molecular pharmaceutics

ARTICLE

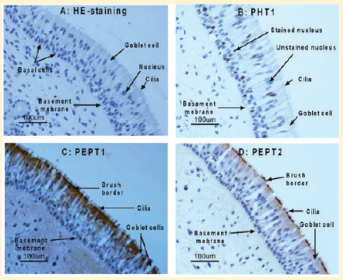
pubs.acs.org/molecularpharmaceutics

# Proton-Coupled Oligopeptide Transporter (POT) Family Expression in Human Nasal Epithelium and Their Drug Transport Potential

Remigius Agu,*[,†] Elizabeth Cowley,[‡] Di Shao,[†] Christopher MacDonald,[‡] David Kirkpatrick,[§] Ken Renton,[∥] and Emad Massoud[§]

[†]Biopharmaceutics and Drug Delivery Laboratory, [‡]Department of Physiology, [§]Department of Surgery, and [∥]Department of Pharmacology, Dalhousie University, Halifax, NS B3H3J5, Canada

**ABSTRACT:** The molecular and functional expression of peptide transporters (PEPT1 and PEPT2, PHT1, PHT2) in human nasal epithelium was investigated. Quantitative/reverse transcriptase polymerase chain reaction (qPCR/RT-PCR), Western blotting and indirect immuno-histochemistry were used to investigate the functional gene and protein expression for the transporters. Uptake and transport studies were performed using metabolically stable peptides [$\beta$-alanyl-L-lysyl-N$\varepsilon$-7-amino-4-methyl-coumarin-3-acetic acid ($\beta$-Ala-Lys-AMCA) and $\beta$-alanyl-L-histidine (carnosine)]. The effects of concentration, temperature, polarity, competing peptides, and inhibitors on peptide uptake and transport were investigated. PCR products corresponding to PEPT1 (150 bp), PEPT2 (127 bp), PHT1 (110 bp) and PHT2 (198 bp) were detected. Immunohistochemistry and Western blotting confirmed the functional expression of PEPT1 and PEPT2 genes. The uptake of $\beta$-Ala-Lys-AMCA was concentration-dependent and saturable ($V_{max}$ = 4.1 ± 0.07 $\mu$mol/min/mg protein, $K_m$ = 0.6 ± 0.07 $\mu$M). The optimal pH for intracellular accumulation of $\beta$-Ala-Lys-AMCA was 6.5. Whereas dipeptides and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) significantly inhibited peptide uptake and transport, L-Phe had no effect on peptide transport. The permeation of $\beta$-alanyl-L-histidine was concentration-, direction-, and temperature-dependent. The uptake, permeation, qPCR/RT-PCR and protein expression data showed that the human nasal epithelium functionally expresses proton-coupled oligopeptide transporters.




**KEYWORDS:** nasal epithelium, peptide transporters, PEPT1, PEPT2, drug permeation, tissue culture

## INTRODUCTION

About 50 years ago Newey and Smyth reported the first studies indicating the transport of intact peptides across the intestinal epithelium.[1] Eighteen years later, Adibi et al. demonstrated active transport of peptides against a concentration gradient,[2] a phenomenon that was later explained to be due to proton-coupled peptide transport by peptide transporters.[3] In mammals, the proton-coupled oligopeptide transporter (POT) family consists of four members including PEPT1, PEPT2, PHT1, and PHT2. Although the roles of PHT1 and PHT2 are not yet fully defined, PEPT1 and PEPT2 transporters are plasma membrane proteins that mediate the cellular uptake of di/tripeptides and a variety of other non-peptides[4] via an inwardly directed proton gradient and negative membrane potential.[5] Each member of the POT family displays a distinct pattern of tissue expression, providing a possible avenue for drug targeting to select tissues,[6] whereas PEPT1 is principally expressed in the intestine, and PEPT2 is expressed mainly in the kidney.[7,8] It has also been demonstrated that PEPT1 and PEPT2 are differentially distributed along the proximal tubule, with the former predominantly expressed in the convoluted segment and more of the latter in the straight segment.[9,10]

Although several articles have been published on drug delivery strategies via peptide transporters expressed in the ocular, intestinal, and pulmonary epithelial cells,[11−13] the potential of these transporters to be exploited for drug delivery via the nasal route is yet to be investigated. In order to explore this possibility, it is important to ask some pertinent questions regarding the expression of drug transporters in the nasal epithelium. Which active drug transporters have been identified in the nasal epithelium? Among the identified transporters, which ones are expressed in the plasma membranes? Both direct and indirect evidence have shown that solutes including amino acids, dopamine, and some polypeptides are actively transported across the nasal epithelium. Studies conducted in the early 1970s[14,15] on the nasal absorption of amino acids suggested the existence of active transport systems for amino acids in nasal mucosa of rodents. Later studies in rodents[16,17] and humans[18] also confirmed these findings. Recently, Monte et al.[19] demonstrated the expression of organic anion transporter family member, OAT6 in murine olfactory mucosa. Similarly, Chemuturi et al. showed that pig nasal mucosa expressed dopamine and organic cation transporters (OCT-1 and OCT-2).[20,21] Although peptide transporters have been recognized as the solute carrier transporter family (SLC) with the most favorable characteristics as drug carriers (due to their substrate versatility and solute-carrying capacity), not much is known about their expression and transport attributes in the nasal epithelium.

**Received:** July 13, 2010
**Accepted:** March 2, 2011
**Revised:** January 5, 2011
**Published:** March 02, 2011

In a study to validate the use of excised bovine nasal mucosa as an *in vitro* model to study drug transport and metabolism, Schmidt et al.[22] found no direct correlation between permeability coefficients of some peptides and their respective molecular masses. This was an indication that other factors such as metabolism and active transport played important roles during the permeation process. In fact, they reported that the permeability of metabolically labile peptides (thymopoietin fragments, Arg-Lys-Asp (TP3) and Arg-Lys-Asp-Val (TP4)) were concentration dependent and saturable. As these compounds are small peptides that are subject to break down to dipeptides, oligopeptides and amino acids, it is possible that the observed concentration-dependency and saturation were due to active transport by peptide transporters in the bovine nasal epithelium. Recently, Quarcoo et al.[23] showed that PEPT2 transporters are expressed in the rat nasal epithelium. The purpose of this study was to provide functional and molecular evidence supporting the existence of peptide transporters in the human nasal epithelium for drug targeting.

