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The Cell Line NCI-H441 Is a Useful *in Vitro* Model for Transport Studies of Human Distal Lung Epithelial Barrier

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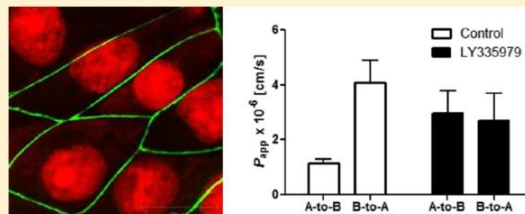
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ABSTRACT: The lack of a well characterized, continuously growing *in vitro* model of human distal lung epithelial phenotype constitutes a serious limitation in the area of inhalation biopharmaceutics, particularly in the context of transepithelial transport studies. Here, we investigated if a human lung adenocarcinoma cell line, NCI-H441, has potential to serve as an *in vitro* model of human distal lung epithelium. The development of barrier properties was studied by immunocytochemistry (ICC) against the junction proteins zonula occludens protein 1 (ZO-1) and E-cadherin and measurement of transepithelial electrical resistance (TEER). Moreover, transport studies with the paracellular marker compounds fluorescein sodium and fluorescein isothiocyanate (FITC)-labeled dextrans of molecular weights ranging from 4 to 70 kDa were carried out. The expression of P-glycoprotein (P-gp; *ABCB1*) and organic cation transporters (OCT/Ns; *SLC22A1-AS*) was investigated by ICC and immunoblot. P-gp function was assessed by monolayer release and bidirectional transport studies using rhodamine 123 (Rh123) and the inhibitors verapamil and LY335979. Uptake of 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide (ASP⁺) was measured, in order to assess organic cation transporter function *in vitro*. Furthermore, the inhibitory potential of several organic cations on ASP⁺ uptake was studied. NCI-H441 cells, when grown under liquid-covered conditions, formed confluent, electrically tight monolayers with peak TEER values of approximately 1000 $\Omega \cdot \text{cm}^2$, after 8–12 days in culture. These monolayers were able to differentiate paracellularly transported substrates according to their molecular weight. Presence of P-gp, OCT1, OCT2, OCT3, OCTN1, and OCTN2 was confirmed by Western blot and ICC and was similar to data from freshly isolated human alveolar epithelial cells in primary culture. Rh123 release from NCI-H441 monolayers was time-dependent and showed low, albeit significant attenuation by both inhibitors. In transport studies, Rh123 exhibited net secretion, which again was inhibitable by *bona fide* P-gp modulators. The uptake of ASP⁺ was time- and temperature-dependent with $K_m = 881.2 \pm 195.3 \mu\text{M}$ and $V_{\max} = 2.07 \pm 0.26 \text{ nmol/min/mg protein}$. TEA, amantadine, quinidine, and verapamil significantly inhibited ASP⁺ uptake into NCI-H441 cells, whereas the effect of D- and L-carnitine and ergothioneine, two OCTN substrates, was less pronounced. NCI-H441 cells are the first cell line of human distal lung epithelial origin with the ability to form monolayers with appreciable barrier properties. Moreover, drug transporter expression and activity in NCI-H441 cells was consistent with what has been reported for human alveolar epithelial cells in primary culture.

KEYWORDS: inhalation biopharmaceutics, absorption, pulmonary drug disposition, drug transporters, P-glycoprotein, organic cation transporters



■ INTRODUCTION

The lung offers great potential as a portal into the systemic circulation for drugs with difficult oral pharmacokinetics or stability issues.¹ Recent examples using this pulmonary route are aerosolized insulin (Adagio, Afrezza) and dihydroergotamine (Levadex). Due to the complex structure of the respiratory tract, however, the fate of inhaled medicines after reaching the pulmonary mucosa remains poorly understood.² One of the less well conceived issues is epithelial transport, particularly in the bronchiolar and alveolar segments (i.e., the distal lung). One reason the study of transport processes across the epithelial barriers of the distal lung has been seriously compromised is the absence of reliable, continuously growing

cell culture models.^{3,4} For other biological barriers, e.g., the intestine or indeed the proximal respiratory tract, a number of well characterized *in vitro* models are available, such as Caco-2, Calu-3, VA10, and 16HBE14o-, respectively.^{5,6}

The respiratory zone, where a significant part of systemic drug absorption is believed to occur, includes the terminal bronchioles and the alveolar sacs. While the bronchioles are mainly populated by ciliated cuboidal epithelial and Clara cells,

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the alveolar barrier is formed by the type I and the type II pneumocytes.⁷ Alveolar type II (ATII) cells are granular and roughly cuboidal in shape and cover approximately 5% of the total alveolar surface area, albeit they contribute to ~60% of the cell population by numbers.⁸ ATII cells participate in lung fluid homeostasis and are responsible for the synthesis and secretion of surfactant.⁹ The extremely thin, squamous alveolar type I (ATI) cell, on the other hand, forms the bulk (~95%) of the alveolar wall and also contributes to water and ion transport.¹⁰ A number of protocols for the isolation and culture of alveolar cells from different species, including human, have been established over the years.¹¹ While these primary cultures best reflect the situation *in situ*, they come with the disadvantage that they are very time-consuming and expensive and require ethical consent (and a well-developed tissue supply chain, in the case of isolation from human specimens). The use of lung tissue from laboratory animals is overcoming some of these disadvantages, but in addition to the obvious animal welfare concerns, yet unknown species differences are posing a risk.

