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Nasal absorption enhancement strategies for therapeutic peptides: an in vitro study using cultured human nasal epithelium

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Abstract

This study examined the potential usefulness of cultured human nasal epithelium as a model to investigate nasal absorption enhancement strategies for therapeutic peptides. The transport of leucine enkephalin (Leu-Enk) in the presence of bestatin and puromycin, respectively and various combinations of these protease inhibitors with absorption enhancers capable of inhibiting proteases or protecting peptides against protease degradation (glycocholate, dimethyl-β-cyclodextrin (DMβCD)) was studied. Epithelial membrane perturbation, protein leakage, bestatin/puromycin absorption and rebound aminopeptidase activity were used as toxicological end-points. The combination of puromycin with glycocholate or DMβCD resulted in a higher absorption enhancement of Leu-Enk (9–14%) than when the absorption enhancers were combined with bestatin (1–3%) or when the inhibitors were used alone (2–4%). The higher absorption enhancement resulting from the combination of protease inhibitors with absorption enhancers caused a significant reduction of epithelial resistance and increased sodium fluorescein transport. Although only puromycin permeated the human nasal epithelium, both protease inhibitors induced a significant rebound aminopeptidase activity (25–61%), which can be associated with protein leakage (21–46%). This study highlighted (i) the potential usefulness of cultured human nasal epithelium as a model to study nasal absorption enhancement of therapeutic peptides; (ii) further studies using in vivo nasal models are required to ascertain whether the membrane perturbation and cytotoxicity observed with various combinations of the protease inhibitors and absorption enhancers really raise safety concerns. © 2002 Published by Elsevier Science B.V.

Keywords: Nasal cell culture; Peptides; Metabolism; Absorption enhancement; Toxicity

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1. Introduction

Proteins and peptides delivery through the nasal route is considerably less effective than through the parenteral route (Ugwoke et al., 2001; Illum et al., 2001a). The bioavailability of therapeutic peptides delivered nasally is often limited by presystemic elimination, due to enzymatic degradation or mucociliary clearance, and by poor mucosal membrane permeability of large polar substrates (Irwin et al., 1994).

The major strategies to enhance transmucosal peptide absorption include (a) co-administration with protease inhibitors, (b) the use of membrane permeation enhancers, (c) co-administration with a combination of absorption enhancers and protease inhibitors, (d) modification of peptide structure to improve metabolic stability or membrane permeation and (e) use of micro particles (Watanabe et al., 1998; Morimoto et al., 2001; Illum et al., 2001b). Reasonable information exists in literature on the stabilization of nasally administered peptides using protease inhibitors (Hussain and Aungst, 1992; Watanabe et al., 1998). On the other hand, information concerning the effectiveness and potential toxicity of this approach of nasal peptide absorption enhancement is scanty and limited to animal studies (Raehs et al., 1988; Schmidt et al., 1998).

The essential protective functions of the nose should remain fully intact and not be compromised following intranasal administration of proteins and peptides using excipients. Other important issues to be considered during nasal drug delivery using excipients include potential interference with normal metabolism and function of the mucosal cells, possible metabolism of enhancer/inhibitor to toxic and carcinogenic substances in the nasal mucosa, and the systemic absorption of enhancer/inhibitor into the blood stream (Lee, 1991).

The use of cultured nasal epithelium to study nasal drug absorption may yield useful information regarding nasal drug delivery using excipients. The usefulness of human nasal epithelium cultured on collagen has been extensively demonstrated in characterization of ion transport in cystic fibrosis patients (Boucher et al., 1987; Widdicombe, 1990). Recently, the potentials of different human nasal primary cultures as in vitro models to study nasal drug absorption have been reported (Werner and Kissel, 1995; Agu et al., 2001a, 2002).

This study was aimed at investigating the relationship between protease inhibition, absorption enhancement and safety of bestatin and puromycin as nasal absorption enhancer using a human nasal model. The transport of Leu-Enk across human nasal mucosa in the presence of bestatin and puromycin, and their various combinations with absorption enhancers capable of inhibiting proteases or protecting leucine enkephalin (Leu-Enk) against protease degradation (sodium glycocholate, dimethyl-β-cyclodextrin (DMβCD)) was studied (Irwin et al., 1994). Cytotoxicity studies of the absorption-enhancing strategies and the possible mechanisms involved were X-rayed. This was done by monitoring bestatin and puromycin permeation across the epithelium, changes in nasal epithelial electrical resistance, rebound aminopeptidase activity (increase in aminopeptidase activity following exposure to protease inhibitors), and protein leakage following exposure of the cultured cells to the excipients.

