Delineation of Human Peptide Transporter 1 (hPepT1)-Mediated Uptake and Transport of Substrates with Varying Transporter Affinities Utilizing Stably Transfected hPepT1/Madin-Darby Canine Kidney Clones and Caco-2 Cells

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ABSTRACT

In the present investigation, the uptake and transport kinetics of valacyclovir (VACV), 5-aminolevulinic acid (5-ALA), and benzylpenicillin (BENZ) were studied in stably transfected Madin-Darby canine kidney (MDCK)/human peptide transporter 1 (hPepT1)-V5&His clonal cell lines expressing varying levels of epitope-tagged hPepT1 protein (low, medium, and high expression) and in Caco-2 cells to delineate hPepT1-mediated transport kinetics. These compounds were selected due to the fact that they are known PepT1 substrates, yet also have affinity for other transporters. Caco-2 cells, traditionally used for studying peptide-based drug transport, were included for comparison purposes. The time, pH, sodium, and concentration dependence of cellular uptake and permeability were measured using mock, clonal hPepT1-MDCK, and Caco-2 cells. A pH-dependent effect was observed in the hPepT1-expressing clones and Caco-2 cells, with an increase of 1.96-, 1.84-, and 2.05-fold for VACV, 5-ALA, and BENZ uptake, respectively, at pH 6 versus 7.4 in the high-expressing hPepT1 cells. BENZ uptake was significantly decreased in Caco-2 and MDCK cells in Na+/H+-depleted buffer, whereas VACV uptake only decreased in Caco-2 cells. Concentration-dependent uptake studies in the mock-corrected hPepT1-MDCK and Caco-2 cells demonstrated hPepT1 affinity ranking of VACV > 5-ALA > BENZ. The apical-to-basal apparent permeability coefficient ($P_{app}$) values of VACV, 5-ALA, and BENZ in mock-corrected hPepT1-MDCK cells showed solely hPepT1-mediated transport in contrast to Caco-2 cells. Lower $K_m$ values and higher $P_{app}$ in Caco-2 cells compared with hPepT1-MDCK cells suggested the involvement of multiple transporters in Caco-2 cells. Thus, hPepT1-MDCK cells corrected for endogenous transporter expression may be a more appropriate model for screening compounds for their affinity to hPepT1.

The intestinal epithelium is the main site of absorption of dietary and therapeutic compounds into systemic circulation. Therefore, understanding the absorption mechanism(s) and the extent to which a compound is transported by the intestinal epithelium is one of the critical factors in determining the overall bioavailability of compounds. Different transporters are known to play a significant role in the uptake of xenobiotics at the intestinal epithelium (Zhang et al., 2002). For example, human peptide transporter 1 (hPepT1) is a 708-amino acid member of the mammalian protein-coupled oligopeptide transporter superfamily that is expressed in the small intestine and present in the brush border of the absorptive epithelial cells (Freeman et al., 1995; Liang et al., 1995; Herrera-Ruiz and Knipp, 2003). The substrates reported to be transported by the proton-coupled hPepT1 are di- and tripeptides and many peptide-based compounds including ß-lactam antibiotics, angiotensin-converting enzyme inhibitors, renin inhibitors, and nonpeptide prodrug, e.g., valacyclovir (Leibach and Ganapathy, 1996; Tamai et al., 1997; Zhang et al., 1997; Zhang et al., 2002; Herrera-Ruiz and Knipp, 2003).

To better screen, predict, and understand the different routes of transport across the intestinal epithelium, several

