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A Novel hPepT1 Stably Transfected Cell Line: Establishing a Correlation between Expression and Function

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Abstract: Stably transfected MDCK/hPepT1-V5&His clonal cell lines expressing varying levels of epitope-tagged hPepT1 protein were established to quantify the relationship between transgene hPepT1 expression levels and its functional kinetics in facilitating peptide and peptide-like drug uptake and transport *in vitro*. The hPepT1 sequence was amplified from Caco-2 cell mRNA, inserted into the pcDNA3.1-V5&His TOPO plasmid, and transfected into MDCK cells. Transgene protein levels were quantified by Western Blot analysis utilizing a standard curve generated with a positive control protein containing a V5&His epitope. Three clones expressing different levels of the hPepT1 fusion protein (low, medium, and high) were selected for the functional characterization with [^{14}C]Gly-Sar and [^{3}H]carnosine. The MDCK/hPepT1 cells expressed a novel hPepT1/epitope tag protein with an apparent molecular mass of 110 kDa. The [^{14}C]Gly-Sar uptake in the transfected cells was sodium-independent and pH-dependent, demonstrating enhanced uptake, the rate of which increased significantly from the weakly to strongly expressing hPepT1 MDCK/hPepT1-V5&His clones as compared to the mock cell line at pH 6.0. The uptake and permeability of [^{14}C]Gly-Sar and [^{3}H]carnosine demonstrated a direct correlation between the hPepT1 level of expression, uptake, and transport capabilities. Molecular and functional characterization of the MDCK/hPepT1-V5&His cell line confirmed a directly proportional relationship between V_{max} and P_{app} versus the molar levels of hPepT1 transgene expression. This stably transfected hPepT1 cell line may serve as a useful *in vitro* model for screening and quantifying peptide and peptide-like drug transport as a function of hPepT1 expression in drug discovery.

Keywords: Human peptide transporter 1; glycylsarcosine; reverse transcriptase polymerase chain reaction; apparent permeability

Introduction

Understanding the absorption mechanisms responsible for peptide transport across the gastrointestinal (GI) tract is of significant pharmaceutical importance. In recent years, there has been considerable research exploring the role of active membrane transporters that facilitate the intestinal transport of di- and tripeptides and peptide-like drugs.[1−3] The intestinal oligopeptide transporter that has been predominantly studied in intestinal cell culture and tissue models is peptide transporter 1 (PepT1), with particular interest focused on the human isoform (hPepT1).[1−5]

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The discovery and design of orally bioavailable peptide-like drugs requires adequate drug transport screening models for assessing GI absorption as part of lead candidate optimization and selection for drug development. Different cell culture and tissue models have been used for estimating oral absorption, whereas the human colon carcinoma Caco-2 cell line is the most widely reported *in vitro* model for predicting GI drug absorption *in vivo*.[6−8] While the Caco-2 model is useful for assessing passive transport of moderate to highly lipophilic compounds, it is much less predictive for very polar compounds or those that are substrates for active membrane transporters.[9−14] The later may be attributed to variable gene expression and Caco-2 cell function, which has been shown to vary in different laboratories.[12,15] Caco-2 cells that variably express endogenous multiple oligopeptide transporters can potentially confound the correlation of peptide and peptide-like drug permeation with *in vivo* absorption data.[16]

A cell line that can selectively account for the role of a specific peptide transporter in a tissue and enable the delineation of its functional properties in relation to its levels of expression would offer significant advantages. Several cell culture models have been established to study the role of PepT1 in the facilitation of peptides and peptide-like drug transport. The established systems express the rat isoform of the PepT1 gene,[17,18] use transient hPepT1 expression systems in CHO cells,[19−21] or utilize pancreatic carcinoma cell lines expressing high levels of endogenous hPepT1.[22] A more appropriate model for the delineation of the contribution of hPepT1 was established by stably overexpressing hPepT1 at different levels in Caco-2 cells by adenoviral transduction.[23] Although these systems have been useful in the identification of PepT1 and PepT2 substrates, they have not been explored in elucidating the relationship between quantifiable, differently expressing PepT1 trans-

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porter protein levels in a cell line to delineate its effects in altering the kinetic parameters of its substrates. On the basis of this fact, this study was designed to develop the hPepT1/V5&His transfected stable MDCK cell line with different levels of hPepT1 protein and to elucidate the relationship between the transgene expression of various stably hPepT1/V5&His transfected clones and its function. Thus, the studies reported here were designed to address some of the limitations of current models, which may potentially lead to the establishment of a better functional and molecular characterization of the *in vitro* expression of hPepT1 and its substrates. The model may be fairly easily applied to the study of other transporters and receptors, and may potentially enable the correlation between their *in vitro* and *in vivo* molecular and functional characterization.