2. Materials and methods

2.1. Chemicals

Leu-Enk and puromycin hydrochloride were purchased from Fluka (Buchs, Germany). Bestatin hydrochloride, *des*-tyrosine leucine enkephalin acetate (*des*-Tyr-Leu-Enk), sodium glycocholate, cholera toxin, bovine serum albumin (BSA) and pronase were supplied by Sigma (St Louis, MO). DMEM-F12 1/1, phenol red, Ultroser G, NU-serum, phosphate buffered saline (PBS) and Hanks' balanced salt (HBBS) were obtained from GIBCO (Paisley, UK). Dimethyl-β-cyclodextrin, Coomasie brilliant blue G-250, Streptomycin/penicillin and physiological saline were bought from UCB (Leuven, Belgium), Merck (Darmstadt, Germany), Boehringer Mannheim GmbH (Germany), and

Braun (Melsungen, Germany), respectively. All other chemicals and reagents used for sample analysis were of analytical grade.

2.2. Cell culture

The cell culture methods used in this study has been described in detail elsewhere (Agu et al., 2001a, 2002). Briefly, normal human nasal epithelial tissues obtained from patients undergoing elective surgery were dissociated enzymatically overnight at 4 °C using 0.1% pronase. The pronase was competitively inhibited with NU-serum and the cells were filtered through 60 or 70 μm nylon mesh or polypropylene filters (Pall, Portsmouth, UK) to remove cell debris and to obtain a homogenous cell suspension. Subsequently, the cells were washed three times in DMEM-F12 1/1 supplemented with streptomycin 100 μg/ml and penicillin 100 U/ml, before counting with a Coulter Multisizer counter (Northwell, UK). Incubating the cells on plastic for 1 h reduced fibroblast contamination. For transport studies, cells were then plated at a density of 1.0×10^6 cells/cm^2 to grow on Cellagen membranes (CD-24, 0.785 cm^2 area, 4000 MW cut off, ICN Biomedical, Costar Mensa, CA). Cellagen membranes are permeable films made entirely of collagen. Prior to cell plating, the membranes were conditioned by placing 1 ml of monolayer medium (DMEM F12 supplemented with 2% Ultroser, 10 ng/ml cholera-toxin, 100 μg/ml streptomycin and 100 IU penicillin) to the basolateral compartment and incubating for 30–60 min at 37 °C. Subsequently, the cells were plated and maintained in this medium for 24 h to allow maximum attachment. Thereafter, the medium was used to maintain the culture at air–liquid interface by changing the basolateral medium everyday until the cells were used for transport studies (8–10 days post seeding). Throughout the culture duration, both the apical and basolateral sides of the cells were carefully washed with DMEM-F12 every 2 days to reduce the accumulation of cell metabolic products.

For rebound aminopeptidase activity, the cells were seeded (5.0×10^5 cells/well) on rat-tail collagen coated multi-well plates (Nunc, Denmark) and maintained under immersion condition using the monolayer medium. The medium was changed three times a week.

2.3. Transport studies

The various combinations of Leu-Enk with bestatin, puromycin, glycocholate and DMβCD

Table 1
Summary of transport of leucine enkephalin across human nasal epithelial cell monolayers in different experimental conditions

Experimental conditions	Permeability coefficient ($cm/s \times 10^{-8}$)	Amount transported (%) after 2 h
3 mM Leu-Enk alone	4.3 ± 1.8	0.10 ± 0.02
3 mM Leu-Enk + 1 mM puromycin	171.0 ± 5.1	4.00 ± 0.24
3 mM Leu-Enk + 1 mM bestatin	101.0 ± 4.0	2.20 ± 0.90
3 mM Leu-Enk + 0.5% GC-Na	135.0 ± 5.8	3.07 ± 0.95
3 mM Leu-Enk + 2.0% DMβCD	6.4 ± 0.4[a]	0.13 ± 0.10
3 mM Leu-Enk + 0.5% GC-Na + 1 mM puromycin	648.0 ± 7.2	14.30 ± 1.59
3 mM Leu-Enk + 0.5% GC-Na + 1 mM bestatin	139.0 ± 4.4	3.13 ± 0.90
3 mM Leu-Enk + 2.0% DMβCD + 1 mM puromycin	425.0 ± 6.6	9.42 ± 1.62
3 mM Leu-Enk + 2.0% DMβCD + 1 mM bestatin	46.7 ± 1.7	1.06 ± 0.39

GC-Na, glycocholate; Leu-Enk, leucine enkephalin; DMβCD, dimethyl-β-cyclodextrin.

[a] Not statisticaly significant from Leu-Enk alone.

tested are summarized in Table 1. The solutions were prepared daily in transport medium,TM, (HBBS supplemented with HEPES buffer and 15 mM glucose, pH 7.4). At the beginning of the transport experiments, the cells were rinsed twice with TM and pre-incubated for 15 min at 37 °C with the test solutions to be investigated, but without Leu-Enk. For transport of Leu-Enk alone, TM was used for pre-incubation. To initiate the transport studies, 250 μl of the test solutions was added to the donor compartment after pre-incubation. At pre-determined time points (10, 20, 30, 40, 60, 90, 120 min), 100 μl aliquots were sampled from the acceptor compartment (750 μl) and were replaced immediately with an equal volume of TM. The samples were immediately frozen at −40 °C to prevent further hydrolysis and were analyzed within 1 h of collection.

