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

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ABBREVIATIONS: HPT1, human oligopeptide transporter; PEPT1, oligopeptide transporter; HeLa/HPT1, HeLa cells over-expressing HPT1; HeLa/PEPT1, HeLa cells over-expressing PEPT1; 4F2hc, cationic amino acid activator; AMV RT, avian myeloblastosis virus reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction; SAPE, streptavidin phycoerythrin; TRIzol, RNA purification reagent; HPT1/pcDNA3.0, pcDNA3.0 expression vector with HPT1 gene insert; CNT2, concentrative purine nucleoside transporter; OCT, organic cation transporter; OAT, organic anion transporter; HPLC, high-performance liquid chromatography; CHO/HPT1, CHO cells over-expressing HPT1

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. However, the role and importance of these transporters in modulating valacyclovir absorption in humans has not been determined. 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 report, 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 HPT1, an oligopeptide transporter expressed at higher levels in the human intestine compared to PEPT1. The validation of HPT1 microarray data with RT-PCR 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-gp) 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 to 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; Han *et al.*, 1998b; 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 (OAT's) and organic cation (OCT's) transporters may also play a role in intestinal valacyclovir uptake (Sinko and Balimane, 1999). However, the relative contribution of oligopeptide and other transporters in overall valacyclovir uptake in humans has not yet been reported.

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 contributed enormously to this endeavor. **However, such reliance on model systems may also lead to the implication of multiple transporters contributing simultaneously to overall intestinal transport. Further, 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 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 in order to identify plausible putative or novel mediators of drug transport and oral drug absorption in the intestinal tissues.

In this report, 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 in order to elicit correlations. Gene expression was analyzed using microarray expression technology

(Affymetrix GeneChip[®]) 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) 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 Gibco BRL (Grand Island, NY). BioArray high yield RNA transcript labeling kit was purchased from Enzo Biochem (New York, NY, USA). Genechips were purchased from Affymetrix (Santa Clara, CA). 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 Becton Dickinson Labware (Bedford, MA). All cell culture medium and reagents were from Gibco BRL (Grand Island, NY, USA). [³H]valacyclovir (sp. act. 5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). All solvents used were 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 21-36 years of age (29.0 ± 5.8 years) and were within 20% of their ideal body weight (75.7 ± 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 prior to 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 prior to 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-hour 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 one half-hour. The subjects remained fasted for the duration of the study, approximately 14 hours. 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 (EGD) 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°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-hour 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 hours and 10 hours 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 (Becton Dickinson, Rutherford, NJ). 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 4h, followed by 5 ml samples at 6, 8, 10, and 12 hours. 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 12h. The blood samples were immediately centrifuged at 3000 rpm for 5-10 minutes 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 high performance liquid chromatography (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. The reversed-phase column used was an Ultrasphere ODS-1 (5µ, 250 x 4.6 mm, Beckman) 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®32 software (Version 3.0.1; Waters Corporation, Milford, MA). Assay 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 minutes. The supernatant was filtered using a 0.45 µm filter cartridge and 100 µl of the filtered supernatant was injected directly onto the column for HPLC analyses. The retention times were ~5 mins and ~10 mins 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 µM 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 μM for both acyclovir and valacyclovir. All samples were assayed in triplicate. The limit of quantitation (LOQ) was set at the lowest concentration of 0.3 μM (~ 0.07 $\mu\text{g/ml}$) used in the standard curve. The limit of detection was ~ 0.1 μM (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[®] was then washed and stained with streptavidin phycoerythrin (SAPE) 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.

Semi-quantitative 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 previously 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 AMV reverse transcriptase (AMV RT) at 48°C for 45 min. This was followed by a 2 min cycle at 94°C to inactivate AMV RT 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 ~1 kb. The reaction mixture was separated on a 4%–20% TBE-polyacrylamide gel (Invitrogen, Carlsbad, CA) 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 modified Eagle medium (DMEM) with high glucose supplemented with 1% nonessential amino acid, 1% L-glutamine, 1% sodium pyruvate, and 10% fetal bovine serum (FBS). Cells were plated onto a 12-well plate (Falcon) for 24 hours before transfection. Transfection was performed after the cells reached 50%-70% confluence. The HPT1/pcDNA3.0 construct (gift of Eli Lilly & 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 DMEM medium with 10% FBS for 48 h before functional assay.