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ABBREVIATIONS: hPepT1, human peptide transporter 1; MDCK, Madin-Darby canine kidney; VACV, valacyclovir; 5-ALA, 5-aminolevulinic acid; BENZ, benzylpenicillin; FBS, fetal bovine serum; MES, 2-(4-morpholino)-ethanesulfonic acid; PIPES, 1,4-piperazine-bis(2-ethanesulfonic acid); AP, apical; BL, basolateral; TEER, transepithelial electrical resistance.
cellular models have been reported to study the oral absorption of pharmaceuticals (Artursson, 1991; Hillgren et al., 1995). The most commonly used are "intestinal like" cell lines including Caco-2, TC-7, HT29-MTX, and 2/4A1 (Knipp et al., 1997; Gres et al., 1998; Hilgendorf et al., 2000; Bohets et al., 2001). Caco-2 cells, a human colon carcinoma-derived cell line, has been the most widely utilized model due to the fact that it forms a microvillous apical surface upon differentiation, which exhibit morphological and functional similarities to the polarized absorptive epithelial cells of the small intestine. However, the utilization of Caco-2 cells for drug screening also offers several disadvantages, including the fact that it is labor intensive to handle the cells due to their long culturing times (2 to 3 weeks) for expressing fully differentiated functions and that they exhibit additional transporter systems that can vary across individual laboratories (Hu, 1993; Mahraoui et al., 1994; Behrens et al., 2004).

The Madin-Darby canine kidney (MDCK) cell model, derived from the distal tubular part of dog kidney, has been investigated as a possible screening tool for absorption studies. The advantages of MDCK cells in contrast to Caco-2 cells is that they form tight monolayers in a shorter culturing time and are more easily transected to delineate the contribution of individual transporters in the absence of significant endogenous transporter "noise" (Pastan et al., 1988; Irvine et al., 1999; Herrera-Ruiz et al., 2004). The ability to isolate, clone, and transfect the cDNAs for peptide transporters (Liang et al., 1995; Liu et al., 1995) has provided a new dimension in developing cell culture models as a screening tool. Studies conducted in either hPepT1 adeno-virally transfected Caco-2 (Hsu et al., 1999) or Chinese hamster ovary/hPepT1 (Han et al., 1999) cells have demonstrated the utility of transfected cell lines for investigation of uptake of peptide transporter substrates. We have recently shown that differently expressing, stably transfected MDCK/hPepT1-V5&His clonal cell lines could be utilized to delineate the functional interaction of PepT1 substrates (Gly-Sar and Carnosine) as a function of the molar levels of hPepT1 transgene expression (Herrera-Ruiz et al., 2004). The limitation of this study was that the substrates used are considered to have high affinity for hPepT1 and lack interaction with other nonpeptide transporters.

To further validate the utility of the MDCK/hPepT1-V5&His clonal cell lines, valacyclovir (VACV), 5-aminolevulinic acid (5-ALA), and benzylpenicillin (BENZ) were utilized as hPepT1 substrates that also have potential affinities for other nonpeptide transporters. Michaelis-Menten kinetics and permeability studies were also correlated with the amount of the transgene hPepT1 protein. Thus, the present study provides a better model for determining hPepT1 affinity and capacity, while minimizing the contributions of other transporters or from endogenous factors. This model can be readily extended to the study of other transporters and enzymes as well.

**Materials and Methods**

**Materials**

[3H]VACV, [3H]5-ALA, and [3H]BENZ were purchased from Moravek Biochemicals (Brea, CA), American Radiolabeled Chemicals (St. Louis, MO), and Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK), respectively. Cold VACV was a gift from GlaxoSmithKline (Research Triangle Park, NC). 5-ALA and BENZ were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), nonessential amino acids, l-glutamine, trypsin, and Hanks’ balanced salt solution were obtained from Mediatech Cellgro Inc. (Herndon, VA). MES, PIPES, HEPES, and all other chemicals were obtained from Sigma-Aldrich.

**Cell Culture**

The mock and stably transfected MDCK/hPepT1-V5&His clonal cells were cultured as described previously (Herrera-Ruiz et al., 2004). Briefly, the cells were maintained in Dulbecco’s modified Eagle’s medium (4.5 g/l D-glucose, 0.7 mM L-glutamine, and 110 mg/l sodium pyruvate) supplemented with 10% FBS, 1% nonessential amino acids, and 200 mM glutamine, containing 0.3 mg/ml G418 to provide selective pressure. The cells were cultured in T-75 flasks at 37°C in 5% CO₂ and at 90% humidity. Cells were harvested and passed at 80 to 90% confluency and used between passages 7 and 17 in these studies, where no changes in the expression of hPepT1-V5&His were observed (Herrera-Ruiz et al., 2004). The Caco-2 cell line was obtained from ATCC (Rockville, MD). Briefly, cells were cultured in T-75 flasks in culture medium, which consisted of Dulbecco’s modified Eagle’s medium with 10% FBS, 1% nonessential amino acids, 100 mg/ml penicillin, and 200 mM glutamine. Cells used in this study were between passages 30 and 45.