At the end of each experiment, 50 μl sample was also taken from the donor compartment. All cell monolayers used were checked for epithelial integrity before and after the experiments by measuring transepithelial electrical resistance (TEER) using Millicel ERS cell voltameter (Millipore, Bedford, MA). Only cells with TEER values $\geq 250\ \Omega\,cm^2$ were used for experiments. In addition, the transport of sodium fluorescein—a paracellular marker—during 1 h after permeation studies, was also used to assess epithelial integrity at the end of each experiment. Transport of Leu-Enk across Cellagen membranes (CD-24) alone was also measured.

Apparent permeability coefficients [P_{app} (cm/s)] for Leu-Enk transport was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{V}{AC_0} \quad (1)$$

where, dQ/dt = amount of Leu-Enk appearing in the basolateral compartment in function of time, C_0 = initial concentration of Leu-Enk in the apical compartment, A = surface area across which transport was measured (0.785 cm^2).

2.4. Aminopeptidase rebound studies

The studies conducted in this section were carried out in different phases (Hussain et al., 1990) using cells cultured in multi-well plates and under aseptic conditions as follows:

- Phase 1: Metabolism of 0.1 mM Leu-Enk alone (control).
- Phase 2: Metabolism of 0.1 mM Leu-Enk in the presence of bestatin and puromycin, respectively 1 h after phase 1 (inhibition phase).
- Phase 3: Metabolism of 0.1 mM Leu-Enk alone 1 h after washout of inhibitors (rebound phase).
- Phase 4: Metabolism of 0.1 mM Leu-Enk 18 h after phase 3 (recovery phase).

The same batch of cells was used for experiments in the four phases.

Two concentrations of bestatin and puromycin were tested (0.02 and 0.1 mM).

At the inception of the rebound studies, each batch of cells was washed three times using TM. After washing and 15 min pre-incubation at 37 °C, the TM was removed and the cells were incubated with 100 μM Leu-Enk (1 ml) or its combination with the various excipients. Samples (50 μl) were taken at pre-determined intervals (0, 10, 20, 30, 40, 60, 90, 120 min) and were immediately diluted with 100 μl citric acid (0.1 M) to prevent further hydrolysis. Samples were stored at −40 °C (for not more than 1 week) until analyzed by HPLC. The chemical stability of Leu-Enk was maintained upon storage.

The remaining concentrations of Leu-Enk in function of time were plotted against time and straight lines through the points were drawn using a least-square fit. The slopes of these lines (normalized for protein content) were taken as the specific activities of the cells in the various phases. The percentage change in aminopeptidase activity was calculated by comparing the specific activities of the cells in phases 2, 3, and 4 relative to the specific activity of the cells in phase 1.

After metabolism study in each phase, the remaining incubation solution was collected and used to estimate the amount of protein released (P_{medium}) according to the method described by Bradford (1976). At the end of the experiments, the collagen coating with cells grown on them

were dissolved in 1 ml 1 M NaOH for 1 h at 60 °C. Aliquots of the cell solution was used to determine the cell protein content (P_{cell}). The actual protein content of the cells was corrected by subtracting the protein due to collagen coating (P_{col}). The rat-tail collagen was polymerized using ammonia vapor, dissolved with 1 ml 1 M NaOH and assayed using the same protocol used to assay the cells. Protein release caused by TM served as a reference to compare induction of protein release by Leu-Enk and its combination with bestatin and puromycin, respectively. Percentage protein release in the various phases was calculated as follows:

$$\text{Protein release (\%)} = \frac{P_{medium}}{\sum P_{medium} + (P_{cell} - P_{col})} \times 100 \quad (2)$$

where, P_{medium}, protein released into the assay medium in each phase; ΣP_{medium}, summation of protein released into the assay medium in phases 1–4, P_{cell}, protein recovered from cells at the end of the experiment; P_{col}, protein content of 1 ml rat-tail collagen used to coat the wells.

2.5. Sample analysis

Sodium fluorescein was analyzed using a Diode array spectrophotometer (HP 8452A, Hewlett Packard, Palo Alto, CA) at a wavelength of 490 nm Leu-Enk and *des*-Tyr-Leu-Enk were analyzed isocratically by HPLC using Hypersil BDS C18 5 μm, 250 × 4.6 μm column (Thermo Hypersil, Cheshire, England). The mobile phase used was a 23:77 (v/v) mixture of acetonitrile and phosphate buffer (0.05 M, pH 2.5). The flow rate and detection wavelength was set at 1.3 ml/min and 210 nm, respectively. The calibration curves were linear with correlation coefficients, $r \geqslant 0.999$. The sensitivity of the HPLC method was 0.1 μM at signal to noise ratio of ≈ 3 and was repeatable with respect to Leu-Enk and *des*-Tyr-Leu-Enk (RSD $\leqslant$ 6%).

2.6. Data presentation and statistical analysis

Unless otherwise stated, each experiment was carried out in triplicate using different cell monolayers and the data were expressed as mean ± SD. Bonferroni's one-way analysis of variance (ANOVA) was used to compare changes in aminopeptidase activity and protein release of phases 2, 3 and 4 relative to phase 1. Differences between cells incubated with TM and those treated with bestatin and puromycin, respectively were also compared using this statistical approach. Simple Students' *t*-test was used to compare the transport of Leu-Enk alone with its transport in the presence of excipients. The level of significance was set at $P = 0.05$.