Immortal, continuously growing cell lines, on the contrary, are much easier to handle, their use can be validated, and they are suitable for high throughput assays.¹² The most commonly used cell line of human alveolar origin is A549. A549 cells were isolated from a human pulmonary adenocarcinoma and exhibit a phenotype similar to that of ATII cells due to the presence of lamellar bodies and surfactant proteins.^{13,14} While routinely used as a surrogate for human pneumocytes *in vitro*, A549 cells lack the ability to form electrically tight monolayers of polarized cells, due to their compromised tight junctions.^{15,16} The recently characterized, transformed human ATI-like cell line (TT1) similarly did not produce physiologically relevant restrictive tight junctions.¹⁷

A cell line with the potential to overcome some of the issues mentioned above is NCI-H441. NCI-H441 cells were originally isolated from the pericardial fluid of a patient with papillary adenocarcinoma of the lung and show characteristics of Clara-like bronchiolar pulmonary epithelium.^{18,19} Expression of markers typical of human ATII cells, e.g., thyroid transcription factor 1 and the surfactant proteins SP-A, SP-B, SP-C, and SP-D, has also been confirmed in NCI-H441 cells.²⁰ NCI-H441 monolayers, when grown on permeable supports, have been reported to generate reasonable transepithelial electrical resistance (TEER) values in monoculture and in co-culture with microvascular endothelial cells.^{18,21}

The aim of this work was to characterize NCI-H441 cells as an *in vitro* model for transport studies of distal lung epithelial barrier. For this purpose, the influence of different culture conditions on cell monolayer formation was determined. Moreover, the expression and activity of prominent drug transporter molecules, which have previously been associated with human alveolar barrier, such as P-glycoprotein (P-gp) and the five cloned members of the polyspecific organic cation transporter family (i.e., OCT1–3, OCTN1, and OCTN2), was investigated and compared to expression in freshly isolated human alveolar epithelial cells (i.e., ATII and ATI-like cells) in primary culture.^{22,23}

■ EXPERIMENTAL SECTION

Cell Culture. NCI-H441 (American Type Culture Collection, HTB-174) cells were obtained from LGC Standards (Teddington, U.K.) and cultured at 37 °C in 5% CO₂ atmosphere in Gibco RPMI-1640 medium (Biosciences, Dun

Laoghaire, Ireland) supplemented with 5% fetal bovine serum (FBS), 1% sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Sigma-Aldrich, Dublin, Ireland). In preliminary studies, the optimal seeding density was established to be 75,000–250,000 cells/cm² (data not shown). NCI-H441 cells were routinely cultured in 75 cm² growth area tissue culture flasks (Greiner BioOne, Frickenhausen, Germany) and passaged when approximately 80% confluence was reached. Twenty-four hours post-seeding, the medium was replaced with RPMI-1640 medium, which in addition contained dexamethasone (200 nM, Sigma-Aldrich) and insulin-transferrin-selenium (ITS) supplement (Roche Diagnostics Limited, West Sussex, U.K.). For liquid-covered culture (LCC), cells were grown on Transwell Clear inserts (12 mm in diameter, pore size 0.4 µm; Corning, VWR, Dublin, Ireland), and apical and basolateral fluid volumes were 500 and 1500 µL, respectively. For cells grown under air-liquid interface culture (AIC) conditions, the apical fluid volume was completely removed, and the volume of the basolateral compartment was adjusted to 700 µL. The culture medium was exchanged every other day. NCI-H441 cells were obtained at passage number 50 and used until passage number 78 in this study.

Human Alveolar Epithelial Cell Isolation and Culture.

Human alveolar type II epithelial cells were freshly isolated from non-tumor lung tissue obtained from patients undergoing lung lobectomy. The use of human material was approved by Saarland State Medical Board, Germany. Isolation of ATII cells was performed according to a protocol adapted from Ehrhardt et al.²⁴ Purified ATII cells were seeded at a density of 600,000 cells/cm² on collagen (Sigma-Aldrich) and fibronectin (BD Biosciences, Oxford, U.K.) coated plastics using complete small airways growth medium (SAGM; Lonza, Verviers, Belgium) supplemented with penicillin, streptomycin, and 1% FBS. ATII cells were either used after 24 h or after transdifferentiation into monolayers of ATI-like phenotype, following 8 days of culture.²⁵

Bioelectric Measurements. TEER values were measured daily with STX01 electrodes connected to a Millicell ERS volt-ohm meter (Millipore, Carrigtwohill, Ireland). TEER values were corrected for the background value contributed by growth support and medium. For cells cultured under AIC conditions, fluid volumes were adjusted to 500 µL apically and 1500 µL basolaterally with prewarmed medium, and cell monolayers were then allowed to equilibrate for 10 min prior to TEER measurement. TEER values were obtained from at least three different passages.

Western Blot. Total cell protein extracts were generated from NCI-H441 monolayers grown on 6-well plates (Greiner BioOne, Frickenhausen, Germany) for 8 days or human primary ATII or ATI-like cells cultured for 24 h and 8 days, respectively. Cell lysis was performed on ice in cell extraction buffer (Invitrogen, Karlsruhe, Germany) containing protease inhibitors (Sigma-Aldrich). Cell samples were sonicated twice for 10 s, and then the lysate was centrifuged (10,000g at 4 °C) for 20 min. The resulting supernatants were collected, and sample concentrations were determined using a standard protein assay (Bio-Rad, Hemel Hempstead, U.K.) according to the manufacturer's instructions. Protein was loaded at equal concentrations and separated by electrophoresis using 6% (P-gp) and 10% (OCTs) polyacrylamide gels, respectively. After electrophoresis, transfer onto immunoblot polyvinylidene fluoride membranes (Bio-Rad) was performed. Membranes were then blocked in 5% bovine serum albumin (BSA) in

phosphate-buffered saline (PBS) with Tween 20 (pH 7.4) for at least 1 h at room temperature, followed by incubation overnight at 4 °C with the relevant primary antibodies. Mouse monoclonal anti-P-gp antibody (clone D-11, sc-55510) and polyclonal goat anti-OCTN1 (sc-19819) and OCTN2 (sc-19822) antibodies were obtained from Santa Cruz (Heidelberg, Germany), polyclonal rabbit anti-OCT1 antibody was purchased from Aviva (San Diego, CA, ARP41640_T100), and polyclonal rabbit anti-OCT2 and OCT3 antibodies from Sigma-Aldrich (AV43847 and AV44026). The antibodies were used at the following concentrations: P-gp (1:100), OCT1 (1:200), OCT2 (1:500), OCT3 (1:2,000), OCTN1 and OCTN2 (1:200). Incubation with primary antibodies was followed by three washings with PBS containing 0.1% Tween 20 and subsequent incubation with the relevant horseradish peroxidase (HRP)-conjugated secondary antibodies (at concentrations of 1:12,500 (anti-mouse (W4021) and anti-rabbit (W4011, Promega, Medical Supply Company, Dublin, Ireland) or 1:22,500 (anti-goat, A5420, Sigma-Aldrich)) at room temperature for 1 h. After three additional washings with PBS containing 0.1% Tween 20, the peroxidase activity was detected with Immobilon Western chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). To ensure equal loading, the level of a protein of interest was normalized to β -actin levels (data not shown).