[³H]Valacyclovir uptake studies in HPT1 transfected HeLa cells

After a 48-hour transfection, cells were washed twice with transport buffer (pH 6, 1 mmol/L CaCl₂, 1 mol/L MgCl₂, 150 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L NaH₂PO₄, 5 mmol/L D-Glucose, 5 mmol/L MES) and incubated with 10 μmol/L valacyclovir (9.94 μmol/L valacyclovir and 0.06 μmol/L [³H]valacyclovir) in 1mL transport buffer for 30 minutes at room temperature. After 30 minutes, the uptake was

JPET #51011

stopped by the addition of 0.5 mL ice-cold transport buffer. Cells were washed 3 times with ice-cold transporter buffer, collected in 0.5 mL 1.5% Triton X-100, and sonicated 3 times for 10 seconds. 200 μ L of sonicated cell suspension 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 non-compartmental 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_{max}) were obtained from the observed plasma concentration-time profiles. The finite and infinite areas under the acyclovir plasma concentration-time curves, AUC_{0-last} and AUC_{0-inf} , respectively, were determined by a trapezoidal method with extrapolation. The valacyclovir levels in all plasma samples were below the detection limit ($0.1\mu M$) at all 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 to 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 to acyclovir administration. A detailed evaluation of the single-dose pharmacokinetics of acyclovir following oral acyclovir and valacyclovir administration will be described elsewhere (Menon et al., 2003; Single-dose pharmacokinetics and evaluation of time-dependent absorption of acyclovir after oral administration of acyclovir and valacyclovir to healthy human volunteers. Submitted to *Antimicrob Agents Chemother*).

Intestinal mRNA expression and variability of selected genes

The mRNA expression data for 12,559 gene sequences from biopsy samples of the ten subjects (excluding Subject 5115) assayed using GeneChip[®] expression analysis and reported previously (Sun et al., 2002) was 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 ten subjects observed with the 281 genes was in the range of 5-148%, 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 transporters (peptide, amino acid, and nucleoside), ion exchangers, and intestinally related genes are shown in Figure 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. Further, the variability in PEPT1 expression levels (25%) was lower than the average variability in expression levels of all transporters in the set (33%), while 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 co-transporter, 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

In order 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 (Figure 2). The areas in red denote the existence of positive correlations, while 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 (Figure 2). Curiously, the PEPT1 expression levels correlated poorly and negatively with valacyclovir pharmacokinetic parameters ($r = -0.147$, $p = 0.710$ with AUC_{0-last} ; $r = -0.132$, $p = 0.740$ with AUC_{0-inf} ; $r = -0.589$, $p = 0.095$ with C_{max}). On the other hand, positive and significant correlations were observed between AUC's following valacyclovir oral administration and the expression levels of HPT1 peptide transporter ($r = 0.794$, $p = 0.011$ with AUC_{0-last} ; $r = 0.766$, $p = 0.016$ with AUC_{0-inf}). The linear correlations of HPT1 and PEPT1 expression levels with AUC_{0-last} following oral valacyclovir administration are shown in Figure 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-inf}), and with PROT, an amino acid transporter ($r = 0.857$, $p = 0.003$ with AUC_{0-last}). A linear correlation plot of 4F2hc expression levels with AUC_{0-last} after valacyclovir oral administration is shown in Figure 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 Na^+/H^+ exchanger gene, NHE-1, exhibited a better positive correlation with $\text{AUC}_{0\text{-last}}$ following valacyclovir administration ($r = 0.680$, $p=0.044$) (Figure 4), compared to that with $\text{AUC}_{0\text{-last}}$ following acyclovir oral administration ($r = 0.230$, $p=0.050$). 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 $\text{AUC}_{0\text{-inf}}$). There were a few other Na^+/H^+ exchanger protein genes such as SLC9A3R2 that also exhibited similar correlations. It was also found that Na^+/K^+ -ATPase proteins on the basolateral membrane may potentially be involved as well. Thus, the Na^+/K^+ -ATPase beta 1 (ATP1B1) and beta 2 (ATP1B2) subunit expression levels, especially the beta1 subunit, exhibited a positive correlation with valacyclovir pharmacokinetic parameters. The highest significant positive correlation observed was between the ATP6V1B1 proton transporting ATPase expression levels and $\text{AUC}_{0\text{-last}}$ following valacyclovir oral administration ($r = 0.780$, $p = 0.013$) and is shown in Figure 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 Figure 6. High negative correlations were obtained with expression levels of efflux proteins such as MDR1 and MRP2 (cMOAT) with pharmacokinetic parameters following either acyclovir or valacyclovir oral administration (Figure 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} ($r = 0.788$, $p = 0.012$), and with AUC_{0-inf} ($r = 0.708$, $p = 0.033$), and with 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.

RT-PCR analysis

The duodenal mRNA expression profiles obtained for PEPT1 and HPT1 from microarray data analyses were validated using semi-quantitative 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 to uptake in normal HeLa cells. The HPT1 mRNA expression in the transfected cells was enhanced compared to control HeLa cells (data not shown). The uptake experiment results are shown in Figure 8. It is seen from Figure 8 that the uptake of [³H]valacyclovir after a 30-minute incubation period was ~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 over-expressing PEPT1. The valacyclovir uptake was found to be ~1.6-fold ($p < 0.05$) greater in HeLa cells over-expressing PEPT1 compared to that obtained in normal HeLa cells. The valacyclovir 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 utilized 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 to 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 (Figures 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; Han et al., 1998b; Oh et al., 1998; 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 to 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, 1999a; Oh et al., 1999b). 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 over-expressed cells transported several di- and tri-peptides 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. 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). Further, Erickson et al. 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. also detected HPT1 protein along the entire human gastrointestinal tract (Dantzig et al., 1994). Sun et al. compared PEPT1 and HPT1 expression levels in Caco-2 cells with that in human duodenum using microarray analyses (Sun et al., 2002). 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., and of Dantzig et al., 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 CHO/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 validated 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 to normal HeLa cells confirmed HPT1 over-expression in the transfected cells. The ~1.8-fold enhancement of [³H]valacyclovir uptake in HeLa/HPT1 cells compared to 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 [³H]valacyclovir uptake in HeLa cells over-expressing 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 to PEPT1 may determine its predominance in valacyclovir transport.**