Experimental Section

Materials. *Pfu* Turbo proofreading polymerase was obtained from Stratagene (La Jolla, CA). The Super Signal Western Femto Maximum Sensitivity Detection kit and the BCA protein quantitation reagent were obtained from Pierce Chemical Co. (Rockford, IL). DNA isolation kits were purchased from Qiagen (Valencia, CA). TRIzol Reagent for RNA extraction, medium (D-MEM, M199), fetal bovine serum (FBS), nonessential amino acids, L-glutamine, trypsin, neomycin, Lipofectamine 2000, OptiMEM, Hank's balanced salts solution (HBSS), the pcDNA3.1/V5&His TOPO cloning kit, the pcDNA/V5&His B vector, the anti-V5 epitope HRP antibody, and the PosiTope protein were obtained from Invitrogen (Carlsbad, CA). [^{14}C]Mannitol, [^{14}C]glycylsarcosine (Gly-Sar), and [^{3}H]carnosine were purchased from Moravek Biochemicals (Brea, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Vector Construction. PCR amplification of the human PepT1 sequence was amplified from Caco-2 mRNA using gene specific primers [5′ AGG AGC CCT GGG AGC CGC CGC CAT GG 3′ (sense) and 5′ CAT CTG TTT CTG TGA ATT GGC CCC TGA CAT G 3′ (antisense)] and *Pfu* Turbo polymerase, a proofreading enzyme. The hPepT1 primers were designed to omit the stop codon (TGA) in the downstream primer to allow the expression of the epitope tag (V5&His). Upon PCR amplification, the sequence was placed into the pcDNA3.1/V5&His TOPO cloning vector (Invitrogen), and plasmids were amplified according to the manufacturer's protocol. The DNA sequencing facility at Rutgers University (University of Medicine and Dentistry of New Jersey, Piscataway, NJ) was utilized to complete the sequencing analysis of the hPepT1 plasmid, and its identity was confirmed by comparing the plasmid hPepT1 sequence with the hPepT1 cDNA sequence reported in GenBank.

Cell Culture. MDCK cells were obtained from ATCC (Manassas, VA). MDCK cells were maintained in D-MEM (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. The cells were cultured in T-75 flasks at 37 °C in 5% CO_2 and 90% humidity. Cells were harvested at 80−90% confluence and then transported. Medium was changed every other day of the culture.

Transfection Protocol. MDCK cells were transfected with the empty pcDNA3.1/V5&His plasmid (to obtain the mock, control cell line) and with the pcDNA3.1-hPepT1/V5&His plasmid using Lipofectamine. Briefly, cells were seeded in 12-well plates at a density of 2.4×10^5 cells/well and incubated at 37 °C. After 24 h, the cells were washed twice and then incubated for 1 h before the transfection in OptiMEM. The DNA/Lipofectamine mixture was prepared with 1.6 μg of DNA and 10 μL of Lipofectamine in 800 μL of OptiMEM per well, according to the manufacturer's protocol. Cells were then incubated overnight with the DNA/liposome mixture at 37 °C in a CO_2 incubator. Transfected cells were counted the next day, and plated in 100 mm tissue culture-treated Petri dishes at different cell densities with antibiotic-free complete medium. Twenty-four hours later, the medium was replaced with complete medium containing 1 mg/mL G418 and changed every 3 days until it was transported. After 7−10 days, colonies were isolated using cloning rings and transferred to 12-well plates for amplification. MDCK cells were maintained in medium containing 0.3 mg/mL G418 to provide selective pressure.

Characterization of Transgene Expression. (1) Messenger RNA Expression. Reverse transcriptase polymerase chain reaction analysis was utilized to confirm hPepT1/V5&His mRNA expression in each of the selected clones. Sequence specific primers for the neomycin resistance gene were designed as a control marker [5′ ACC TGT CCG GTG CCC TGA ATG AAC T 3′ (sense) and 5′ ACT CGT CAA GAA GGC GAT AGA AGG CGA T 3′ (antisense)]. Messenger RNA for hPepT1 was identified with sequence specific designed primers for hPepT1 [5′ ATG GGA ATG TCC AAA TCA CAC AGT TTC TTT G 3′ (sense) and 5′ CAA AAC TTT AAT TTG GAC TTC GTT TCC 3′ (antisense)]. Five micrograms of total RNA and 0.5 μg of oligo(dT) were used for the reverse transcriptase reaction. The positive control for PCR was a hPepT1 plasmid (kindly donated by F. H. Leibach, Medical College of Georgia, Augusta, GA), and Caco-2 mRNA was employed as a positive reference control for the relative level of expression. PCRs were performed under optimized conditions: denature at 94 °C for 2 min, annealing at 65 °C for 1 min, and elongation at 72 °C for 1 min (30 cycles). Reaction products were electrophoretically separated in 1% agarose gels.

Real-time PCR analysis (QPCR) of hPepT1 expression was performed using the following procedure. Two micrograms of total RNA harvested from cells using the SV Total RNA Isolation Kit (Promega) was converted into cDNA (with or without reverse transcriptase) using the Superscript II First Strand Synthesis Kit (Invitrogen). A commercially available human gastrointestinal tract cDNA panel was used in this study (Clontech). QPCR was performed in a Bio-

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Rad Icycler using Quantitative PCR SuperMix-UDG (Invitrogen). HPepT1 specific PCR primers (L-CTTGTTACCCACTGGCCTTT and R-GGAACATCACCCTCGTAACC) and a TaqMan probe were utilized (6FAM-CCAAGAGGGAGCACTGGCTGG-TAMRA, Applied Biosystems). Standards were prepared with 10-fold serial dilutions of hPepT1 plasmid DNA (10^6, 10^5, 10^4, 10^3, and 10^2 copies). Minus RT and no input controls (nuclease-free H_2O) were included. GAPDH expression was assessed using Bio-Rad's SYBR-Green QPCR mix. Results are presented as the number of copies per microgram of RNA normalized to the ratio of hPepT1 to GAPDH threshold cycle (TC) values ± the standard error of the mean.