Immunocytochemistry. Lab-Tek chamber slides (Nunc, Roskilde, Denmark) or Transwell Clear inserts were used to grow cell monolayers for immunocytochemistry. Cells were cultured for 24 h (ATII) or 8 days (ATI-like, NCI-H441), before they were fixed with 4% paraformaldehyde solution for 10 min and then incubated for 10 min in PBS containing 50 mM NH_4Cl , followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100 in PBS. After 60 min of incubation with 200 μL of a 1:50 (OCT1, OCTN1 (OAAB03385) and OCTN2 (OAAB05449); all from Aviva), 1:100 (E-cadherin; MCA1482, AbD Serotec, Oxford, U.K.), 1:250 (OCT2; Sigma-Aldrich), 1:300 (ZO-1; 610966, BD Biosciences, Oxford, U.K.), 1:500 (P-gp; Santa-Cruz), or 1:1000 (OCT3; Sigma-Aldrich) dilution of the relevant primary antibodies, cell monolayers were rinsed three times with PBS, before incubation with 200 μL of a 1:300 dilution of the respective Alexa Fluor-labeled F(ab')_2 fragment (A11017, A11070, and A21222, Biosciences, Dun Laoghaire, Ireland) in PBS containing 1% BSA. Propidium iodide or DAPI (both at 1 $\mu\text{g/mL}$ in PBS) was used to counterstain cell nuclei. After 30 min of incubation, the specimens were again rinsed three times with PBS containing 1% BSA and embedded in FluorSave anti-fade medium (Merck, Nottingham, U.K.). Images were obtained using a confocal laser scanning microscope (CLSM, Zeiss LSM 510, Göttingen, Germany) with the instrument's settings adjusted so that no positive signal was observed in the channel corresponding to the fluorescence of the relevant isotypic controls (data not shown).

Transport Studies. Transport experiments were performed in freshly prepared bicarbonated Krebs-Ringer buffer (KRB; pH 7.4) composed of 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4 , 25 mM NaHCO_3 , 1.8 mM CaCl_2 , 0.81 mM MgSO_4 and 5.55 mM glucose, unless stated otherwise. NCI-H441 cells were grown under LCC conditions on Transwell Clear membranes for at least 8 days and were only used when TEER values exceeded $350 \Omega \cdot \text{cm}^2$.²⁶ Prior to the experiment, both sides of the cell monolayers were washed twice with prewarmed KRB solution, followed by 60 min

equilibration in KRB at 37 °C. To initiate transport studies, the incubation buffer was replaced with a solution of the relevant compound in KRB (i.e., fluorescein sodium (FNa, 50 μM), fluorescein isothiocyanate (FITC)-dextran 4,000 (FD4k, 1 mg/mL), FITC-dextran 10,000 (FD10k, 1 mg/mL), FITC-dextran 20,000 (FD20k, 1 mg/mL), FITC-dextran 70,000 (FD70k, 1 mg/mL), and rhodamine 123 (Rh123, 50 μM) in the respective donor chamber. For apical-to-basolateral transport, 0.52 mL of donor solution was added; for basolateral-to-apical transport, 1.52 mL. The initial donor concentration was determined by taking a 20- μL sample directly after addition of the donor fluids. The respective acceptor volumes were 1.5 mL in a-to-b and 0.5 mL in b-to-a transport studies. Cell monolayers were kept at 37 °C during the experiment, and 200- μL samples were drawn serially from the receiver compartments at 15, 30, 45, 60, 75, and 90 min. After each sampling, fresh transport buffer of an equal volume was returned to the receiver side to maintain a constant volume. At the end of transport studies, another 20- μL sample was collected from the donor side for determination of mass balance. Each experiment was run at least in triplicates. TEER values were recorded before and after the transport studies, in order to verify the cell layer integrity. In P-gp inhibition experiments, LY335979 (at 10 μM) was added to donor and receiver fluids.

The fluorescence activity of samples was assessed in 96-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively. Samples were diluted with KRB solution, where appropriate.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{\text{app}} = (\Delta Q / \Delta t) / (A C_0) \quad (\text{Eq. 1})$$

where ΔQ is the change in concentration of Rh123 over the designated period of time (Δt), A is the nominal surface area of the growth supports (1.13 cm^2), and C_0 is the initial concentration of Rh123 in the donor fluids.

4-(4-(Dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺) Uptake Studies. Uptake studies were carried out using NCI-H441 cell monolayers grown for 8 days on 24-well plates. The cationic fluorescent probe ASP⁺ was used to investigate time-, concentration-, temperature-, pH-, and sodium ion concentration-dependence of organic cation uptake as described before.²⁷ All experiments were carried out in bicarbonated KRB solution unless described otherwise. Time-dependence of ASP⁺ uptake was studied by incubating NCI-H441 cell monolayers with ASP⁺ (at 10 μM final concentration) for up to 90 min at 37 °C. The uptake reaction was stopped at certain time points by rinsing the cells twice with ice-cold KRB. Subsequently, monolayers were solubilized with 1% Triton X-100, and the fluorescence activity of ASP⁺ in the cell lysate was measured (see below). Concentration-dependence of ASP⁺ uptake into NCI-H441 cells was studied at a range from 10 to 1000 μM at 37 and 4 °C for 20 min. The effect of varying pH values (i.e., pH 5.7, 6.5, and 8.2) on ASP⁺ uptake into NCI-H441 cells was studied in order to assess pH-dependence. In addition, Na⁺-free medium was prepared by replacing NaCl, NaH_2PO_4 , and NaHCO_3 salts with equimolar concentrations of KCl, KH_2PO_4 , and KHCO_3 (i.e., 5.4 mM $[\text{Na}^+]$ and 142 mM $[\text{K}^+]$) or N-methyl-D-glucamine (NMG), KH_2PO_4 , and KHCO_3 (i.e., 5.4 mM $[\text{Na}^+]$ and 137 mM $[\text{NMG}]$), respectively, to investigate sodium ion concentration-dependence of ASP⁺ uptake into NCI-H441 cells.