## MATERIALS AND METHODS

**Chemicals.** β-Alanyl-L-histidine (carnosine), Gly-Phe, Gly-Sar, L-Phe, cephadroxil, sodium fluorescein, bovine serum albumin (BSA), Pronase, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), CCCP, isopropanol, Hanks balanced salt (HBBS), Mayer's hematoxylin and penicillin/streptomycin were supplied by Sigma (St. Louis, MO, USA). DMEM-F12 1/1, M-MVL reverse transcriptase, cDNA buffer, TriZol and dNTPs were purchased from Invitrogen (Burlington, ON, Canada). Oligo dT primers and Ultroser G were from Promega (Madison, WI, USA) and Biosepra (St-Germain-en-Laye Cedex, France), respectively. β-Ala-Lys-AMCA was custom-made by Biotrend GmbH (Cologne, Germany); Taq polymerase and the gene ladder (100 bp GeneRuler) were from Fermentas (Burlington, ON, Canada). Reverse transcription kit and SYBR green mix were purchased from Qiagen (Mississauga, ON, Canada). Universal biotin link, streptavidin-HRP and protein block were from Dako (Mississauga, ON, Canada). Polyclonal antibodies for PEPT1, PEPT2, and PHT1 were from Abcam (Cambridge, MA, USA). Secondary anti-rabbit and anti-goat antibodies were purchased from Jackson Immunoresearch (West Grove, PA, USA). Chemiluminescent HRP substrate was from Millipore (Billerica, MA, USA).

**Cell Culture.** The cell culture method used for the study has been described in detail elsewhere.[24] Normal nasal epithelial cells were extracted from tissues obtained from patients that underwent endoscopic trans-nasal skull base surgery. Smokers and patients with chronic inflammatory respiratory diseases were excluded from the study. The use of human biopsies was approved by QEII Regional Hospital Research Ethics Board (REB # CDHA-RS/2006-352). The tissues were transported in DMEM-F12 1/1 culture medium supplemented with streptomycin 100 μg/mL and penicillin 100 IU/mL and used for cell culture. Biopsies were washed three times with physiological saline solution supplemented with antibiotics. The cells were dissociated enzymatically for a period of 16−24 h at 4 °C using 0.1% Pronase. The Pronase was deactivated with 10% NU-serum prior to cell washing with DMEM-F12 1/1. The washing solution was removed after centrifugation at 170*g* (1500 rpm) for 5 min on each occasion. The resulting suspension of cells was preplated on plastic for 1 h at 37 °C in a 95% $O_2$ and 5% $CO_2$ environment to reduce fibroblast contamination. Subsequently, the cells were counted and seeded at a density of $5.0 \times 10^4$ cell/well on Cellagen CD-24 inserts (MP Biomedicals, OH, USA) for transport studies. Cells were also seeded on 24 well plates (Fisher Scientific, ON, Canada) and Lab-Tek 6-chambered borosilicate # 1.0 cover slides (Fisher Scientific, ON, Canada) for uptake and immunohistochemical studies, respectively. The cells were incubated at 37 °C in a 95% $O_2$ and 5% $CO_2$ environment using DMEM F12 supplemented with Ultroser G 2%. The medium was changed every other day. Cells for transport studies were maintained as immersion culture for the first 3 days and were switched to air−liquid interface until used for experiments (10−14 days after culture).

**Quantitative and Nonquantitative RT-PCR Studies.** Total RNA was extracted from human nasal turbinates using TriZol according to the manufacturer's instructions. In brief, cells were lysed in 1 mL of TriZol containing 200 μL of chloroform was added per 1 mL of TriZol and vortexed. Three phases were separated by centrifuging for 15 min at 13,000 rpm at 4 °C. Only the colorless upper aqueous RNA phase was removed and vortexed with isopropanol to precipitate the RNA. Samples were incubated at room temperature for 10 min before centrifugation for 10 min at 13000 rpm at 4 °C. The resulting RNA pellet was washed twice with ice cold 75% ethanol and then resuspended in $ddH_2O$. Concentration and purity of RNA was assessed using spectrophotometry. All samples had $A_{260}/A_{280}$ absorbance readings greater than 1.6 confirming high RNA purity. Subsequently, 20 μL of cDNA was synthesized from 0.5 μg of total RNA using Qiagen reverse transcription kit as recommended by the manufacturer.

PCR amplification was performed in a total volume of 20 μL containing 1 μL of cDNA sample, 10 μL of 2x SYBR green mix, and 0.5 μM forward and reverse primers (Table 1). Following 95 °C incubation for 15 min, forty cycles of PCR (94 °C/15 s; 58 °C/20 s; 72 °C/30 s) were then performed on an ABI Prism 7900HT sequence detection system. Threshold cycles ($C_T$) for duplicate reactions were determined using Sequence Detection System software (version 2.2.2) and relative transcript abundance calculated following normalization with a β-actin PCR amplicon. Amplification of only a single species was verified by a dissociation curve for each reaction. Reactions with RNA alone were used as negative control. Amplified DNA was electrophoresed on a 1% agarose gel containing ethidium bromide.

**Western Blot.** Cultured nasal cells were washed with PBS and then scraped off the plates and collected by centrifugation. Cell pellet was resuspended in radioimmunoprecipitation assay buffer (RIPA) with Halt protease inhibitor cocktail (Pierce Biotechnology, IL, USA) and incubated on ice for 30 min. The lysate was centrifuged at 15,000 rpm for 20 min. The remaining supernatant was removed, and the protein concentration was determined using BCA protein assay kit (Pierce Biotechnology, IL, USA) according to the manufacturer's protocol. Aliquots of 50 μg of proteins were boiled for 3 min in loading buffer and separated on a 10% SDS−polyacrylamide gel. Transfer onto a nitrocellulose membrane was performed over a 2 h period on ice. The membrane was then blocked in 5% skimmed milk in Tris-buffered saline Tween-20 (TBST) for 1 h before overnight incubation with rabbit polyclonal anti-PEPT1 and anti-PEPT2 at 4 °C with gentle agitation. The membranes were then washed 3 times for 15 min in TBST before incubation with anti-rabbit IgG-HRP. The blots were washed three more time for 15 min in TBST before antibodies were detected with the enhanced chemiluminescence (ECL) method.

**Table 1. Primers for Nonquantitative/Quantitative PCR Studies**[a]

| transporter | forward/reverse primer | position | amplicon size (bp) |
|---|---|---|---|
| hPEPT1 | CAATCCTGATTCTGTACTTCAC | 200 | 150 |
| | ACAATGGTCTTGAACTTTCC | 350 | |
| hPEPT2 | TCACTTCCACCTGAAATATCAC | 1428 | 127 |
| | CTGTATCCTTTACCATCATGCT | 1555 | |
| hPHT1 | GAGTAATGGTGAAGGCATTGG | 886 | 110 |
| | ACATCTTCCACTTTCTCTTCTG | 996 | |
| hPHT2 | GATCAGTGAGATCTTTGCCA | 1640 | 198 |
| | ATTGTTGATGTTCCCAAAGTCC | 1838 | |

[a] Gene sequences for primer design were obtained from the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/). Primers were designed using OligoPerfect Designer from Invitrogen.