Recently, the non-linear absorption of valacyclovir as a function of dose was simulated using ACAT (GastroPlus[®]) (Bolger et al., 2003 Accurate simulation of the nonlinear absorption of valacyclovir depends on a uniform small intestinal distribution of oligopeptide transporter. 1st International Conference on Molecular Biopharmaceutics, Waikiki, Hawaii, Poster #208). 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-dependency of the oligopeptide transporters. Thus, enhanced expression of Na^+/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 co-transporters and require a proton gradient that is maintained by Na^+/H^+ exchangers on the luminal membrane, while the Na^+/K^+ -ATPases on the basolateral membrane regulate the

cellular 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 towards understanding the roles and interdependence of these factors.

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REFERENCES

- Balimane PV, Tamai I, Guo A, Nakanishi T, Kitada H, Leibach FH, Tsuji A and Sinko PJ (1998) Direct evidence for peptide transporter (Pept1)-mediated uptake of a nonpeptide drug, valacyclovir. *Biochem Biophys Res Commun* **250**:246-251.
- Beauchamp LM and Krenitsky TA (1993) Acyclovir prodrugs: the road to valacyclovir. *Drugs Future* **18**:619-628. (1993)
- Beauchamp LM, Orr GF, de Miranda P, Burnette TC and Krenitsky TA (1992) Amino acid ester prodrugs of acyclovir. *Antiviral Chem Chemother* **3**:157-164.
- Botka CW, Wittig TW, Grauland RC, Nielsen CU, Higaka K, Amidon GL and Sadee W (2000) Human proton/oligopeptide transporter (POT) genes: identification of putative human genes using bioinformatics. *AAPS PharmSci* **2**:E16.
- Chu XY, Sanchez-Castano GP, Higaki K, Oh DM, Hsu CP and Amidon GL (2001) Correlation between epithelial cell permeability of cephalexin and expression of intestinal oligopeptide transporter. *J Pharmacol Exp Ther* **299**:575-582.
- Covitz KM, Amidon GL and Sadee W (1996) Human dipeptide transporter, hPEPT1, stably transfected into Chinese hamster ovary cells. *Pharm Res* **13**:1631-1634.
- Dantzig AH, Hoskins JA, Tabas LB, Bright S, Shepard RL, Jenkins IL, Duckworth DC, Sportsman JR, Mackensen D, Rosteck Jr. PR and Skatrud PL (1994) Association of intestinal peptide transport with a protein related to the cadherin superfamily. *Science* **264**:430-433.
- Dantzig AH, de Alwis DP and Burgess M (2003) Considerations in the design and development of transport inhibitors as adjuncts to drug therapy. *Adv Drug Deliv Revs* **55**:133-150.

- Erickson RH, Gum Jr. JR, Lindstrom MM, McKean D and Kim YS (1995) Regional expression and dietary regulation of rat small intestine peptide and amino acid transporter mRNAs. *Biochem Biophys Res Commun* **216**:249-257.
- Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Leibach FH, Romero MF, Singh SK, Boron WF, Hediger MA (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **368** :563-6.
- Grauland RC and Sadee W (1997) Sequence alignments of the H(+)-dependent oligopeptide transporter family PTR: inferences on structure and function of the intestinal PET1 transporter. *Pharm Res* **14**:388-400.
- Han H, de Vrueth RL, Rhie JK, Covitz KM, Smith PL, Lee CP, Oh DM, Sadee W and Amidon GL (1998a) 5'-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm Res* **15**:1154-1159.
- Han HK, Oh DM and Amidon GL (1998b) Cellular uptake mechanism of amino acid ester prodrugs in Caco-2/hPEPT1 cells overexpressing a human peptide transporter. *Pharm Res* **15**:1382-1386.
- Han HK, Rhie JK, Oh DM, Saito G, Hsu CP, Stewart BH and Amidon GL (1999) CHO/hPEPT1 cells overexpressing the human peptide transporter (hPEPT1) as an alternative in vitro model for peptidomimetic drugs. *J Pharm Sci* **88**:347-350.
- Herrera-Ruiz D, Wang Q, Gudmundsson OS, Cook TJ, Smith RL, Faria TN and Knipp GT (2001) Spatial expression patterns of peptide transporters in the human and rat gastrointestinal tracts, Caco-2 in vitro cell culture model, and multiple human tissues. *AAPS PharmSci* **3**:E9.