3. Results and discussion

The cell culture system used for this study exhibited differentiated morphological features and expressed ciliated and non-ciliated cuboidal to columna pseudostratified monolayer epithelium. Details of the morphological characteristics of the cells can be found in literature (Agu et al., 2001a,b, 2002).

3.1. Transport studies

In this study, cultured human nasal epithelium was used as a model to investigate the relationship between the efficacy and cytotoxicity/membrane perturbation of protease inhibitors (bestatin, puromycin) and their combination with absorption enhancers (glycocholate, DMβCD) using Leu-Enk as a model peptide. Leu-Enk possesses interesting characteristics that make it a useful substrate to investigate transmucosal permeation and metabolism of peptides (Hussain et al., 1990). For instance, this compound is rapidly inactivated by nasal peptidases, but still chemically stable up to 3 h over the time course of incubation at 37 °C. Further, Leu-Enk may be degraded by different proteases including aminopeptidase, enkephalinase and carboxypeptidase (Schwartz et al., 1981). This makes it suitable for the identification of the presence of different proteases in the cultured cells. Selection of concentrations of the protease inhibitors investigated in this study was based on literature information (Hussain et al., 1990).

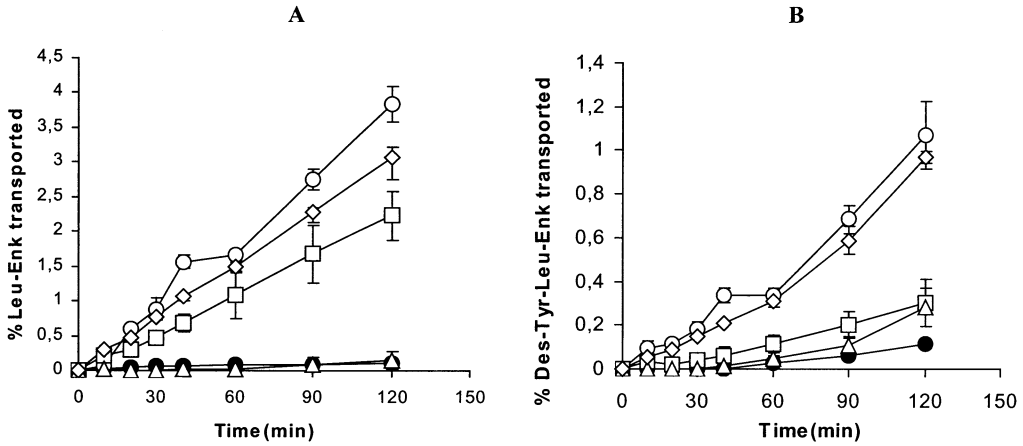

Fig. 1. Transport of Leu-Enk (A) and its major metabolite *des*-Tyr-Leu-Enk (B) across human nasal cell monolayers in the presence of protease inhibitors and absorption enhancers. Each point represents the mean ± SD, $n = 3$ monolayers. (●) Control (3 mM leucine enkephalin); (○) 1 mM Puromycin; (□) 1 mM Bestatin; (△) DMCD (2.0% dimethyl-β-cyclodextrin); (◇) 0.5% Sodium glycocholate.

Fig. 1 (a) and (b) illustrate the transport of Leu-Enk and its major metabolite, *des*-Tyr-Leu-Enk across human nasal epithelial monolayers in the presence and absence of enzyme inhibitors and absorption enhancers. Consistent with reported degradation studies (Hussain et al., 1985; Kashi and Lee, 1986; Chun and Chien, 1993) the transport of Leu-Enk alone was accompanied with significant enzymatic degradation to *des*-Tyr-Leu-Enk. The percentages of Leu-Enk and *des*-Tyr-Leu-Enk detected on the donor compartment after 2 h were ≈ 51 and 46%, respectively. The remaining amount of Leu-Enk ($\approx 3\%$) may be accounted for by degradation to other metabolites and transport to the acceptor compartment.

In the absence of enzyme inhibition and absorption enhancement, only 0.1% of Leu-Enk was transported after 2 h. Except for 2% DMβCD that resulted in only $0.3 \pm 0.1\%$ transport, stabilization of Leu-Enk with 1 mM puromycin, 0.5% glycocholate and 1 mM bestatin resulted in 4.0 ± 0.2, 3.0 ± 0.3 and $2.0 \pm 0.7\%$ transport, respectively. In the presence of the excipients, the percentage of Leu-Enk remaining on the apical side after 2 h were 93, 83, 80 and 68% in the presence of 1 mM puromycin, 0.5% GC-Na, 1 mM bestatin and 2% DMβCD, respectively. Comparing the extent of Leu-Enk stabilization by excipients and subsequent low penetration across the epithelium suggests that permeation barrier plays a more dominant role with respect to nasal absorption of Leu-Enk.

