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Evaluation of air-interfaced Calu-3 cell layers for investigation of inhaled drug interactions with organic cation transporters *in vitro*

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ABSTRACT

A physiologically pertinent *in vitro* model is urgently needed for probing interactions between inhaled drugs and the organic cation transporters (OCT) in the bronchial epithelium. This study evaluated OCT expression, functionality, inhibition by common inhaled drugs and impact on formoterol transepithelial transport in layers of human bronchial epithelial Calu-3 cells grown at an air–liquid interface. 21 day old Calu-3 layers expressed OCT1, OCT3, OCTN1 and OCTN2 whereas OCT2 could not be detected. Quantification of the cellular uptake of the OCT substrate ASP^+ in presence of inhibitors suggested several OCT were functional at the apical side of the cell layers. ASP^+ uptake was reduced by the bronchodilators formoterol, salbutamol (albuterol), ipratropium and the glucocorticoid budesonide. However, the OCT inhibitory properties of the two β_2-mimetics were suppressed at therapeutically relevant concentrations. The absorptive permeability of formoterol across the cell layers was enhanced at a high drug concentration shown to decrease ASP^+ uptake by ~50% as well as in presence of the OCT inhibitor tetraethylammonium (TEA). Secretory transport was unaffected by the drug concentration but was reduced by TEA. Our data indicate air-interfaced Calu-3 layers offer a low-cost *in vitro* model suitable for assessing inhaled drug–OCT interactions in the bronchial epithelium.

1. Introduction

Membrane transporters are known to have a significant impact on the absorption, distribution, elimination and toxicity of a large number of drugs (Ayrton and Morgan, 2008; Szakacs et al., 2008). Accordingly, active transport mechanisms in the intestine, liver, kidneys, brain as well as their role in drug pharmacokinetic/pharmacodynamic (PKPD) and safety profiles are currently being intensively investigated. In contrast, although the lungs express a broad range of both uptake and efflux transporters (Bosquillon, 2010), the evaluation of their influence on the disposition of inhaled drugs has received little attention.

Organic cation transporters (OCT) are members of the super family of solute-link carriers, SLC22A. The five predominant OCT found in humans are divided into subtypes based on the driving force for cation transport: (i) the electrogenic transporters OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3) and (ii) the pH-dependent novel transporters OCTN1 (SLC22A4) and OCTN2 (SLC22A5). OCT transport mainly positively charged molecules across the cell membrane in both directions and their involvement in the intestinal absorption and renal excretion of many cationic drugs is well established (Ciarimboli, 2008; Koepsell et al., 2007; Nies et al., 2011). OCT have been localised on the luminal surface of airway epithelial cells although the relative expression levels of each subtype in the respiratory epithelium remain unclear (Bosquillon, 2010). Lips et al. (2005) reported the presence of OCT1, OCT2, OCT3 in both tracheal and bronchial epithelial cells. Horvath et al. (2007a) compared the mRNA levels of each of the five subtypes in the human airway epithelium with those in tissues known to highly express the transporter. They measured high levels of both OCTN1 and OCTN2 transcripts relative to the liver or kidney and very low levels of OCT1, OCT2 and OCT3 as compared to the liver, kidney or placenta, respectively.

Several common inhaled drugs are positively charged at physiological pH and have been reported to interact with the OCT. The short-acting β_2-agonist salbutamol (albuterol) was actively transported in the absorptive direction across layers of the two human

Abbreviations: OCT, organic cation transporters; PKPD, pharmacokinetic/pharmacodynamic; NHBE, normal human bronchial epithelial; RT-PCR, reverse transcriptase polymerase chain reaction; TEER, transepithelial electrical resistance; ASP^+, 4-(4-(diethylamino)styryl)-N-methylpyridinium iodide] (4-Di-2-ASP); ALI, air–liquid interface; DMSO, dimethyl sulphoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; PI, piperidine iodide; TEA, tetraethylammonium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); P_{app}, coefficient of apparent permeability.

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bronchial epithelial cell lines Calu-3 and 16HBE14o-, allegedly *via* an OCT-mediated mechanism (Ehrhardt et al., 2005). Both salbutamol and formoterol reduced the uptake of the OCT substrate ASP^+ by a transporter identified as OCTN2 in undifferentiated normal human bronchial epithelial (NHBE) cells (Horvath et al., 2007a). In a related study in human airway smooth muscle cells, formoterol and the glucocorticoid budesonide were shown to be, respectively, substrate and inhibitor for OCT3 (Horvath et al., 2007b). Budesonide and fluticasone had in addition the ability to modulate the activity of OCT2 in transfected *Xenopus laevis* cells (Lips et al., 2005). Finally, the antimuscarinic ipratropium decreased the uptake of the OCTN2 substrate L-carnitine in human proximal tubule Caki-1 cells (Glube et al., 2006) and, more recently, its internalisation by human bronchial epithelial BEAS-2B cells was demonstrated to be predominantly mediated by OCTN2 and to a lesser extent by OCTN1 (Nakamura et al., 2010).

Cells used in the majority of the aforementioned studies were either of a non-pulmonary origin or they were grown on cell culture plates under submerged conditions, neither of which provide conditions that mimic the absorption barrier properties of the airway epithelium. Therefore, there is a need for a reliable and economical physiologically relevant *in vitro* representation of the bronchial epithelium that would allow (a) further exploration of the interactions of inhaled drugs with the OCT, (b) evaluation of the impact of the transporters on drug disposition in the lungs, (c) identification of drug candidates that are actively transported by OCT at an early stage of clinical development and (d) investigation of possible OCT-related drug interactions.