**Immunohistochemistry.** Human nasal turbinate tissue was dissected and fixed in formalin within an hour of excision. Subsequently, the tissues were embedded in paraffin wax and several 5 $\mu$m tissue sections were cut out using a Reichert−Jung rotary microtome, and placed on silinated slides. Sections were deparaffinized through xylene and an alcohol gradient. After the slides were washed in PBS, endogenous peroxidase activity was quenched with 2% hydrogen peroxide in PBS. The slides were then incubated with protein block to block nonspecific antigen binding sites followed by rabbit polyclonal antibodies (PEPT1, PEPT2, PHT1) overnight in a humid chamber. After the incubation, the slides were washed in PBS and then further incubated for an additional 1 h with universal biotin link. After another PBS wash, slides were incubated with streptavidin-HRP for 1 h. Peroxidase activity was visualized with diaminobenzidine-tetrachloride chromogen and counterstained with Mayer's hematoxylin. Control staining sections were incubated with PBS instead of the primary antibody. Photographs were taken with a high-resolution digital microscopic camera (AxioCamHR, Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

**Drug Uptake Studies.** Uptake studies were conducted using coumarin-tagged peptide probe ($\beta$-Ala-Lys-AMCA) as a substrate for peptide transporters according to the method described by Groneberg et al.[25] Following a brief wash with Hanks balanced salt solution (HBSS, pH 6.5), the nasal cells were incubated with a specific concentration of the peptide for 10 min. The cells were rapidly washed twice with 1 mL of ice cold HBSS to stop the uptake. This was followed by cell lyses with a solution of 0.1% Triton-X100 in 0.1 N NaOH. Inhibition studies were conducted by preincubating the cells for 15 min with competing compounds. The effect of pH on the uptake of the model peptide was investigated by conducting Ala-Lys-AMCA uptake in buffers of varying pH (4.0, 5.0, 6.0, 6.5, 7.4, 8.4). Nasal cells that were cultured for 10−14 days were used for the studies because preliminary PCR studies showed higher gene expression within this period compared to cells that were grown for 1 week.

**Transport Studies.** $\beta$-Alanyl-L-histidine (PEPT1/2 substrate) was used as a model compound for transport studies. The influence of concentration (0.1−5 mM), temperature (4 °C, 37 °C), competing peptides (Gly-Phe, Gly-Sar, L-Phe) and polarity on the transport of the compound was investigated. Prior to the transport studies, the cells were rinsed twice and preincubated with transport medium, TM (Hanks balanced salt supplemented with HEPES buffer 10 mM and 25 mM glucose, pH 6.5), for 15 min at 37 °C. An additional 15 min apical preincubation with the competing peptides was allowed for inhibition studies. Transport studies were initiated by adding 250 $\mu$L of test solutions to the donor compartment. At predetermined time points (0−120 min), 100 $\mu$L aliquots were sampled from the acceptor compartment (750 $\mu$L) and were replaced immediately with an equal volume of TM. All cell monolayers were checked for epithelial integrity before and after the experiments by TEER measurement and sodium fluorescein permeation at the end of the experiments. The average pre- and post-transport study TEER values for all the data points were $407 \pm 22$ and $398 \pm 18$ ($n = 120$), respectively. Sodium fluorescein permeation at the end of the experiments was $\leq 1.0\%$. Details of the characterization and validation of the cell culture model used for the transport studies can be found in the literature.[24,26] Cell protein content was estimated using BCA protein assay kit.

**HPLC and Fluorescence Analysis.** Sodium fluorescein was analyzed with a Cary 50 UV−vis spectrophotometer (Varian Inc., CA, USA) at 490 nm wavelength. Carnosine samples were analyzed by HPLC using Waters 2690 separations modules coupled to a Waters 996 photodiode array detector. The HPLC conditions were as follows: column (Brava ODS 5 $\mu$m), wavelength (210 nm), flow rate (1 mL/min), mobile phase (60:40 methanol/water), and software (Breeze). $\beta$-Ala-Lys-AMCA samples were measured with a Modulus single tube multimode reader (Turner Biosystems, CA, USA) using a fluorescent UV optical kit.

**Data and Statistical and Analyses.** Apparent permeability coefficients [$P_{app}$ (cm/s)] for $\beta$-alanyl-L-histidine transport were calculated using the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0}$$

where $dQ/dt$ = time-dependent carnosine flux, $C_0$ = $\beta$-alanyl-L-histidine initial concentration in the apical compartment, $A$ = Cellagen membrane (CD-24) surface area across (0.785 cm$^2$).

In order to calculate kinetic parameters ($K_m$, $V_{max}$), dipeptide uptake/transport data were fitted to Michaelis−Menten equation using Prism 5.0 (GraphPad, San Diego, CA, USA). Passive diffusion was accounted for by subtracting the rate constant for $\beta$-alanyl-L-histidine passive diffusion at 4 °C using the following equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} + K_d[S]$$

where $K_m$ = Michaelis−Menten constant ($\mu$M), $V_{max}$ = maximum $\beta$-alanyl-L-histidine transport rate (nmol/min/mg protein), [S] = $\beta$-alanyl-L-histidine concentration ($\mu$M), $V$ =

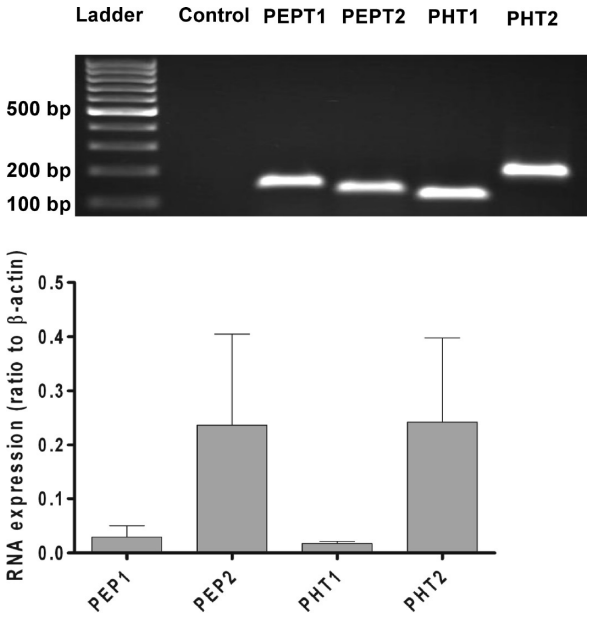



**Figure 1.** Qualitative and quantitative expression of PEPT1, PEPT2, PHT1 and PHT2 mRNA in human nasal epithelium. Bands depict expected transcripts (PEPT1 = 150 bp, PEPT2 = 127 bp, PHT1 = 110 bp, PHT2 = 198 bp). Bar chart shows quantitative gene expression relative to $\beta$-actin ($n$ = 6).