(2) Protein Expression. Ten micrograms of hPepT1/V5&His-MDCK and mock cell membrane protein fractions were obtained after lysis with freshly prepared RIPA (radioimmunoprecipitation assay, 1× PBS, 1% Nonidet P-40, and 0.5% SDS, with freshly added protease inhibitors) buffer. The membrane protein fractions were separated on 7.5% SDS−polyacrylamide gels and electrophoretically blotted onto PVDF membranes. The membranes were blocked with 5% powdered nonfat milk in PBST (1× PBS and 0.5% Tween 20) and probed with the V5-HRP-conjugated antibody (Invitrogen). Detection of the immunoreactions of interest were accomplished using the Femto Super Signal Western detection kit (Pierce Chemical Co.). Densitometric analysis of the blots was performed utilizing a Bio-Rad (Hercules, CA) chemiluminescence imaging system (ChemiGel Documentation System), equipped with a 16-bit, peltier-cooled digital camera for detecting band signal intensities.

To quantify the level of expressed V5 epitope-labeled hPepT1 protein, PosiTope protein (Invitrogen), a multiple-epitope protein containing a single V5 epitope site, was used to prepare a standard curve on the Western blots. Picomolar HPepT1/V5&His levels were calculated for two protein loading levels (5 and 10 μg) of each clone by interpolation of the band intensity values from the standard curve.

Uptake Studies. Transfected cells were grown under the conditions specified above. As a control, mock cells (pcDNA3.1/V5&His-MDCK cells) were seeded under the same conditions as hPepT1/V5&His stably transfected cells. Cells were seeded at a density of 5×10^4 cells/cm^2 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, BRS [15 mM MES (pH 6.0) or 5 mM HEPES (pH 7.4)]. The appropriate substrate solutions were added for the assessment of uptake. Washing the cells twice with ice-cold PBS then stopped the uptake. The cells were then solubilized by adding 200 μL of 1% TX-100 per well and then shaking for 1.5 h. A 150 μL aliquot was used for scintillation counting, and a 10 μL sample was used for the protein assay. Protein concentrations were determined by the bicinchoninic acid (BCA) assay following the microtiter plate protocol (Pierce).

(1) Time Dependence Assay. hPepT1/V5&His-MDCK stably transfected cells and their mock controls were seeded as specified above for the uptake studies. Subsequently, the cells were incubated with 1 μCi/mL [^{14}C]Gly-Sar (20 μM) at 37 °C for 1, 5, 10, 20, 60, and 90 min. The complete time course was obtained at pH 6.0.

(2) pH Dependence Assay. hPepT1/V5&His-MDCK stably transfected cells and their mock controls were seeded as specified for the uptake studies. Subsequently, the cells were incubated with 1 μCi/mL [^{14}C]Gly-Sar (20 μM) at 37 °C for 15 min at pH 6.0 and 7.4.

(3) Concentration Dependence Assay. The concentration dependence of [^{14}C]Gly-Sar or [^{3}H]carnosine uptake was studied at pH 6.0 over a concentration range of 0.01−50 mM (0.5 μCi/mL). Cells were incubated with the substrate for 15 min. The ratio of labeled to unlabeled Gly-Sar or carnosine was kept at 1:50 throughout the experiment. The same study was performed in the mock cell line to account for any endogenous and nonspecific transport activity. Michaelis−Menten-like kinetic parameters were determined (K_m and V_{max}) by fitting the specific uptake results to the following equation using nonlinear curve fitting (Origin, version 6.0):

$$V_0 = (V_{max}[S])/(K_m + [S])$$

where V_0 is the initial uptake velocity, V_{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. Initial K_m and V_{max} values were obtained from the initial fit of the concentration dependence data. Since the K_m is a function of the transporter and not the transporter amount, the mean K_m obtained for each fit was utilized to perform a weighted fit fixed with the mean K_m to provide a more accurate assessment of V_{max} in each system. The K_m and V_{max} values generated for each substrate in each clone were evaluated for statistical significance using analysis of variance (ANOVA). A p value of ≤ 0.05 was considered significant.

(4) Inhibition Studies. Competitive hPepT1 inhibitors enalapril, cephalexin, and valacyclovir were selected for evaluation. Inhibitor solutions (0.01−20 mM) containing 20 μM [^{14}C]Gly-Sar (pH 6.0) were added to each well containing high hPepT1 expressing cells and incubated for 15 min at 37 °C. The IC_{50} for each inhibitor was determined and compared for each cell line.

Permeability Studies. All transport experiments were carried out for 2 h in triplicate with the three hPepT1/V5&His-MDCK cell lines and the mock cells. Studies were performed in 12 mm tissue culture collagen-coated polycarbonate membranes [0.4 μm pores, Transwells (Costar)] at a cell density of 5×10^4 cells/cm^2. After seeding, the medium was changed every other day until the day of the study. On the day of the study, the culture medium was removed and the cells were washed twice with transport buffer. The cells were equilibrated in the transport buffer for 30 min prior to each study. After this time, a working buffer solution was added and cells were kept on a rocker platform inside a CO_2 incubator at 37 °C.