Amongst currently available respiratory cell culture models (Forbes and Ehrhardt, 2005), Calu-3 cells form differentiated layers that are morphologically representative of the bronchial epithelium when grown on permeable filters at an air–liquid interface (ALI) for over 10 days (Grainger et al., 2006). ALI Calu-3 layers produce mucus, exhibit a high transepithelial electrical resistance (TEER) indicative of functional tight junctions and show permeability characteristics similar to those of the native epithelium (Foster et al., 2000; Grainger et al., 2006; Mathias et al., 2002). They express the range of transporters found in the lungs (Bosquillon, 2010) but although OCT mRNA have been detected in undifferentiated Calu-3 cells (Endter et al., 2009), the presence of functional OCT in ALI layers has not been probed till date.

The aim of the present work was to evaluate the suitability of air-interfaced Calu-3 layers as a simple but nevertheless, physiologically adequate cell culture model for investigating the interactions of inhaled drugs with the OCT in the respiratory epithelium. OCT gene expression in the cell line was compared with that in layers of NHBE cells and the presence of transporter proteins was confirmed in ALI Calu-3 layers. The functionality of the transporters was evaluated by quantifying the uptake of the OCT model substrate ASP^+ in the presence of inhibitors. The ability of common inhaled drugs to modulate the OCT activity in Calu-3 layers, as reported in less relevant cell culture systems, was determined and the permeability of the bronchodilator formoterol across the layers was measured at different concentrations in presence or absence of an OCT inhibitor, to assess the impact of the transporters on drug absorption across the bronchial epithelium.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all materials were purchased from Sigma–Aldrich, Dorset, UK.

2.2. Cell culture and maintenance

Calu-3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). They were grown in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham supplemented with 10% FBS, 100 UI/ml penicillin and 100 μg/ml streptomycin, 20 mM L-glutamine and 1% non-essential amino acids. Upon reaching 90% confluence, the cells were trypsinised using 0.25% trypsin/0.1% EDTA. Post trypsinisation, Calu-3 cells were seeded at a density of 10^5 cells/cm^2 onto 0.4 μm pore size, 1.13 cm^2 surface area polyester Transwells® cell culture inserts (CoStar Corning, Corning, UK) and after 24 h, were cultured under ALI conditions for up to 21 days.

NHBE cells (B-ALI certified batch 139014 from a 61 year old Hispanic male) were obtained from Lonza (Slough, Berkshire, UK) and cultured according to the supplier's protocol. In brief, cells were grown in the proprietary bronchial air–liquid interface B-ALI™ basal medium to which SingleQuots™ supplements and growth factors were added (Lonza). Once they reached ~90% confluence, cells were harvested using the supplier's subculture reagents (Lonza) and seeded onto 0.4 μm pore size, 0.33 cm^2 polyester Transwell® inserts previously coated with rat tail collagen type I (BD Biosciences, Oxford, Oxfordshire, UK) at a density of 16,500 cells/cm^2. On day 3 after seeding, cells were raised to an ALI and subsequently cultured in the supplier's differentiation medium (Lonza) for 21 days.

All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere and supplied with fresh culture medium every 2–3 days. The monolayer integrity was verified by measuring the transepithelial electrical resistance (TEER) using an epithelial voltohmmeter with chopstick electrodes (World Precision Instruments, Stevenage, UK) and only cell layers with TEER values above 400 Ω cm^2 were used for experimentation.

2.3. OCT gene expression

The expression of OCT in Calu-3 and NHBE cells was assessed by reverse transcription polymerase chain reaction (RT-PCR). Calu-3 cells at 90% confluency in a T75 flask or grown at an ALI on Transwells for 14/21 days and NHBE cells cultured at an ALI for 21 days were all harvested using Non-enzymatic Cell Dissociation Solution. cDNA was synthesised following muMacs RNA extraction using a cDNA synthesis kit (Multenyi Biotech, UK). 0.5 μl of the respective cDNA was used for each PCR reaction (final volume of 20 μl, 35 cycles). Primers for all five human OCT and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed on Primer3 software and checked with Primer-Blast (Table 1). All primers (Invitrogen, Paisley, UK) were validated on human lung cDNA and the OCT2 primer was additionally validated on cDNA from the intestinal epithelial cell line Caco-2. The PCR products for each reaction were separated on a 1% agarose gel and stained with ethidium bromide at a concentration of 0.5 μg/ml.

2.4. OCT protein expression

OCT protein expression was assessed in 21-day old ALI Calu-3 layers by immunocytochemistry. Cell layers were washed with ice-cold PBS, fixed with 4% (w/v) paraformaldehyde for 10 min and permeabilised using PBS containing 1% BSA and 0.15% Triton-X100 at room temperature. The layers were blocked with human serum (#H4522, Sigma) diluted in PBS (1:25), followed by a second blocking with 10% goat serum (#G9023, Sigma) for 30 min each, at 37 °C. Primary antibodies for OCT1–3, OCTN1 and OCTN2 (Alpha Diagnostics, TX, USA, Cat #OCT1–1A, #OCT2–1A, #OCT3–1A, #OCTN1–1A and #OCTN2–1A) were diluted 1:1000 in PBS with 10% goat serum.

Table 1
Details of custom designed primers used for RT-PCR.