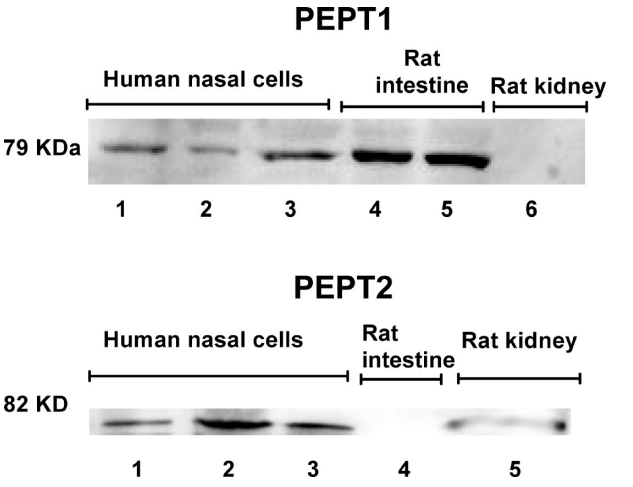



**Figure 2.** Expression of functional protein for PEPT1 and PEPT2 transporters in nasal epithelium. Lanes 1−3 were based on 50 $\mu$g of aliquot protein from human nasal epithelium that were subjected to 10% SDS−polyacrylamide gel followed by post nitrocellulose transfer and 1 h incubation with anti-PEPT1 and anti-PEPT2 antibodies. Rat intestine (jejunum) and kidney tissues were used as positive or negative controls for PEPT1 and PEPT2, depending on the protein. The bands were visualized with HRP conjugated anti-rabbit antibodies and chemoluminescence.

rate of $\beta$-alanyl-L-histidine (nmol/min/mg protein), $K_d$ = rate constant for $\beta$-alanyl-L-histidine passive diffusion ($\mu$M/min).

Unless stated otherwise, all experiments were performed in triplicate and data presented as mean ± SD. Where appropriate, statistical significance of the results was determined using ANOVA (inhibition studies) followed by post-test analysis using InStat 3.0 (GraphPad, San Diego, CA, USA). $P$ values lower than 0.05 were considered significant.

## RESULTS

**Gene Expression Studies.** The isolated RNA from normal human nasal turbinates, which was transcribed to cDNA using oligo dT primers, was used for the studies. As negative control, RNA was used as a template to confirm lack of genomic DNA contamination. The result of the gene expression studies using PCR is summarized in Figure 1. No band was seen in the control reaction. However, the predicted PCR products for PEPT1 (150 bp), PEPT2 (127 bp), PHT1 (110 bp) and PHT2 (198 bp) were seen. These results represent the first molecular evidence supporting the expression of proton-coupled oligopeptide transporter family in the nasal epithelium. Quantitatively, we also compared the expression levels of the genes in the epithelium (Figure 1). The levels of PEPT2 and PHT2 were comparable and significantly higher than the levels shown by PEPT1 and PHT1 ($p < 0.05$). Based on the quantitative PCR data, it appears that the epithelium expressed similar levels of PEPT1 and PHT1 genes.

**Protein Expression and Immunohistochemical Studies.** To further verify the expression data from PCR results, Western blot of cultured nasal tissues was used to probe the expression of PEPT1 and PEPT2 proteins. Furthermore, immunohistochemistry of fresh tissues was used not only to confirm the protein expression but also to identify the location the transporters within the epithelium. Figure 2 shows the Western blot analysis for PEPT1 and PEPT2 proteins from human nasal epithelium. As expected, immunoreactive bands of PEPT1 (79 kDa) and PEPT2 (82 kDa) were detected. PEPT1 protein was detected in rat jejunum (positive control for PEPT1/negative control for PEPT2), but not in rat kidney (positive control for PEPT2/negative control for PEPT1). Similarly, PEPT1 was detected in the human nasal samples (lanes 1−3), but with less dense bands than the rat small intestine. Conversely, PEPT2 was detected in the rat kidney (positive control) and the human nasal samples (lanes 1−3), but not in the rat intestine (negative control). These results buttressed the fact that the functional uptake and transport of Ala-Lys-AMCA and $\beta$-alanyl-L-histidine observed in this study could be linked to PEPT1 and PEPT2 transporters. However, for solute transporters to participate in drug uptake and transport across the biological epithelia, they must be located on the apical or basolateral side of the cells.

Figure 3 shows the results of immunohistochemical staining of the human nasal epithelial tissues using specific antibodies for PEPT1, PEPT2 and PHT1. Differentiated morphological features of mature nasal epithelium including goblet cells, ciliated cells, basal cells and basement membrane are evident in panels A to D. Panel A is the control where PBS was added instead of antibodies. The tissue was only stained with hematoxylin−eosin staining. In panel B, staining was only found within the cell nucleus for PHT1 protein. No PHT1 was observed on either the apical or basolateral regions of the cells, thus highlighting the fact that PHT1 transporters may not play a significant role in solute absorption and transport across the nasal epithelium. In contrast, panels C and D indicate positive staining for PEPT1 and PEPT2, respectively along the brush border regions of the cells. No staining for the protein was seen along the basement membrane, which suggests that these transporters are located on the apical region of the cells. Although qPCR studies suggested higher expression levels for the PEPT2 genes, immunohistochemical studies yielded more intense staining for PEPT1 compared to PEPT2.

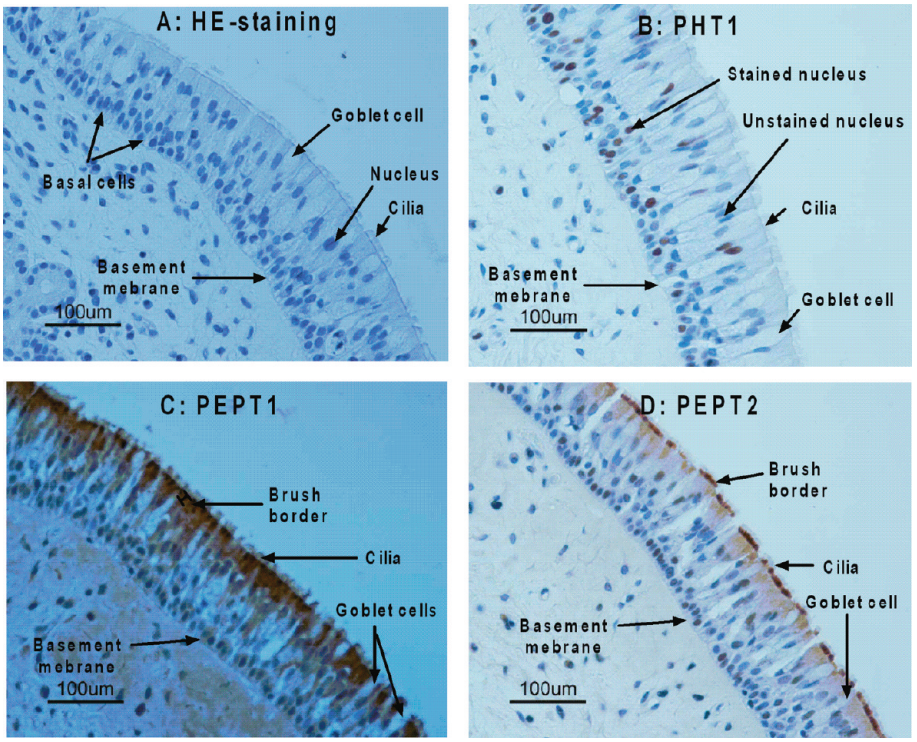



**Figure 3.** Immunohistochemical staining of human nasal epithelial tissues embedded in paraffin using specific antibodies for PEPT1, PEPT2 and PHT1. The tissues were transferred to the lab from the hospital within 1 h of surgery in ice-cold PBS, supplemented with 25 mM glucose. Sections were assayed with the HRP method and counterstained with Mayer's hematoxylin. A, Control (HE-staining only); B, PHT1; C, PEPT1; D, PEPT2.

