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Permeability Dominates in Vivo Intestinal Absorption of P-gp Substrate with High Solubility and High Permeability

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Abstract: Three purposes are presented in this study: (1) to study the in vivo regional dependent intestinal absorption of a P-gp substrate with high solubility and high permeability, (2) to study the gene expression difference in the various regions of the intestine, and (3) to study the contributions of P-gp or any other transporters for the absorption of a P-gp substrate. The in vivo permeability of verapamil and propranolol were determined by single-pass in situ intestinal perfusion in rat. The gene expression profiles were measured using Affymetrix GeneChip. Correlation analysis between drug in vivo permeability and expression of 3500 genes was performed with nonparametric bootstrap and ANOVA analysis. The permeability of verapamil and propranolol did not demonstrate regional dependency even though significant differences in gene expression were observed in various regions of the intestine. Verapamil permeability significantly correlates with propranolol permeability in both jejunum and ileum, but did not correlate with the permeability of other hydrophilic compounds (valacyclovir, acyclovir, and phenylalanine). Four different regions (duodenum, jejunum, ileum, and colon) showed distinct gene expression patterns with more than 70−499 genes showing at least 5-fold expression differences. Interestingly, P-gp expression is gradually increased by 6-fold from the duodenum to colon. Despite the distinct gene expression patterns in the various regions of the intestine, verapamil permeability did not correlate with any gene expression from 3500 expressed genes in the intestine. A 2−6-fold P-gp expression difference did not seem to associate verapamil permeability in the various intestinal regions in vivo. These data suggest that P-gp plays a minimal role in the in vivo intestinal absorption process of verapamil with high water solubility and high membrane permeability. The intestinal absorption of verapamil in vivo is primarily dominated by its high permeability. However, it is important to note that the findings in this paper do not undermine the importance of P-gp in oral drug bioavailability, drug disposition from the liver, drug efflux from the blood−brain barrier, and drug−drug interaction.

Keywords: Permeability; P-gp; intestine; gene expression; verapamil; correlation; microarray

Introduction

Oral bioavailability (F) is a function of the fraction of drug absorbed (F_a), fraction of drug escaped from the intestinal metabolism (F_g), and fraction of drug escaped from the liver metabolism (F_h) ($F = F_aF_gF_h$).[1,2] Therefore, the oral bioavailability of a drug is largely a function of its solubility characteristics in gastrointestinal fluids, absorption into the systemic circulation, and metabolic stability. Oral drug

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absorption and the fraction of drug absorbed in the intestine are a function of drug's solubility and permeability.[3] If both drug solubility and permeability are enhanced, there will be a great increase in the rate and extent of oral absorption. Oral drug absorption processes occur mainly in small intestinal regions; such processes include passive transcellular diffusion, carrier-mediated transport processes, paracellular transport, and endocytosis. In general, lipophilic compounds are usually absorbed by passive diffusion through the intestinal epithelium. Many hydrophilic compounds are absorbed through a carrier-mediated process, while some small hydrophilic compounds may be transported through the paracellular junction. Under physiological conditions, the fastest absorption process may dominate the absorption for a particular compound.

With the development of modern molecular biology studies and the completion of the human genome project,[4] more and more transporters have been discovered to be involved in drug absorption processes.[5] It has been estimated that 5−10% of the human genome may encode transporter proteins and channels. To date, more than 350 transporters have been studied for their physiological, pathological, and pharmaceutical functions.[6−9] These transporters are classified into 44 different carrier families (SLC 1−43 and ABC transporters). These transporters include peptide transporters, amino acid transporters, organic cation transporters, organic anion transporters, bicarbonate transporters, glucose transporters, neurotransmitter transporters, ion transporters and exchangers, bile salt transporters, carboxylate transporters, urea transporters, amine transporters, folate transporters, fatty acid transporters, nucleoside transporters, phosphate transporters, nucleoside-sugar transporters, and ABC transporters. Many clinically used drugs are substrates of these transporters such as β-lactam antibiotics, ACE inhibitors, anticancer drugs, and antiviral drugs.[6−8,10,11] In addition, many in vitro cell systems with overexpression or downregulations of transporters were established to test drug flux by these transporters.[11−15] However, due to the differences of the in vitro cell culture system and in vivo animal models, drug absorption through transporters often shows some discrepancy between in vitro and in vivo system.[11]

A total of 49 ABC transporters have been cloned and studied.[16] In this family, multidrug resistant gene (MDR1, ABCB1) encodes a P-glycoprotein (P-gp) that is a 170 kDa glycosylated plasma membrane protein with two ATP binding cassettes (ABC).[17−19] It is a dimer consisting of 1280 amino acids with 12 transmembrane domains. It was first characterized as the ATP-dependent transporter responsible for efflux of chemotherapeutic agents from multidrug

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resistant (MDR) cancer cells. By reducing the intracellular concentration of chemotherapeutic agents, P-gp can confer multidrug resistance to cancer cells.[8,20]

P-gp is widely expressed in many tissues, such as the membrane of endothelial cells in the intestine,[21−23] liver,[22] kidney,[15] placenta, blood−brain barrier, and blood−testis barriers.[24] Therefore, P-gp plays important roles in drug intestinal absorption, drug disposition by liver and kidney, and drug flux through the blood−brain barrier and other barriers. P-gp is present on the villus tip of the apical brush border membrane of gut enterocytes and actively causes efflux of drugs from gut epithelial cells back into the intestinal lumen.[21−23] P-gp is also expressed in the epithelial apical membrane of the biliary canalicular membranes of hepatocytes and the proximal tubules of the kidney.[25−30] The unique localization of P-gp facilitates excretion of many drugs or their metabolites from liver to bile or from kidney to urine. It has been reported that P-gp can actively transport a wide range of hydrophobic and amphipathic drugs out of the cell. Compounds transported by P-gp include anticancer drugs like *Vinca* alkaloids, anthracyclines, epipodophyllotoxins and taxanes, HIV-1 protease inhibitors, immunosuppressants, antibacterial reagents, flavonoids, antifungals, and cardiac glycosides.[31−35]

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Although P-gp has been exclusively studied to affect oral bioavailability of many lipophilic compounds, the in vivo intestinal absorption of the P-gp substrate has not been well characterized. Since oral bioavailability $F = F_a F_g F_h$, many factors that influence drug disposition (metabolism and/or excretion) in the liver may not affect the drug absorption process in the intestine. In this paper, we isolate the in vivo oral drug absorption process from bioavailability for a P-gp substrate (verapamil) to differentiate the P-gp function on drug absorption and drug bioavailability.