Gene	Gene symbol	Forward primer (5′ → 3′)	Reverse primer (3′ → 5′)	Tm	Size (bp)
GAPDH	GAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC	53.8/53.1	101
OCT1	SLC22A1	TGAAGGACGCCGAGAACC	AGGAAGAATACAGAGAAGTGAAGG	55.7/55.6	188
OCT2	SLC22A2	AAGTTGCCTATACAGTTGG	GCGATGTGCTTAATGATTC	48.9/49.3	196
OCT3	SLC22A3	CCACTCCACCATCGTCAG	ACACCAAGGCAGGATAGC	53.5/53	168
OCTN1	SLC22A4	TGTCATCACCCGTAGTTG	ACATACCATTGAAGCCATTG	50.3/50.9	156
OCTN2	SLC22A5	TTACTTCATCCGAGACTGG	CTGCTTCTTGGAACTTAGG	50.3/49.7	229

Tm = melting temperature; bp = base pairs.

The layers were exposed to the primary antibody solutions for 2 h at 37 °C before thorough rinsing with 0.05% Tween in PBS. They were then incubated with a FITC-labelled goat anti-rabbit IgG secondary antibody at a 1:100 dilution in 10% goat serum (#F0382, Sigma) for 1 h at 37 °C. Cells were finally washed (3 × 15 min) with 0.05% Tween in PBS, in the dark and counter-stained with 1 μg/ml piperidine iodide (nuclear staining). The membranes were carefully excised from the Transwell inserts, mounted on a glass slide with 80% glycerol and covered with a cover slip. The slides were examined under an Inverted Zeiss LSM 510 META Confocal Microscope (Carl Weiss, Germany) and the images were processed using the recommended Zeiss LSM Image viewer version 3.5.0.359. Cell layers exposed to the secondary antibody only were used as negative controls.

2.5. ASP^+ uptake studies

[4-(4-(Diethylamino)styryl)-N-methylpyridinium iodide] (4-Di-2-ASP or ASP^+, Invitrogen, Oregon, USA) is a fluorescent cation whose cellular uptake is predominantly governed by the OCT (Cetinkaya et al., 2003; Ciarimboli et al., 2004). The apical pole of the Calu-3 layers (passage 29–33) were exposed to 100 μM of ASP^+ in Hank's balanced salt solution (HBSS) supplemented with 20 mM HEPES (pH 7.4), or in HBSS/HEPES with 0.1% dimethyl sulphoxide (DMSO) for 20 min at 4 °C and at 37 °C in presence or absence of competitive OCT inhibitors: tetraethylammonium (TEA, 5 mM), decynium-22 (20 μM), L-ergothioneine (100 μM), L-carnitine (10 μM); and common inhaled drugs: the inhaled bronchodilators salbutamol/albuterol, formoterol (Chemos GmbH, Regenstauf, Germany), ipratropium (5 μM, 500 μM) and the inhaled glucocorticoid budesonide (5 μM, 30 μM, 50 μM). Budesonide solutions were prepared in HBSS/HEPES with 0.1% DMSO. Cell layers were rinsed with ice cold PBS. The layers were then fixed with 4% (w/v) paraformaldehyde and treated with 4% (v/v) Trypan blue to quench the external fluorescence. After a final wash with PBS, the membrane filters were carefully cut off the cell culture insert and mounted in 4% glycerol. Cell layers were visualized under a LEICA DM IRB fluorescence microscope (Milton Keynes, UK), images were captured with a Qcam FAST1394 camera (Surrey, Canada) and the intracellular fluorescence was quantified using a QCapturePro software version 6.0. The dye emits at a wavelength of 550 nm (green) in a membrane-bound state and at 580–630 nm (red) while intracellular (Stachon et al., 1997). Hence, the dye intracellular uptake was quantified using the ratio between the detected red and green fluorescence. ASP^+ uptake data are presented as a percentage of the control.

2.6. Permeability studies

The permeability of [^{3}H]-formoterol (677.1 GBq. $mmol^{-1}$,7.4 MBq. ml^{-1}, radiochemical purity 99%, Vitrax, Placentia, USA) across the Calu-3 monolayers (passage 26–28) was measured in both apical to basolateral (AB) and opposite directions at three different concentrations (0.05 μM, 5 μM and 500 μM) and in presence of 5 mM TEA. The donor solutions were made up in HBSS at pH 6.8 or HBSS/HEPES at pH 7.4 for AB or BA permeability measurement, respectively, and contained 6.5 μM of the paracellular marker [^{14}C]-mannitol (2.25 GBq. $mmol^{-1}$, 7.4 MBq. ml^{-1}, radiochemical purity 98.6%, Amersham Biosciences, Amersham, UK). Cold formoterol was added to the solutions to achieve a final concentration of 5 μM or 500 μM. Cell layers were equilibrated in HBSS/HEPES at 37 °C for 30 min before the TEER was recorded. Test solutions were added to the donor chambers and HBSS at pH = 6.8 or HBSS/HEPES at pH = 7.4 to the apical and basolateral receiver chambers, respectively. Donor chambers were immediately sampled for determination of the initial formoterol/mannitol concentration. Cell layers were incubated at 37 °C on an orbital shaker (60 rpm). Samples were withdrawn from the receiver compartments every 30 min over 2 h and replaced with the same volume of corresponding buffer. The concentration of TEA was maintained constant throughout the inhibitory experiments. The final TEER was measured directly after collection of the last samples. The coefficient of apparent permeability P_{app} was calculated as $P_{app} = (dQ/dt)/AC_0$ where dQ/dt (mol s^{-1}) is the transport rate, A (cm^2) is the surface area of the filter supporting the cell layer, and C_0 (mol cm^{-3}) is the initial substrate concentration in the donor chamber.