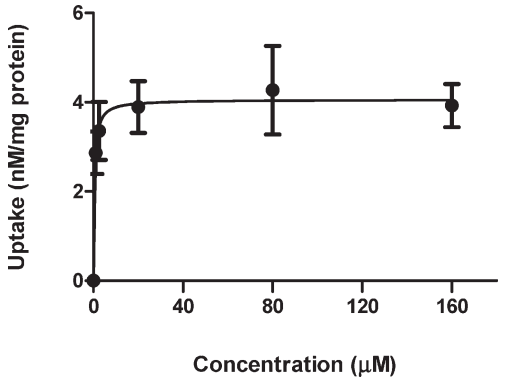



**Figure 4.** Concentration-dependent uptake of Ala-Lys-AMCA in human nasal epithelium. Each point represents mean ± SD of triplicate measurements.

**Uptake Studies.** In the uptake studies, the mechanism of intracellular accumulation of β-Ala-Lys-AMCA in human nasal epithelium was investigated. For this aspect of the studies, the effect of concentration and competing dipeptides on the uptake of the dipeptide were investigated (Figure 4). The dipeptide uptake was rapid, concentration-dependent, and saturable and followed Michaelis−Menten kinetics ($V_{max}$ = 4.1 ± 0.07 μmol/min/mg protein, $K_m$ =0.6 ± 0.07 μM). Substrate specificity studies showed that other dipeptides and cephadroxil (Figure 5A) significantly reduced the dipeptide uptake. Gly-Phe, Gly-Sar and β-alanyl-L-histidine (1 mM) reduced Ala-Lys-AMCA uptake by approximately 50%. Similarly, cephadroxil also significantly reduced the dipeptide uptake. To further characterize the active transport mechanism, the effect of EIPA, a potent inhibitor of $Na^+/H^+$ exchanger and CCCP, a potent protonophore were investigated (Figure 5B). Although EIPA had no effect on the peptide uptake, CCCP significantly reduced its uptake ($p < 0.05$). These observations implied that electrogenic proton pump rather than $Na^+/H^+$ exchanger was involved in the uptake of the compound. In order to investigate this hypothesis, the effect of pH or proton gradient on Ala-Lys-AMCA uptake was investigated (Figure 6). Most published reports on PEPT1 and PEPT2 transporters were performed using buffers with pH of either 6.0 or 6.5. In this study we adopted pH 6.5 as our standard pH for all uptake studies. So for studies involving the effect of pH, uptake at other pHs was compared to uptake at pH 6.5. Figure 6 summarizes the results of the studies. Maximum uptake occurred at pH 6.0, followed by pH 5.0. Although uptake occurred at pH 4.0, it was significantly lower than pH 6.5 ($p < 0.01$). Similarly, the uptake of the compound was significantly lower at neutral (7.4) and basic (8.4) pHs compared to pH 6.5 ($p < 0.05$). These observations clearly support the evidence that proton pumps were involved in the uptake of the Ala-Lys-AMCA in human nasal epithelium.

**Transport Studies.** Metabolically stable dipeptide derivative β-alanyl-L-histidine was used to demonstrate peptide-transporter-assisted solute transport across the human nasal epithelium. Validation studies in our laboratory using β-alanyl-L-histidine and human nasal cells confirmed that the peptide was intact and not metabolized during the experiment as L-histidine was not detected by HPLC. For the transport studies, the effects of concentration (Figure 7A,B), temperature (Figure 8A), polarity (Figure 8B) and other peptides (Figure 9) on β-alanyl-L-histidine transport were investigated. Figure 4A shows that transport of the compound was concentration-dependent and saturable. The permeability of the compound was inversely related to

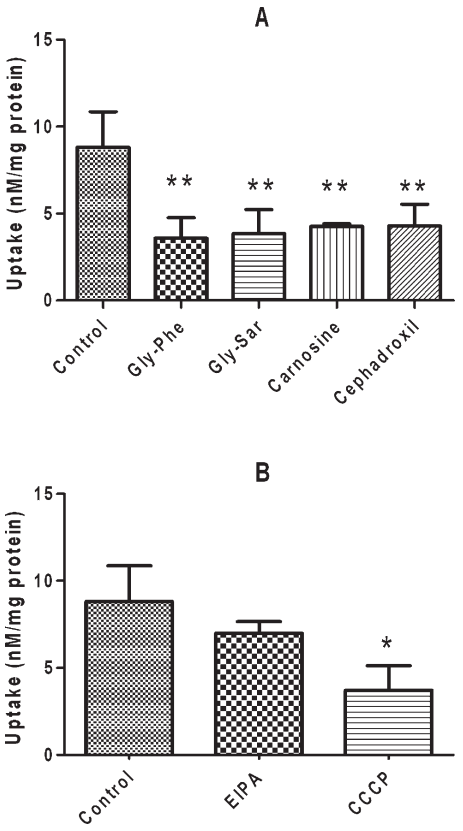



**Figure 5.** Effect of dipeptides and cephadroxil (A) and biological inhibitors (B) on intracellular uptake of Ala-Lys-AMCA in human nasal epithelium. Uptake was measured at 37 °C for 10 min in HBSS buffer supplemented with 25 mM glucose, pH 6.5. Each point represents mean ± SD of triplicate uptake measurements, * $P < 0.05$ vs control, ** $P < 0.01$ vs control.

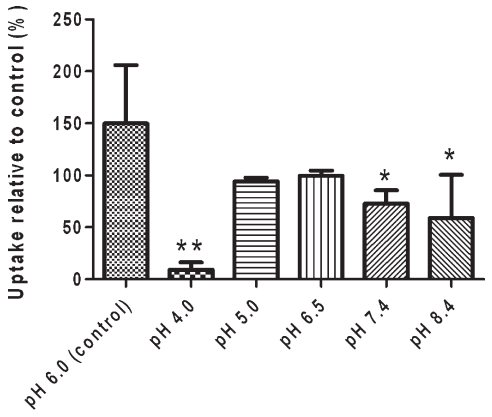



**Figure 6.** Effect of pH on the uptake of Ala-Lys-AMCA across human nasal epithelium. Uptake at pH 6.5 was used as control. Data are presented as a percentage of control. Each point represents mean ± SD of triplicate uptake measurements, * $P < 0.05$ vs control, ** $P < 0.01$ vs control.

concentration (Figure 7B). The lowest concentration (0.1 mM) had the highest permeability coefficient; whereas the highest concentration (10 mM) resulted in the least permeation coefficient, which suggested possible transporter saturation as concentration increased. Furthermore, the transport of the dipeptide (50 $\mu$m) exhibited polarity- and temperature-dependency