(1) Monolayer Integrity. Transport studies were performed using the paracellular marker [^{14}C]mannitol. Mannitol permeability was determined across hPepT1 medium and

Table 1. Kinetic Parameters {V_{max} [nmol (15 min)$^{-1}$ (mg of protein)$^{-1}$] and K_m (mM)} of [^{14}C]Gly-Sar and [^{3}H]Carnosine Measured over the Concentration Range of 0.01−50 mM Using the Different hPepT1/V5&His-MDCK Transfected Cells[a]

hPepT1/V5&His-expressing MDCK cells	hPepT1/V5&His level (fmol of hPepT1/mg of protein)	[^{14}C]Gly-Sar		[^{3}H]carnosine	
		V_{max}	K_m	V_{max}	K_m
low	2.6 ± 0.2	20.77 ± 4.38	0.47 ± 0.18	2.75 ± 0.56	4.16 ± 2.95
medium	8.0 ± 0.3	31.82 ± 2.96	0.44 ± 0.09	6.86 ± 0.83	4.13 ± 1.76
high	24.2 ± 1.2	48.02 ± 3.15	0.41 ± 0.07	16.4 ± 2.25	3.81 ± 1.87

[a] Uptake of [^{14}C]Gly-Sar and [^{3}H]carnosine was assessed after incubation for 15 min at 37 °C and pH 6. The amount of expressed hPepT1 protein was quantified with Western blotting using antibodies against the V5 epitope of the PosiTope protein. Values represent means ± the standard error of the mean ($n = 3$).

high expressing clones of the hPepT1/V5&His-MDCK cell line, MDCK wild-type cells, and the collagen-coated filter support in the absence of cells. A [^{14}C]mannitol solution (0.01 mM, 0.5 μCi/mL) was added in the apical (AP) side and HBSS to the basolateral (BL) compartment. Samples (100 μL) at 15, 30, 45, and 60 min were collected from the BL solution, and the volume was replaced with prewarmed HBSS. Transepithelial electrical resistance (TEER) was also measured to determine the intactness of monolayers,[24] and corrected for background (40−70 Ω cm^2).

(2) Peptide Permeability Studies. Human PepT1 low, medium, and high expressing clones along with the mock cells were analyzed for peptide permeability using [^{14}C]Gly-Sar, or a [^{3}H]carnosine buffer solution at pH 6.0 (1 μCi/mL) was added to the AP side and transport buffer (pH 7.4) to the BL compartment. Samples (100 μL) at 15, 30, 45, 60, 90, and 120 min were collected from the BL solution, and the volume was replaced with prewarmed HBSS. Apparent permeabilities from AP to BL compartments were determined. BL (pH 6.0) to AP (pH 7.4) permeability was also evaluated, and 50 μL samples were collected from the AP side at the specified times.

The apparent permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = dQ/dt \times 1/AC_o$$

where dQ/dt is the steady-state appearance rate of the compound on the serosal side (BL), C_o the initial concentration of the compound on the mucosal side of the membranes (AP), and A the surface area of the membrane exposed to the compound (1 cm^2). Sink conditions were maintained throughout each of the studies.

Results

Amplification of the hPepT1 sequence was achieved using *pfu* Turbo polymerase, a proofreading enzyme. The mRNA sequence that was amplified from Caco-2 cells contained the coding frame and the *Kozak* consensus sequence,[25] but not the stop codon to ensure translation of the epitope. Sixty-two percent of the selected clones expressed the hPepT1 mRNA. The MDCK clones demonstrating positive expression of hPepT1 mRNA (data not shown) were selected and further analyzed for the identification of protein expression. The transfected cell lines expressed a novel hPepT1/epitope-tagged protein with an apparent molecular mass of 110 kDa. Western Blot analysis of the MDCK cells (Figure 1) showed different levels of hPepT1 expression in the clones. Comparison of the 110 kDa band intensities was used for the selection of three hPepT1/V5&His-MDCK clones with different levels of hPepT1 protein expression: clone M for low, clone C for medium, and clone F for high.

The standard PosiTope protein containing a single copy of the V5 epitope was utilized to quantify the amount of hPepT1 protein expressed in the selected MDCK cell lines. The standard curve showed a linear range within the working protein levels that were tested ($r^2 = 0.998$). Interpolation of the densitometry measurements for the hPepT1/V5&His total protein extracts allowed the quantification of the hPepT1/V5&His protein content (Table 1). Expression of the hPepT1/V5&His protein was found to be fairly stable with an increasing passage number in the low, medium, and high hPepT1 expressing cells, demonstrating little variation in its protein levels (≤15%) after 16 passages (data not shown). PepT1 mRNA levels were determined in the three cell lines that were selected and in normal human tissues by real-time quantitative PCR analysis (QPCR, Figure 2), showing increased levels of expression of PepT1 RNA.