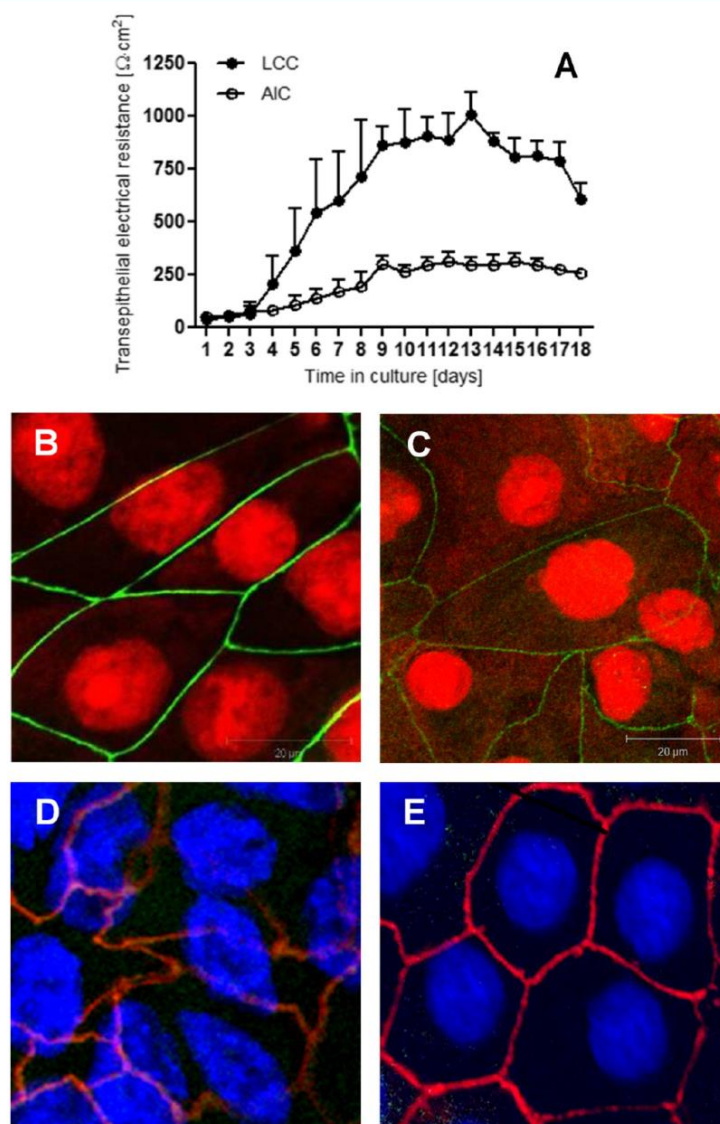


Figure 1. (A) Time course of TEER development in NCI-H441 cell monolayers. Cells were seeded at a density of 2.5×10^5 cells/cm² on Transwell Clear inserts and grown under liquid-covered culture (LCC, ●) or under air-interfaced culture (AIC, ○) conditions from day 1 onward. Each data point represents the mean $\pm$ SD ($n \geq 12$) from at least three different passages. (B, D, E) Immunostaining of cell junctional proteins ZO-1 and E-cadherin (C) in NCI-H441 cell monolayers. Cells were seeded on chamber slides (B and C) or Transwell Clear membranes (D and E) and cultured for 8 days under liquid-covered (B, C, and E) or air-interfaced conditions (D). Cell nuclei were counterstained with propidium iodide (red) or DAPI (blue). Bar represents 20 μ m.

It was also determined whether the presence of inhibitors of organic cation transporters, i.e., acetylcholine (1 mM), amantadine (100 and 500 μ M), corticosterone (100 μ M), D-carnitine (100 and 500 μ M), L-ergothioneine (100 and 200 μ M), hemicholinium-3 (HC-3, 100, and 500 μ M), quinidine (100 and 500 μ M), tetraethylammonium (TEA, 1 and 20 mM), and verapamil (10 and 200 μ M) affected ASP⁺ accumulation in NCI-H441 monolayers. Concentrations of these organic cations were chosen according to their published K_m or K_i values.^{28,29} Furthermore, the effect of two β_2 -receptor agonists (i.e., formoterol and salbutamol) as well as two inhaled glucocorticosteroids (ICS; i.e., budesonide and beclomethasone) on ASP⁺ uptake into NCI-H441 cells was determined. The β_2 -receptor

agonists were used at final concentrations of 100 and 500 μ M. The inhibitory potential of budesonide and beclomethasone was studied at concentrations of 10 and 30 μ M. All compounds were dissolved in KRB (pH 7.4). The ICS drugs were freshly prepared in KRB containing 0.01% DMSO as cosolvent. A similar amount of DMSO was used in the relevant controls and found to not significantly affect the results (data not shown). In all cases, cell monolayers were incubated simultaneously with ASP⁺ (10 μ M) and the compound of interest for 20 min.

In order to open tight junctions and allow better access to basolaterally localized transporters, calcium complexation was carried out by incubation with 5 mM EDTA for 15 min prior to initiation of the ASP⁺ uptake study.²⁷

The fluorescence activity of samples was assessed in 24-well plates using an automated plate reader (FLUOstar Optima) at excitation and emission wavelengths of 485 and 590 nm, respectively. The samples were diluted with KRB where appropriate. For standardization, the total protein amount of cell layers was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, USA).