Hoffman K and Stoffel W (1993) TMbase-A database of membrane spanning protein segments. *Biol Chem Hoppe Seyler* **347**:166.

Liang R, Fei YJ, Prasad PD, Ramamoorthy S, Han H, Yang-Feng TL, Hediger MA, Ganapathy V and Leibach FH (1995) Human intestinal H⁺/peptide cotransporter. Cloning, functional expression, and chromosomal localization. *J Biol Chem* **270**:6456-6463.

Oh DM and Amidon GL (1999a) Overview of membrane transport. *Pharm Biotechnol* **12**:1-27.

Oh DM, Han HK and Amidon GL (1999b) Drug transport and targeting. Intestinal transport. *Pharm Biotechnol* **12**:59-88.

Sandusky GE, Mintze KS, Pratt SE and Dantzig AH (2002) Expression of multidrug resistance-associated protein 2 (MRP2) in normal human tissues and carcinomas using tissue microarrays. *Histopathology* **41**:65-74.

Shin HC, Landowski CP and Amidon GL (2003) Transporters in the GI tract, in *Drug bioavailability/estimation of solubility, permeability and absorption (Series: Methods and principles in Medicinal Chemistry)*, (Van de Waterbeemd H, Lennernas H and Artursson P eds), Wiley-VCH Verlag GmbH, Weinheim Germany. In press.

Sinko PJ and Balimane PV (1998) Carrier-mediated intestinal absorption of valacyclovir, the L-valyl ester prodrug of acyclovir: 1. Interactions with peptides, organic anions and organic cations in rats. *Biopharm Drug Dispos* **19**:209-217.

Smith C, Klein A and Zimmerman T (1993) Influx of valacyclovir into cynomolgous

- monkey intestinal brush border membranes is transporter mediated and enhanced over acyclovir. *33rd Interscience Conference on Antimicrobial Agents and Chemotherapy* Abstract 1705.
- Soul-Lawton J, Seaber E, On N, Wootton R, Rolan P and Posner J (1995) Absolute bioavailability and metabolic disposition of valciclovir, the L-valyl ester of acyclovir, following oral administration to humans. *Antimicrob Agents Chemother* **39**:2759-2764.
- Sun D, Landowski CP, Chu X, Wallsten R, Komorowski TE, Fleisher D and Amidon GL (2001) Drug inhibition of Gly-Sar uptake and hPepT1 localization using hPepT1-GFP fusion protein. *AAPS PharmSci* **3**:E2.
- Sun D, Lennernas H, Welage LS, Barnett JL, Landowski CP, Foster D, Fleisher D, Lee KD and Amidon GL (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. *Pharm Res* **19**:1400-1416.
- Surendran N, Covitz KM, Han H, Oh DM, Amidon GL, Williamson RM, Bigge CF and Stewart BH (1999) Evidence for overlapping substrate specificity between large neutral amino (LNAA) and dipeptide (hPEPT1) transporters for PD 158473, an NMDA antagonist. *Pharm Res* **16**:391-395.
- Takamatsu N, Welage LS, Idkaidek NM, Liu DY, Lee PI, Hayashi Y, Rhie JK, Lennernas H, Barnett JL, Shah VP, Lesko L and Amidon GL (1997) Human intestinal permeability of piroxicam, propranolol, phenylalanine, and PEG 400 determined by jejunal perfusion. *Pharm Res* **14**:1127-1132.
- Thwaites DT, Kennedy DJ, Raluda D, Anderson CM, Mendoza ME, Bladen CL and

- Simmons NL (2002) H/dipeptide absorption across the human intestinal epithelium is controlled indirectly via a functional Na/H exchanger. *Gastroenterology* **122**:1322-1333.
- Weller S, Blum MR, Doucette M, Burnette T, Cederberg DM, de Miranda P and Smiley ML (1993) Pharmacokinetics of the acyclovir pro-drug valaciclovir after escalating single- and multiple-dose administration to normal volunteers. *Clin Pharmacol Ther* **54**:595-605.
- Yang CY (1998) Studies on the human intestinal di-/tri-peptide transporter HPT-1 as a potential carrier for orally administered drugs. Ph.D. Thesis, Purdue University, West Lafayette, Indiana.
- Yang CY, Dantzig AH and Pigeon C (1999) Intestinal peptide transport systems and oral drug availability. *Pharm Res* **16**:1331-1343.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Variability and expression of selected transporters, ion exchange proteins, ATPases and intestinal protein genes in human duodenal biopsies. (n=10). Shaded box indicates 25–75% of expression range, the line within the box marks the median, and error bars indicate 10–90% of expression range. PEPT1 (di-/tri-peptide 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 (Na⁺/H⁺ exchanger protein); NHE-1 (SLC9A1; sodium/hydrogen exchanger); ATP6V1B1 and ATP6V1B2 (proton transporting ATPases); MDR1 (P-gp); Claudin-7 (tight junction protein).

