JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 883–890
© 2009 The Authors
Received February 23, 2009
Accepted April 6, 2009
DOI 10.1211/jpp/61.07.0007
ISSN 0022-3573

Research Paper

An improved primary human nasal cell culture for the simultaneous determination of transepithelial transport and ciliary beat frequency

Roel Mallants[a], Valerie Vlaeminck[b], Mark Jorissen[b] and Patrick Augustijns[a]

[a]Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven and
[b]Laboratory for Experimental Otorhinolaryngology, Universitaire Ziekenhuizen Leuven, Belgium

Abstract

Objectives The aim was to establish a preclinical in-vitro system of the nasal mucosa for the simultaneous evaluation of nasal absorption and effects on ciliary activity.
Methods Human nasal epithelial cells were grown in collagen-coated transport inserts with transparent polyethylene terephthalate membranes (3 μm). Transepithelial transport and ciliary beat frequency values were measured every 15 min for 1 h.
Key findings The apparent permeability coefficients (P_{app}) for atenolol (mainly paracellular transport) and propranolol (transcellular transport) amounted to 0.1 ± 0.1 and $23.7 \pm 0.6 \times 10^{-6}$ cm/s, respectively, illustrating that the system can be used to discriminate between high permeability and low permeability compounds. Transport of talinolol (substrate for the P-glycoprotein efflux carrier) did not reveal polarity (0.3 ± 0.2 and $0.2 \pm 0.1 \times 10^{-6}$ cm/s for absorptive and secretory transport, respectively) and was not affected by verapamil (10 μM), suggesting the absence of P-glycoprotein in the nasal cell culture. No significant effects of atenolol, propranolol and talinolol on ciliary beat frequency were observed (98 ± 20% of the control condition after 60 min). Chlorocresol significantly decreased the ciliary activity but this decrease was not accompanied by effects on the transepithelial transport of atenolol, propranolol and talinolol.
Conclusions A new system was developed which offers possibilities as a fast screening tool for studying the potential of compounds for nasal drug administration, since permeability and a possible cilio-toxic effect can be assessed simultaneously.
Keywords ciliary beat frequency; human nasal epithelial cell culture; nasal transport

Introduction

Drugs that suffer from poor absorption after oral administration due to chemical or metabolic decomposition in the gastrointestinal tract are possible compounds for nasal administration. Over the last two decades, there has indeed been an increase in the number of drugs that have been reported for nasal administration (e.g. drugs for the treatment of migraine, pain, nausea and vomiting). The advantages of nasal drug delivery include rapid absorption and fast onset of action due to a relatively large absorption surface and high vascularization, avoidance of the hepatic and intestinal first-pass metabolism, and avoidance of chemical and enzymatic degradation in the gastrointestinal tract. Another advantage of nasal drug delivery is that it is cheaper than parenteral drug administration. In addition, nasal drug delivery is needle-free and can be performed without trained personnel, which facilitates self-medication and improves patient compliance compared to the parenteral route.[1]

The rapid absorption and fast onset of action of nasally applied drugs offers possibilities for drug administration in emergency situations as an alternative to parenteral administration.[2] Examples of drugs that have been administered nasally for this purpose are naloxone (for the treatment of opioid overdose),[3] benzodiazepines (for the treatment of children suffering from intractable seizures)[4,5] and glucagon (for the treatment of insulin-induced hypoglycaemia).[6,7]

One of the drawbacks of nasal drug delivery is the possible effect of the drugs or formulation compounds on ciliary activity. As the mucociliary clearance is one of the most important defence mechanisms of the respiratory tract, impairment of the mucociliary

Correspondence: Professor Patrick Augustijns, Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven, Belgium. E-mail: Patrick.Augustijns@pharm.kuleuven.be

clearance results in increased contact time between noxious particles and the nasal mucosa and can result in deleterious effects on the mucosa. Malfunction of the mucociliary clearance can also cause bacterial colonization of the nasal mucosa, resulting in acute or chronic infections. It is therefore important that drugs and formulation compounds do not interfere with this defence mechanism, which is characterized by the presence of mucus and ciliary activity. The effect of compounds on the ciliary activity can be assessed by measuring the ciliary beat frequency (CBF).

In order to screen compounds for potential systemic exposure after nasal administration, different in-vitro systems have been described. Basically, these in-vitro systems can be categorized into cell lines and primary cell cultures. Three human epithelial cell lines have been reported for permeability assessment: Calu-3 cells[8] and 16HBE14o$^-$ cells[9] are of bronchial origin while RPMI 2650 cells are of nasal origin.[10] Thus, the only human nasal cell line is RPMI 2650, of which it is generally believed that the cells grow in clusters rather than as a monolayer, although Bai *et al*.[11] reported the formation of tight junctions between the epithelial cells.

As none of the cell lines mentioned above express ciliary activity, we decided to develop and evaluate an in-vitro model for performing transport experiments, using primary human nasal epithelial cell cultures. The advantages of these primary cell cultures are that they attain confluency and express both tight junctions and ciliary activity. Human nasal epithelial cells were preferred over cells from animal species to limit inter-species differences.

Other authors have previously reported the use of primary cell cultures to explore the potential of compounds for nasal systemic delivery. Agu *et al*.[12] have described an in-vitro model in which the transport of sodium fluorescein across the nasal epithelium was determined. Although the authors determined CBF during the cell culture period, CBF was not determined during the transport experiment. The in-vitro model was also used to investigate nasal absorption of therapeutic peptides and to determine absorption enhancement strategies.[13] Another study, performed by Lin *et al*.,[14] also reported on the use of human nasal epithelial cell monolayers. However, these authors did not determine the effect of the compounds tested on ciliary activity.