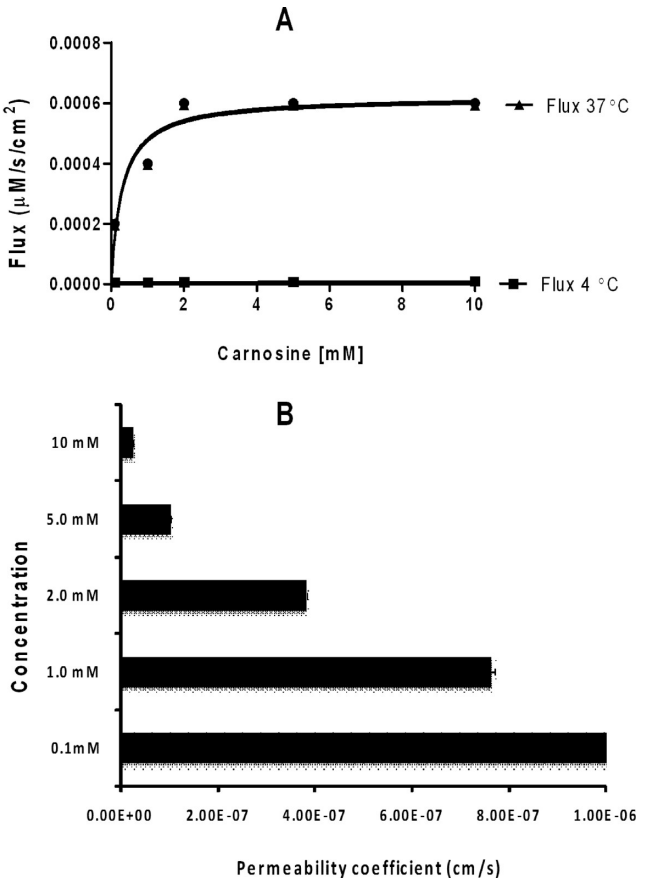



**Figure 7.** Effect of concentration on the flux (A) and permeability coefficient (B) of $\beta$-alanyl-L-histidine across human nasal epithelium. Each point represents mean ± SD of triplicate uptake measurements.

(Figure 8A,B). The apical to basolateral transport and transport at 37 °C were approximately 3 times higher than basolateral to apical transport and transport at 4 °C, respectively. This suggests that the peptide transporters responsible for the transport of $\beta$-alanyl-L-histidine are possibly located on the apical side of the cells. Additional studies conducted to confirm that the active transport of $\beta$-alanyl-L-histidine occurred via peptide transporters involved the permeation of the compound in the presence of competing dipeptides (Gly-Sar, Arg-Phe, Gly-Phe, L-Phe). The result of the studies is shown in Figure 9. As anticipated, Gly-Sar, Arg-Phe and Gly-Phe (10 mM) significantly reduced the transport of the dipeptide ($p < 0.05$). Although the dipeptides with phenylalanine residue significantly reduced the transport, phenylalanine, an amino acid, had no effect on the transport.

## DISCUSSION

In this study, we tested the hypothesis that the human nasal epithelium expresses POT transporter family (PEPT1, PEPT2, PHT1, PHT2) and that some of the isoforms are capable of binding and transporting solutes.

Genetic and molecular methods were used to identify and confirm the specific transporters that were involved in solute transport. Quantitatively, the expression levels of the four POT family gene transcripts were different. However, the functional significance of this observation remains to be seen in terms of solute absorption and transport. Moreover, the level of gene

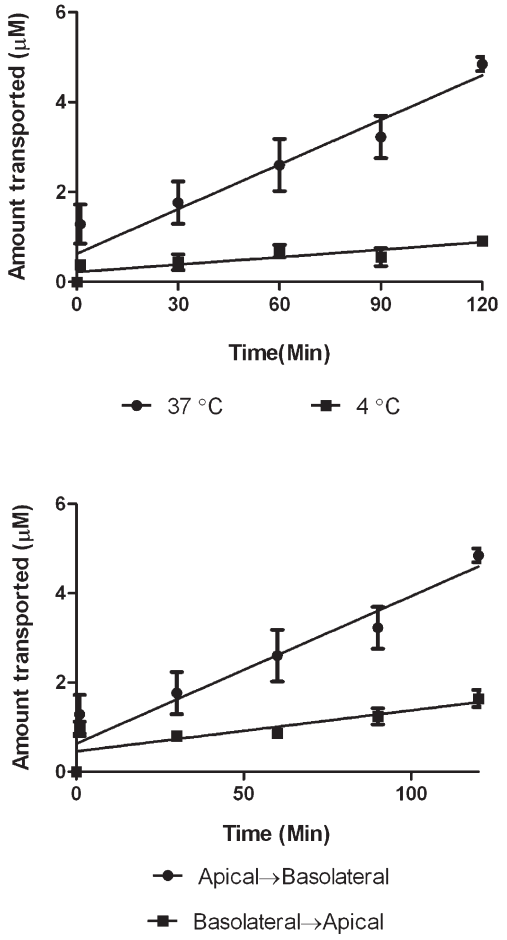



**Figure 8.** Transport of $\beta$-alanyl-L-histidine across human nasal epithelium. (A) Effect of polarity (apical → basolateral vs basolateral → apical) on transport. (B) Effect of temperature on transport. Each point represents mean ± SD of triplicate uptake measurements.

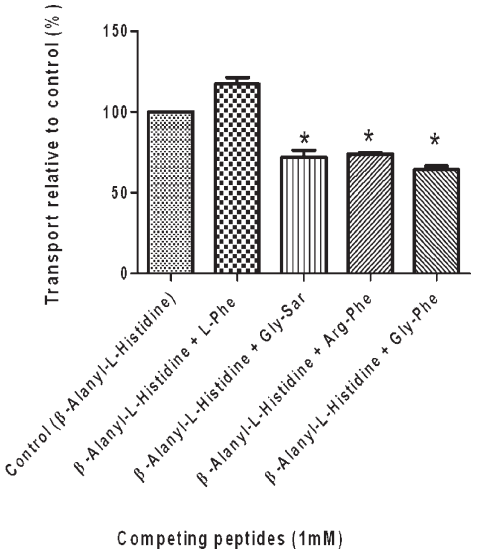



**Figure 9.** Effect of competing peptides on the apical to basolateral transport of $\beta$-alanyl-L-histidine transport across the human nasal epithelium. Each point represents mean ± SD of triplicate uptake measurements, * $P < 0.05$ vs control.

expression, especially in an *in vitro* cell culture system, may not necessarily reflect the level of functional protein expression *in vivo*. Based on our studies, one may assert that PEPT1 and PEPT2 genes were functionally linked to the transport of the dipeptides because the functional proteins corresponding to these genes were detected by Western blot and confirmed by immunohistochemistry.