2.7. Statistical analysis

Each uptake and permeability experiment was performed on 4 cell layers and results are presented as mean ± standard

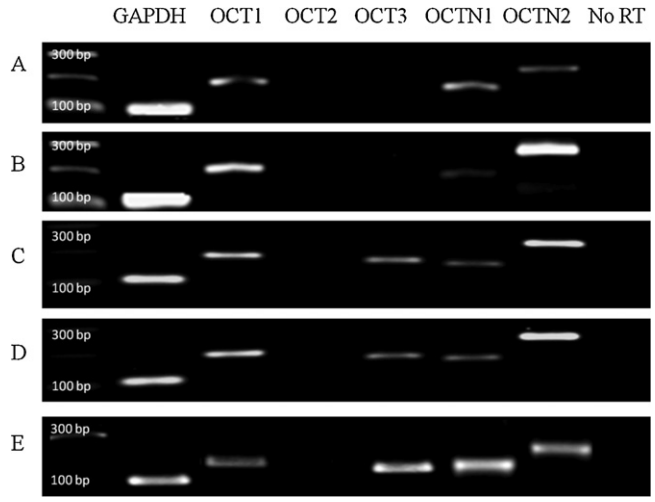

Fig. 1. RT-PCR analysis of the mRNA expression of the different OCT isoforms in undifferentiated Calu-3 cells at passage 27 (A), in 14-day old ALI Calu-3 layers at passage 27 (B), 21-day old ALI Calu-3 layers at passage 27 (C) or 47 (D) and in 21 day old ALI NHBE cell layers (E). GAPDH was used as the house-keeping gene control. No RT indicates that the reverse transcription step was omitted.

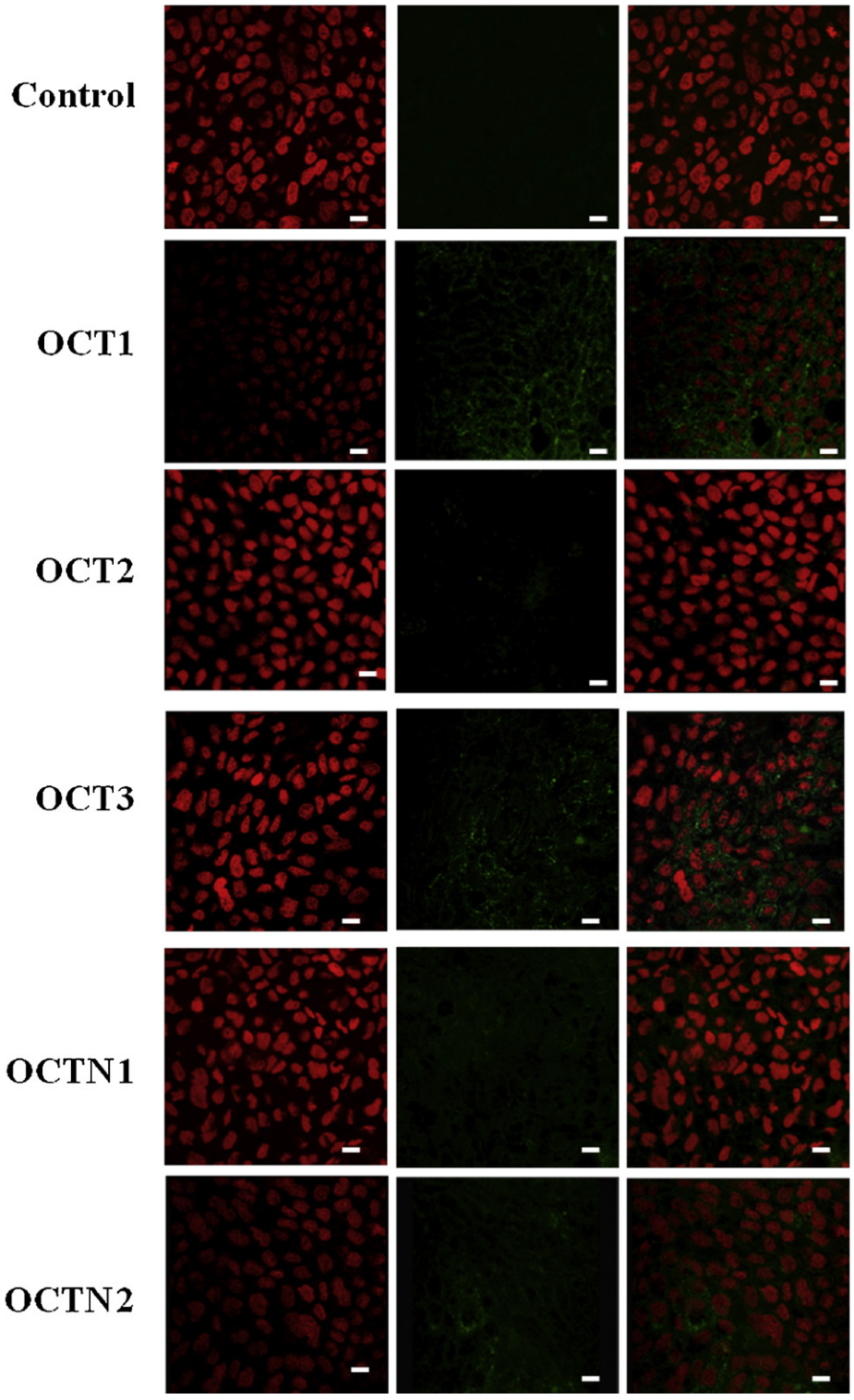

Fig. 2. Immunohistochemistry of OCT proteins in 21-day old ALI Calu-3 layers. The red channel (left) shows the PI nuclear counterstain and the green channel (FITC, middle) the localisation of OCT proteins. The overlays between the red and green channels are presented on the right. The scale bar represents 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

deviation (SD) or standard error of the mean (SEM). Two groups were compared using an unpaired *t*-test, while ANOVA was used for comparing more than two groups followed by a Bonferroni *post hoc* correction. A p value < 0.05 was considered to be statistically significant. The software used for the statistical analysis was GraphPad Prism Version 5.