Rhodamine 123 Release Studies. Release studies of the fluorescent P-gp probe Rh123 were performed in NCI-H441 cells cultured in 24-well plates for 8–12 days as described above. All experiments were carried out in KRB at 37 °C. Cell monolayers were initially loaded with Rh123 solution (50 μ M) for 60 min, which was then replaced by fresh KRB or KRB containing either LY335979 (10 μ M) or verapamil (50 μ M), and Rh123 release was followed for 1 h. At 0, 15, 30, 45, and 60 min 200 μ L samples were taken from the cell supernatant. Immediately afterward, all remaining supernatant was removed from the relevant cell layers, which were washed twice with ice cold KRB before being left for lysis in KRB containing 1% (w/v) Triton X-100. Rh123 activity was measured in the cell lysate using an automated plate reader (FLUOstar Optima) at excitation and emission wavelengths of 485 and 520 nm, respectively. The samples were diluted with KRB, where appropriate. For standardization, the total protein amount of cell layers was determined by BCA assay according to the manufacturer's instructions (see above).

Statistical Analysis. Results were expressed as means $\pm$ SD. The significance of differences between the mean values was determined by unpaired, two-tailed Student's *t*-test. *P* < 0.05 was considered significant. All experiments were carried out at least in triplicate.

■ RESULTS AND DISCUSSION

Barrier properties. Transepithelial electrical resistance values of NCI-H441 monolayers were slightly lower than what is commonly reported for monolayers of ATI-like cells in primary culture.^{15,30} When grown under LCC condition on permeable polyester supports, NCI-H441 cells were able to form electrically tight monolayers, reaching peak TEER values of $1010 \pm 105 \Omega \cdot \text{cm}^2$ after 13 days in culture (Figure 1A).

Very regular signals for ZO-1 and E-cadherin were visible along the cell–cell contacts in the confocal laser scanning micrographs (Figure 1B–E), confirming polarization in cells cultured submersed both on plastic (Figure 1B and C) as well as on Transwell Clear membranes (Figure 1E). TEER was significantly lower when cell monolayers were cultured at an air–liquid interface; the ZO-1 signal, nevertheless, remained unchanged (Figure 1D). In AIC culture no clear peak in monolayer resistance was observed; instead, a plateau with values ranging from 280 to 315 $\Omega \cdot \text{cm}^2$ was reached between days 9 and 17 in culture (Figure 1A). In preliminary experiments, we tested a cell culture medium without dexamethasone, but neither LCC nor AIC grown cells were able to develop TEER above 100 $\Omega \cdot \text{cm}^2$ in the absence of the corticosteroid (data not shown). While the underlying mechanism is not exactly known, there is a lot of evidence that glucocorticosteroids have an effect on tight junction structure and function by modulating the localization, stability, or assembly of junctional proteins to membrane sites of intercellular contact. For example, in subconfluent Madin-Darby canine kidney (MDCK) cells a marked increase in junctional content for occludin and ZO-1 with no changes in

their total expression was observed.³¹ Neuhaus et al. recently reported that dexamethasone was necessary to obtain stable NCI-H441 cell layers.²¹ Moreover, dexamethasone increased expression of cell type I markers (i.e., caveolin-1, receptor for advanced glycation end-products (RAGE)) and the type II cell marker SP-B. This was concordant with results from Ladenburger et al., who also showed that dexamethasone was able to significantly increase SP-B levels in NCI-H441 cells.³² Neuhaus and co-workers reported peak TEER values of $438 \pm 9 \Omega \cdot \text{cm}^2$, whereas in another study Hermanns et al. observed resistances of $218 \pm 83 \Omega \cdot \text{cm}^2$ for NCI-H441 cell monolayers in monoculture.^{18,21} The significantly higher values in our work can have several different causes. The use of various membrane materials (i.e., polyester vs polycarbonate vs collagen-coated polyethylene terephthalate) and different cell seeding densities (i.e., 250,000 vs 20,000 vs 23,000 cells/ cm^2) will have an impact on expression of adhesion molecules and cellular architecture, which in turn will affect cell–cell junctional structure. Moreover, Neuhaus et al. conclusively demonstrated the effect of dexamethasone concentration on the formation of tight NCI-H441 monolayers. When comparing the results of all three studies, it appears plausible that the chosen seeding density together with dexamethasone supplementation are the main factors for the increased TEER values. The observation that AIC gives rise to respiratory epithelial cell layers exhibiting lower bioelectric properties has been made before on a number of occasions. For Calu-3 human airway cells, which have many similarities to submucosal gland serous cells, TEER values for cells grown in LCC were also approximately three times higher than for cells grown in AIC.³³ The two immortalized human bronchial epithelial cell lines 16HBE14o- and CFBE41o-, on the other hand, when grown air–liquid interfaced, resulted in cell layers with very moderate bioelectrical properties (i.e., TEER values $\leq 250 \Omega \cdot \text{cm}^2$).^{34–36} It can be speculated that respiratory epithelial cells have to be of a surfactant or mucus producing phenotype to be able to cope with AIC conditions; however, confirming this speculation is beyond the scope of this work.

In Figure 2A, the cumulative transport of various paracellular marker compounds across monolayers of NCI-H441 cells is shown. The apparent permeability coefficients (P_{app}) were calculated to be $4.85 \pm 0.33 \times 10^{-7} \text{ cm/s}$ for FNa, $1.51 \pm 0.17 \times 10^{-7} \text{ cm/s}$ for FD4k, $1.58 \pm 0.63 \times 10^{-7} \text{ cm/s}$ for FD10k, $1.17 \pm 0.27 \times 10^{-7} \text{ cm/s}$ for FD20k, and $2.52 \pm 0.98 \times 10^{-8} \text{ cm/s}$ for FD70k. Thus, passive fluxes across NCI-H441 cell monolayers were more restricted than across bronchial 16HBE14o-³⁵ but less restricted than across monolayers of cultured AT-like cells (Figure 2B),¹⁵ supporting the hypothesis that TEER is very closely related to trans-monolayer permeability of passively diffusing solutes.²⁷ Grainger and colleagues conducted a similar study using Calu-3 monolayers and, although their data is presented in a different format, report comparable values for paracellular diffusion of FITC dextrans.³⁷

P-Glycoprotein Expression and Function. The main feature of a *bona fide* organotypic cell-based *in vitro* model is that it most closely resembles the biological barrier of interest, and while passive permeation is fairly comparable across, e.g., epithelial cells of the lung and the intestine,³⁸ major differences have to be acknowledged when it comes to drug transporter mediated permeability.³⁹ Drug transporters have only recently moved into the center of attention of pulmonary drug delivery research,^{39,40} and of the over 400 identified transporter