Uptake Studies. All of the transport activity studies were completed before passage 8, and the results were normalized to the amount of protein expressed in these cells. [^{14}C]Gly-Sar uptake in the transfected cells was pH-dependent (pH 6.0 vs pH 7.4), and the level was found to be significantly increased up to 4.1-fold (hPepT1/high expression clone) when compared to that of the control cell line at pH 6.0. This pH dependence profile is characteristic of those observed for PepT1.[20] The level of [^{14}C]Gly-Sar uptake in low, medium, and high expressing cells was measured for 90 min, which determined that the linear range for the measurement of uptake kinetics was within the first 20 min (Figure 3). An incubation period of 15 min was selected on the basis of this observation, and used in the subsequent experiments to approximate the initial uptake rate. The uptake kinetics manifested between the hPepT1 clones analyzed and

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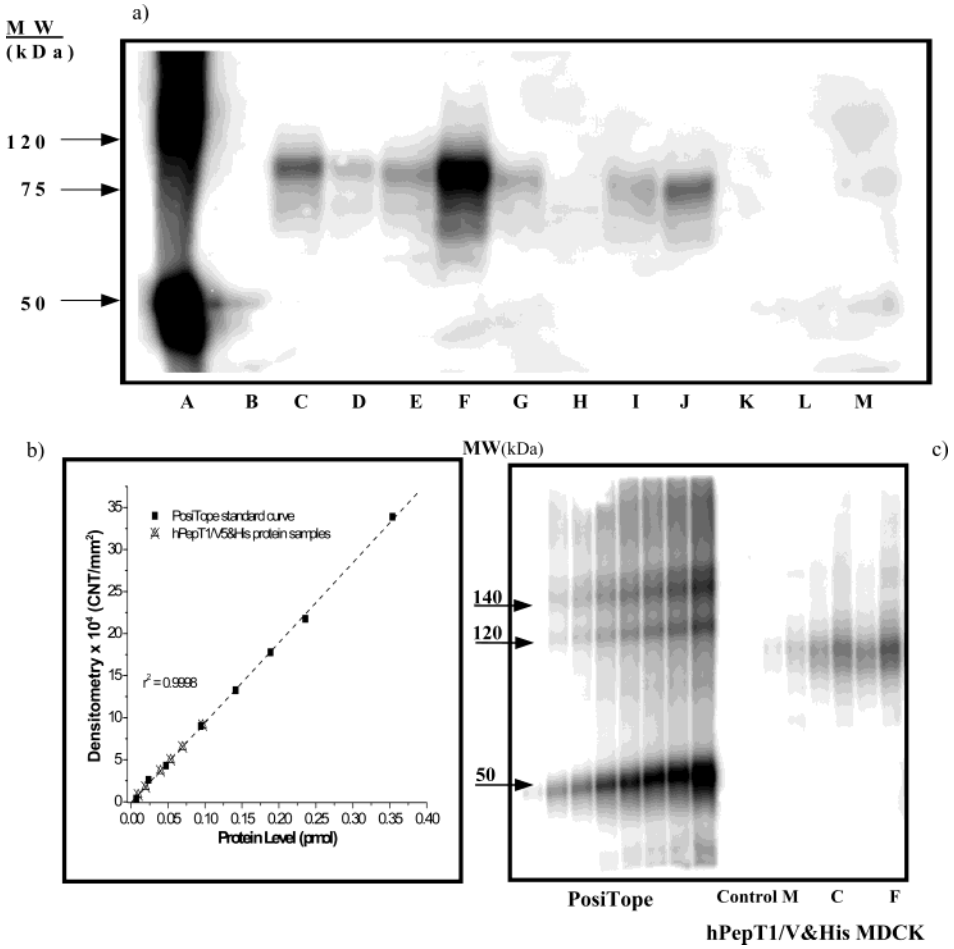

Figure 1. (a) Expression of the human PepT1/V5&His protein in MDCK transfected cells. Western Blot analysis using a V5 epitope antibody for the detection of 20 μg of protein lysate from MDCK stably transfected cells: (A) PosiTope, standard protein, (B) pcDNA3.1/V5&His-MDCK control cell line, and (C−M) hPepT1/V5&His-MDCK cells. (b) Standard curve showing regression analysis of PosiTope densitometry and interpolation of samples. (c) Western blot analysis: left, standard PosiTope protein; and right, MDCK control cell line (10 μg of total protein) and hPepT1/V5&His-MDCK cells (5 and 10 μg of total protein) at different levels of expression (M, low; C, medium; and F, high).

the control cell lines were determined to be significantly different ($p < 0.05$).

Concentration dependence profiles of Gly-Sar and carnosine uptake were determined in the three different hPepT1/V5&His-MDCK cell lines and in the mock cells to elucidate the Michaelis−Menten kinetic parameters. After correction for nonspecific uptake, i.e., after the uptake values measured in the mock cells had been subtracted, the K_m and V_{max} values for [^{14}C]Gly-Sar and [^{3}H]carnosine were determined in the hPepT1/V5&His-MDCK cell lines (Table 1). The concentration dependence profile of [^{14}C]Gly-Sar and [^{3}H]carnosine is shown in Figure 4. The potential of using the hPepT1/V5&His-MDCK cell line as a screening tool for peptide and peptide-based drug transport was further examined through inhibition studies utilizing valacyclovir, enalapril, and cephalexin that are well-established as hPepT1 substrates.[1,3−5] The valacyclovir, enalapril, and cephalexin dose dependably inhibited the uptake of [^{14}C]Gly-Sar and showed the IC_{50} values of 1.1, 4.6, and 10.2 mM, respectively.