The goal of this research study was to develop and characterize an in-vitro system which used human nasal epithelial cells for the simultaneous assessment of transepithelial transport and ciliary activity. In order to characterize the transepithelial transport system we have selected three beta-adrenoreceptor antagonists with different transport properties: atenolol as a marker for mainly paracellular transport, talinolol as a substrate for the P-glycoprotein (P-gp) efflux carrier and propranolol as a marker for passive transcellular transport. The physicochemical characteristics of these compounds are summarized in Table 1. In addition, we were interested in whether a modulation of the ciliary activity could affect transepithelial transport. Chlorocresol was selected as cilio-modulatory compound.

Materials and Methods

Materials

Protease type XIV and penicillin–streptomycin solution (10 000 IU/ml and 10 000 μg/ml, respectively) were purchased from Sigma-Aldrich (St Louis, MO, USA). DMEM-Ham's F12 (1 : 1) medium and Ultroser G and NU-serum were obtained from Life Technologies Ltd (Paisley, UK).

The transport medium (TM) consisted of Hanks' balanced salt solution supplemented with glucose (final concentration of 25 mM; Sigma-Aldrich) and 10 mM HEPES (Cambrex Bio Science, Verviers, Belgium). Sodium hydroxide (NaOH; BDH Laboratory Supplies, Poole, UK) was used to adjust the pH to 7.4.

Sodium fluorescein and chlorocresol were provided by UCB (Brussels, Belgium). Talinolol was a kind gift of Arzneimittelwerk Dresden (Radebeul, Germany). Atenolol, propranolol, verapamil, fexofenadine and triprolidine were purchased from Sigma-Aldrich. Lidocaine, diazepam and bacitracin were obtained from Certa (Braine-l'Alleud, Belgium). For the transmission electron microscopy (TEM) pictures, the following compounds were used: glutaraldehyde and sodium cacodylate were obtained from Sigma-Aldrich; osmium tetroxide, propylene oxide, dodecenyl succinic anhydride and methyl nadic anhydride were purchased from Agar Scientific (Stansted, UK); lead citrate was prepared from lead nitrate and sodium tricitrate obtained from Merck (Darmstadt, Germany); uranyl acetate was obtained from Electron Micrscopy Sciences (Fort Washington, PA, USA).

Cell isolation and culture

Human nasal epithelial cells were isolated from nasal biopsies according to a previously described method.[19,20] This study was approved by the Committee of Medical Ethics of the

Table 1 Summary of the physicochemical properties of the selected marker compounds and test compounds

Compound	MW	pK_a	logP	Reported P_{app} (cm/s)	Obtained P_{app} (cm/s)
Atenolol	266	9.6	0.16	$0.30 \pm 0.02 \times 10^{-6}$ [a]	$0.1 \pm 0.01 \times 10^{-6}$
Propranolol	259	9.5	3.20	$27 \pm 0.6 \times 10^{-6}$ [a]	$23.7 \pm 0.6 \times 10^{-6}$
Talinolol	364	9.4	3.38	$0.6 \pm 0.4 \times 10^{-6}$ [a]	$0.3 \pm 0.2 \times 10^{-6}$
Diazepam	285	3.3	2.7	$7 \pm 1 \times 10^{-6}$ [b]	$11 \pm 2 \times 10^{-6}$
Fexofenadine	538	4.2 and 9.5	0.49	$0.24 \pm 0.08 \times 10^{-6}$ [c]	$1.1 \pm 0.3 \times 10^{-6}$
Lidocaine	271	7.9	–0.26	$52 \pm 8 \times 10^{-6}$ [d]	$18.5 \pm 0.9 \times 10^{-6}$
Triprolidine	315	6.5	3.9	$17 \pm 3 \times 10^{-6}$ [e]	$18.6 \pm 0.9 \times 10^{-6}$

Apparent permeability values (P_{app}) as reported in literature are included. References are as follows: [a]Augustijns & Mols,[15] [b]Maitani *et al*.,[16] [c]Lin *et al*.,[14] [d]Wadell *et al*.[17] and [e]Kandimalla & Donovan.[18]

University Hospitals, Leuven. Briefly, the human nasal epithelial tissues were enzymatically dissociated using 0.1% protease solution in DMEM–Ham's F12 (1 : 1) medium, supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin, for a period of 16–24 h at 4°C after rinsing the tissue three times in saline (0.9% NaCl). At the end of the protease incubation, the large pieces of tissue were removed, and the protease activity was inhibited by adding 10% NU-serum. The cells were washed three times in DMEM–Ham's F12 (1 : 1) medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 2% Ultroser G by centrifugation (800 rpm, 5 min, 4°C). After the last centrifugation, the cell pellet was resuspended in 10 ml of the medium and incubated for 1 h in a 25 cm^2 plastic tissue culture flask in a CO_2 incubator (5% CO_2, 95% air, 37°C) to allow selective attachment of the contaminating fibroblasts and macrophages. The cell number was determined using a Bürker chamber. Cell viability was assessed by trypan blue exclusion.