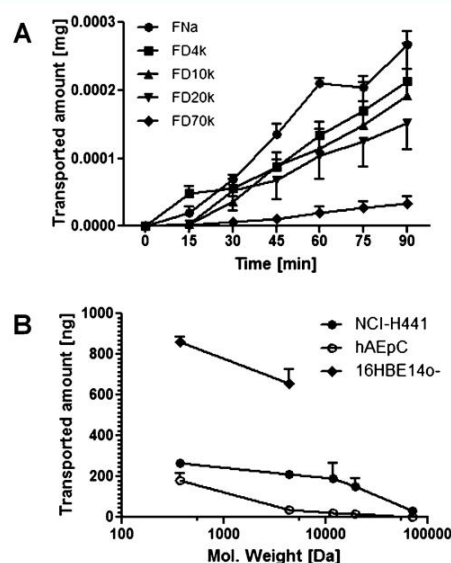


Figure 2. (A) Time courses of paracellular marker transport in the apical-to-basolateral direction across NCI-H441 cell monolayers. Cells were seeded at a density of 2.5×10^5 cells/cm² on Transwell Clear membrane inserts and grown under liquid-covered culture conditions for 12 days before transport experiments with fluorescein sodium (FNa) and FITC-labeled dextrans of molecular weight 4000 (FD4k), 10,000 (FD10k), 20,000 (FD20k), and 70,000 (FD70k) were carried out. Each data point represents the mean $\pm$ SD for $n = 3-4$. (B) Comparison of paracellular marker transport across monolayers of NCI-H441 cells (●), human alveolar epithelial type I-like cells (○, from ref 15), and 16HBE14o- bronchial epithelial cells (◆, from ref 35).

proteins, P-gp is probably the most prominent one. In Figure 3A, a representative immunoblot of P-gp in NCI-H441 cell monolayers is shown. Bands from LCC and AIC grown cells showed similar appearances and intensities, and thus culture conditions have only negligible effects on abundance of the transporter protein. A punctate staining pattern for P-gp, mostly localized at the apical membrane, was observed by CLSM (Figure 3B). Intensity and pattern of the signal were comparable to previously published data of human alveolar epithelial barrier *in vitro*.^{22,41} Expression levels of P-gp in the lung are generally well below what is found in other biological barriers, e.g., the liver, intestine, or blood-brain barrier.⁴¹ The lower expression levels of P-gp in lung epithelium is paralleled by a lower transporter activity in respiratory epithelial cells *in vitro*.^{6,42,43} In Figure 3D release of Rh123 from NCI-H441 monolayers is shown. Over time, the amount of fluorophore in the cells decreases, while at the same time the concentration in the supernatant increases. Addition of the specific P-gp inhibitor LY335979 resulted in attenuated Rh123 translocation. Although this decrease was significant at 60 min, the effect was much less pronounced as, for example, in Caco-2 cells.⁴⁴ Of note, the inhibitory efficiency of the broad-spectrum inhibitor verapamil was not different from that of the specific LY335979, suggesting that Rh123 efflux from NCI-H441 is predominantly mediated by P-gp. Despite the relatively low effect of the P-gp inhibitors in the release studies, bidirectional transport experiments of Rh123 showed significant net secretion, which was again sensitive to inhibition by LY335979 (Figure 3C). The more pronounced P-gp effect in the transport versus the release

might have its reason in a higher transporter activity in membrane-grown cells, albeit we currently have no experimental proof to support this speculation. The calculated Rh123 efflux ratio was 3.58 in NCI-H441 cells, which was very similar to the value of 3.09 obtained in monolayers of human ATI-like cells²² but higher than the efflux ratio of bronchial epithelial cell type, e.g., 1.43 (VA10) or 2.40 (Calu-3).^{6,45} Generally, all lung-derived cells showed significantly lower efflux ratios than intestinal epithelial Caco-2 cells (Rh123 efflux ratio 10.5).⁴⁶

Organic Cation Transporters Expression and Function

This is the first report detailing expression of the five cloned members of the human polyspecific organic cation transporter (OCT/N) family in primary human alveolar epithelial cells and in the NCI-H441 cell line. These transporters might have therapeutic relevance, as many drugs used in inhalation therapy (e.g., bronchodilators) are organic cations and thus potential substrates of OCT/Ns.^{23,47} In addition, it was previously shown that ICS were inhibitors of the polyspecific organic cation transporter 2 (hOCT2).⁴⁸ In Figure 4, representative immunoblots and the corresponding immunocytochemistry data for OCT1, OCT2, OCT3, OCTN1, and OCTN2 are shown. Generally, expression of all transporter proteins was confirmed in all cell types investigated. Robust bands were observed in Western blots from NCI-H441, ATII, and ATI-like cells in the case of OCT1 (Figure 4A). In CLSM of NCI-H441 monolayers, OCT1 showed a comparably weaker signal with predominant localization along the lateral membranes and a number of additional intracellular spots. OCT1 immunostaining in freshly isolated ATII cells and in ATI-like cell monolayers was considerably stronger. In both primary cell types the exact determination of the protein locus by CLSM proved difficult, as freshly isolated ATII pneumocytes are not yet polarized, and ATI-like cells are extremely thin. OCT2 was also found in the three cell models studied (Figure 4B). These data are in agreement with recently published observation from our laboratory on OCT2 expression in alveolar A549 cells.²⁷ In the same study, OCT2 was found to be absent from cell lines of bronchial origin (i.e., Calu-3 and 16HBE14o-). Figure 4B shows a relatively weak and diffuse CLSM signal in the case of NCI-H441 cells. Nonetheless, clearly visible OCT2 protein bands were detected by Western blot in NCI-H441, ATII, and ATI-like cell lysates. Data for OCT3 were generally very similar to what was described for OCT2 (Figure 4C). In the case of the ergothioneine and carnitine transporters OCTN1 and OCTN2, respectively, Western blot analysis revealed rather weak bands in all cell types. This was probably related to the suboptimal suitability of the antibodies for immunoblotting (Figure 4D and 4E), since immunocytochemistry yielded micrographs of reasonable quality, confirming the expression of both transporters in all three cell types studied. Horvath and colleagues previously presented immunohistological evidence for OCTN1 and OCTN2 expression in alveolar epithelial cells of human parenchyma *in situ*.⁴⁹ However, using immunofluorescence technique in an intricate tissue such as lung parenchyma renders it difficult to allocate the staining to a distinct cell type. Our data support Dr. Horvath's findings and moreover suggest that OCTN1 and OCTN2 are localized to both human alveolar epithelial cell types.