3. Results

3.1. OCT expression in Calu-3 cells

The expression of OCT was assessed at the mRNA level in both undifferentiated and air-interfaced Calu-3 layers as well as in 21

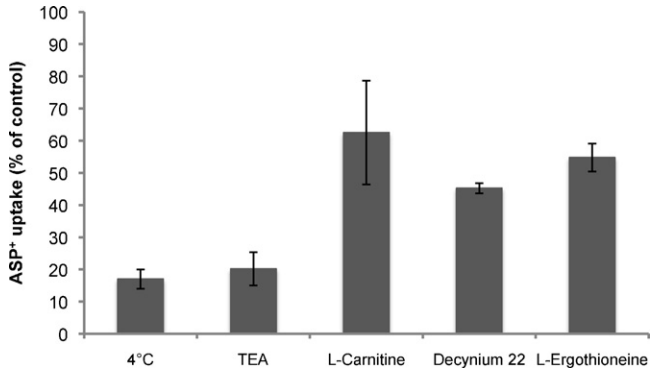

Fig. 3. ASP^+ uptake at 4 °C or in presence of the OCT inhibitors TEA, Decynium 22, L-ergothioneine and L-carnitine. Data are expressed as the percentage of the dye uptake measured in absence of inhibitors at 37 °C and are presented as the mean ± SEM ($n = 4$ layers).

day old ALI NHBE layers for comparison. RT-PCR analysis for the five OCT is presented in Fig. 1. Undifferentiated cells harvested from 90% confluent cell culture flasks and cells cultured on Transwells at an ALI for 14 days expressed OCT1, OCTN1 and OCTN2 while OCT2 and OCT3 mRNA could not be detected. After 21 days under ALI culture, Calu-3 layers expressed OCT3 levels above the analytical threshold in addition to OCT1, OCTN1 and OCTN2. OCT2 mRNA remained undetectable in 21 day old ALI Calu-3 layers while a positive staining for that transporter was obtained in Caco-2 layers cultured on Transwell for 21 days (data not shown). The OCT expression pattern in Calu-3 layers cultured on permeable filters for 21 days was similar to that in 21 day old NHBE cell layers and was stable over long-term passaging with no apparent variations in transporter expression levels between passage 27 and 47.

OCT protein expression in 21-day old ALI Calu-3 layers was confirmed by immunohistochemistry. Positive staining was obtained for OCT1, OCT3 and OCTN2 while probing for OCTN1 resulted in a faint signal that was nevertheless above background level. In agreement with gene expression data, no staining was observed for OCT2 (Fig. 2).

3.2. OCT functionality in differentiated Calu-3 layers

OCT functionality was evaluated in 21 day old Calu-3 layers by quantifying the internalisation of the cationic dye ASP^+ in the presence and absence of a panel of OCT inhibitors. The intracellular fluorescence was 83 ± 3% ($p < 0.05$) lower when the dye uptake was measured at 4 °C as compared to 37 °C (Fig. 3), indicating the involvement of an active transport mechanism. The non selective OCT inhibitor TEA reduced ASP^+ uptake by 80 ± 5% ($p < 0.05$), which suggested the presence of an OCT activity at the apical side of Calu-3 layers (Fig. 3). The inhibitors decynium 22 (OCT1–3), L-ergothioneine (OCTN1) and L-carnitine (OCTN2) at subtype-selective inhibitory concentrations (Koepsell et al., 2007) decreased the internalisation of the dye by 55 ± 2%, 45 ± 4% and 38 ± 10% respectively (Fig. 3, $p < 0.05$).

3.3. OCT inhibition by common inhaled drugs

Previous studies in non respiratory cells and undifferentiated human bronchial epithelial cells have reported interactions between common inhaled drugs and OCT (Ehrhardt et al., 2005; Horvath et al., 2007a,b; Nakamura et al., 2010). In the present study, the ability of those inhaled drugs to inhibit the transporters in ALI Calu-3 layers was evaluated by measuring their impact on the internalisation of ASP^+. All drugs tested: the β_2-agonists formoterol and salbutamol, the antimuscarinic ipratropium and the