In most reported studies with peptide transporters, metabolically stable dipeptides or dipeptide derivatives, especially Gly-Sar, Ala-Lys-AMCA and $\beta$-Ala-L-histidine, were used to characterize peptide transport in various epithelia.[27−29] In the present study, we chose Ala-Lys-AMCA and $\beta$-Ala-L-histidine as our model peptides for two reasons. First, studies involving radioactive tracers were not allowed on the floor level where our laboratory is located. So it was not possible to work with tritium-labeled Gly-Sar, the compound preferred by most researchers. Two, since the major future application of our study is to target nasally expressed peptide transporters for systemic delivery of neuropeptides, it was important to characterize the transporters using a peptide derivative ($\beta$-Ala-L-histidine) in addition to a fluorescently labeled dipeptide (Ala-Lys-AMCA).

Ala-Lys-AMCA accumulated intracellularly in the nasal epithelium with a $K_m$ and $V_{max}$ of 0.6 ± 0.07 $\mu$M and 4.1 ± 0.07 $\mu$mol/min/mg protein, respectively. The fact that the uptake of the compound was significantly reduced by competing peptides (carnosine, Gly-Sar) and cephadroxil was an indication of the involvement of either PEPT1 or PEPT2 transporters. However, considering the low $K_m$, it seems that Ala-Lys-AMCA accumulated via the low capacity PEPT2 transporter. The lack of effect on peptide uptake observed for EIPA, a potent inhibitor of $Na^+/H^+$ exchanger, and inhibition of the peptide uptake by CCCP, a potent protonophore, suggested that the uptake of Ala-Lys-AMCA was driven by proton pumps and not by $Na^+/H^+$ exchanger. The remaining uptake might come from unspecific binding of Ala-Lys-AMCA to the cells and/or uptake through endocytosis. Also there could be some unknown mechanisms. This hypothesis was confirmed by the effect of pH on the peptide uptake. While maximum uptake occurred at pH 6.0, significantly lower uptake was observed at more acidic (pH 4.0) and basic (8.5) pHs. The observed effect of pH on peptide transport was important because it showed that hydrogen ions provided the driving force for the active peptide transport via an inwardly directed $H^+$ gradient and an inside-negative membrane potential maintained by the combined action of a $Na^+/K^+$ ATPase at the basolateral membrane and the $Na^+/K^+$ exchanger at the apical membrane.

The immunohistochemical data localized the expression of PEPT1 and PEPT2 transporters to the brush border regions of the nasal epithelium with none on the basolateral side or along the basement membrane (Figure 3). These data also shed some light on the polarity of $\beta$-alanyl-L-histidine transport. Previous studies with respiratory cells showed that peptide transporters are localized at various regions of individual cell types. For instance PEPT2 transporters are localized on the apical membrane of bronchial and tracheal epithelial cells and in cytoplasm of pneumocytes type II.[4] In the nasal mucosa one might speculate that these transporters are responsible for reabsorption of physiological protein metabolic products following degradation by nasal protease enzymes. Physiological functions of peptide transporters may depend not only on the tissue expressing the transporters but also on the location of the transporters within

the tissue. In order for peptide transporters in the nasal epithelium to be targeted for drug delivery, it was pertinent to investigate their involvement in the flux of a model dipeptide across polarized nasal epithelium. As shown in Figure 7, the transport of β-alanyl-L-histidine was concentration-dependent and saturable. This observation, together with its polarity in transport, was a clear indication that apically located peptide transporters were responsible for transporting the dipeptide. The present of competing dipeptides (Gly-Sar, Arg-Phe, Gly-Phe) instead of amino acid (phenylalanine) further buttressed this argument.

Generally, both our molecular and functional studies suggested the expression of peptide transporters, PEPT1 and PEPT2, in the human nasal epithelium and their involvement in substrate transport that occur predominantly in the brush border membranes. These results are in accordance with published studies of other epithelial cells in the small intestine, lung, choroid plexus, and kidney which have been summarized in the Introduction. Although PHT1 and PHT2 do express in the human nasal epithelium, the fact that at least PHT1 is not transmembrane protein implies they possibly have no significant roles in drug uptake and transport process across the nasal epithelium.

## CONCLUSIONS

Drugs used for treatment of various diseases must reach specific parts of the body where they act in order to be effective. This may be challenging, especially for peptide drugs due to poor absorption and rapid degradation in the intestinal walls. Discovering peptide transporters in nondigestive epithelial cells could be a strategy for improving drug permeation. The expression of PEPT2 transporters in the human nasal cells was in agreement with the published studies reported for the rat nasal epithelium.[23] This study provided the first documented evidence that peptide transporters are expressed in the human nasal epithelium. Based on the location of PEPT1 and PEPT2 transporters on the brush boarder regions of the epithelium and their involvement in the uptake and transport of Ala-Lys-AMCA and β-alanyl-L-histidine these transporters can potentially act as surrogate carriers for drug molecules. Such a targeted trans-membrane drug delivery strategy may not be possible with PHT1 and PHT2 transporters because of their intracellular location. The nasal route has good prospects for peptide transporter-targeted drug delivery because of its noninvasiveness, circumvention of first-pass metabolism, and lower protease enzyme activity in comparison to the gastrointestinal tract. Moreover, the possibility of food-related drug interactions such as competition between dietary dipeptides and dipeptide drugs or peptidomimetics is not applicable. The identification of PEPT1 and PEPT2 transporters in the human nasal epithelium may result in new drug targeting strategies based on these transporters. However, further studies using radioactive-labeled dipeptides of diverse structural features including Gly-Sar are necessary to understand the structure activity relationship necessary for rational drug design and targeting for the nasal peptide transporters.

## AUTHOR INFORMATION

### Corresponding Author

*Biopharmaceutics and Drug Delivery Laboratory, College of Pharmacy, Faculty of Health Professions, Dalhousie University, Halifax, NS, Canada B3H 3J5. Tel: 1-902-494-2092. Fax: 1-902-494-1396. E-mail: Remigius.agu@dal.ca.

## ACKNOWLEDGMENT

We thank Caren Wyse for liaison between QEII hospital and our laboratory, and Dr. Kerry Goralski for PCR instrumentation. Funding for this project was provided by Nova Scotia Health Research Foundation (D2006-44, MED-Project-2008-4691) and Pharmacy Endowment Fund.

## REFERENCES

(1) Newey, H.; Smyth, D. H. The intestinal absorption of some peptides. *J. Physiol.* **1959**, *135*, 43–44.

(2) Adibi, S. A.; Krzysik, B. A.; Drash, A. L. Metabolism of intravenously administered dipeptides in rats: effects on amino acid pools, glucose concentration and insulin and glucagon secretion. *Clin. Sci. Mol. Med.* **1977**, *52*, 193–204.

(3) Ganapathy, V.; Mendicino, J. F.; Leibach, F. H. Transport of glycyl-L-proline into intestinal brush border vesicles from rabbit. *J. Biol. Chem.* **1981**, *256*, 118–124.

(4) Rubio-Aliaga, I.; Daniel, H. Mammalian peptide transporters as targets for drug delivery. *Trends Pharmacol. Sci.* **2002**, *23*, 434–440.

(5) Smith, D. E.; Johanson, C. E.; Keep, R. F. Peptide and peptide analog transport systems at the blood-CSF barrier. *Adv. Drug Delivery Rev.* **2004**, *56*, 1765–1791.