ASP⁺ has regularly been used as fluorescent substrate in the functional expression analysis of OCT/N transporters in a number of respiratory epithelial cell models.^{27,49,50} Here, we determined if ASP⁺ uptake was time- and concentration-

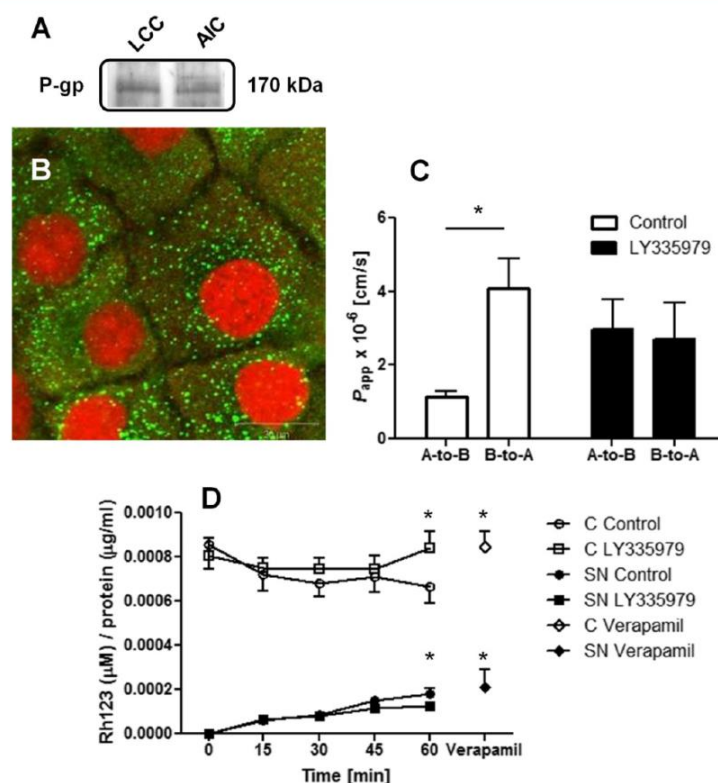


Figure 3. (A) Representative Western blot of P-glycoprotein (P-gp) in NCI-H441 human distal lung epithelial cells. Cells were cultured under LCC or AIC conditions for 8 days on Transwell Clear inserts. (B) Immunostaining of P-gp (green) in NCI-H441 cell monolayers. Cells were grown on chamber slides and stained on day 8. Cell nuclei were counterstained with propidium iodide (red). (C) Permeability of rhodamine 123 (Rh123) across NCI-H441 cell monolayers. Cells were cultured for 12 days on Transwell Clear inserts under liquid-covered culture conditions. Transport studies were performed in the presence and absence of LY335979 (10 μ M). Data are represented as means $\pm$ SD, $n = 3-4$, $*P < 0.05$. (D) Inhibitory effects on Rh123 release from NCI-H441 cell monolayers grown in 24-well plates. Rh123 decrease in cells (C) and increase in supernatant (SN) was measured in the presence of LY335979 (10 μ M) or verapamil (50 μ M) at pH 7.4, 37 $^{\circ}$ C for 60 min. Values are represented as means $\pm$ SD, $n = 7-16$, $*P < 0.05$ versus control.

dependent, and if it was controlled by extracellular proton and sodium concentrations. As shown in Figure 5A, accumulation of ASP⁺ in NCI-H441 cells was more or less linear over 90 min. Uptake of ASP⁺ into NCI-H441 cells, when studied after the tight junctional complexes were opened using EDTA and thus transporter sites localized to the basolateral membranes became better accessible to ASP⁺, was ~ 6 -fold increased (Figure 5B). Uptake rates into NCI-H441 cells, as well as the tight junction-related phenomenon, were very similar to what was previously observed in Calu-3 cell monolayers.²⁷ In Figure 5C, the concentration-dependence of ASP⁺ uptake into NCI-H441 cell monolayers is illustrated. ASP⁺ uptake was a saturable process with $K_m = 881.2 \pm 195.3$ μ M and $V_{max} = 2.07 \pm 0.26$ nmol/min/mg protein. Horvath et al. reported a K_m of 394.4 ± 15.4 μ M for ASP⁺ uptake into human airway epithelial cells in primary culture, which is in the similar order of magnitude.⁴⁹ ASP⁺ uptake into NCI-H441 cells was further found to be significantly reduced at lower extracellular pH values (Figure 5D) and decreased Na⁺ concentrations (Figure 5E) and was significantly reduced at 4 $^{\circ}$ C (Figure 6). Also in Figure 6, data from inhibition studies using various modulators of organic cation transport are shown. Extracellular TEA resulted in a $\sim 35\%$ decrease of ASP⁺ uptake, and verapamil diminished ASP⁺ accumulation by $\sim 70\%$ (at 200 μ M). Both substances

have the potential to inhibit all five OCT/Ns in the concentration range used.²⁸ Quinidine also showed a concentration-dependent inhibition of more than 50%. The steroids corticosterone, beclometasone, and budesonide, on the other hand, did not have significant effects. Amantadine, like the steroids a relatively selective inhibitor of OCT2 function, showed a more pronounced inhibition, but the concentrations used were also considerably higher. ASP⁺ uptake was attenuated by 20–30%, when the choline transporter substrates HC-3 and acetylcholine were present in the transport buffer, and a concentration-dependent inhibition of ASP⁺ uptake into NCI-H441 cells was also achieved with formoterol and salbutamol. The OCTN substrates L-ergothioneine and D-carnitine did not reduce ASP⁺ uptake, suggesting that organic carnitine transporters had only a minor involvement in the process. Horvath and colleagues previously proposed OCTN2 as the responsible transporter for ASP⁺ uptake into the human airway epithelium.⁴⁹ However, direct evidence of ASP⁺ transport by OCTNs, for example, using heterologous expression systems, is still outstanding. In addition, a publication by Grigat and co-workers comes to the conclusion that OCTN2 is not a general drug transporter and hence does not translocate ASP⁺.⁵¹