Cell plating

ThinCerts with a transparent polyethylene terephthalate membrane of a pore size of 3 μm were used as transport inserts (Greiner Bio-One, Wemmel, Belgium). The cell culture surface was 1.13 cm^2. The transport inserts were placed in Cellstar 12-well tissue culture plates (Greiner Bio-One). The polyethylene terephthalate membrane was coated with a home-made rat tail collagen solution. The coating solution was obtained by twice diluting a collagen solution of 0.2% in acetate buffer (pH 4.5) with a concentrated (10×) phosphate-buffered saline solution. The solution was kept on ice and the pH was adjusted to 7.4 using sodium hydroxide. Each transport insert was coated with 150 μl of the coating solution. After coating, the inserts were kept at room temperature for 2–3 h in a laminar airflow; subsequently, they were incubated overnight in a CO_2-incubator (5% CO_2, 95% air, 37°C) to allow the collagen gel to dry. The next day, the coatings were rinsed five times with an isotonic saline solution. One hour prior to seeding of the cells in the transport inserts, the isotonic saline solution was replaced by cell culture medium. The cells were plated at a density of 1–1.3 × 10^6 cells/insert. During the rinsing procedure 0.5 ml isotonic saline was added in the apical compartment and 1.5 ml in the basolateral compartment. In the cell culture period, the same quantities of DMEM–Ham's F12 were added to the two compartments. The medium was changed daily. Five days after plating, the epithelial cells formed microscopically confluent layers consisting of ciliated and non-ciliated cells. Transport experiments were performed on day 11 after plating.

Transport experiments

Experimental set-up

Before initiating the transport experiment, the nasal cells were washed three times with TM at room temperature in order to remove the cell culture medium. After 30 min of pre-incubation of the nasal cell culture with transport medium, the transepithelial electrical resistance (TEER) values were measured. Only inserts with TEER values above 600 Ω cm^2 were included in the transport experiments. Control CBF values were determined, after which the transport experiment was initiated. Transport inserts were placed in new wells containing 1.5 ml of transport medium every 15 min to assure sink conditions and CBF values were determined immediately after the transfer. Transport was monitored for 1 h. Afterwards, the monolayer integrity was assessed by measuring the TEER values over the nasal cell layer and by determining the transport of a paracellular marker compound (sodium fluorescein 1 mg/ml) over 1 h. The transport values of sodium fluorescein were typically below 0.5%. TEER values were also measured at the end of the experiment.

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

$$P_{app} = dQ/dt \times 1/AC_0 \quad (1)$$

with dQ/dt being the transport rate of the compounds across the cell layer, A the surface area available for transport (1.13 cm^2) and C_0 the initial concentration at the donor compartment.

Analysis of marker compounds

Atenolol, propranolol and talinolol were analysed using a high-performance liquid chromatographic system equipped with a Waters 600E controller and pump, a Waters 717plus autosampler and a Waters 2475 multi λ fluorescence detector (Waters, Milford, MA, USA) as described by Augustijns and Mols.[15] Excitation and emission wavelengths were set at 271 and 302 nm, respectively, for atenolol and 249 and 333 nm, respectively, for talinolol and propranolol. Fluorescence signals were monitored and the obtained peaks integrated using a digital personal computer running Waters Empower 2 software. The column used was a Waters Novapak C-18 column (4 μm). The flow rate amounted to 1.65 ml/min. Mobile phase A consisted of sodium acetate (50 mM; pH adjusted to 3.3 with acetic acid) and methanol (85 : 15 v/v). Mobile phase B consisted of sodium acetate (50 mM; pH adjusted to 3.3 with acetic acid) and methanol (20 : 80 v/v). The column was initially equilibrated at 85% mobile phase A and 15% mobile phase B. After injection, the concentration of mobile phase B was increased to 55% and the flow rate decreased to 1.55 ml/min over 6 min. After 10 min, the system was returned to the initial conditions and equilibrated for 2 min before the next injection. All water was purified by a Maxima system (Elga Ltd, High Wycombe, UK). The volume injected amounted to 50 μl. The retention times of atenolol, talinolol and propranolol were 2.6, 8.3 and 8.8 min, respectively. Samples from the human nasal epithelial cell system were analysed by direct injection into the HPLC system.

Analysis of the transport compounds

Lidocaine, diazepam and triprolidine were analysed with spectrophotometry (Tecan Infinite M200 platereader, Tecan Group Ltd, Männedorf, Switzerland) at 230 nm. Sodium fluorescein was determined using spectrophotometry at a wavelength of 486 nm. For the analysis of fexofenadine, fluorescence detection was used (Kontron SFM 25, Kontron Instruments, Watford, UK): the excitation wavelength was set at 230 nm and the emission wavelength at 290 nm.

Measurement of ciliary beat frequency

Data acquisition

Unless stated otherwise, experiments were performed at a temperature of 22°C. An inverted microscope (Olympus IX70) was used at a magnification of 600 times. A MotionScope high-speed digital camera and PCI application software, running in a Windows 2000 environment (Redlake MASD Inc., San Diego, CA, USA), were used for image acquisition. The images were captured at a frame rate of 500 frames per second with a sampling interval of 2 ms. A sequence of 1024 images was recorded for each area. Each sequence of frame-by-frame images was stored in a file folder containing 1024 TIF format files for later analysis.

Ciliary beat frequency calculation

CBF was calculated as described elsewhere.[20–22] Briefly, a CBF value is computed separately for each pixel by spectral analysis of the variation of the pixel intensity over time. First, a region of interest (ROI) is selected, which is defined as all pixels for which the standard deviation of the intensity variation over time exceeds a threshold value of 5 dB. For each pixel in the ROI, the influence of noise on the CBF computation is reduced by spatial averaging of the intensity signal at each time point within a 3×3 pixel region centered around that pixel. Fast Fourier transformation (FFT) analysis is then applied to the smoothed intensity signal. The CBF is then computed as the frequency corresponding to the maximal FFT amplitude value in the range 0–20 Hz. The analysis method was implemented in Matlab (The Mathworks, Inc., Natick, MA, USA) and a graphical user interface was developed for CBF measurement and histogram analysis, which allowed derivation of overall statistics (mean, standard deviation and median) for the CBF of all beating cilia in the image.

