEPT1 and rPT1 in rat intestine that were found to be evenly distributed in various small intestine regions (Erickson et al., 1995). Furthermore, Erickson et al. (1995) found that a high-protein diet induced a 1.5- to 2-fold increase in rPEPT1 and rPT1 mRNA expression in the mid and distal regions of intestine suggesting a role for the two transporters in peptide transport. Dantzig et al. (1994) also detected HPT1 protein along the entire human gastrointestinal tract. Sun et al. (2002) compared PEPT1 and HPT1 expression levels in Caco-2 cells with that in human duodenum using microarray analyses. These microarray results indicated that in differentiated Caco-2 cells PEPT1 expression levels were 45-fold lower than HPT1 expression levels. Interestingly, HPT1 levels in differentiated Caco-2 cells and in human duodenum were similar (Sun et al., 2002). The findings of Herrera-Ruiz et al. (2001) and of Dantzig et al. (1994) suggest that HPT1 may play an important role in peptide and peptidomimetic transport. Indeed, in Dantzig's pioneering study (Dantzig et al., 1994), up to 90% of cephalixin uptake in Caco-2 cells was attributed to HPT1. Additionally, the uptake of bestatin into Chinese hamster ovary/HPT1 cells has also been demonstrated (Dantzig et al., 1994). The HPT1 mediated uptake in the two cell systems was found to be proton dependent and inhibited by dipeptides. The active transport of cephalixin and *p*-hydroxyloracarbef into liposomes reconstituted with purified HPT1 protein further supports its capacity to transport peptidomimetic substrates independent of regulatory factors (Yang, 1998).

The PEPT1 and HPT1 expression levels in the human biopsy samples obtained from microarray data were vali-

dated with RT-PCR. The excellent correlation between the microarray and RT-PCR mRNA patterns indicates the reliability of the microarray analyses. The positive correlation observed between HPT1 expression and valacyclovir-related pharmacokinetics suggested that valacyclovir might be a HPT1 substrate. We therefore investigated this previously unreported relationship *in vitro*. The functionality of the HPT1 transporter in facilitating valacyclovir uptake was examined using HeLa cells that were transfected with a HPT1/pcDNA3.0 construct. Enhanced mRNA expression in the transfected cells compared with normal HeLa cells confirmed HPT1 overexpression in the transfected cells. The  $\sim 1.8$ -fold enhancement of [ $^3$ H]valacyclovir uptake in HeLa/HPT1 cells compared with the controls suggests quite clearly the ability of HPT1 to transport valacyclovir. The ability of PEPT1 to transport valacyclovir was determined as a positive control. The observed 1.6-fold enhancement of [ $^3$ H]valacyclovir uptake in HeLa cells overexpressing PEPT1 was comparable to that reported by Balimane et al. (1998). These results demonstrate that valacyclovir is a substrate for both transporters and that they appear to have similar valacyclovir transport abilities. Therefore, it is quite likely that *in vivo*, the much higher expression levels of HPT1 compared with PEPT1 may determine its predominance in valacyclovir transport.

Recently, the nonlinear absorption of valacyclovir as a function of dose was simulated using ACAT (GastroPlus) (Bolger et al., 2003). The authors found that a uniform transporter distribution predicted absorption better than one whose expression decreased aborally in the intestine. These modeling results also point to the possibility that valacyclovir absorption in humans might be influenced by HPT1. Besides PEPT1 and HPT1, the peptide transporters PTR3 and PHT1 are also known to be expressed in human intestine (Herrera-Ruiz et al., 2001). The expression levels of PTR3 and PHT1 were not determined in this study and their possible involvement in valacyclovir transport cannot be ruled out. The combined results presented here are consistent with suggestions that more than one peptide transporter may be involved in facilitating transport of peptides and peptidomimetics (Grauland and Sadee, 1997; Botka et al., 2000; Herrera-Ruiz et al., 2001).

In evaluating other potential factors that may contribute to valacyclovir absorption, we investigated the role of channels, exchangers, and metabolizing enzymes. The positive correlations observed between pharmacokinetic parameters and proton and ion exchanger expression levels may be the result of their modulating effects on the proton-dependence of the oligopeptide transporters. Thus, enhanced expression of  $\text{Na}^+/\text{H}^+$  exchangers such as NHE-1 and NHE-3 that reside on the apical enterocyte membrane could produce a larger proton gradient across the intestinal membrane and contribute to more active peptide transport (Thwaites et al., 2002). Similarly, ion channels and exchanger proteins that may not be directly involved in valacyclovir transport may contribute to ion gradient generation that could potentially influence the ion coupled transporters. For instance, oligopeptide transporters are proton cotransporters and require a proton gradient that is maintained by  $\text{Na}^+/\text{H}^+$  exchangers on the luminal membrane, whereas the  $\text{Na}^+/\text{K}^+$ -ATPases on the basolateral membrane regulate the cellular  $\text{Na}^+$  concentration. The significant negative correlations of pharmacokinetic parameters with expression levels of MDR1, MRP2

(cMOAT), and the cytochrome P450 IIIA subfamily member genes may indicate that these genes are involved in valacyclovir efflux and metabolism (Sandusky et al., 2002; Dantzig et al., 2003).

The overall absorption parameters of valacyclovir and acyclovir following oral administration undoubtedly are determined by several interdependent processes such as intestinal transport, gut, and liver metabolism, efflux, as well as secondary effects such as ion and pH gradients, and regulatory and transcription factors. The simple univariate correlation results of microarray expression analyses of human duodenal biopsies with absorption parameters following oral valacyclovir and acyclovir administration presented in this study are a first step toward understanding the roles and interdependence of these factors.

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