The results of our study seem comparable with literature information on nasal absorption enhancement using protease inhibitors and absorption enhancers. Several studies have been reported in literature, which indicate that protease inhibition may result in significant increase in nasal absorption of peptides. Morimoto et al. (1991), showed that camostat mesilate which is slowly absorbed (8%/h) and could inhibit the proteolytic activity in the nasal mucosa resulted in enhanced nasal absorption of vasopressin and its analogue. Similarly, Hussain and Aungst (1992) showed by determining the percentage of Leu-Enk remaining unabsorbed that protease inhibition resulted in increased nasal absorption of Leu-Enk. In another study, Sayani et al. (1993) demonstrated that a combination of protease inhibitors (amastatin, thiomerosal, EDTA) with dihydrofusidates resulted in improved transmucosal delivery of Leu-Enk. Using Caco-2 monolayers Quan et al., (1999) reported that co-administration of Leu-Enk with aminopepti-

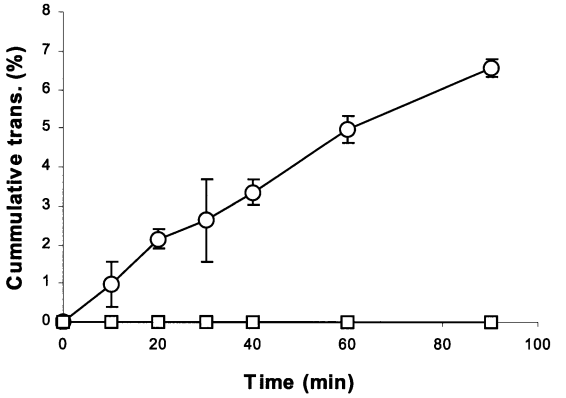

Fig. 2. Permeation of bestatin and puromycin across human nasal cell monolayers during absorption enhancement of leucine enkephalin. Each point represents the mean ± SD, $n = 3$ monolayers. (○) 1 mM Puromycin; (□) 1 mM Bestatin.

dase inhibitors such as puromycin, bacitracin and amastatin resulted in a 2.2, 3.4, 6.0 -fold increase in the amount of Leu-Enk transported. Further the authors showed that that co-administration of a protease inhibitor (amastatin) with an absorption enhancer (EDTA) enhanced the transport of Leu-Enk compared with amastatin alone.

Fig. 2 shows that while 6.6 ± 0.2% of puromycin was transported across the cells during absorption enhancement, bestatin was not detected on the basolateral side. This observation provides unique information regarding the potential mechanism of nasal absorption enhancement of bestatin and puromycin. Given the fact that the amount of Leu-Enk transported in the presence of puromycin was higher than recorded when bestatin was present, one can claim that the ability of puromycin to suppress Leu-Enk degradation before and during permeation process was due to its ability to permeate the epithelium (Taki et al., 1995). Bestatin can only suppress Leu-Enk metabolism before permeation due to its restricted diffusion (Fig. 2) (Giros et al., 1986).

Fig. 3 (a) and (b) highlight the results of Leu-Enk transport in the presence of various combinations of bestatin and puromycin with glycocholate and DMβCD, respectively. Compared to transport experiments with either protease inhibitors or absorption enhancers alone, there was a significant increase in the transport of Leu-Enk and *des*-Tyr-Leu-Enk. The higher amount of *des*-Tyr-Leu-Enk that was seen with puromycin in comparison to other ex-