Verapamil is a very well-studied P-gp substrate. Combination use of verapamil and other P-gp substrates significantly increased their oral bioavailability.[36−38] In Caco-2 cells, the B−A permeability of verapamil is 3−5-fold higher than its A−B permeability due to the drug efflux by apical membrane localized P-gp.[39,40] In this study, we intend to isolate the in vivo drug absorption process from the bioavailability for studying the P-gp contribution for in vivo oral absorption of verapamil. The drug permeability was measured by in situ intestinal perfusion in rat. Gene expression in rate intestine was determined by GeneChip. The correlation of drug permeability and gene expression was analyzed by nonparametric bootstrap and ANOVA analysis. Propranolol that is absorbed by passive diffusion is used as a control.

Materials and Methods

Materials. SD rats were purchased from Charles River laboratories (Wilmington, MA). Verapamil and propranolol were purchased from Sigma (St. Louis, MO). ^{14}C-PEG 4000 was from Amersham Biosciences (Piscataway, NJ). All drugs were dissolved in MES perfusion buffer (NaCl 140 mM, KCl 5 mM, MES 10 mM, NaOH 5 mM, pH 6.5). The rat diets were purchased from TestDiet Inc. (Richmond, IN). The diets' compositions were as follows. High-protein diet: 65% protein, 10% carbohydrate, 10% fat, 3.8% fiber, 48 ppm cholesterol, 2.5% minerals, and all essential multivitamins; energy, 3.9 kcal/g. High-fat diet: 10% protein, 18.5% carbohydrate, 60% fat, 6.3% fiber, 285 ppm cholesterol, 2.5% minerals, and all essential multivitamins; energy, 6.55 kcal/g. Normal diet: 19% protein, 60.6% carbohydrate, 10% fat, 4.3% fiber, 48 ppm cholesterol, 2.5% minerals, and all essential multivitamins; energy, 4.08 kcal/g.

Single-Pass in Situ Intestinal Perfusion. Rats (250−300 g) were fed on different diets for 10 days. The animals had free access to water, and consumption of diet was monitored. On the day of experiment, 16 rats (250−300 g) were anesthetized with ketamine/xylyazine (1 mL/kg) and pentobarbital (20−40 mg/kg), and a surgery was performed to isolate 10 cm segments of jejunum and ileum, respectively. The perfusion tubes were connected to each 10 cm segment. MES buffer with 100 μM propranolol, verapamil, and 7 μCi/L ^{14}C-PGE 4000 as a mixture was perfused through the intestinal segment at a flow rate of 0.16 mL/min. The experiments were repeated four times in each group. All perfusion solutions and animals were maintained at a 30 °C environment during the perfusion. After absorption reached steady state at 60 min perfusion, the outlet flow was collected every 15 min for 1 h and 15 min. Small portions of perfusate (0.4 mL) were used for scintillation counting for ^{14}C-PEG 4000 concentration measurements to correct for water absorption and secretion during the perfusion. The rest of the perfusate was immediately frozen on dry ice for HPLC analysis. After perfusion, the jejunum and ileum of the rat intestine were removed, cut open, and washed in saline 5 times. The mucosal layer was scraped off using a glass slide. The mucosal sample was frozen in liquid N_2 and saved for RNA isolation.

HPLC Analysis of Drug Concentration. The perfusate was centrifuged at 15 000 rpm for 15 min, then supernatant was filtered with a 0.45 μm filter, and 50 μL of sample was injected onto HPLC for drug concentration analysis. The column was a C18 Supelco column (5 μm, 4.6 × 250 mm). The HPLC pump (model 515) and auto injector were from Waters (WISP model 712). The mobile phase was 35% acetonitrile in pH 3.5 ammonium acetate buffer for propranolol and verapamil analysis. The PDA detector (Waters 996 photodiode array detector) was used for the detection. The absorbance at 235 nm was used for verapamil and propranolol detection.

Drug Intestinal Effective Permeability Calculation. The intestinal effective permeability was calculated under steady-state perfusion. The perfusate was collected and analyzed after 60 min perfusion at a flow rate of 0.16 mL/min. The inlet drug concentration was measured by sham perfusion tube at 60, 75, 90, 105, and 120 min, and the outlet drug

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concentrations were measured at the perfusion tube after passage through 10 cm isolated intestinal segment at 60, 75, 90, 105, and 120 min. The outlet drug concentration was normalized by the nonabsorbable marker ^{14}C-PEG 4000 outlet and inlet concentrations to correct for water absorption or secretion.

$$C'_{out} = C_{out(drug)}C_{in(PEG\ 4000)}/C_{out(PEG\ 4000)}$$

$$P_{eff} = Q(1 - C'_{out}/C_{in})/2\pi RL$$

P_{eff} is effective permeability, Q is perfusion flow rate (0.16 mL/min), C'_{out} is corrected outlet drug concentration, C_{in} is inlet drug concentration, R is the radius of rat small intestine (0.18 cm), and L is the 10 cm intestinal segment.