(6) Christopher, W.; Botka; Wittig, T. W.; Graul, R. C.; Nielsen, C. U.; Sadée, W. Human Proton/Oligopeptide Transporter (POT) Genes: Identification of Putative Human Genes Using Bioinformatics. *AAPS PharmSci.* **2000**, *2*, 76–97.

(7) Freeman, T. C.; Bentsen, B. S.; Thwaites, D. T.; Simmons, N. L. H+/Ditripeptide transporter (PEPT1) expression in the rabbit intestine. *Eur. J. Physiol.* **1995**, *430*, 394–400.

(8) Ogihara, H.; Saito, H.; Shin, B. C.; Terada, T.; Takenoshita, S.; Nagamachi, Y.; Inui, K. I.; Takata, K. Immuno-localization of $H^+$/peptide cotransporter in rat digestive tract. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 848–852.

(9) Smith, D. E.; Pavlova, A.; Berger, U. V.; Hediger, M. A.; Yang, T.; Huang, Y. G.; Schnermann, J. B. Tubular localization and tissue distribution of peptide transporters in rat kidney. *Pharm. Res.* **1998**, *15*, 1244–1249.

(10) Shen, H.; Smith, D. E.; Yang, T.; Huang, Y. G.; Schnermann, J. B. Brosius FC III. Localization of PEPT1 and PEPT2 proton-coupled oligopeptide transporter mRNA and protein in rat kidney. *Am. J. Physiol.* **1999**, *276*, 658–665.

(11) Talluri, R. S.; Samanta, S. K.; Gaudana, R.; Mitra, A. K. Synthesis, metabolism and cellular permeability of enzymatically stable dipeptide prodrugs of acyclovir. *Int. J. Pharm.* **2008**, *361*, 118–124.

(12) Groneberg, D. A.; Fischer, A.; Chung, K. F.; Daniel, H. Molecular mechanisms of pulmonary peptidomimetic drug and peptide transport. *Am. J. Respir. Cell Mol. Biol.* **2004**, *30*, 251–260.

(13) Brandsch, M.; Knütter, I.; Bosse-Doenecke, E. Pharmaceutical and pharmacological importance of peptide transporters. *J. Pharm. Pharmacol.* **2008**, *60*, 543–585.

(14) Munzel, M. Tierexperimentelle autoradiographische untersuchungen zur resorption von aminosäuren in die nasenschleimhaut "Experimental autoradiographic studies on the amino acid resorption of nasal mucosa. *Arch. Klin. Exp. Ohren-, Nasen- Kehlkopfheilkd.* **1972**, *202*, 364–368.

(15) Munzel, M.; Hochstrasser, K. Tierexperimentelle untersuchungen zur aufnahme von aminosäuren nach applikation auf die nasenschleimhaut, "Animal experimental studies on uptake of amino acids applied to the nasal mucosa. *Laryngol., Rhinol., Otol.* **1973**, *52*, 144–151.

(16) Yang, C.; Gao, H.; Mitra, A. K. Chemical stability, enzymatic hydrolysis, and nasal uptake of amino acid ester prodrugs of acyclovir. *J. Pharm. Sci.* **2001**, *90*, 617–624.

(17) Yang, C.; Mitra, A. K. Nasal absorption of tyrosine-linked model compounds. *J. Pharm. Sci.* **2001**, *90*, 340–347.

(18) Agu, R.; Dang, H. V.; Jorissen, M.; Willems, T.; Vandoninck, S.; Van Lint, J.; Vandenheede, J. V.; Kinget, R.; Verbeke, N. In vitro

polarized transport of L-phenylalanine in human nasal epithelium and partial characterization of the amino acid transporters involved. *Pharm. Res.* **2003**, *20*, 1125–1132.

(19) Monte, J. C.; Nagle, M. A.; Eraly, S. A.; Nigam, S. K. Identification of a novel murine organic anion transporter family member, OAT6, expressed in olfactory mucosa. *Biochem. Biophys. Res. Commun.* **2004**, *323*, 429–436.

(20) Chemuturi, N. V.; Haraldsson, J. E.; Prisinzano, T.; Donovan, M. Role of dopamine transporter (DAT) in dopamine transport across the nasal mucosa. *Life Sci.* **2006**, *79*, 1391–1398.

(21) Chemuturi, N. V.; Donovan, M. D. Role of organic cation transporters in dopamine uptake across olfactory and nasal respiratory tissues. *Mol. Pharmaceutics* **2007**, *4*, 936–942.

(22) Schmidt, M. C.; Simmen, D.; Hilbe, M.; Boderke, P.; Ditzinger, G.; Sandow, J.; Lang, S.; Rubas, W.; Merkle, H. P. Validation of excised bovine nasal mucosa as in vitro model to study drug transport and metabolic pathways in nasal epithelium. *J. Pharm. Sci.* **2000**, *89*, 396–407.

(23) Quarcoo, D.; Fischer, T. C.; Heppt, W.; Lauenstein, H. D.; Pilzner, C.; Welte, T.; Groneberg, D. A. Expression, Localisation and Functional Implications of the Transporter Protein PEPT2 in the Upper Respiratory Tract. *Respiration* **2009**, *77*, 440–446.

(24) Agu, R. U.; Jorissen, M.; Willems, T.; Augustijns, P.; Kinget, R.; Verbeke, N. In-vitro nasal drug delivery studies: comparison of derivatised, fibrillar and polymerised collagen matrix-based human nasal primary culture systems for nasal drug delivery studies. *J. Pharm. Pharmacol.* **2001**, *53*, 1447–1456.

(25) Groneberg, D. A.; Döring, F.; Eynott, P. R.; Fischer, A.; Daniel, H. Direct visualization of peptide uptake activity in the central nervous system of the rat. *Neurosci. Lett.* **2004**, *364*, 32–36.

(26) Agu, R. U.; Jorissen, M.; Willems, T.; de Witte, P.; Verbeke, N. Derivatized and fibrillar collagen films as scaffold for nasal epithelial cells to study nasal drug absorption in vitro. *STP Pharma Sci.* **2002**, *12*, 81–87.

(27) Lin, H.; King, N. Demonstration of functional dipeptide transport with expression of PEPT2 in guinea pig cardiomyocytes. *Pfluegers Arch.* **2007**, *453*, 915–922.

(28) Groneberg, D. A.; Döring, F.; Eynott, P. R.; Fischer, A.; Daniel, H. Intestinal peptide transport: ex vivo uptake studies and localization of peptide carrier PEPT1. *Am. J. Physiol.* **2001**, *281*, 697–704.

(29) Omkvist, D. H.; Larsen, S. B.; Nielsen, C. U.; Steffansen, B.; Olsen, L.; Jørgensen, F. S.; Brodin, B. A Quantitative Structure-Activity Relationship for Translocation of Tripeptides via the Human Proton-Coupled Peptide Transporter, hPEPT1 (SLC15A1). *AAPS J.* **2010**, *12*, 385–396.