Morphology of the cell layer

The morphology of the cultured cells was characterized by transmission electron microscopy (TEM). Cell layers cultured in the transport inserts were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C, and maintained in 0.1 M sodium cacodylate buffer until further processing (4°C). The samples were postfixed for 1 h in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), and dehydrated in a graded series of ethanol (25, 50, 75% and absolute). After 1 h incubation in Epon : propylene oxide (1 : 1), the membranes were embedded in fresh Epon. The Epon was polymerized for 24 h in an oven at 65°C. Ultrathin sections were cut (Ultramicrotome System 2128, LKB), mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips (CM 10) electron microscope.

Data presentation and statistical analysis

Transport data are presented as the mean ± SD of the apparent permeability coefficients. The CBF data are presented as mean ± SD of the percentage of the average CBF value of the corresponding control condition (mean ± SD). These data were analysed using a one-way ANOVA coupled with Dunnett's multiple comparison test. The difference in conditions between test and control was considered to be statistically significant when $P < 0.05$.

Results and Discussion

Characterization of the transport system

Cell support materials and coatings commonly used in culturing nasal epithelial cells have recently been discussed by Dimova *et al*.[13] In a previous unpublished study from our laboratory, different cell-support materials were compared, revealing the efficiency of polyethylene terephthalate membranes with 3 μm pores as cell support. Culturing human nasal epithelial cells on collagen-coated polyethylene terephthalate membranes resulted in a polarized cuboidal-to-columnar epithelium with well-differentiated ciliated cells with stable and reactive ciliary activity. These results were obtained by culturing the cells both in a CO_2 incubator and using a cell culture perfusion system.

In the present study, we explored whether similar conditions could be implemented using a standard insert set-up. Commercially available transport inserts with a polyethylene terephthalate membrane (pore size 3 μm) were selected for further studies. In a first set of experiments, we examined whether a cell layer was formed after seeding the cells on the collagen pre-coated transport inserts. Cell layer integrity was determined by measuring the transepithelial electrical resistance (TEER) as a function of the culturing period (Figure 1). After a short lag-time, TEER values increased and then levelled off after approximately 8 days. These data suggest that intact cell layers were formed. Based on these data, transport studies could be performed starting from day 8 after seeding.

A second set of preliminary experiments was designed to explore whether a threshold TEER value needed to be defined, below which the inserts should be disregarded. We therefore studied transport of the markers atenolol (mainly paracellular) and propranolol (transcellular) across the nasal cell layer over a broad range of TEER values. The results of

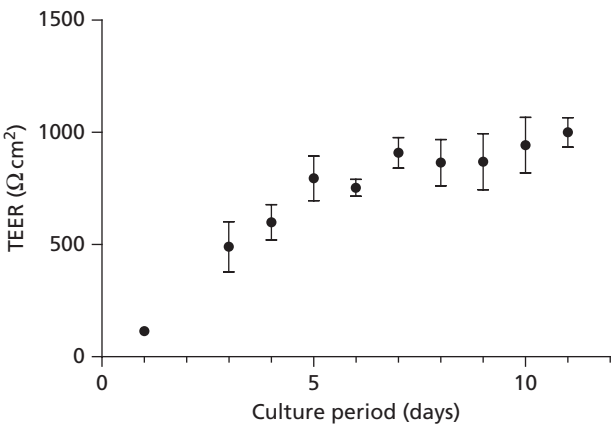

Figure 1 Typical transepithelial electrical resistance values (Ω cm^2) across the nasal cell culture as a function of culture time (days). Data are presented as mean ± SD ($n = 6$). TEER, transepithelial electrical resistance.

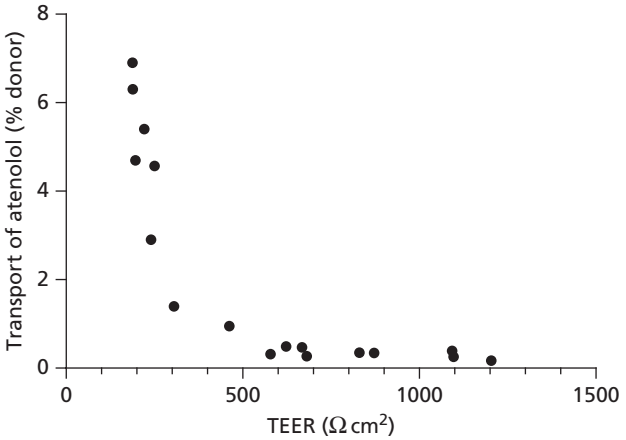

Figure 2 Transport of atenolol as function of transepithelial electrical resistance across the nasal epithelial cell layer. Low transepithelial electrical resistance (TEER) values result in high transport of atenolol, while increasing TEER values result in lower transport of atenolol. TEER values above 600 Ω cm^2 appear not to affect the transport of atenolol.