RNA Isolation and Purification from Rat Intestinal Mucosal Tissue. All mucosal tissues of the rat intestine were immediately scraped with a glass slide, transferred to a new frozen vial, and dipped into liquid N_2. A total of 100 mg of tissue was added to 1 mL of TRIzol reagent (Gibco/BRL, Grand Island, NY), and the mixture was homogenized with a tissue razor at maximum speed for 20 s 3 times on ice. The homogenate was transferred to a new tube, and then 200 μL of chloroform was added to the TRIzol mixture. After the mixture was centrifuged at 12 500 rpm for 15 min at 4 °C, the aqueous phase was transferred to a new tube, and the RNA was precipitated with 500 μL of 2-propanol and washed with 80% ethanol. The RNA pellet was resuspended in 30−50 μL of DNase/RNase-free water, and the concentration was measured at 260 nm. The absorbance ratio at wavelength 260/280 nm should be at least greater than 1.6. The RNA was further purified with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's manual. The purified RNA concentration was measured at 260 nm. The absorbance ratio at 260/280 nm of the purified RNA should be 1.7−1.8. The purified RNA was checked in an agarose/formaldehyde gel for quality before further cRNA labeling. A total 5 μg of purified RNA was mixed with RNA loading buffer and heated at 75 °C for 15 min. After cooling on ice for 5 min, the RNA was loaded on 1% agarose/formaldehyde gel in 1 × MOPS buffer. The gel was run at 80 V for 50 min, and two sharp 18S and 28S bands should be visible under UV irradiation.

cRNA Labeling and GeneChip Analysis. First-strand cDNA was transcribed from 8 μg of total RNA using T7-$(dT)_{24}$ oligomer primer, the primer was annealed at 70 °C for 10 min, and then SSII reverse transcriptase was used for reverse transcription at 42 °C for 1 h. The second-strand cDNA was synthesized from first-strand cDNA using DNA ligase, DNA polymerase I, and T4 DNA polymerase at 16 °C for 2 h using a SuperScript Choice System for cDNA synthesis kit (Gibco/BRL, Grand Island, NY), and the reaction was stopped by adding 10 μL of 0.5 M EDTA. The double-strand cDNA was then cleaned by phenol/chloroform extraction with phase-locking gel and ethanol precipitation in the presence of 1 μg of glycogen.

Biotin-labeled cRNA was synthesized from the double-strand cDNA using T7 RNA polymerase-catalyzed in vitro transcription in the presence of biotin-labeled NTP using BioArray high-yield RNA transcription labeling kit (Enzo Biochem, New York, NY) at 37 °C for 5 h, and the labeled cRNA was purified using an RNeasy Mini Kit (Qiagen). The concentration of labeled cRNA was measured at 260 nm. A total of 20 μg of labeled cRNA was fragmented at 95 °C. Biotin-labeled cRNA was heated at 99 °C for 5 min in hybridization cocktail including hybridization control (Bio B, C, D, and Cre) and hybridized with GeneChip U34A (Affymetrix, Santa Clara, CA) at 42 °C for 16 h. The GeneChip was then washed with nonstringent wash buffer at 50 °C and stained with streptavidin phycoerythrin (SAPE) solution. After washing at 25 °C, the GeneChip was scanned with a laser scanner (Affymetrix). The gene expression profiles were analyzed by Affymetrix Microarray Suite and Data Mining Tool software.

Correlation Analysis. Data used for this statistical analysis consisted of gene expression from Affymetrix GeneChip, diet and intestinal region information, and effective permeability of verapamil and propranolol. Gene expression levels were first normalized using MAS5 software and then log base 2 transformed. ANOVA for linear regression models (*t*-test and *F*-test) was used to assess correlations between permeability with diet and intestinal region to test whether the data from different intestinal region with different diet could be combined for correlation analysis.

To determine genes that were involved in drug permeability, we utilized ANOVA for different linear models and tried to analyze drug permeability through gene expression, diet, and intestinal region. The linear models we used were

model 1: permeability = $a + b \times$ gene expression level

model 2: gene expression level = $a + b \times$ diet

model 3: permeability = $a + b \times$ diet

model 4: permeability = $a + b \times$ diet $+ c \times$ gene expression level

where diet was either 0 or 1 depending upon whether the sample was on normal diet or high protein diet.

The first model helped us identify genes with good correlation with drug permeability (i.e., b is significantly different from zero). Alternatively, we tested the same association using the correlation coefficient (R) between gene expression levels and drug permeability. In this case the significance of the association was calculated using nonparametric bootstrap analysis for sample labels. Bonferroni adjustment for significance of the p values was used in both cases.[7,41,42] Also, a comparison between model 3 and model

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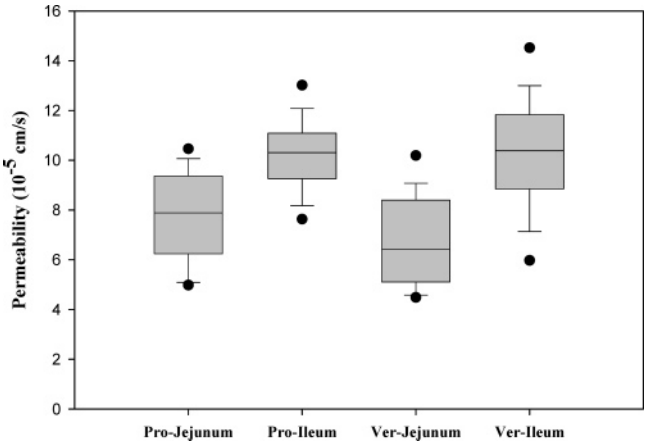

Figure 1. The permeability of propranolol and verapamil in the different regions of the intestine ($n = 16$). Their permeability did not show any significant regional dependency between the jejunum and ileum. Boxes represent the data between 25% and 75% quartiles. The whisker distance is 1.5 IQR (interquartile range), which is the distance between 25% and 75% quartiles. Pro: propranolol. Ver: verapamil.