Transport Studies. Determination of transepithelial electrical resistance (TEER) and mannitol permeation were evaluated to characterize the monolayer integrity. The TEER values did not significantly change after the first 3 days until the period of the experiments performed by 7 days (data not shown). The [^{14}C]mannitol apparent permeability coefficient (P_{app}) in the hPepT1/V5&His-MDCK cell line was found to be insignificantly different from the P_{app} values observed in the parental and control cell lines (Table 2). The [^{14}C]Gly-Sar and [^{3}H]carnosine apparent permeability coefficients demonstrated a statistically significant difference ($p < 0.05$) between the control cell line and the hPepT1/V5&His MDCK cells after being cultured for 7 days (Table 2). The rate of transepithelial transport of [^{14}C]Gly-Sar and [^{3}H]carnosine in the stably transfected cells was from 1.2 to 8.7 and 1.22 to 2.3 times, respectively, higher in low to high expressing cells than the values observed in the mock cells. Basolateral to apical [^{14}C]Gly-Sar [$(2.3 \pm 0.4) \times 10^{-6}$ cm/s] and [^{3}H]-carnosine [$(6.06 \pm 0.2) \times 10^{-6}$ cm/s] permeability coefficients were calculated in the mock cell line and were found to be insignificantly different when compared to the basolateral to apical permeability coefficients of the hPepT1/V5&His-expressing cell lines.

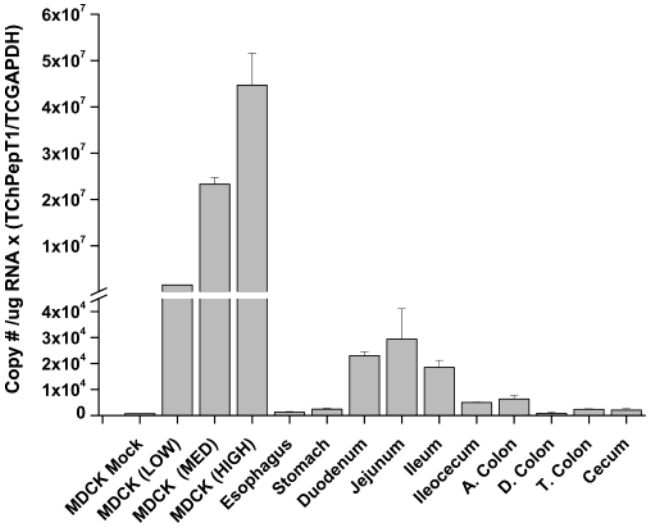

Figure 2. Real-time PCR analysis (QPCR) of hPepT1 expression in three cell lines. The TC values were converted to copy number by using a standard curve built with serial dilutions of hPepT1 plasmid DNA. The data were normalized for GAPDH expression. Minus RT and no input controls were included. Results are presented as the number of copies per microgram of RNA normalized to the ratio of hPepT1 to GAPDH threshold cycle (TC) values ± the standard error of the mean.

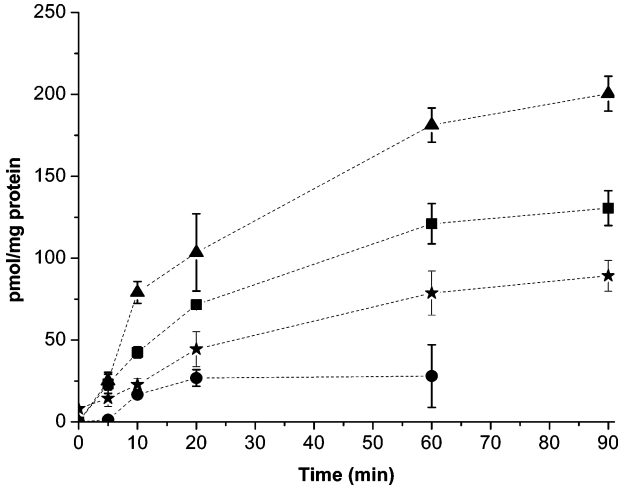

Figure 3. [^{14}C]Gly-Sar uptake kinetics in hPepT1/V5&His-MDCK cells. Analysis in 24-well plates (5×10^4 cells/cm^2) at pH 6.0. Data are presented as means ± the standard error of the mean ($n = 3$): (●) mock cells and (★) low expressing, (■) medium expressing, and (▲) high expressing hPepT1 clones.

Discussion

While active routes of transport across cellular barriers have been characterized,[26] it has been difficult to delineate the extent to which a solute permeates a cell monolayer by any one transporter. Several factors contribute to this difficulty, including (i) the fact that it is nearly impossible to exactly quantify the endogenous expression levels of the functional active transporter(s) responsible for solute permeation, (ii) differences in the specificities and activities of endogenously expressed transporter isoforms that may obfuscate kinetic assessments, (iii) families of other transporters that may be present and may have overlapping specificities, and (iv) the fact that solutes may have several potential routes of permeation across a biological barrier.