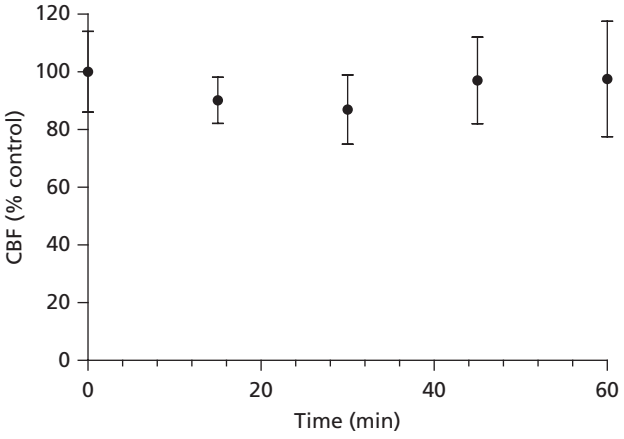

Figure 3 The time-dependent effect of a combination of atenolol (100 μM), talinolol (100 μM) and propranolol (50 μM) on ciliary beat frequency. No significant decrease in ciliary beat frequency (CBF) could be observed. Data are presented as mean ± SD (n = 6).

this study are presented in Figure 2. Transport of propranolol was independent of TEER values. However, low TEER values resulted in a higher concentration of atenolol in the basolateral compartment due to an incomplete formation of tight junctions. An increase in atenolol flux could be observed starting from TEER values below 600 Ω cm^2; above this value, transepithelial transport of atenolol remained stable. Based on these data, we decided to use only inserts with TEER values above 600 Ω cm^2. Based on this criterion, 16% of the cultured transport inserts could not be used for further experiments.

This cut-off value of 600 Ω cm^2 may appear rather high in comparison with the TEER values reported for excised nasal mucosa from different animal species. However, other authors have also reported TEER values as high as 500 Ω cm^2 for serially passaged human nasal cell cultures[24] and 1000 Ω cm^2 for the human pulmonary Calu-3 cell line.[25]

After these preliminary experiments, the apparent permeability coefficients (P_{app}) for atenolol and propranolol were determined using inserts with TEER values above the cut-off value of 600 Ω cm^2. P_{app} coefficients amounted to $0.1 \pm 0.1 \times 10^{-6}$ and $23.7 \pm 0.6 \times 10^{-6}$ cm/s for atenolol and propranolol, respectively. These data are comparable with data obtained in the Caco-2 system ($0.30 \pm 0.02 \times 10^{-6}$ and $27 \pm 3 \times 10^{-6}$ cm/s for atenolol and propranolol, respectively).[15]

One of the advantages of the present insert system is that the effect of compounds on the ciliary activity and the possible modulating effect of ciliary activity on transepithelial transport can be assessed simultaneously. First, we explored the effect of the cell culture period on the ciliary activity: CBF was not affected by the cell culture period and remained stable for at least 12 days (data not shown). Figure 3 illustrates that a combination of atenolol (100 μM), propranolol (100 μM) and talinolol (100 μM) does not affect cilairy beating: at none of the a priori defined time points (15, 30, 45 and 60 min after initiating the transport experiment) could a significant decrease in CBF be observed. Preliminary data had indicated there were no differences in the effects of atenolol, talinolol and propranolol when they were applied separately or in combination. An inhibitory effect of propranolol has been described by Boek *et al.*,[26] who reported a severe non-reversible ciliostatic effect of propranolol in both human and chicken ciliated mucosa; the reason for this discrepancy is probably the much higher concentration used by Boek *et al.* (34 mM).[15] Kanthakumar *et al.*[27] have tested the effect of both atenolol (1 μM) and propranolol (0.1 μM) on CBF in the human nasal ciliated epithelium using the cytology brush technique; in this study, no effect on CBF compared to control could be observed.

Effect of P-glycoprotein related efflux carriers on the bidirectional transport of talinolol

The presence of P-gp related efflux carriers in the nasal epithelium has been suggested by Graff and Pollack.[28] We therefore explored whether these carriers were functionally active in our experimental set-up by assessing (1) the bidirectional transport of talinolol (a known substrate of P-gp) across the nasal cell layers and (2) the effect of pre-incubation with verapamil (10 μM), a well-established inhibitor of P-gp. The P-gp inhibitory effect of verapamil on transport of talinolol has previously been shown in the Caco-2 system. However, no polarity in transport of talinolol could be observed using the nasal transport system: P_{app} values amounted to $0.3 \pm 0.2 \times 10^{-6}$ cm/s for absorptive transport and $0.2 \pm 0.1 \times 10^{-6}$ cm/s for secretory transport. The transport of talinolol was also not affected by the inclusion of verapamil (10 μM).

As these transport studies were performed at room temperature (our experimental set-up to measure CBF is operational at 22°C) and as the activity of transporters (e.g. P-gp) is presumably reduced by lower temperature, these permeation studies were repeated at 37°C. For the absorptive transport of talinolol, P_{app} values amounted to $0.27 \pm 0.07 \times 10^{-6}$ cm/s and $0.3 \pm 0.1 \times 10^{-6}$ cm/s in the absence and the presence of verapamil respectively, while for secretory

transport P_{app} values were $0.3 \pm 0.1 \times 10^{-6}$ cm/s and $0.27 \pm 0.02 \times 10^{-6}$ cm/s, respectively. These data illustrate that the effect of temperature (37 versus 22°C) is negligible. In the same set of experiments, the effect of temperature on the permeability of atenolol and propranolol was tested: for these compounds, no temperature dependence could be revealed.

The absence of polarity in transport and the fact that no effect was observed by inclusion of verapamil might be explained by the localization of the P-gp efflux carrier in the nasal mucosa: P-gp is probably mainly present in the olfactory epithelium. The presence in the nasal respiratory epithelium appears to be less pronounced.[28] The data obtained in our study confirm the results of Kandimalla and Donovan,[18] who reported on the localization of P-gp in both bovine olfactory and nasal respiratory mucosae; using immunohistochemistry, the expression appeared to be greater in the olfactory epithelium than in the nasal respiratory epithelium. In view of the limited surface area of the olfactory region as compared to the respiratory epithelium, the effect of P-gp on transepithelial transport is expected to be negligible or very low. Direct nose-to-brain transport via the olfactory mucosae has recently been proposed as an alternative pathway for drugs to reach the brain, hereby circumventing the blood–brain barrier.[29] It is obvious that in studies dealing with transport via the olfactory epithelium, the effect of P-gp cannot be ignored.