4 was performed using ANOVA to identify if adding gene expression to the model would improve the analysis outcome, which indicates the strong association between drug permeability and gene expression.

For the *t*-test to detect the diet effect on transporter gene expression levels,

$$t = \frac{\overline{x_1} - \overline{x_2}}{\sqrt{\frac{{s_1}^2}{n_1} + \frac{{s_2}^2}{n_2}}}$$

$\overline{x_1}$ and $\overline{x_2}$ were the average expression levels; ${s_1}^2$ and ${s_2}^2$ denoted the sample variances in the n_1 and n_2 samples for the two phenotypes, respectively. To account for multiple hypothess testing, we adjusted the significance of the *p* values, controlling the false discovery rate (FDR) at a level of 25%.

Results

Verapamil and Propranolol Show High Permeability in Both Jejunum and Ileum under Different Diets. To determine the drug permeability in different regions of rat intestine, 16 samples from rat jejunum and 16 samples from ileum were used to measure the effective permeability of verapamil and propranolol under different diets. Four different dietary conditions (high protein, high fat, normal diet, and fasted state) were used to create more variability for future correlation analysis between drug permeability and gene expression. As shown in Figure 1, verapamil and propranolol showed high permeability in both jejunum and ileum (greater than $5-12 \times 10^{-5}$ cm/s). Two statistical tests, the *t*-test and the *F*-test, were employed to assess the permeability difference in these two intestinal regions. Although the permeability of both verapamil and propranolol showed a slight increase in the ileum compared to the jejunum (1.5−1.9-fold) under normal diets, the statistical analysis showed no significant difference in their permeability in these two regions under all four different dietary conditions. Therefore, we combined drug permeability from both jejunum and ileum in the following correlation analysis between drug permeability and gene expression to increase the sample size and statistical power ($n = 16$).

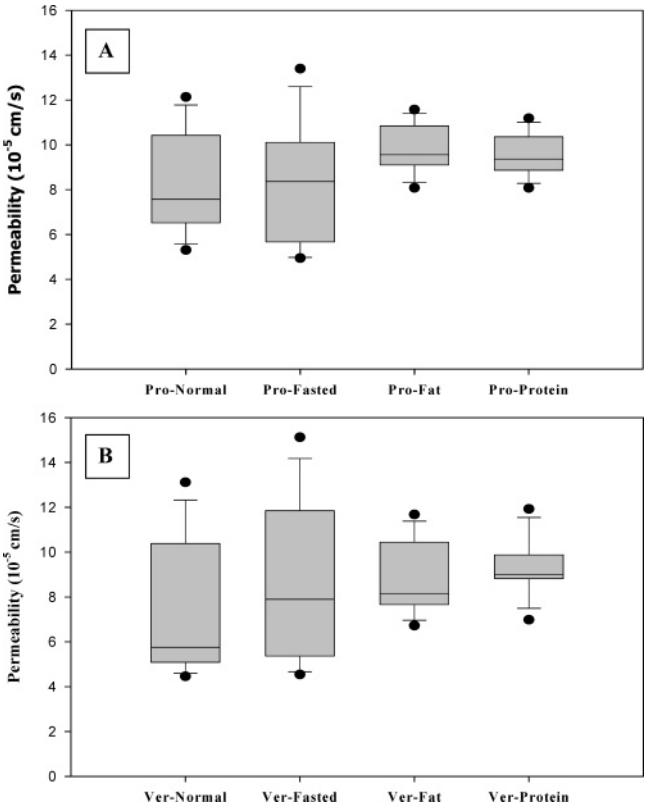

Figure 2. (A) Propranolol permeability and (B) verapamil permeability in both the jejunum and ileum under four different dietary conditions (normal diet, fasted state, high-fat diet, and high-protein diet) ($n = 8$). No significant diet dependency was observed for the intestinal permeability of propranolol and verapamil. Boxes represent the data between 25% and 75% quartiles. The whisker distance is 1.5 IQR. Pro: propanolol. Ver: verapamil.

Using the same statistical analysis, we evaluated whether different diets would affect the drug permeability. Both the *t*-test and the *F*-test indicate that the permeabilities of verapamil and propranolol did not show any diet dependency in both the jejunum and ileum although the high-protein diet and the high-fat diet slightly increased their permeability by 1.4−1.8-fold (Figure 2). These results indicate that oral absorption of verapamil and propranolol does not show regional and dietary dependency.

The Permeability of Verapamil Significantly Correlates with Permeability of Propranolol in Both the Jejunum and Ileum. Verapamil is a P-gp substrate, while propranolol is absorbed in intestine by passive diffusion. In order to test if verapamil uses a different absorption mechanism from propranolol, the correlation analysis of the permeabilities of verapamil and propranolol in the jejunum and ileum was

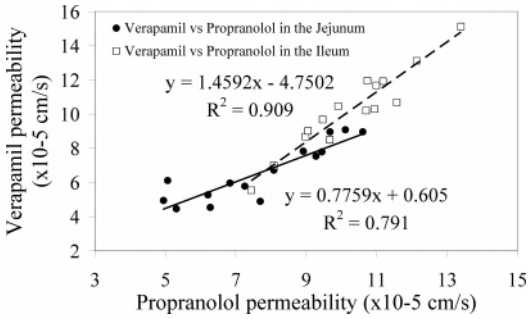

Figure 3. Correlation analysis of the intestinal permeability of verapamil and propranolol in rat jejunum (●) and ileum (□).

performed in 16 rats under different diets. Very interestingly, verapamil permeability correlated well with propranolol permeability in both the jejunum and ileum with correlation coefficients (R^2) of 0.791 and 0.909, respectively (Figure 3). However, the permeabilities of both verapamil and propranolol did not show any correlation with permeability of other hydrophilic drugs such as valacyclovir, acyclovir, and phenylalanine (data not shown). These results indicate that the absorption mechanism of verapamil and propranolol is very similar. Even though verapamil is a substrate of P-gp and propranolol is believed to be absorbed through passive diffusion,[27−29] it is possible that both verapamil and propranolol are mainly absorbed effectively by the passive diffusion in intestine in vivo due to their high lipophilicity.