**Uptake Studies**

Transfected cells were grown under the conditions specified above and described previously (Herrera-Ruiz et al., 2004). Cells were seeded at a density of 5 x 10⁴ cells/cm² per well in 24-well plates. Uptake studies were performed in triplicate 2 days postseeding. On the day of the experiment, the cells were washed twice with buffered Ringer’s solution (15 mM MES, pH 6.0 or 5 mM HEPES, pH 7.4). The appropriate substrate solutions (see below) were added for the measurement of uptake. The uptake was then stopped by washing the cells two times with ice-cold phosphate-buffered saline. The cells were solubilized by adding 200 µl of 1% Triton X-100 per well. A 150-µl aliquot was used for scintillation counting and a 20-µl sample was used for the protein assay. Protein concentration was determined by the bicinchoninic acid assay following the microtiter plate protocol (Pierce, Rockford, IL). For Caco-2 experiments, the same procedure was followed, except seeding cell density was 1 x 10⁴ cells/cm² per well in 24-well plates, and the uptake studies were performed on the 21st day.

**Time Dependence Assay.** HPept1/V5&His-MDCK stably transfected cells, mock, and Caco-2 cells were seeded as specified above. Subsequently, the cells were incubated with 1 µCi/ml VACV, 5-ALA, or BENZ at 37°C for 5, 10, 20, 60, and 90 min. The complete time course was determined at pH 6.

**pH Dependence Assay.** HPept1/V5&His-MDCK stably transfected cells, mock controls, and Caco-2 cells were seeded as specified above. Subsequently, the cells were incubated for 15 min, with 1 µCi/ml VACV, 5-ALA, or BENZ at 37°C at pH 6 and pH 7.4. The proton-dependent uptake of VACV at various pH values (5.5, 6, 6.5, 7, 7.5, and 8) was also studied using cloned and Caco-2 cells. To rule out the possibility of buffer component affecting VACV uptake, different buffer systems (MES, PIPES, and HEPES) were also studied.
Sodium Dependence Assay. H PepT1/V5&His-MDCK stably transfected cells, mock controls, and Caco-2 cells were incubated for 15 min at 37°C with 1 μCi/ml VACV, 5-ALA, and BENZ at pH 6 and pH 7.4 with and without Na⁺-containing buffer. For Na⁺-depleted medium, sodium chloride and sodium bicarbonate in MES and HEPES buffer at pH 6 and 7.4, respectively, were replaced by choline chloride and choline bicarbonate.

Concentration Dependence Assay. The concentration dependence of VACV, 5-ALA, and BENZ was studied at pH 6 over a concentration range of 0.01 to 50 mM. The mock, clonal MDCK/h PepT1/V5&His (low, medium, and high), and Caco-2 cells were incubated with each individual substrate for 15 min. The mock cell line kinetic values were subtracted from those observed in each h PepT1/V5&His-containing clone to account for any endogenous and nonspecific transport activity. Michaelis-Menten kinetic parameters were determined (Km and Vmax) using the following equation with GraphPad Prism version 4.02 (GraphPad Software Inc., San Diego, CA).

\[ V_0 = \frac{V_{\text{max}}[S] + K_m [S]}{K_m + [S]} \]

where \( V_0 \) is the initial uptake velocity, \( V_{\text{max}} \) is the maximal uptake velocity at saturating substrate concentrations, \( K_m \) is a constant analogous to the Michaelis-Menten constant, and \( S \) is the substrate concentration.

To estimate the kinetic values of the saturable uptake by Caco-2 cells, the uptake rate was fit to the following equation that consists of both saturable and nonsaturable components. Modeling was performed using a nonlinear regression program of GraphPad Prism version 4.02.

\[ V_0 = V_{\text{max}}[S] + K_s[S] \]

where \( K_s \) is the rate constant for the nonsaturable uptake, \( V_0 \), \( V_{\text{max}} \), \( K_m \), and \( S \) are mentioned above.