Morphological study of the nasal cell layers

As transport of the paracellular marker atenolol in the system established was comparable with that obtained in the Caco-2 system, there was an expectation that the nasal cell culture had grown as a monolayer. In order to test this hypothesis, we performed a morphological study of the nasal cell culture by TEM. The purpose of this study was mainly to determine whether the cells had grown as multilayers or monolayers. TEM studies were performed on samples derived from four different cell cultures. No morphological variation between the different cell cultures could be observed. A typical example of the pictures obtained is presented in Figure 4, illustrating that the cells existed as a monolayer, consisting of ciliated and non-ciliated cells.

Transport of selected drugs

In the next set of experiments, we selected several drugs for which permeability data were available in the literature. The drugs selected were diazepam, fexofenadine, lidocaine and triprolidine. The physicochemical characteristics of these compounds are summarized in Table 1. The compounds were selected since they cover a broad range of reported permeability values ($0.24–52 \times 10^{-6}$ cm/s). All transport experiments with test compounds were performed in triplicate. Lidocaine transport is presented in Figure 5. Transport appeared to be linear over the time period studied. Lidocaine did not affect ciliary activity over the time period studied: CBF amounted to $96 \pm 7\%$ of the initial values after 60 min. Also, for diazepam and triprolidine, no effect on CBF could be observed: CBF values after 60 min were $100 \pm 10\%$ and $88 \pm 15\%$ of the initial CBF values, respectively. On the other hand, fexofenadine decreased CBF to $74 \pm 15\%$ of the initial values after 15 min and this decrease was maintained during the exposure time: CBF amounted to $70 \pm 22\%$, $78 \pm 20\%$ and $74 \pm 20\%$ of the initial values after 30, 45 and 60 min, respectively. At each time point, CBF was significantly lower ($P < 0.05$) compared to the initial CBF values, while the values at the different time points did not differ significantly compared to each other. Although fexofenadine has been studied for nasal application, no data with respect to its effect on the ciliary activity are available.

The apparent permeability coefficients (P_{app}) amounted to $11 \pm 2 \times 10^{-6}$, $1.1 \pm 0.3 \times 10^{-6}$, $18.5 \pm 0.9 \times 10^{-6}$ and $18.6 \pm 0.9 \times 10^{-6}$ cm/s for diazepam, fexofenadine, lidocaine and triprolidine, respectively. In Table 1 we have included the apparent permeability coefficients as reported in the literature.

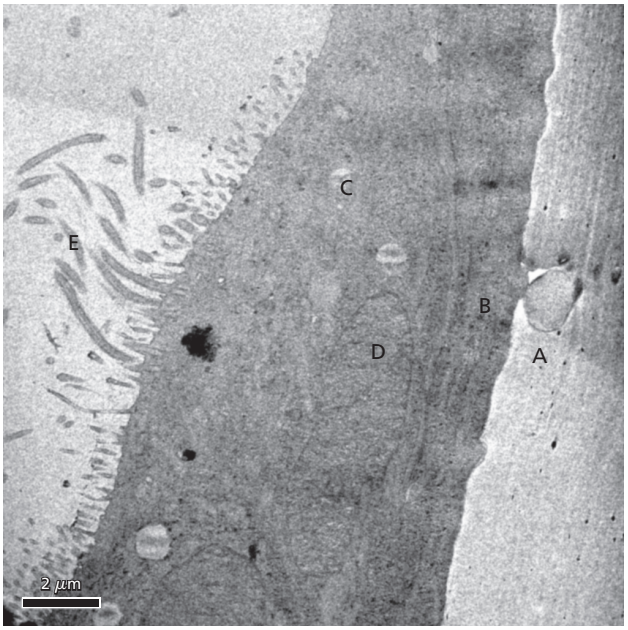

Figure 4 Transmission electronic microscopy picture of the nasal cell layer. A, cell-support; B, collagen coating; C, cell monolayer; D, cell nucleus; E, cilia.

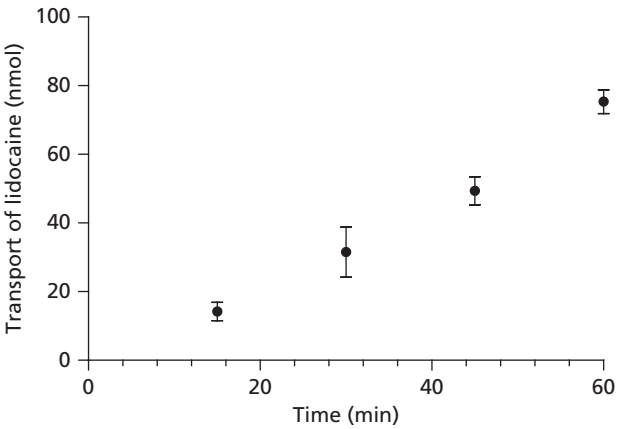

Figure 5 Time-dependent transport of lidocaine across the human nasal epithelial cell culture. Data are presented as mean ± SD ($n = 3$).

These data were obtained using different experimental set-ups and therefore cannot be compared directly with the corresponding data as observed in our system. These data are set out in order to discuss the data obtained in an illustrative way. For diazepam and triprolidine, the P_{app} values obtained in our system are within the range of data obtained for other nasal transport systems, while the apparent permeability coefficient for fexofenadine is approximately 4-fold higher and for lidocaine 3-fold lower (Table 1).