Different Regions of the Intestine Show Distinct Overall Expression of 8799 Gene Sequences (Including 719 Transporters and Metabolizing Enzymes) by GeneChip. The gene expression in different regions of intestine may have distinct patterns. To determine if different gene expression levels may contribute to intestinal drug absorption in vivo, we measured expression levels of 8799 gene sequences in four different regions (duodenum, jejunum, ileum, and colon) of the rat intestine by GeneChip. The results showed that similar numbers of genes (~3500 genes) were detected in four different regions of the rat intestine (Table 1). After normalizing gene expression levels, boxplots and Q−Q plots of individual arrays against each other or against the standard normal distribution showed that different arrays had similar distributions (Figures 4 and 5). Therefore, data from all of 19 samples were used for the following analysis.

As expected, small intestine regions showed distinct gene expression profiles from the colon, while different small intestinal regions (duodenum, jejunum, and ileum) showed much less difference. For instance, a total of 340−499 gene sequences showed at least a 5-fold difference between the small intestinal regions and the colon, and only 70−233 gene sequences showed more than 5-fold expression differences among different regions of the small intestine (Table 2).

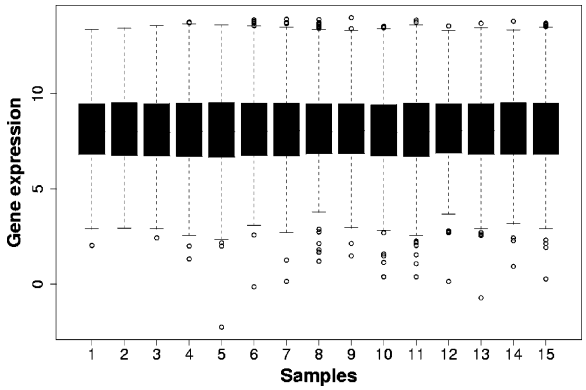

Figure 4. Boxplots of the gene expression levels after log transformation in different samples. Boxes represent the data between 25% and 75% quartiles. The whisker distance is 1.5 IQR.

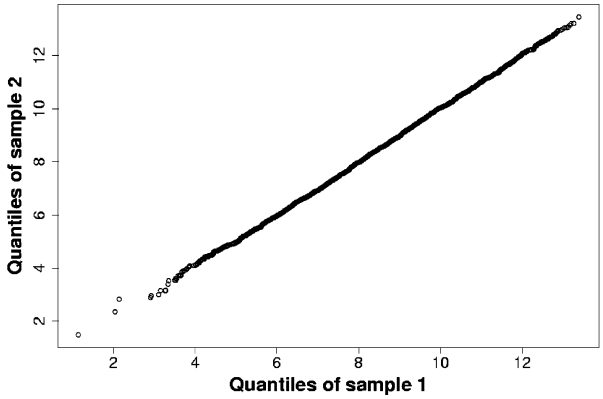

Figure 5. Quantile−quantile plot for comparison between two different samples. The linear trend of this graph suggests that the gene expression levels from the two experiments follow the same distribution.

Within the small intestinal regions, the duodenum and the jejunum showed very similar gene expression profiling with only 70 gene sequences showing more than 5-fold expression differences. Greater differences were observed between the duodenum and ileum (233 genes with more than 5-fold differences) than in other comparisons within the small intestinal regions (70 genes with more than 5-fold differences between the duodenum and the jejunum, 127 genes with more than 5-fold expression differences between the jejunum and the ileum). These data further confirm that the small intestine is dramatically different from the colon in overall gene expression. Within small intestinal regions, the duodenum is very similar to the jejunum and different from the ileum in overall gene expression profiles.

The expression profiles of transporters and metabolizing enzymes were also observed in the different regions of rat intestine. On the rat GeneChip, 719 sequences of transporters,

Table 1. The Number of Gene Sequences Expressed in Different Regions of Rat Intestine

	duodenum	jejunum	ileum	colon
no. of detected gene sequences	3409 ± 124	3630 ± 169	3840 ± 44	3850 ± 68
total no. of analyzed sequences	8739	8739	8739	8739

Table 2. The Number of Gene Sequences with More Than 2−5-fold Expression Differences Compared to the Various Regions of Rat Intestine

	>2-fold		>3-fold		>5-fold	
	increase	decrease	increase	decrease	increase	decrease
duodenum vs colon	487	776	372	440	254	245
jejunum vs colon	542	646	345	373	228	211
ileum vs colon	462	482	282	233	193	148
jejunum vs duodenum	190	159	67	67	28	42
ileum vs duodenum	544	411	253	198	124	109
jejunum vs ileum	264	293	124	133	61	66

Table 3. The Number of Transporters and Metabolizing Enzymes with More Than 2-fold Expression Differences Compared to the Various Regions of Rat Intestine

	>2-fold		
	increase	decrease	total 2-fold change
duodenum vs colon	57	19	76
jejunum vs colon	54	18	72
ileum vs colon	35	16	51
jejunum vs duodenum	5	9	14
ileum vs duodenum	12	39	51
jejunum vs ileum	24	11	35

exchangers, channels, and metabolizing enzymes were printed. A total of 199 transporters and metabolizing enzymes were detected in the intestine. Again, significant expression differences of transporters and metabolizing enzymes were observed between the small intestine and the colon, in which 51−76 gene sequences showed at least 2-fold expression differences (Table 3). Within small intestinal regions, the duodenum is very similar to the jejunum with only 14 gene sequences showing more than 2-fold expression differences, while the duodenum showed slightly greater differences from the ileum, in which 51 gene sequences showed more than 2-fold expression differences (Table 3).