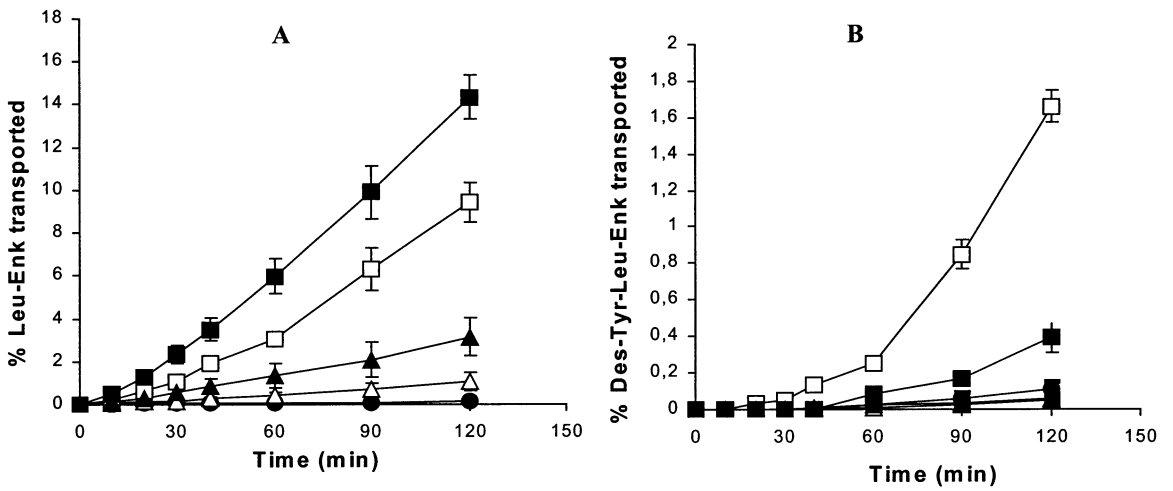

Fig. 3. Transport of Leu-Enk (A) and its major metabolite *des*-Tyr-Leu-Enk (B) across human nasal cell monolayers in the presence of combinations of protease inhibitors and absorption enhancers. Each point represents the mean ± SD, $n = 3$ monolayers. (●) Control (3 mM leucine enkephalin alone); (□) 2.0% Dimethyl-β-cyclodextrin + 1 mM puromycin; (△) 2.0% Dimethyl-β-cyclodextrin + 1 mM bestatin; (■) 0.5% Sodium glycocholate + 1 mM puromycin; (▲) 0.5% Sodium glycocholate + 1 mM bestatin.

cipients despite the protease inhibitory effect of this compound may be related to its higher effect in opening the tight junctions, thus allowing the passage of both the peptide and its metabolites. The order of increasing absorption enhancing efficacy of the various combinations of enzyme inhibitors with absorption enhancers was as follows: 0.5% glycocholate + 1 mM puromycin > 2% DMβCD + 1 mM puromycin > 0.5% glychocholate + 1 mM bestatin > 2% DMβCD + 1 mM bestatin. The permeability coefficients and percentages of Leu-Enk transported under the various conditions investigated are summarized in Table 1. Though DMβCD alone did not result in a significant increase in Leu-Enk transport, cyclodextrins are known to be good absorption enhancer due to their multiple action including peptidase inhibition (Sigurjonsdottir et al., 1999).

The flux of Leu-Enk in the absence of excipients ($4.3 \pm 1.8 \times 10^{-8}$ cm/s) estimated in this study is comparatively lower than that reported by Kissel and Werner (1998) for a closely related compound, methionine enkephalin ($4.4 \pm 0.5 \times 10^{-7}$ cm/s). The disparity in the permeability coefficient may not only hinge on differences between both cell culture systems, but also on other variables that affect the use of permeability coefficients to estimate nasal absorption of peptides and proteins. The major differences between our culture system for permeation and the cell culture model reported by Werner and Kissel (1995) is that while their cell culture was established on teflon using culture medium that contains serum, our cells were grown at air–liquid interface on a membrane made entirely of collagen type I matrix (Cellagen) using serum-free medium. These attributes may result in differences in metabolic cleavage of the peptide during transport. Other factors such as pH gradient, additional diffusion barriers (i.e. unstirred water layer, type of filter support), analyte concentration and detection method may result in differences in permeability coefficient (Caldwell et al., 1998).

Based on literature information, the permeability of peptides and macromolecules across cultured cells is comparatively lower in comparison with intact nasal epithelium (Kubo et al., 1994; Kissel and Werner, 1998; Schmidt et al., 1998). This may be linked to the basic morphological and functional differences between cultured cells and leaky intact epithelium. On the other hand, a selection of effective permeability coefficients for therapeutic oligo-and polypeptides across excised nasal tissue (human, cattle and rabbit) indicate that there is no obvious correlation between molecular weight and effective permeability in various intact tissues models (Jorgensen and Bechgaard, 1994; Schmidt et al., 1998). Therefore, the interpretation and extrapolation to in vivo situation of results of proteins and peptides absorption based on in vitro models requires a reasonable level of caution.

Fig. 4 (a) and (b) illustrate changes in TEER induced by the various combinations of excipients tested and the transport of sodium fluorescein, a paracellular marker (during 1 h) at the end of each experiment.

Except for the combination of Leu-Enk with bestatin and DMβCD, respectively, the reduction of TEER by the excipients was significantly different from that caused by Leu-Enk alone (control). TEER reduction was remarkably higher when protease inhibitors were combined with absorption enhancers. Under these conditions TEER reduction was between 62 and 93%. Interestingly, the change in TEER upon exposing the cells to the excipients alone did not correlate well with transport of sodium fluorescein at the end of each study. For instance, the amount of sodium fluorescein transported after Leu-Enk transport in the presence of bestatin, puromycin, GC–Na, and DMβCD were not significantly different from control. On the other hand, permeation of sodium fluorescein in the presence of combinations of protease inhibitors and absorption enhancers had a better correlation with appreciable sodium fluorescein transport (3–21%). Generally, enhancers are thought to act by one or a combination of several mechanisms including (1) increasing membrane fluidity, either by creating disorder in the phospholipid domain or by facilitating the leaching of proteins from the membrane, (2) re-