Maitani *et al.* have determined the nasal *in vitro* transport of diazepam using rabbit mucosa mounted in diffusion cells.[16] When using an isotonic Krebs–Ringer solution (pH = 7.4) as medium, the permeability coefficient for diazepam amounted to $7 \pm 1 \times 10^{-6}$ cm/s, which was in the same range as data obtained in our system ($11 \pm 2 \times 10^{-6}$ cm/s).

Kandimalla and Donovan[18] have examined the transport of triprolidine across bovine olfactory mucosa using diffusion cells. The permeability coefficient amounted to $17 \pm 3 \times 10^{-6}$ cm/s, which was in agreement with the result obtained in this study ($P_{app} = 18.6 \pm 0.9 \times 10^{-6}$ cm/s).

The permeability for fexofenadine has been studied by Lin *et al.* using passaged human nasal epithelial cell monolayers cultured in an air–liquid interface.[14] They observed a permeability coefficient of $0.24 \pm 0.08 \times 10^{-6}$ cm/s and the authors suggested that fexofenodine crosses the nasal mucosa by the paracellular route. Although a higher P_{app} value was obtained in our study ($1.1 \pm 0.3 \times 10^{-6}$ cm/s), this result also suggests primarily paracellular transport for this compound.

Transport of lidocaine was studied by Wadell *et al.*[17] using a diffusion chamber model in which mucosal specimens of porcine nasal mucosa were mounted. The apparent permeability coefficient observed by these authors amounted to $52 \pm 8 \times 10^{-6}$ cm/s for lidocaine. The permeability observed in our study was lower ($18.5 \pm 0.9 \times 10^{-6}$ cm/s). In the same study, Wadell *et al.*[17] also determined the permeability coefficient for propranolol ($P_{app} = 20 \pm 8 \times 10^{-6}$ cm/s), which corresponds with the permeability values obtained in this study ($23.7 \pm 0.6 \times 10^{-6}$ cm/s). Although the rank order of the latter two compounds is not the same, both compounds would probably be categorized as high permeability compounds in both studies.

Effect of chlorocresol on transepithelial transport

The mucociliary clearance limits the residence time of a formulation in the nasal cavity to 15–30 min.[30] This short residence time is likely to influence transepithelial transport, especially for compounds with low permeability. The effect of inclusion of cilio-modulatory compounds on the transport of the marker compounds atenolol, talinolol and propranolol was examined, as we wanted to know whether a modulation of the CBF could affect the transepithelial transport. The lipophilic preservative chlorocresol was selected as the cilio-inhibitory compound. Chlorocresol has been previously reported to induce cilio-inhibition at a concentration of 0.005%.[31,32] This decrease in CBF is based on the disappearance of the central pair in the ciliary ultrastructure (data not shown). Exposing the nasal cell layer to chlorocresol significantly decreased CBF to $44 \pm 3\%$ of the corresponding control condition. However, inclusion of chlorocresol did not significantly alter transport of atenolol, talinolol and propranolol across the nasal cell layer: apparent permeability coefficients amounted to $0.2 \pm 0.2 \times 10^{-6}$, $0.2 \pm 0.1 \times 10^{-6}$ and $15 \pm 2 \times 10^{-6}$ cm/s versus $0.2 \pm 0.1 \times 10^{-6}$, $0.2 \pm 0.1 \times 10^{-6}$ and $13 \pm 3 \times 10^{-6}$ cm/s in the control condition without chlorocresol, respectively. Based on these data, cilio-modulation appeared to have only a minor or no effect on the transepithelial transport.

In vitro, the donor concentration remains constant regardless of the ciliary activity, while *in vivo* the mucociliary clearance results in a conveyer belt effect, leading to decreased concentrations over time. It may, however, be possible that *in vivo*, chlorocresol results in a slower decrease in donor concentration compared to the control condition due to impairment of ciliary activity. The data from this study suggest that cilio-modulation has no effect on permeability.

Conclusions

In this study, a system has been described which offers the possibility of determining the transepithelial transport of compounds across human nasal epithelial cell culture, in combination with an assessment of the effect of compounds on the ciliary beat frequency. As control transport compounds, atenolol and propranolol were studied as they are markers for different transport pathways: paracellular transport and transcellular transport, respectively. Overall, the permeability values obtained in the nasal transport experiments are comparable with data obtained in the Caco-2 system, illustrating the discriminative power of the system. The bidirectional transport of talinolol was assessed since this compound is a substrate of the P-gp efflux carrier. Talinolol did not exhibit polarity in absorptive and secretory transport, and the inclusion of verapamil (a well-established inhibitor of P-gp) did not affect talinolol transport, suggesting an absence of the P-gp efflux carrier. Atenolol, propranolol and talinolol did not affect CBF. Morphological studies revealed the presence of a monolayer consisting of ciliated and non-ciliated cells.

The system established was useful in order to rank test compounds with respect to their permeability characteristics. However, the absence of in-vivo nasal bioavailability studies prohibits the making of any statements about in-vitro/in-vivo correlations.