Cluster Analysis Demonstrates Different Patterns of Transporter Expression Profiles and Distinguishes Tissue Origins from Various Regions of the Intestine. Since we observed the dramatic gene expression difference in the four regions of the intestine, we performed the cluster analysis to visualize the distinct transporter expression patterns and to cluster the various tissue origins. Very interestingly, cluster analysis showed that overall transporter expression profiles in the duodenum and the jejunum clustered together, while the expression profiles in the ileum and the colon clustered together. This suggests that cluster analysis with transporter expression is able to distinguish the tissue origin from duodenum, jejunum, ileum, and colon. The results also indicate that the duodenum and the jejunum exhibit a similar transporter expression pattern while the ileum and the colon show a similar transporter expression pattern. However, different groups of transporters showed distinct expression patterns in various regions of the intestine as depicted in Figure 6. Group A showed transporters with high expression levels in the colon and low expression levels in the small intestinal regions. Group B showed transporters with high expression levels in the ileum and the colon but low expression levels in the duodenum and jejunum. Group C showed transporters with high expression levels in the duodenum and the jejunum but low expression levels in the colon and the ileum. Group D showed transporters with low expression levels in the colon but high expression levels in the small intestinal regions.

Permeability of Verapamil and Propranolol Did Not Correlate with Expression Levels of 3500 Expressed Genes in the Rat Intestine. To test if any gene expression may relate to drug permeability, the correlation analysis between drug permeability and expression of 3500 expressed genes in the intestine was performed by four different statistical models as described in Materials and Methods. To our surprise, no significant correlation was found with false detective rate (FDR) at a reasonable level (up to 10−20%) between the permeability of verapamil and propranolol with expression of 3500 genes. More surprisingly, even though verapamil was reported to be a substrate of P-gp,[43−45] its permeability showed no correlation with Mdr1 expression in both the jejunum and ileum. Since propranolol is considered to be absorbed by passive diffusion in the intestine,[46,47] no correlation was found between its permeability and any gene expression. These data suggest that verapamil and propranolol may have a similar absorption mechanism, in which the passive diffusion may dominate the absorption process.

Different Regions of Intestine Exhibit Distinct Expression Profiles of ABC Transporters. Since verapamil is a P-gp substrate, we also analyzed the expression levels of ABC transporters in the intestine to explore the mechanism

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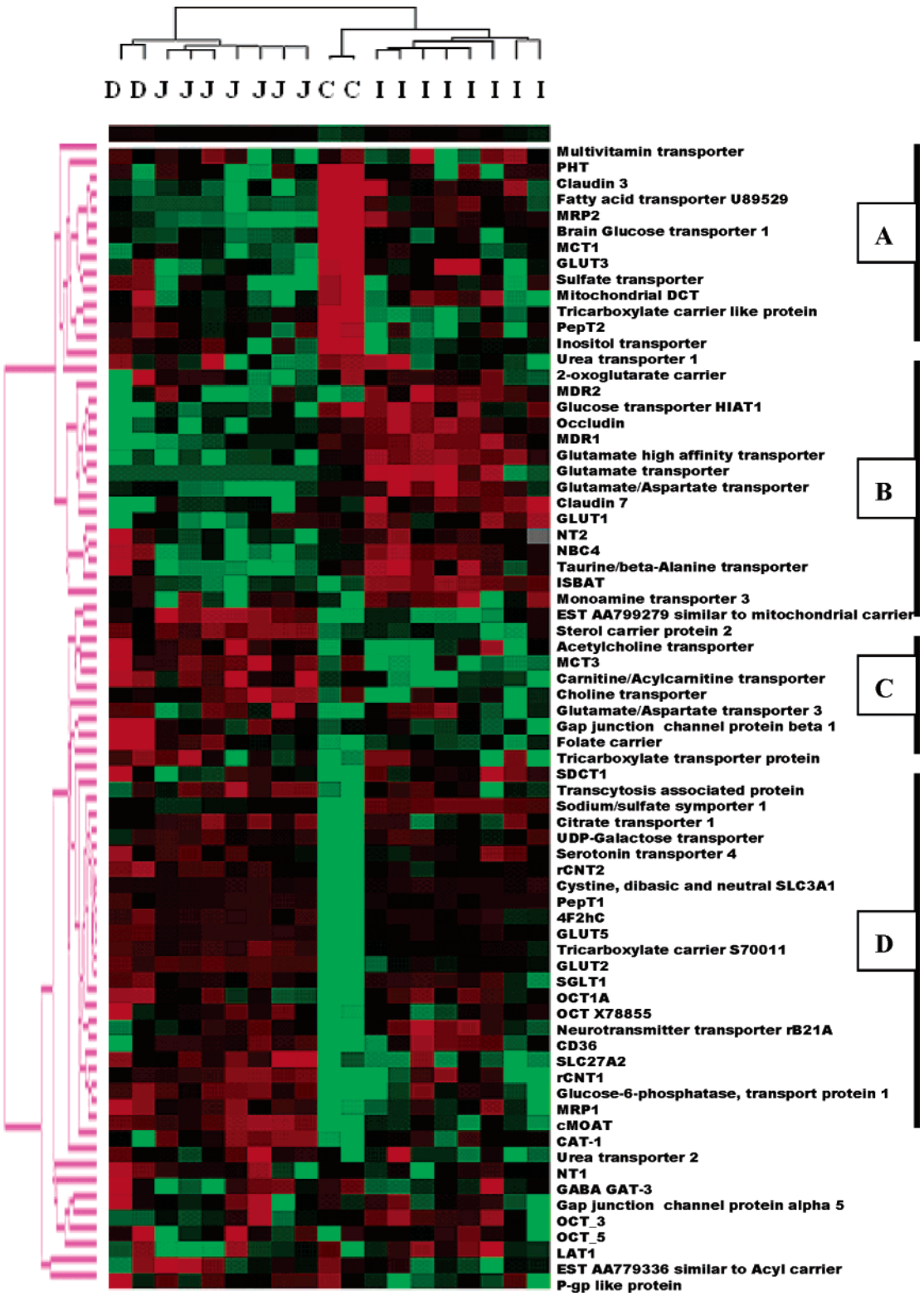