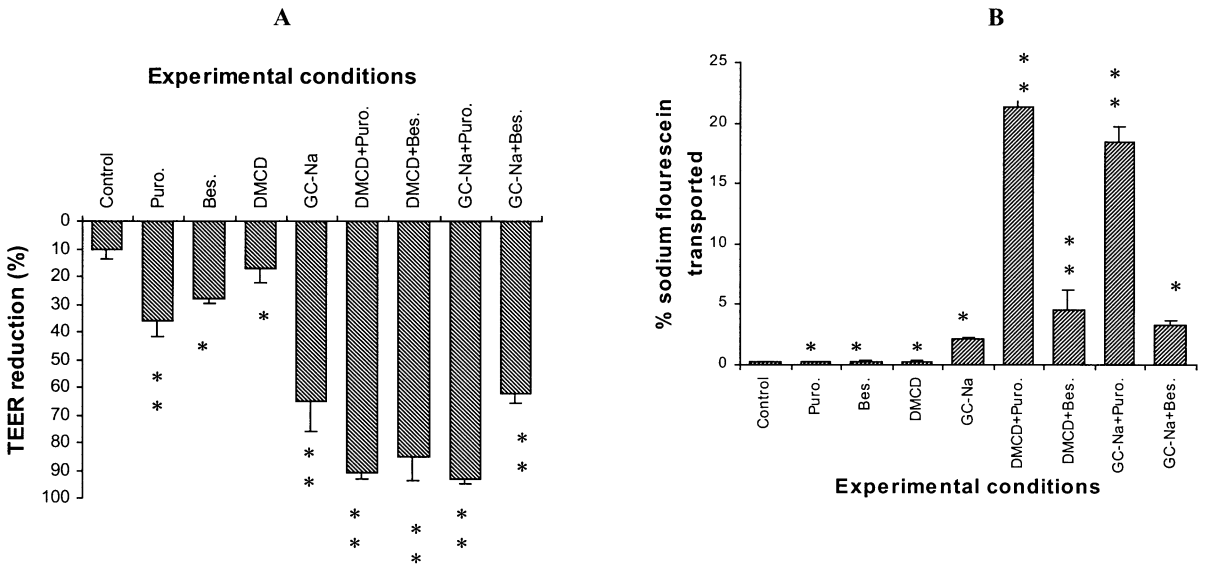

Fig. 4. Change in epithelial electrical resistance (A) and transport of sodium fluorescein (B) after Leu-Enk transport. Each bar represents the mean ± SD, $n = 3$ monolayers. Control = 3 mM leucine enkephalin alone, Puro = 1 mM puromycin, Bes = 1 mM bestatin, GC-Na = 0.5% Sodium glycocholate, DMCD = 2.0% dimethyl-β-cyclodextrin. ((**) Statistically significant; (*) not statistically significant).

ducing the viscosity of the mucus layer, (3) interfering with calcium ions in the intercellular spaces thereby opening up the tight junctions, and (4) inhibiting proteolytic activity (Quan et al., 1999; Hayashi et al., 1999; Aungst, 2000). Therefore, a combination of two or more enhancers that act through one or more of these mechanisms could result in higher deleterious effect on epithelial membrane compared to when one enhancer is employed.

3.2. Aminopeptidase rebound studies

One of the safety considerations when using bestatin and puromycin as nasal absorption enhancer is the induction of rebound aminopeptidase activity (Hussain et al., 1990). Unlike other cytotoxicity assays such as LDH and propidium iodide, rebound aminopeptidase assay gives more specific information with respect to the release of membrane-bound and cytosolic aminopeptidases following cell exposure to peptides and/or excipients. Currently there is no evidence that this phenomenon occurs in human nasal epithelium. In this study, cultured human nasal epithelium was used to monitor changes in aminopeptdase activity following the metabolism of Leu-Enk in the presence of different concentrations of bestatin and puromycin. The experiments were conducted in four phases: Phase 1 (control), phase 2 (inhibition phase), phase 3 (reversible phase), phase 4 (recovery phase). The results of this study are summarized in Table 2.

The specific activity for Leu-Enk degradation in phase 1 was 1.15 ± 0.02 μM/min/mg protein. In phase 2, the activity was reduced to 94.0 ± 7.2 and $55.0 \pm 5.6\%$ upon exposure to 0.02 and 1 mM bestatin, respectively. Similarly, 0.02 and 1 mM puromycin reduced the enzyme activity to 92.0 ± 8.0 and $44.0 \pm 5.6\%$, respectively.

Following washout of inhibitors and 1 h equilibration period (Phase 3) the specific activity of the cells exposed to 0.02 mM bestatin and puromycin was not statistically different from phase 1 ($P > 0.05$). While the specific activity of cells exposed to 1 mM puromycin increased to

125.0 ± 8.1%, the activity of cells exposed to 1 mM bestatin decreased to 44.0 ± 8.3%. The enzyme activity seen with 1 mM bestatin in phase 3 was comparable to phase 2. The lower enzyme activity seen with 1 mM bestatin in phase 3 may be explained by slower reversibility of the inhibitory effect of bestatin within 1 h. In phase 4, the aminopeptidase activity of both bestatin and puromycin did not return to baseline values, but rebounded to higher values. The rebound in aminopeptidase activity were as follows: 0.02 mM puromycin = 125 ± 6.5%, 1 mM puromycin = 110.0 ± 7.5%, 0.02 mM bestatin = 161.0 ± 8.4%, 1 mM bestatin = 129.0 ± 7.5%. The rebound aminopeptidase activity found in this study though differing in magnitude was comparable to that reported using the rat in situ perfusion technique (Hussain et al., 1990).