Figure 2. Cluster diagram of correlation of transporter expression levels with AUC's 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.

Figure 3a. Correlation of expression intensities of HPT1 in human duodenal biopsies with AUC_{0-last} following oral administration of valacyclovir. (n=9).

Figure 3b. Correlation of expression intensities of PEPT1 in human duodenal biopsies with AUC_{0-last} following oral administration of valacyclovir. (n=9).

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

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

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

Figure 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).

Figure 8. Direct uptake of [3H]valacyclovir in HeLa/HPT1 and HeLa/PEPT1 cells. (Uptake in HeLa cells over-expressing HPT1 or PEPT1 significantly higher compared to control HeLa cells; $p < 0.05$).

Table 1. Acyclovir pharmacokinetic parameters following oral administration of 400 mg acyclovir to humans (n=11).

Subject	C_{max} (µg/ml)	AUC_{0-last} (µg.h/ml)	AUC_{0-inf} (µg.h/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	28	28

Table 2. Acyclovir pharmacokinetic parameters following oral administration of 500 mg valacyclovir to humans (n=10).

Subject	C_{max} (µg/ml)	AUC_{0-last} (µg.h/ml)	AUC_{0-inf} (µg.h/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	a	a	a
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	19	18

^a Subject did not participate in this Phase.

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 #	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

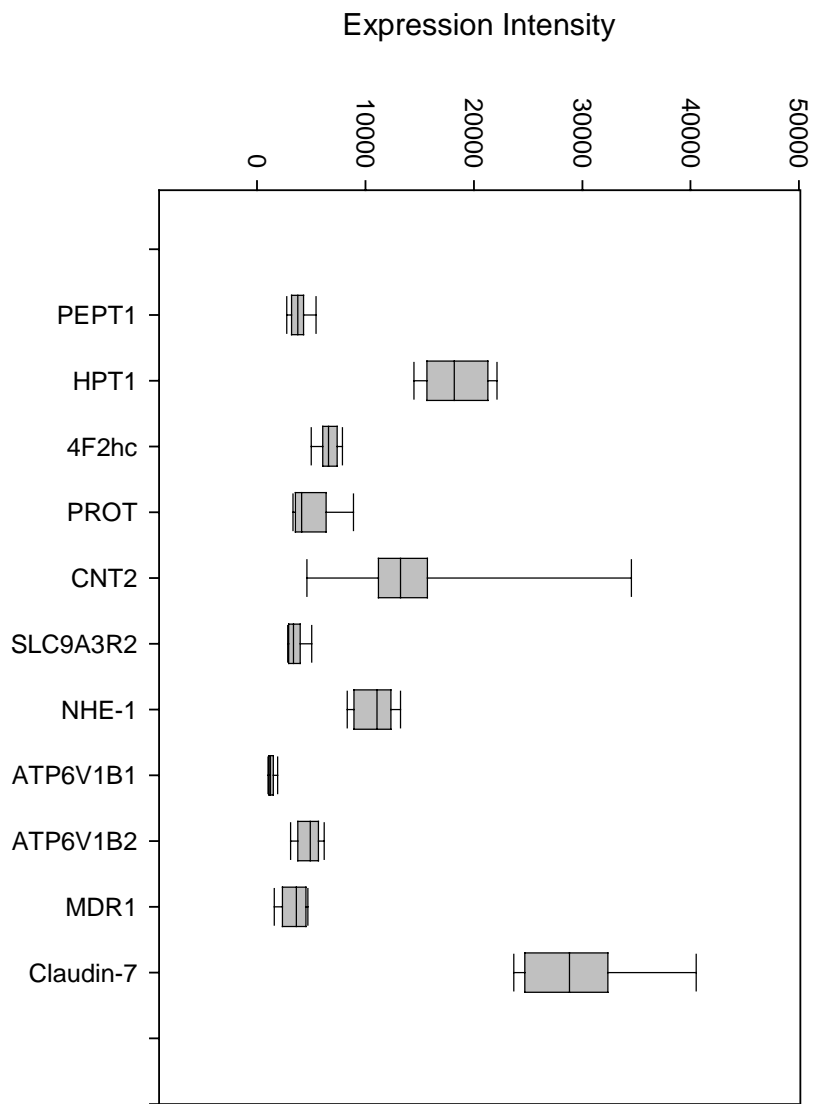


Figure 1.

Figure 2.