Figure 6. Cluster analysis of the expression levels of transporters in different regions of the intestine. The red color indicates the high expression levels, and the green color indicates the low expression levels. D: duodenum. J: jejunum. I: ileum. C: colon. Group A: highly expressed in colon only. Group B: highly expressed in both ileum and colon. Group C: highly expressed in both duodenum and jejunum. Group D: poorly expressed in colon.

of verapamil absorption in vivo (Figure 7). Three multidrug resistance genes were detected in the intestine. Mdr1 expression gradually increased along the GI tract from the duodenum to the colon, and its expression in the colon was similar to that in the ileum, which was 6-fold higher than in the duodenum. MDR2 was expressed at similar levels in all

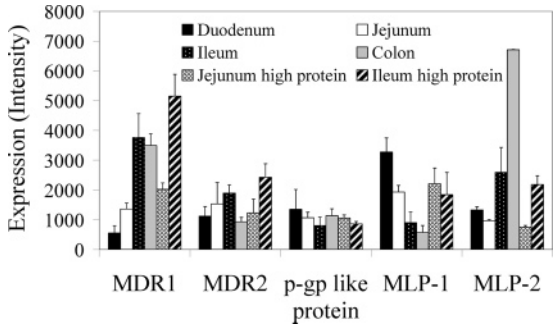

Figure 7. Expression profiles of ABC transporters in the duodenum, jejunum, ileum, and colon under normal diet and high protein diet. Expression levels were quantified by the intensity of hybridization signal. MDR: multidrug resistance gene (P-glycoprotein, P-gp). MLP: multidrug resistance associated protein-like protein.

regions of the intestine although there appeared to be a slight increase from the duodenum to the ileum by 1.7-fold. P-gp-like protein expression showed no difference among all regions of the intestine. In contrast to MDR1, the expression of multidrug resistance associated protein-like protein 1 (MLP-1) decreased along the GI tract from the duodenum to the colon by 5-fold. However, MLP-2 expression increased gradually along the GI tract from the duodenum to the colon by 7-fold (Figure 7).

Discussion

Different regions of the intestine have different physiological characteristics, and the drug absorption in different regions may have different patterns.[48−51] It is generally believed that the drug absorption process occurred mainly in the small intestinal region. However, different regions of small intestine, the duodenum, jejunum, and ileum, may also possess different expression patterns of transporter expression, and thus may have distinct drug absorption properties.[11,52,53] Understanding these differences may provide valuable information for the evaluation and prediction of drug intestinal absorption, as well as for the design of drug molecules and formulations to enhance drug bioavailability, especially for the drugs that are absorbed through a transporter-mediated process.

However, since the intestinal drug absorption is determined by both drug solubility and permeability,[1] the intestinal absorption of drugs with different solubility and permeability may demonstrate distinct regional dependency. In this paper, we determined the regional dependency of the permeability for drugs with high solubility and high permeability. We also compared the in vivo absorption of a P-gp substrate (verapamil) to propranolol with passive diffusion to study the P-gp effect on absorption of this class of compounds. The correlation analysis between drug permeability and expression levels of 3500 genes in the different regions of rat intestine was performed to explore if any gene is associated with drug absorption.

Verapamil is a P-gp substrate, and it competes with other P-gp substrates for transport to increase their bioavailability by drug−drug interaction.[8,17−23,36−40] Propranolol is believed to be absorbed by passive diffusion[46,54] although some reports also indicate that propranolol also interacts with P-gp.[43,44,55] Both drugs have high water solubility (the salt form is more than 1−2 mg/mL). Our results confirm that both drugs have very high membrane permeability in both jejunum and ileum. Even though four different regions (duodenum, jejunum, ileum, and colon) of rat intestine showed distinct gene expression profiles, verapamil and propranolol did not exhibit regional dependency for their permeability. Surprisingly, verapamil permeability correlates well with propranolol permeability in both jejunum and ileum with a correlation coefficient of more than 0.8−0.9, but did not correlate with the permeability of other hydrophilic compounds under the same conditions. Although P-gp showed a 6-fold difference in the different regions of rat intestine, verapamil showed no regional dependent permeability and no correlation with P-gp expression. These results indicate that these two drugs may be absorbed from intestine by a very similar mechanism. It is very possible that the passive diffusion dominates their intestinal absorption even though verapamil is a P-gp substrate.

To confirm our hypothesis that passive diffusion is the determinant factor for the absorption of verapamil and propranolol in vivo, we also performed the correlation analysis between drug permeability and expression levels of

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3500 genes in the intestine to explore if other transporters may be involved in verapamil absorption. Very surprisingly, none of the 3500 expressed genes was correlated with the permeability of verapamil and propranolol.