Permeability Studies

All transport experiments were run for 2 h in triplicate with the three h PepT1/V5&His-MDCK cell lines (low-, medium-, and high-expressing clones), mock, and Caco-2 cells. Studies were performed in 12-mm tissue culture collagen-coated polycarbonate membranes (0.4-μm pores, Transwells; Costar, Cambridge, MA) at a cell density of 5 × 10⁴ cells/cm². After seeding, the media were changed every other day, and the study was performed on the 7th day. On the day of the study, culture media were removed, and cells were washed two times with transport buffer. The cells were equilibrated in the transport buffer for 30 min prior to each study. After this time, the substrate containing buffer solution was added and cells kept on a rocker platform inside a humidified culture incubator at 37°C.

VACV, 5-ALA, and BENZ in the high and mock h PepT1/V5&His-MDCK cells (a) and Caco-2 cells (b). Cells were plated in 24-well plates and incubated for 15 min for uptake studies. The reaction was stopped using ice-cold phosphate buffer, and the amount of radioactivity was measured using liquid scintillation counter with final values normalized to protein quantity. Data are expressed as mean ± S.D., n = 3. ***, \( p < 0.001 \); **, \( p < 0.01 \); *, \( p < 0.05 \), as determined by the Student’s t test.

Results

Uptake Studies. The levels of h PepT1 transgene protein expression in the selected h PepT1/V5&His-MDCK clones (low, medium, and high) have been previously determined (Herrera-Ruiz et al., 2004) and did not change during these studies. The values obtained in the uptake studies were normalized to the amount of protein expressed in these cells. VACV, 5-ALA, and BENZ uptake in low-, medium-, and high-expressing cells was measured for 90 min, showing a linear range of uptake over the first 20 min (data not shown). The uptake of VACV, 5-ALA, and BENZ in the transfected cells was pH-dependent (pH 6 versus pH 7.4), with significant increases observed for VACV (\( p < 0.001 \)), 5-ALA (\( p < 0.01 \)), and BENZ (\( p < 0.01 \)) at pH 6 in the high h PepT1-V5&His-expressing cells when compared with pH 7.4 (Fig. 1a). Similar results were also obtained with Caco-2 cells,
which also demonstrated higher uptake at pH 6 compared with pH 7.4 (Fig. 1b). Mock cells demonstrated no significant difference in the uptake between pH 6 and pH 7.4 (Fig. 1a).

The pH dependence of VACV uptake was further studied in the pH range of 5.5 to 8 in the various hPepT1-expressing cells. The uptake of VACV was found to be the highest at pH 6 and showed significantly higher uptake in the rank order of high, medium, and low hPepT1-expressing cells (Fig. 2a). In the pH-dependent study, HEPES buffer appeared to increase the uptake of VACV at pH 7 in contrast to the values obtained with MES buffer at pH 6.5, suggesting non-pH-dependent contribution of the buffer components to VACV uptake. To elucidate the buffer effect on the uptake of VACV, different buffers (MES, PIPES, and HEPES) were used, showing significantly higher uptake at pH 6 compared with pH 7.5 (Fig. 2b). In contrast, PIPES at pH 7 demonstrated a pH-dependent profile that was consistent with the changes observed with the MES buffer and had a statistically significant reduction in uptake at pH 7 when compared with those observed with HEPES. Similar buffer-dependent results were obtained with Caco-2 cells when the uptake of VACV at different pH values was measured using MES and HEPES (Fig. 2c). In these studies, higher uptake at pH 6 compared with pH 7.5 was observed, with no apparent change elucidated between MES at pH 6.5 and HEPES at pH 7.0 (Fig. 2c).