Clearly, a need exists for the development of cell lines capable of focusing the transport kinetics of therapeutic agents on the expression of a single transporter found in a tissue to enable the correlation of its functional properties to its levels of expression. The current research utilizes an epitope tagging strategy to enable the development of such correlations between *in vitro* functional and molecular characteristics of transporters. An hPepT1-epitope/tag transgene system was established that enabled quantification of the changes in the uptake and permeation of hPepT1 substrates due to varying transgene transporter expression

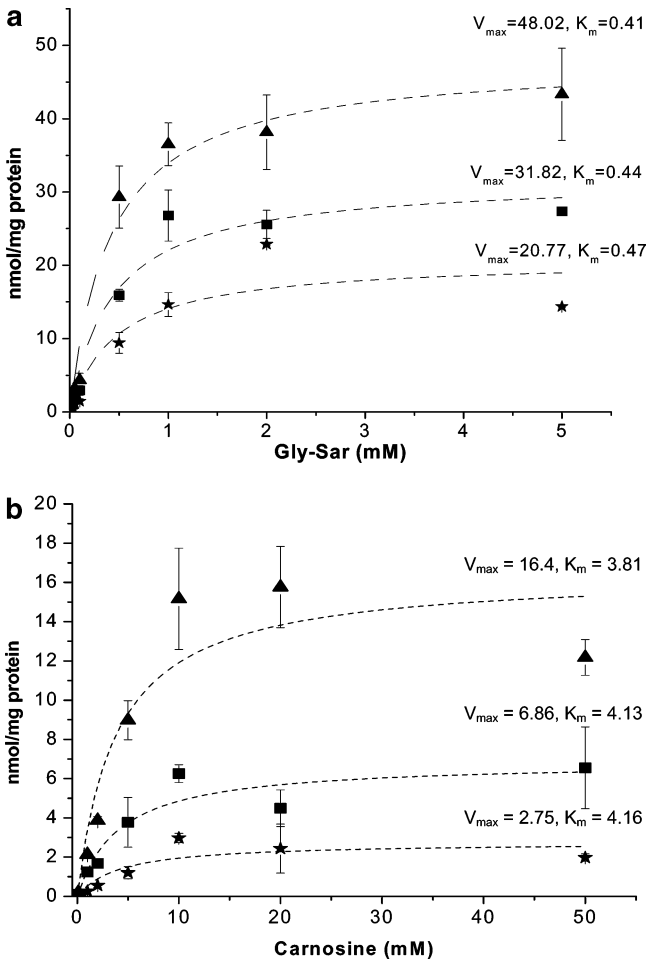

Figure 4. Concentration dependence of [^{14}C]Gly-Sar (a) and [^{3}H]carnosine (b) uptake in hPepT1/V5&His-MDCK cells. Analysis in 24-well plates (5×10^4 cells/cm^2) after incubation for 15 min at pH 6.0. Data are presented as means ± the standard error of the mean ($n = 3$): (★) low expressing, (■) medium expressing, and (▲) high expressing hPepT1 clones.

(26) *Membrane Transporters As Drug Targets*; Amidon, G. L., Sadee, W., Eds.; Kluwer Academic/Plenum Publishers: New York, 1999.

Table 2. Permeability Coefficients (Apical to Basolateral) for [^{14}C]Mannitol, [^{14}C]Gly-Sar, and [^{3}H]Carnosine Crossing MDCK Cell Monolayers[a]

	P_{app} ($\times 10^6$ cm/s)		
	[^{14}C]mannitol	[^{14}C]Gly-Sar	[^{3}H]carnosine
wild-type MDCK cells	2.1 ± 0.4	ND	ND
mock MDCK cells	3.7 ± 0.5	1.7 ± 0.2	6.78 ± 0.2
hPepT1/V5&His MDCK cells			
low level of expression	ND	2.0 ± 0.3	8.31 ± 0.4[b]
medium level of expression	ND	4.3 ± 0.5[c]	10.3 ± 2.39[c]
high level of expression	2.0 ± 0.4	15.0 ± 1.9[c]	15.6 ± 2.25[c]

[a] Transport studies were performed at day 7 of the culture using Transwell precollagenated filters (0.4 μm, 1 cm^2). Data are presented as means ± the standard error of the mean ($n = 3$). ND, not determined. [b] A p of <0.05 shows the significant change in the permeability coefficients (apical to basolateral) as compared to the mock permeability coefficient value (apical to basolateral). [c] A p of <0.01 shows the significant change in the permeability coefficients (apical to basolateral) as compared to the mock permeability coefficient value (apical to basolateral).

and to correct for the uptake or permeation that is attributed to endogenous factors (endogenous transporter expression and passive diffusion).

The Mandin-Darby canine kidney (MDCK) cell line is the one of the most widely utilized *in vitro* models for assessing drug transport.[27−30] These cells possess low-affinity peptide transport activity, although the protein associated with the transport has not been identified.[31] Recently, MDCK monolayers were evaluated as an alternative to Caco-2 cells for the study of peptide transport activity.[32] The bidirectional endogenous transport of Gly-Sar and cephalexin was assessed in Caco-2 and MDCK cells. MDCK cells were shown to be an alternative for assessing peptide and peptide-like drug permeability. However, the level of endogenous expression of peptide transporters in the MDCK cell line appears to be lower than the levels found *in vivo*, and thus, the quantified transport activity using this model seems to be underestimated relative to the *in vivo* drug absorption.[32] Accordingly, our analysis of the endogenous peptide transport activity obtained in the parental and control MDCK (mock cells) cell lines demonstrated a very low level of peptide uptake.

The analysis of both the hPepT1 and neomycin mRNA expression in the hPepT1/V5&His-MDCK transfected cells showed that the transfection and the selection methods employed were highly efficient (62% of the neomycin positive clones expressed hPepT1). In general, the established transfection/selection protocol could be used for the expression and stabilization of other nutrient/drug transporters in MDCK cells. The utilization of V5 epitope tag antibodies further enabled the determination of hPepT1 transgene protein expression concentrations so that the clones could be divided into low, medium, and high expressing cells (Figure 1). Furthermore, the hPepT1/V5&His-MDCK cell line exhibited a stable hPepT1/V5&His protein expression for at least 20 passages.