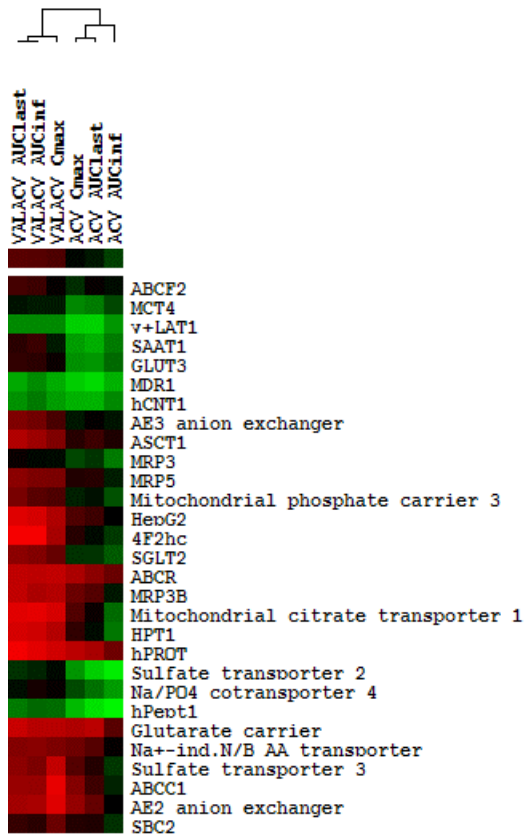


Figure 3a.

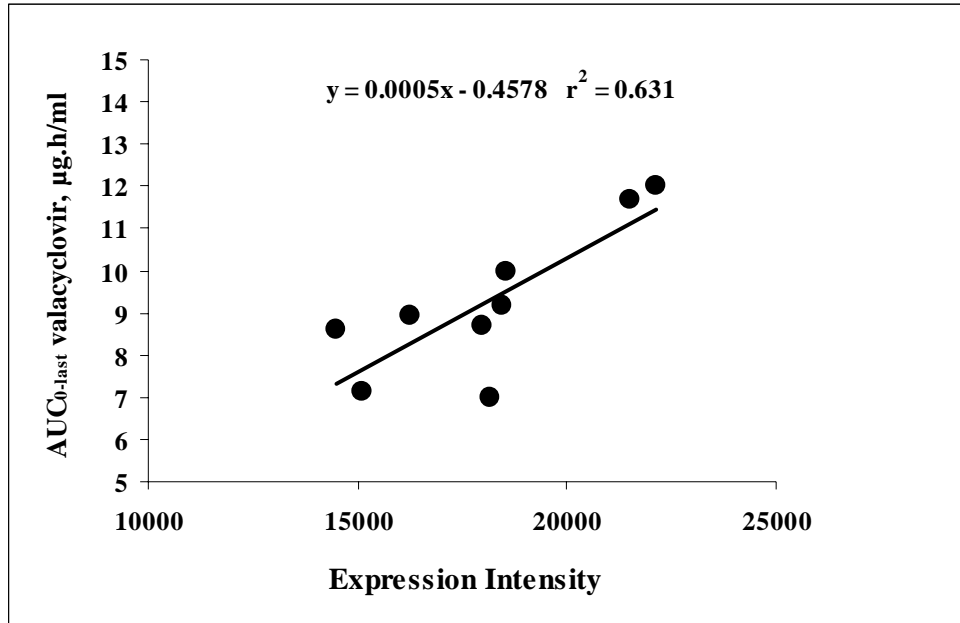


Figure 3b.