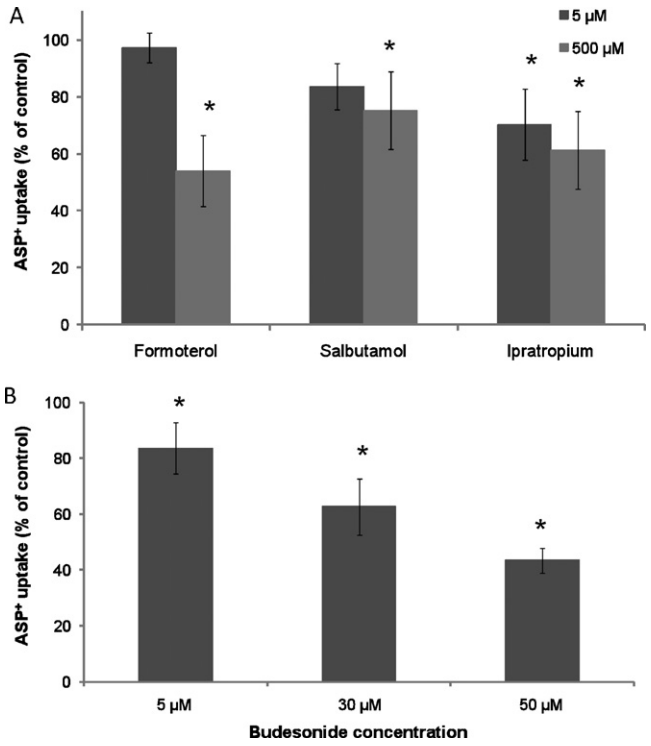

Fig. 4. ASP^+ uptake in presence of (A) the bronchodilators formoterol, salbutamol, ipratropium and (B) the glucocorticoid budesonide. Data are expressed as the percentage of the dye uptake measured in the absence of inhaled drugs and are presented as the mean ± SEM ($n = 4$ layers). *Indicates the decrease in ASP^+ uptake is statistically significant ($p < 0.05$).

glucocorticoid budesonide, significantly decreased ASP^+ uptake at the highest concentration studied (Fig. 4, $p < 0.05$). The reduction in intracellular fluorescence ranged from 46 ± 13% for formoterol to 25 ± 14% for salbutamol. These data confirmed that inhaled drugs are capable of inhibiting one or several OCT in a morphologically close representation of the bronchial epithelium *in vitro* as well as in isolated broncho-epithelial cells. Budesonide reduced OCT transport capacity in Calu-3 layers at the three concentrations tested (Fig. 4B, $p < 0.05$). At the more therapeutically relevant concentration of 5 µM, ipratropium maintained an inhibitory activity on ASP^+ uptake (Fig. 4A, $p < 0.05$). In contrast, the dye internalisation was not affected by the two β_2-agonists at the same concentration (Fig. 4A, $p > 0.05$).

3.4. Formoterol permeability across Calu-3 layers

The impact of OCT on the transport of [^{3}H]-formoterol across Calu-3 layers was assessed by permeability measurements at three different drug concentrations and in presence of TEA. All cell layers used in the permeability study maintained a high TEER throughout the experiment and exhibited a P_{app} for the paracellular marker ^{14}C-mannitol below 1×10^{-6} cm/s (data not shown). At the lowest concentration of 0.5 µM, formoterol P_{app} in the AB direction was determined as $1.3 \pm 0.3 \times 10^{-6}$ cm/s. No variation in the drug permeability was observed at a concentration of 5 µM (Fig. 5A, $p > 0.05$). In contrast, a 1.8 fold enhancement in formoterol transepithelial transport was measured at the highest concentration tested (500 µM) (Fig. 5A, $p < 0.05$). Since formoterol at a concentration of 500 µM was shown to reduce the OCT activity in Calu-3 layers by ~50% (Fig. 4), an increase in the drug permeability at that concentration suggested OCT inhibition enhances formoterol absorption across the cell layers. When formoterol permeability was quantified at the intermediate concentration in presence of TEA, a slight

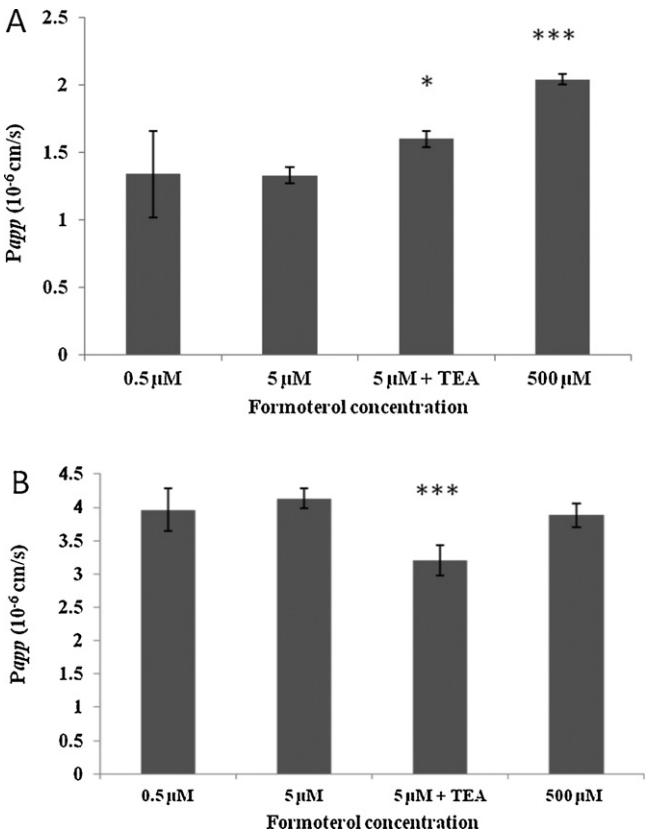

Fig. 5. Permeability of formoterol across Calu-3 layers in the AB (A) and BA (B) directions. Data are presented as mean ± SD ($n = 4$ layers). * and ***indicate P_{app} is significantly higher; $p < 0.05$ or $p < 0.001$, respectively.

but statistically significant increase in P_{app} was observed (Fig. 5A, $p < 0.05$), which confirmed that hypothesis. In the BA direction, a P_{app} of $4.0 \pm 0.3 \times 10^{-6}$ cm/s was measured at the lowest formoterol concentration tested. This was unchanged at higher concentrations (Fig. 5B, $p > 0.05$) but it was diminished upon addition of TEA in both Transwell chambers (Fig. 5B, $p < 0.01$).