The QPCR tissue expression data confirm our previous nonquantitative analysis,[16] which reported that PepT1 was mostly expressed in the upper human GI. The hPepT1 mRNA levels achieved in the MDCK transfected cells were in all cases superior to the levels observed in these tissues, suggesting that the hPepT1/V5&His-MDCK cells may be a better model than other cell lines for correlating peptide and peptide-based drug uptake and transport with *in vivo* absorption. There was a linear correlation ($r = 0.96$) found between the mRNA copy numbers and extrapolated protein levels for the three hPepT1/V5&His-MDCK clones.

Our studies have quantitatively evaluated the enhancement of the peptide transport activity in hPepT1/V5&His-MDCK cells through the kinetic evaluation of Gly-Sar and carnosine uptake and transport in three selected clones expressing different hPepT1 levels (low, medium, and high). Endogenously corrected, Michaelis−Menten K_m values for Gly-Sar and carnosine uptake were obtained, and these results demonstrated that the hPepT1 affinity was the same in the three clones. Differences in hPepT1 capacity were evident; for Gly-Sar, the high hPepT1 expressing cell line had a V_{max}/K_m ratio that was 1.6- and 2.6-fold higher than those of the medium and low hPepT1 expressing cells, respectively, whereas the ratio was 2.5 and 6.5 times higher with carnosine, respectively. Moreover, the uptake kinetics indicated that the hPepT1/V5&His-MDCK cell lines increased the dipeptide uptake capacity to an extent nearly proportional to the levels of expression of the hPepT1/V5&His protein,

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while retaining its substrate specificity. Thus, this system may be useful as an *in vitro* model for the rapid screening of hPepT1-mediated uptake of peptide-like drugs and the characterization of hPepT1 substrate affinity.

Uptake studies are not always a good predictor for the actual cellular absorption of drug candidates, since binding to a transporter does not necessarily guarantee drug absorption into and across the cells.[21] Therefore, the hPepT1/V5&His-MDCK cell monolayer was further evaluated for its transepithelial transport capabilities. TEER and [^{14}C]-mannitol permeability analyses were carried out to determine the monolayer integrity. Parental MDCK cells have a reported TEER value of ~200−300 Ω cm^2 [24] and a mannitol P_{app} value of ~1.9−5.0 × 10^{-6} cm/s. The results obtained in the hPepT1/V5&His-MDCK cells demonstrated that the transfection procedure did not modify the ability of MDCK cells to form a tight monolayer. The TEER (307 ± 40 Ω cm^2) as well as the mannitol P_{app} [(2.0 ± 0.4) × 10^{-6} cm/s] values in the transfected cells were not significantly different than the estimates in the parental cell line after 7 days of growth.

The Gly-Sar and carnosine apparent permeability coefficients after 7 days of culture demonstrated a statistically significant difference between the mock and hPepT1/V5&His-MDCK cells ($p < 0.05$; Table 2). The rate of transepithelial transport of Gly-Sar and carnosine in the stably transfected cells was from 1.2- to 8.7-fold and from 1.5- to 1.8-fold higher in the MDCK low and higher transgene expressing lines than in the mock MDCK cells, respectively, confirming the ability of the cell line to elucidate the interaction and transport capabilities of the hPepT1 transporter. Basolateral to apical [^{14}C]Gly-Sar [(2.3 ± 0.4) × 10^{-6} cm/s] and [^{3}H]carnosine [(6.06 ± 0.2) × 10^{-6} cm/s] permeability coefficients were calculated in the mock cell line and were determined to be insignificantly different when compared to the basolateral to apical permeability coefficients of hPept1-expressing cell lines (data not shown).

The estimated Gly-Sar and carnosine P_{app} in the hPepT1/V5&His-MDCK clones also increased with the increasing hPepT1 protein levels that were used (Table 2). These data demonstrated that changes in the observed transport levels were due primarily to changes in hPepT1 protein expression. This is confirmed when comparing the Gly-Sar and carnosine V_{max} and P_{app} values, where a relationship is observed between the log V_{max} of Gly-Sar (or carnosine) plotted against the log P_{app} of Gly-Sar (or carnosine) in the hPepT1/V5&His-MDCK clones (both demonstrating a linear correlation coefficient r of 0.98). On the other hand, comparison of K_m values between Gly-Sar and carnosine showed that the former has a higher affinity for hPepT1, which may lead to this system being used as a better tool in assessing and screening various substrates for their affinity for a designated transporter.

Overall, these results illustrate that the hPepT1/V5&His cell line could be utilized to study the ability of a drug to specifically interact with hPepT1 during uptake and transport processes. The model described in this research has provided the basis for its extension into other transporter systems. These efforts are the first step in establishing standardized transporter or receptor cell lines as *in vitro* models for drug transport studies. The transport kinetic estimations, coupled with the quantitative assessments of endogenous expression, will eventually help in the establishment of *in silico* models for assessing peptide-based drug absorption phenomena across any biological barrier. Furthermore, these results provide a proof of principle for the ability of our novel, stably transfected cell line methodology to relate the contributions of a transporter or a receptor to the permeation of drugs and its correlation with the level of expression of the transporter.

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