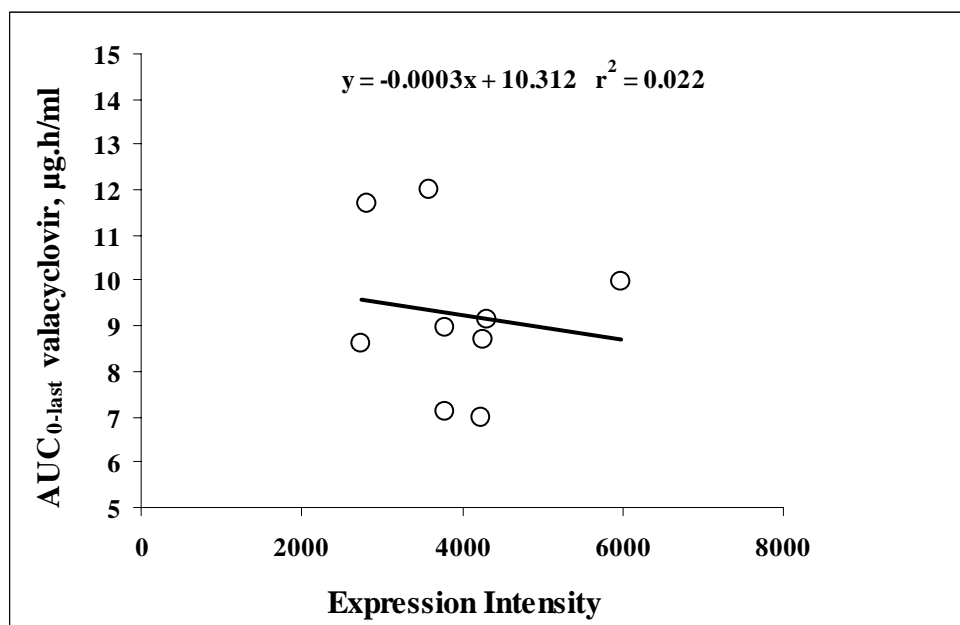


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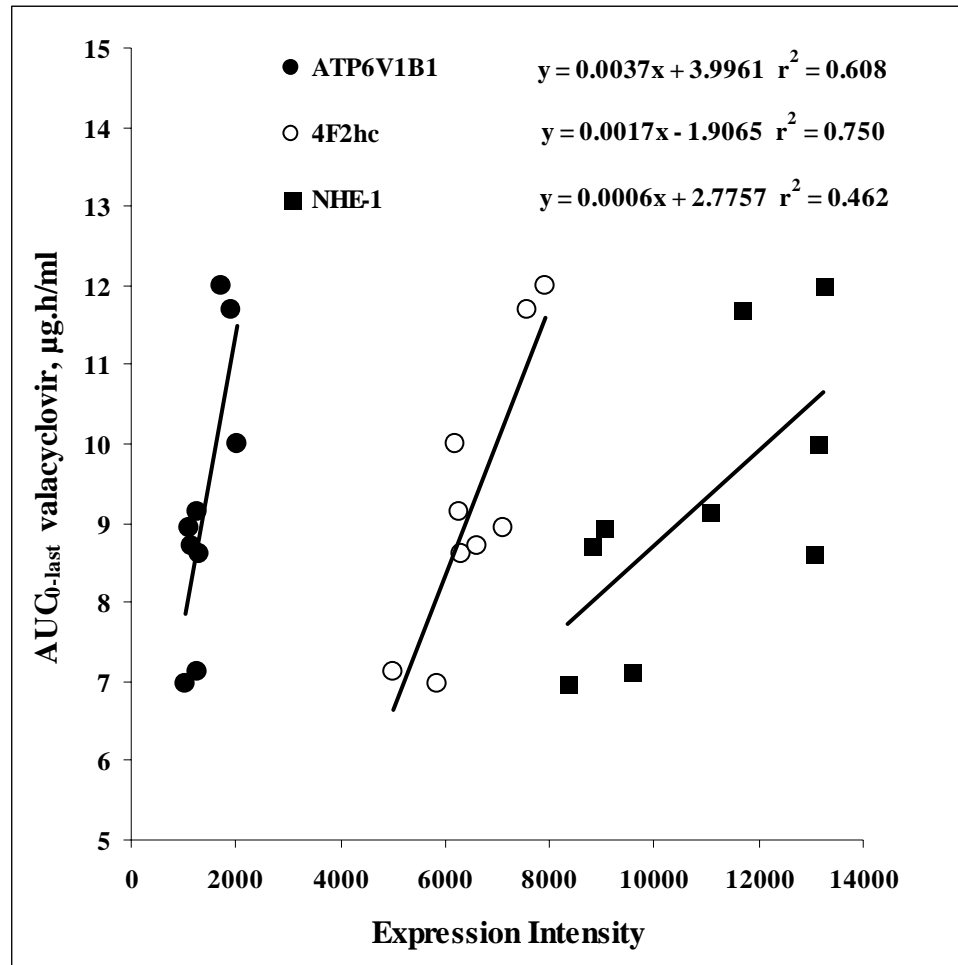


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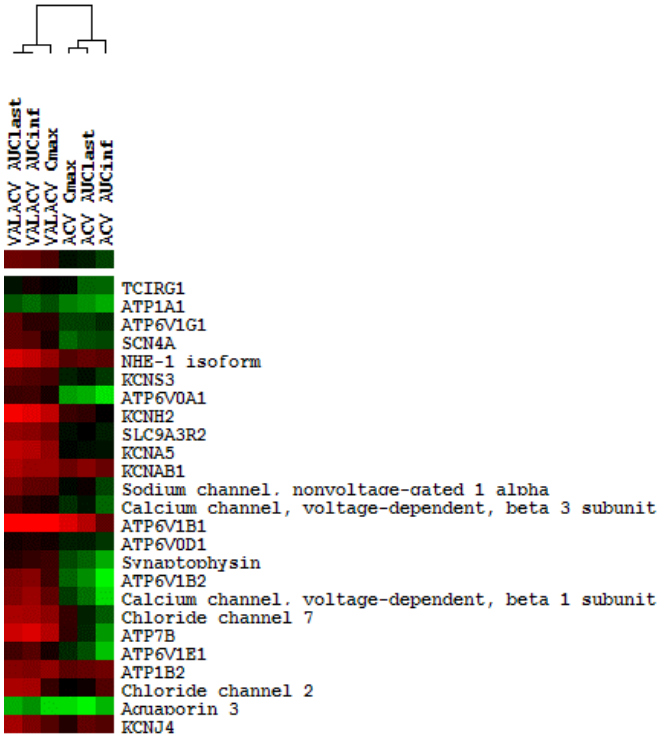