The results from the sodium-dependent studies on the uptake of VACV, 5-ALA, and BENZ at pH 6 are illustrated in Fig. 3. At pH 6, the absence or presence of sodium had no apparent effect on the uptake values determined for VACV.
and 5-ALA in the mock and high hPepT1/V5&His-MDCK cells. Interestingly, BENZ revealed a 33.88 and 47.09% (p < 0.05) decrease in the uptake in Na+-depleted buffer in mock and high hPepT1/V5&His-MDCK cells, respectively (Fig. 3a). Sodium-dependent studies performed at pH 7.4 showed no change in uptake of VACV and 5-ALA, whereas a significant (p < 0.05) decrease in Na+-depleted buffer was seen for BENZ in the mock and high hPepT1/V5&His-MDCK cells (data not shown). In Caco-2 cells at pH 6, VACV and BENZ showed a significant (p < 0.05) sodium-dependent decrease in uptake of 21.17 and 13.82%, respectively. Similar decreases in uptake were also observed for VACV (33.75%) and BENZ (54%) at pH 7.4 in absence of sodium, whereas uptake of 5-ALA was not affected by the presence or absence of sodium (Fig. 3b).

Concentration-dependent profiles of VACV, 5-ALA, and BENZ uptake determined in low, medium, and high hPepT1/V5&His-MDCK cell lines after correcting for mock uptake are shown in Fig. 4. $K_m$ and $V_{max}$ values for VACV, 5-ALA, and BENZ were determined in the hPepT1/V5&His-MDCK cell lines (Table 1). Concentration-dependent studies of VACV, 5-ALA, and BENZ uptake in Caco-2 are shown in Fig. 5. The active (saturable) uptake of each substrate is calculated after subtracting the passive (nonsaturable) uptake values from the total uptake. The $K_m$ and $V_{max}$ values for Caco-2 uptake were also determined and are illustrated in Table 1.

**Transport Studies.** TEER and [14C]mannitol permeability were used to characterize each monolayer’s integrity. The TEER values for the MDCK and Caco-2 cells that were included in the studies were found to be more than 300 and 450 $\Omega$cm$^{-2}$, respectively. The [14C]mannitol apparent permeability coefficient ($P_{app}$) in the hPepT1/V5&His-MDCK cell line was found to be similar to the $P_{app}$ values observed in the parental and control cell lines. In Caco-2 cells, the [14C]mannitol permeability coefficient (AP to BL and BL to AP) was found to be $0.9 \times 10^{-6}$ cm/s. The AP to BL permeability values of VACV, 5-ALA, and BENZ using low, medium, and high hPepT1/V5&His-expressing cells, after subtracting the values from mock $P_{app}$, are shown in Table 2. The AP to BL apparent permeability coefficients of VACV, 5-ALA, and BENZ exhibited a statistically significant difference (p < 0.05) between the mock and high hPepT1/V5&His-expressing MDCK cell lines. However, the BL to AP permeability coefficients across the low, medium, and high hPepT1/V5&His-expressing cell lines for VACV, 5-ALA, and BENZ were found to be similar to the BL to AP permeability coefficient of mock cells. Furthermore, the AP to BL permeability for the transport of VACV, 5-ALA, and BENZ in Caco-2 cells are also listed in Table 2.

**Discussion**

The previously developed (Herrera-Ruiz et al., 2004) stably transfected hPepT1/MDCK cells were utilized to evaluate the functional contribution of hPepT1 on the uptake and transport kinetics of three substrates (VACV, 5-ALA, and BENZ) that also presumably share affinities for other transporters. Similar experiments were performed using the Caco-2 cell culture model and compared with the results from the cloned cells. The cells studied for time dependence showed a linearity of 20 min in cloned and Caco-2 cells for VACV, 5-ALA, and BENZ, which led to our selection of 15 min of incubation time. An inwardly driven proton gradient (extracellular pH = 6) increased 5-ALA and BENZ uptake 1.84- and 2.04-fold, respectively, compared with pH 7.4 in high PepT1/MDCK-expressing cells (Fig. 1a). This is consistent with the findings of Doring et al. (1998) and Poschet et al. (1996), who demonstrated higher uptake of 5-ALA and BENZ at pH 6 versus 7.4.