4. Discussion

Considering all conventional classes of drugs administered by inhalation have been reported to interact with OCT with a possible impact on their PKPD profiles, an economical cell culture model of the human bronchial epithelium suitable for defining the nature of those interactions and their influence on pulmonary drug disposition would be of immense value. Layers of the human respiratory cell line Calu-3 grown under ALI conditions are a well established, physiologically pertinent system for conducting broncho-epithelial permeability studies *in vitro* (Forbes and Ehrhardt, 2005; Sporty et al., 2008). However, their usefulness for OCT-focussed biopharmaceutical investigation in the airway epithelium has not been fully evaluated. For the first time, this study reveals that 21 day old ALI Calu-3 layers express four OCT subtypes at both the gene and protein levels. It indicates they possess an apically located OCT activity that is reduced by common inhaled drugs and demonstrates they are suitable for studying OCT-mediated transport across the bronchial epithelium.

RT-PCR analysis indicated that OCT gene expression in the Calu-3 cell line is driven by cell differentiation and in line with that in NHBE cells (Fig. 1). The presence of four OCT, *i.e.*, OCT1, OCT3, OCTN1 and OCTN2 in Calu-3 cells was evident after 21 days of ALI culture on semi-permeable membranes (Figs. 1 and 2), while no OCT3 transcripts could be observed in undifferentiated cells or cell layers maintained at an ALI for 14 days (Fig. 1). Similarly, Madlova et al. (2009) observed the P-glycoprotein substrate digoxin was effluxed from ALI Calu-3 layers after 21 days in culture but not after 14 days, suggesting the transporter was not expressed in earlier cultures. Transporter expression in layers of the human intestinal Caco-2 cell line is well known to increase with time in culture and consequently, cells are typically used for permeability studies after 21 days on Transwell inserts (Press and Di Grandi, 2008; Sun et al., 2008). Like Caco-2 cells, the Calu-3 cell line might require at least 3 weeks of culture at an ALI for expression of a broad spectrum of transporters. No apparent variations in OCT expression pattern was noticed in 21 day old differentiated Calu-3 layers at passage over 45 as compared to that in cells at a passage below 30 (Fig. 1). This offers a favourable experimental window for OCT-related studies in the cell line and eliminates the need for periodic validation of the model. OCT2 could not be detected in either Calu-3 or NHBE cell layers (Fig. 1). This is in agreement with previously published studies that showed OCT2 was absent in undifferentiated airway epithelial cell culture models (Endter et al., 2009) and in the human nasal epithelium (Agu et al., 2011). Furthermore, an extensive gene microarray analysis of transporter levels in various human tissue samples revealed OCT2 expression in the lungs was much lower than that of the four other OCT subtypes (Bleasby et al., 2006). In contrast to cell layers used in this study, undifferentiated NHBE (Horvath et al., 2007a) and BEAS-2B (Nakamura et al., 2010) cells were reported to express OCTN1 and OCTN2 but not OCT1–3, indicating those models might not be as reliable as air-interfaced cell layers for *in vitro* investigation of drug–OCT interactions in the bronchial epithelium. As the presence of the OCT members and their relative expression levels in the human bronchial epithelium remain unclear to date, it is nevertheless premature to conclude ALI Calu-3 and NHBE layers exhibit an OCT expression profile representative of bronchial cells *in vivo*.

The presence of an OCT activity on the apical side of Calu-3 layers was investigated by measuring the effect of OCT inhibitors on the cellular uptake of the OCT substrate ASP^+. Since the dye was not significantly transported across Calu-3 layers (data not shown), a basolateral internalisation following transfer into the basolateral compartment of the Transwell chambers from the apical donor compartment is excluded. ASP^+ is a positively charged fluorescent dye that is transported by OCT1 (Ciarimboli et al., 2004) and OCT2 (Cetinkaya et al., 2003). Evidence also suggests ASP^+ is translocated by OCTN2 in NHBE cells (Horvath et al., 2007a) and by OCT3, OCTN1 or OCTN2 in the human nasal epithelium (Agu et al., 2011). Conversely, a recent study has shown the dye is not a substrate for OCTN2 (Grigat et al., 2009). ASP^+ uptake in undifferentiated NHBE cells was reduced by ~80% in presence of the OCTN2 inhibitor L-carnitine while it was unaffected by the OCTN1 inhibitor L-ergothioneine and the OCT1–3 inhibitor corticosterone, suggesting, as mentioned above, that it was mediated essentially by OCTN2 (Horvath et al., 2007a). In the present study, L-carnitine but also L-ergothioneine and decynium 22 (OCT1–3) reduced the internalisation of the dye in ALI Calu-3 layers and only TEA (OCT1–3 and OCTN1–2) was capable of nearly abolishing its uptake by the cells (Fig. 3). This might indicate that, in contrast to that in NHBE grown on cell culture plates, the OCT activity in ALI Calu-3 cells is mediated by more than one transporter. The relative contribution of each OCT member can nevertheless not be confidently delineated since, although inhibitors were used at a sub-type selective concentration, overlaps in OCT substrate/inhibitor affinity has been demonstrated (Koepsell et al., 2007).