The rebound aminopeptidase activity may be linked to epithelial damage, improved access of Leu-Enk to cytosolic aminopeptidases and aminopeptidase release into the incubation medium (Hussain et al., 1990; Donnelly et al., 1998).

It was necessary to relate the changes in the aminopeptidase activity to protein leakage following cell exposure to the inhibitors. Table 3 shows that incubation of the cells with TM for 2 h resulted in only 1.4 ± 0.3% protein release. A comparison of protein release due to puromycin and bestatin revealed that only 1 mM puromycin caused a significant protein release ($P < 0.05$) after incubation for 2 h. To obtain an idea on the influence of multiple excipients exposure on protein release, the summation of the total protein released in the four phases was obtained. The summation of total protein released in the four phases was as follows: TM = 5.7 ± 0.3%, Leu-Enk alone = 7.0 ± 1.3%, Leu-Enk + 0.02 mM bestatin = 19.6 ± 3.3%, Leu-Enk + 1 mM bestatin = 23.7 ± 4.1%, Leu-Enk + 0.02 mM puromycin = 24.8 ± 4%, Leu-Enk + 1mM puromycin = 46.2 ± 7.3%. These data suggest a significant ($P < 0.05$%) cumulative protein release following cell exposure to Leu-Enk alone or its combination with enzyme inhibitors relative to TM. Although the high protein release observed in this study is not expected under in vivo situation due to mucus protection and mucociliary clearance, it highlights the fact that caution may be required when these excipients are used in formulating drugs for multiple dosing in humans, especially for sub-acute or chronic use.

4. Conclusions

This study showed that cultured human nasal epithelium may be used to study strategies to improve nasal absorption of peptides via protease inhibition and absorption enhancement. Additionally, cytotoxicity and membrane pertur-

Table 2
Rebound aminopeptidase activity in human nasal epithelium

Metabolic phases	Specific activity relative to phase 1 (%)			
	Bestatin		Puromycin	
	0.02 mM	1 mM	0.02 mM	1 mM
Phase 2 (inhibition phase)	94.0 ± 7.2↓	55.0 ± 5.6[a]↓	92.0 ± 8.0↓	44.0 ± 5.6[a]↓
Phase 3 (rebound phase)	92.0 ± 4.8↓	44.0 ± 8.3[a]↓	102.0 ± 4.6↑	125.0 ± 8.1[a]↑
Phase 4 (recovery phase)	161.0 ± 8.4[a]↑	129.0 ± 7.5[a]↑	125.0 ± 6.5[a]↑	110.0 ± 7.5↑

Each data represents the mean ± SD, $n = 3$ monolayers. $P < 0.05$, (↑) increase, (↓) decrease.
[a] Significantly different from control.

Table 3
Induction of protein leakage by Leu-Enk, bestatin and puromycin

Compounds	% protein release relative to total cell protein content			
	Phase 1	Phase 2	Phase 3	Phase 4
TM	1.43 ± 0.28	1.43 ± 0.28	1.43 ± 0.28	1.43 ± 0.28
Leu-Enk	7.03 ± 1.30	–	–	–
Leu-Enk + 0.02 mM bestatin	–	5.89 ± 0.82	5.87 ± 0.92	0.85 ± 0.32
Leu-Enk + 1 mM bestatin	–	7.76 ± 1.62	6.11 ± 1.18	2.80 ± 0.40
Leu-Enk + 0.02 mM puromycin	–	6.66 ± 1.46	5.45 ± 1.18	5.68 ± 0.99
Leu-Enk + 1 mM puromycin	–	11.98 ± 2.59[a]	7.89 ± 1.37	19.32 ± 2.55[a]

Each data represents the mean $\pm$ SD, $n = 3$ monolayers, $P < 0.05$, TM, transport medium.
[a] Significantly different from control.

bation with respect to nasal drug delivery using excipients may also be elucidated using human nasal primary cultures.

Based on the results of this study, a combination of protease inhibitors (bestatin, puromycin) with absorption enhancers possessing peptide stabilization characteristics (DMβCD or glycocholate) in some cases resulted in a significant increase in nasal Leu-Enk permeation. It is yet to be seen whether the membrane perturbation and cytotoxicity observed with the various combinations of protease inhibitors with absorption enhancers really raise safety concerns when used under in vivo condition. This is because the literature is full or articles using in vitro cell cultures with absorption enhancers for oral delivery in which cytotoxicity was seen even at very low concentrations. On the other hand, using far higher concentrations of the same enhancers under in vivo situation did not produce any real toxicity (Brayden et al., 1997). Furthermore, human nasal vaccine trials have been carried out with reasonable safety profiles with adjuvants such as medium chain fatty acids in milli-molar concentrations which would almost certainly be cytotoxic to nasal cell cultures (Aggerbeck et al., 1997). Therefore, further studies using in vivo nasal models are required to ascertain whether the membrane perturbation and cytotoxicity observed with the various combinations of protease inhibitors and absorption enhancers seen in this study really raise safety concerns.

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