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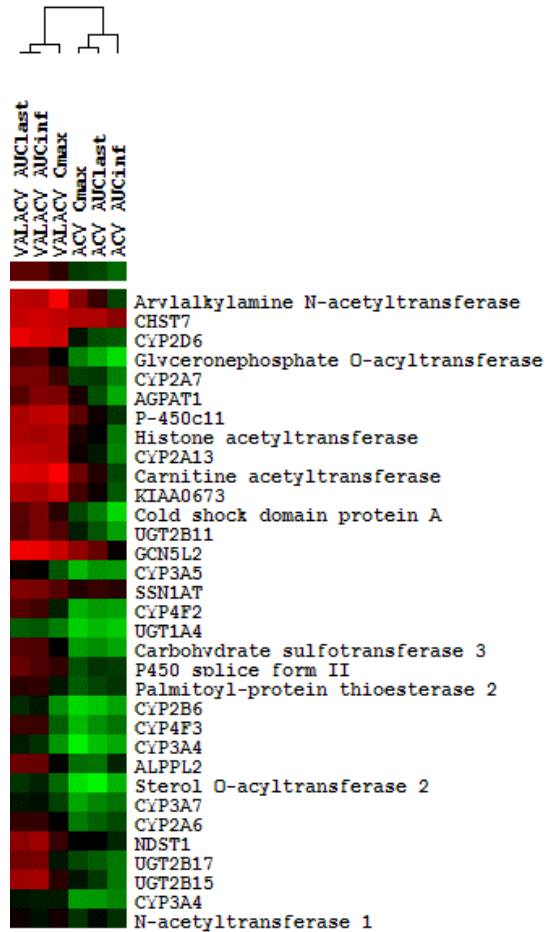


Figure 7.

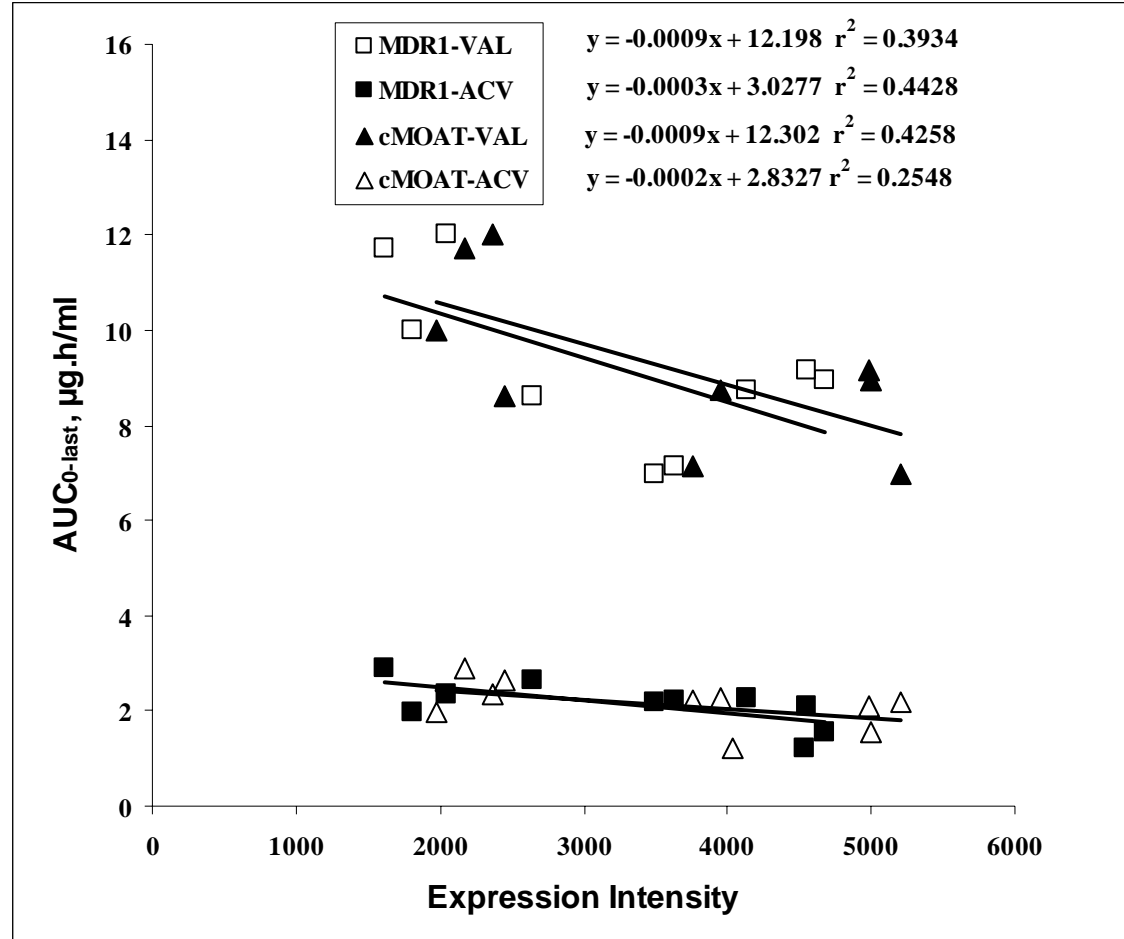


Figure 8.