Since P-gp is believed to export drug from the intestinal epithelial cells back to the intestinal lumen,[21−23] we further analyzed the expression levels of the ABC transporters in the intestine. The data showed that MDR1 expression was increased by 6-fold from the duodenum to the colon, and 2.7-fold higher in the ileum than in the jejunum. In such a case, if P-gp contributes to the absorption of verapamil, its permeability would be expected to be lower in the ileum than in the jejunum. However, verapamil permeability did not show any statistical significance between jejunum and ileum. In contrast, its permeability is slightly higher (1.9-fold higher) in the ileum than in the jejunum. These results indicate that the P-gp effect on the absorption process for high-permeable drugs like verapamil is minimal in vivo, although P-gp affects the bioavailability of those drugs due to the drug efflux function in the liver and other organ systems.

Because oral bioavailability is a function of the fraction of drug absorbed, fraction of drug escaped from intestinal metabolism, and fraction of drug escaped from liver metabolism ($F = F_a F_g F_h$),[1] it is reasonable to assume that factors that influence F_h will affect oral bioavailability, but will not necessarily affect oral absorption (F_a). Therefore, P-gp may have a very minimal effect on the verapamil absorption process, even though it affects the oral bioavailability of its substrates due to the drug efflux in the liver. In addition, since verapamil has such a high membrane permeability, it will have 100% of the fraction of drug absorbed from the intestine no matter whether the P-gp is present or not. The absorption process of verapamil may be dominated by the passive diffusion due to its high permeability.

A well-established cell model (Caco-2 cells), which is derived from a human colon carcinoma, has been widely used to investigate P-gp efflux function.[11−15] P-gp is highly expressed at the apical membrane of the Caco-2 cells, and undoubtedly recognizes and causes efflux of verapamil. In this model, the P-gp substrates usually demonstrate higher basolateral to apical drug transport (B−A) than apical to basolateral drug transport (A−B).[39,40] Verapamil B−A permeability is usually 3−5-fold higher than A−B permeability due to the efflux function of P-gp. Verapamil also reduces the B−A permeability of other P-gp substrates (e.g., vinblastine) due to the competition with P-gp.[39,40] Despite these facts, the in vivo intestinal absorption process of some P-gp substrates may not be greatly influenced by P-gp due to the larger surface area of intestine and high lipophilicity of the compounds. This is especially true for the P-gp substrates (such as verapamil) with high water solubility and high membrane permeability. In this case, the high permeability may dominate the drug oral absorption process, thus generating the in vitro and in vivo discrepancy in drug permeability. Indeed, this type of discrepancy between in vitro Caco-2 cells and in vivo intestine was also found by other data.[11,35,56] For instance, human intestine and Caco-2 cells expressed similar levels of P-gp; however, the normalized permeability ratios (drug permeability/propranolol permeability) of verapamil and cyclosporin in human intestine are 2−10-fold higher than the normalized permeability ratios in Caco-2 cells. The P-gp expression levels between human intestine and Caco-2 cells could not explain in vivo/in vitro permeability differences of verapamil and cyclosporin.[11] The in vivo absorption of 13 P-gp substrates is also not consistent with the in vitro absorption studies mediated by P-gp because these P-gp substrates showed complete oral absorption with no apparent dose-dependent kinetics in vivo.[35]

Conclusions

In summary, the intestinal permeability was determined by in situ single-pass perfusion in rat intestine to study the regional dependent absorption of a P-gp substrate (verapamil) and a passive diffusively absorbed drug (propranolol). The gene expression profiles were measured by GeneChip to distinguish the regional differences in various intestinal regions. Correlation analysis was performed to explore if any gene expression may be associated with drug intestinal absorption in vivo. The results showed that permeability of verapamil and propranolol did not demonstrate any regional dependency even though significant differences in gene expression were observed in different regions of the intestine. Surprisingly, verapamil permeability significantly correlates with propranolol permeability in both jejunum and ileum, but did not correlate with the permeability of other hydrophilic compounds (valacyclovir, acyclovir, and phenylalanine).

The small intestinal regions showed significant gene expression differences from the colon region with more than 340−499 genes showing 5-fold expression differences. In contrast to the comparison between small intestine and colon, various small intestinal regions showed much less gene expression difference. However, distinct gene expression patterns were observed among duodenum, jejunum, and ileum with 70−233 genes showing at least 5-fold differences. Interestingly, P-gp expression is gradually increased by 6-fold from the duodenum to the colon.

Despite these distinct gene expression patterns in the different regions of the intestine, rigorous statistical analysis indicates that verapamil permeability did not correlate with any gene expression in the intestine, which includes 3500 expressed genes in the intestine. A 2−6-fold P-gp expression difference in the various intestinal regions did not seem to associate with verapamil permeability in vivo. These data suggest that P-gp plays a minimal role in the in vivo intestinal absorption process of verapamil with high water solubility and high membrane permeability. The intestinal absorption

(56) Svensson, U. S.; Sandstrom, R.; Carlborg, O.; Lennernas, H.; Ashton, M. High in situ rat intestinal permeability of artemisinin unaffected by multiple dosing and with no evidence of P-glycoprotein involvement. *Drug Metab. Dispos*. **1999**, *27*, 227−32.

of verapamil in vivo is primarily dominated by its high permeability.

However, it is important to note that the findings in this paper do not undermine the importance of P-gp in oral drug bioavailability, since the drug efflux in the liver may greatly contribute to the decrease of the bioavailability of the P-gp substrates. In addition, the conclusion in this paper does not rule out the importance of drug efflux from the blood−brain barrier and the drug−drug interaction mediated by P-gp to increase drug toxicity and reduce drug bioavailability.

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