We also explored the effect of cilio-modulation on transepithelial transport: chlorocresol (a compound which had been used previously to lower CBF) decreased the ciliary activity but this decrease was not accompanied with effects on transepithelial transport of atenolol, propranolol and talinolol, probably due to the fact that our in-vitro system should be considered as a static system. The system presented here does not express mucus. In conclusion, the system described (1) can discriminate between the different transport pathways, a determination which is required for a correct assessment of the absorption potential of compounds, (2) revealed decreased CBF values after exposure to chlorocresol, suggesting that the system can be used to study the effect of compounds on ciliary activity, and (3) consists of a monolayer with ciliated and non-ciliated cells, thereby mimicking the nasal barrier. We can therefore state

that this system offers possibilities as a fast screening tool for studying the potential of compounds for nasal drug administration, since both the permeability across the nasal monolayer and the possible effect of the compounds on the ciliary activity can be assessed simultaneously.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

1. Jadhav K *et al*. Nasal drug delivery system: factors affecting and applications. *Curr Drug Ther* 2007; 2: 27–38.
2. Costantino HR *et al*. Intranasal delivery: physicochemical and therapeutic aspects. *Int J Pharm* 2007; 337: 1–24.
3. Kendall JM, Latter VS. Intranasal diamorphine as an alternative to intramuscular morphine: pharmacokinetic and pharmacodynamic aspects. *Clin Pharmacokinet* 2003; 42: 501–513.
4. Fişgin T *et al*. Nasal midazolam effects on childhood acute seizures. *J Child Neurol* 2000; 15: 833–835.
5. Kendall JL *et al*. Intranasal midazolam in patients with status epilepticus. *Ann Emerg Med* 1997; 29: 415–417.
6. Freychet L *et al*. Effect of intranasal glucagon on blood glucose levels in healthy subjects and hypoglycaemic patients with insulin-dependent diabetes. *Lancet* 1988; 1: 1364–1366.
7. Pontiroli A. Peptide hormones: review of current and emerging uses by nasal delivery. *Adv Drug Deliv Rev* 1998; 29: 81–87.
8. Foster KA *et al*. Characterization of the calu-3 cell line as a tool to screen pulmonary drug delivery. *Int J Pharm* 2000; 208: 1–11.
9. Forbes B *et al*. The human bronchial epithelial cell line 16hbe14o- as a model system of the airways for studying drug transport. *Int J Pharm* 2003; 257: 161–167.
10. Audus KL *et al*. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm Res* 1990; 7: 435–451.
11. Bai S *et al*. Evaluation of human nasal RPMI 2650 cells grown at an air-liquid interface as a model for nasal drug transport studies. *J Pharm Sci* 2008; 97: 1165–1178.
12. Agu RU *et al*. 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.
13. Agu RU *et al*. Nasal absorption enhancement strategies for therapeutic peptides: an in vitro study using cultured human nasal epithelium. *Int J Pharm* 2002; 237: 179–191.
14. Lin H *et al*. Enhancing effect of surfactants on fexofenadine HCl transport across the human nasal epithelial cell monolayer. *Int J Pharm* 2007; 330: 23–31.
15. Augustijns P, Mols R. HPLC with programmed wavelength fluorescence detection for the simultaneous determination of marker compounds of integrity and P-gp functionality in the caco-2 intestinal absorption model. *J Pharm Biomed Anal* 2004; 34: 971–978.
16. Maitani Y *et al*. In vitro nasal transport across rabbit mucosa: effect of oxygen bubbling, pH and hypertonic pressure on permeability of lucifer yellow, diazepam and 17-β-estradiol. *Int J Pharm* 1997; 146: 11–19.
17. Wadell C *et al*. Permeability of porcine nasal mucosa correlated with human nasal absorption. *Eur J Pharm Sci* 2003; 18: 47–53.
18. Kandimalla KK, Donovan MD. Localization and differential activity of p-glycoprotein in the bovine olfactory and nasal respiratory mucosae. *Pharm Res* 2005; 22: 1121–1128.
19. Jorissen M *et al*. Ciliogenesis and coordinated ciliary beating in human nasal epithelial cells cultured in vitro. *Acta Otorhinolaryngol Belg* 1989; 43: 67–73.
20. Mallants R *et al*. Effect of preservatives on ciliary beat frequency in human nasal epithelial cell culture: single versus multiple exposure. *Int J Pharm* 2007; 338: 64–69.
21. Dimova S *et al*. High-speed digital imaging method for ciliary beat frequency measurement. *J Pharm Pharmacol* 2005; 57: 521–526.
22. Mallants R *et al*. Beneficial effect of antibiotics on ciliary beat frequency of human nasal epithelial cells exposed to bacterial toxins. *J Pharm Pharmacol* 2008; 60: 437–443.
23. Dimova S *et al*. The use of human nasal in vitro cell systems during drug discovery and development. *Toxicol In Vitro* 2005; 19: 107–122.
24. Yoo J *et al*. Serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. *Pharm Res* 2003; 20: 1690–1696.
25. Johnson LG *et al*. Enhanced Na+ transport in an air-liquid interface culture system. *Am J Physiol* 1993; 264: L560–L565.
26. Boek WM *et al*. Validation of animal experiments on ciliary function in vitro. i. The influence of substances used clinically. *Acta Otolaryngol* 1999; 119: 93–97.
27. Kanthakumar K *et al*. Effect of salmeterol on human nasal epithelial cell ciliary beating: inhibition of the ciliotoxin, pyocyanin. *Br J Pharmacol* 1994; 112: 493–498.
28. Graff CL, Pollack GM. Functional evidence for p-glycoprotein at the nose-brain barrier. *Pharm Res* 2005; 22: 86–93.
29. Illum L. Is nose-to-brain transport of drugs in man a reality? *J Pharm Pharmacol* 2004; 56: 3–17.
30. Merkus F *et al*. Nasal mucociliary clearance as a factor in nasal drug delivery. *Adv Drug Deliv Rev* 1998; 29: 13–38.
31. Agu RU *et al*. Effects of pharmaceutical compounds on ciliary beating in human nasal epithelial cells: a comparative study of cell culture models. *Pharm Res* 1999; 16: 1380–1385.
32. Dimova S *et al*. Stable ciliary activity in human nasal epithelial cells grown in a perfusion system. *Int J Pharm* 2005; 292: 157–168.