In agreement with observations in non respiratory cells or undifferentiated bronchial cells, all inhaled drugs tested significantly reduced ASP^+ internalisation by Calu-3 cells (Fig. 4). This confirmed those compounds have the ability to modulate

OCT-mediated transport in cell layers morphologically similar to the native bronchoepithelial barrier *in vivo*. The inhibitory potency of salbutamol and formoterol at the same concentration of 500 μM was nevertheless not as pronounced as in undifferentiated NHBE cells (Horvath et al., 2007a). As discussed previously, OCTN2 was largely responsible for ASP^+ uptake in those cells while OCT1, OCT3 and OCTN1 might contribute to its intracellular transport in ALI Calu-3 layers. Taken together, the data suggest the two β_2-agonists are mainly inhibitors of OCTN2. Due to the complexity of the lung anatomy, it is extremely challenging to determine concentrations reached in the lung fluid after drug inhalation. Nevertheless, considering the therapeutic doses as well as the delivery efficiency of inhalers and assuming that the fraction of the dose entering the lung deposits in 1 ml of fluid, it can be grossly estimated that the maximum concentration achieved locally is ~5 μM for the bronchodilators and ~100 μM for budesonide. However, due to solubility issues, the highest test concentration for the glucocorticoid was limited to 50 μM. At a therapeutically relevant concentration, budesonide and ipratropium maintained OCT inhibitory properties while those of formoterol and salbutamol were abolished (Fig. 4). Considering OCT might have fundamental functions in the respiratory epithelium (Lips et al., 2005), the clinical implications of OCT inhibition by common inhaled drugs will need to be determined. Those are likely to be negligible for the two β_2-agonists formoterol and salbutamol. However, this might not be the case for the permanent cations ipratropium and tiotropium, inhaled glucocorticoids or inhaled drug candidates currently under development if they are positively charged at physiological pH.

Formoterol is a long acting β_2 agonist with a quick onset of action. As it possesses two pKa of 7.9 (amine group) and 9.2 (phenol group), it is principally in its cationic form at a pH below 7 and in its zwitterionic form in the range of pH between 7 and 9. For formoterol transport studies, the pH was set at 6.8 and 7.4 in the apical or basolateral Transwell chambers, respectively, in order to mimic the pH gradient across the airway epithelium *in vivo*. Drug permeability was higher in the secretory than in the absorptive direction (Fig. 5) as expected due to the higher proportion of neutral and therefore, more hydrophobic molecules in the basolateral donor solution. A similar effect of the pH on formoterol transport has been previously observed in layers of the human bronchial cell line 16HBE14o- where increasing the alkalinity of the donor solution over the pH range 6–8 resulted in a permeability enhancement (Forbes, 2006). Formoterol AB transport was increased in presence of the OCT inhibitor TEA and at a high drug concentration that was previously shown to reduce the OCT activity in Calu-3 layers by approximately 50% (Figs. 4A and 5A). Furthermore, transport in the BA direction was reduced by TEA but unaffected by the concentration (Fig. 5B). Similarly, OCTN inhibitors were recently reported to facilitate formoterol absorptive transport across NHBE cell layers grown at an ALI while they decreased that of salbutamol (albuterol) and tiotropium (Horvath et al., 2010). Our data therefore demonstrates the usefulness of ALI Calu-3 layers for evaluation of OCT involvement in drug transport across bronchial epithelial cells. OCT are capable of translocating their substrates from the external environment into the cell cytoplasm and in the opposite direction (Koepsell, 2004; Koepsell et al., 2007). Their extracellular and cytoplasmic large binding domains are not identical and present overlapping binding sites for different ligands (Koepsell et al., 2007). Hence, OCT substrates and inhibitors typically have variable affinities for inward and outward interaction sites. This makes formoterol transport data challenging to interpret, especially since our data showed that more than one OCT subtype is likely to be functional in Calu-3 layers. It can nevertheless be envisaged that the drug has the highest affinity for an inhibitory site and is actively transported only at a high concentration or in presence of an inhibitor when this site is saturated. TEA is both a substrate and inhibitor of the OCT (Koepsell et al., 2007). Being a permanent cation, it is likely to have a greater affinity than formoterol for OCT transporting sites, which would explain the marginal increase in drug AB transport in presence of the inhibitor. Further investigation would be required to fully evaluate the actual contribution of OCT in formoterol transport across bronchial epithelial cells *in vitro*. However, our data suggests the potential involvement of OCT in formoterol pulmonary absorption *in vivo* is unlikely to be clinically significant. This assumption is indeed confirmed by pharmacokinetic data collected in healthy volunteers after inhalation of formoterol and budesonide in combination which showed no evidence of active absorption mechanisms or of drug–drug interactions (Eklund et al., 2008) although both compounds interact with OCT (Fig. 3; Horvath et al., 2007a,b; Lips et al., 2005).

5. Conclusion

Calu-3 layers grown in ALI conditions for 21 days were shown to express OCT1, OCT3, OCTN1 and OCTN2 as well as to exhibit an OCT activity likely mediated by more than one OCT at their apical site. The OCT inhibitory properties of common inhaled drugs as well as a possible OCT involvement in formoterol transport across the bronchial epithelium were highlighted in the Calu-3 model as previously reported in undifferentiated bronchial epithelial cells or in differentiated layers of primary cells, respectively. As compared with other respiratory cell culture models, Calu-3 layers provide a system in which to study OCT-inhaled drug interactions that is collectively physiologically relevant, reproducible, low-cost and easy to handle.

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