Consistent with the findings of Sinko et al. (1998), a proton gradient (pH 6) increased the uptake of VACV (Fig. 2a) when compared with pH 7.4 in the different hPepT1-expressing cells (low, medium, and high). Further studies using different buffer systems showed increased uptake at pH 6 thereby confirming the role of proton gradient in increasing the uptake of VACV at pH 6 (Fig. 2b). However, other reports (Guo et al., 1999; Balimane and Sinko, 2000) demonstrated there...
was no proton gradient effect on the uptake of VACV. This discrepancy is most likely due to differences in the cell systems, with the possibility that the VACV uptake was affected by the involvement of various transporters in different studies.

The role of Na⁺ on the uptake of PepT1 substrates in the high hPepT1-expressing and mock MDCK cells was also investigated. At pH 6 and 7.4, VACV and 5-ALA showed an insignificant change in the uptake with and without Na⁺ in both high and mock cells suggesting the inability of sodium-dependent transporters to affect their uptake. However, BENZ at pH 6 (Fig. 3a) and pH 7.4 both showed significant (p < 0.05) decreases in the uptake in Na⁺-depleted buffer. The decrease in the uptake could be due to the influence of the Na⁺-dependent exchange mechanism affecting its transport. Skowronski et al. (1999) demonstrated that the addition of amiloride and quabain, an inhibitor of Na⁺/H⁺ exchanger and Na⁺/K⁺ ATPase, respectively, reduced the BENZ transport by 90%, suggesting the role of Na⁺. Busch et al. (1996) reported the interaction of BENZ with a Na⁺-dependent phosphate cotransporter. Recently, Burkhart et al. (2004) reported the interaction of BENZ with the Na⁺-dependent dicarboxylate (NaDC-3) transporters located in the basolateral membrane of renal proximal tubule cells. In Caco-2 cells at pH 6 and pH 7.4, VACV showed a significant (p < 0.05) Na⁺-dependent decrease in uptake (Fig. 3b). The role of other transporters affecting the uptake of VACV in Caco-2 cannot be ruled out. Hatanaka et al. (2004) have shown that the transport of VACV was demonstrable in heterologous expression systems expressing Na⁺- and Cl⁻-coupled amino acid transporter ATB⁰⁻¹⁻¹. Moreover, Sinko and Balimane (1998) suggested that VACV can interact with organic anion and organic cation transporters. Furthermore, the uptake of VACV was observed in human organic anion transporter 3-expressing cells (Takeda et al., 2002), and VACV had an inhibition constant Kᵢ value of 0.22 mM for rPepT2 when blocking the uptake of glycyrlsarcosine (Sawada et al., 1999).

Although several different routes of active transport across cellular barriers have been characterized (Amidon and Sadee, 1999), it is still difficult to determine the contribution of a single transporter to the permeation of a solute across monolayers. The present study attempts to address the need to develop cell lines expressing a transporter of interest and to functionally evaluate its substrates by establishing and comparing the high hPepT1-expressing cell line to the low/hPepT1. Student’s t-test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>VACV</th>
<th>5-ALA</th>
<th>BENZ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Vₘₐₓ</td>
<td>Kₘ</td>
<td>Vₘₓ</td>
</tr>
<tr>
<td>Low</td>
<td>5.58 ± 0.21</td>
<td>5.05 ± 0.61</td>
<td>6.87 ± 0.78</td>
</tr>
<tr>
<td>Medium</td>
<td>14.96 ± 0.08**</td>
<td>5.03 ± 0.09</td>
<td>12.30 ± 1.65</td>
</tr>
<tr>
<td>High</td>
<td>16.23 ± 0.71**</td>
<td>3.77 ± 0.59</td>
<td>16.20 ± 0.46*</td>
</tr>
<tr>
<td>Caco-2</td>
<td>2.93 ± 0.24</td>
<td>1.55 ± 0.48</td>
<td>5.61 ± 0.18</td>
</tr>
</tbody>
</table>

*p < 0.05 and **p < 0.01 show the significant change in Vₘₐₓ compared with the low/hPepT1. Student’s t-test.
The results of this functional evaluation of hPepT1 substrates, using the hPepT1/V5&His cell line, illustrates that the uptake and transport kinetics of PepT1 substrates is mainly due to hPepT1 protein activity. This approach could also be utilized to determine the effect of any transporter on drug absorption. Ultimately, this system could be used as a better tool to assess and screen various substrates and their affinity for designated transporters.

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References


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