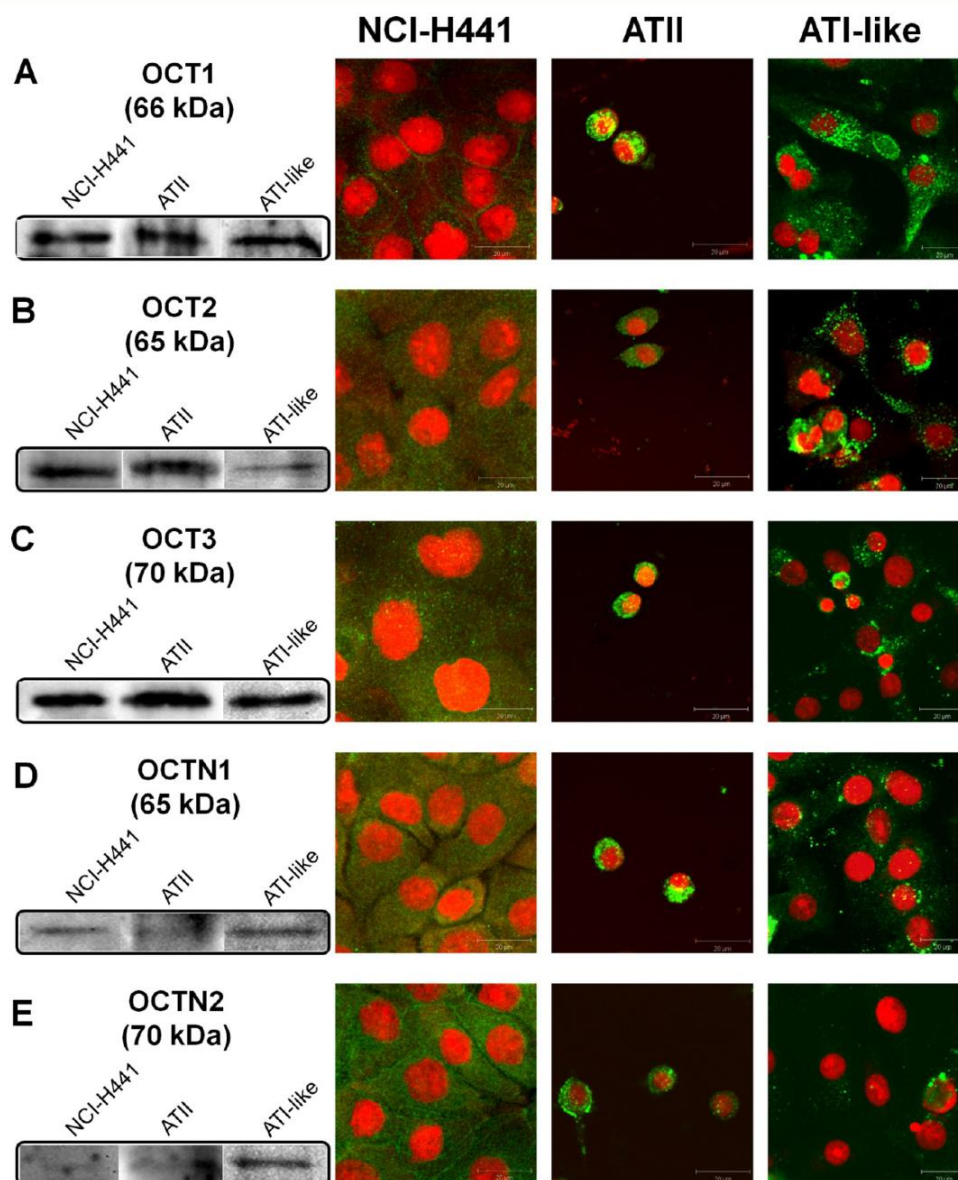


Figure 4. Expression of organic cation transporter proteins in NCI-H441 human distal lung cell line and human alveolar epithelial cells in primary culture (ATII and ATI-like) grown on plastic. The left panel shows representative Western blot, and the right panels show the immunocytochemistry of organic cation transporters OCT1 (A), OCT2 (B), OCT3 (C), OCTN1 (D), and OCTN2 (E). Cells were either used 24 h post isolation (ATII) or cultured for 8 days (NCI-H441 and ATI-like). The relevant protein of interest displays a green signal. Nuclei were counterstained with propidium iodide (red). Bars represent 20 μ m.

We previously reported that ASP^+ accumulation into A549 occurred via two distinct transporter sites, whereas comparably lower uptake levels and no saturation of ASP^+ absorption were observed in polarized Calu-3 and 16HBE14o- monolayers. In the same study, most pharmacological inhibitors showed only relatively weak activities in the bronchial models.²⁷ The exception to this was the lipophilic compound verapamil. The observations in NCI-H441 cells fall in between what was seen in the alveolar and bronchial cell lines. OCT-mediated uptake reached saturation (albeit with a rather high K_m), but opening of tight junctions also had an enhancing effect. Moreover, most compounds investigated as modulators displayed significantly

lower inhibitory activities than in A549 cells.²⁷ This allows the speculation that in the bronchiolar region a transition of organic cation transport occurs. From data generated *in vitro*, it can be speculated that in airway cells mostly basally localized OCT1 (and probably apical OCTN2) are the main transporter sites, whereas apical OCT2 and basal OCT1 are likely the key transporters in the alveolar epithelium. *In situ*, OCT1 was reported by Lips et al. to be expressed mainly within bronchial epithelial cells with weak apical staining of ciliated cells, whereas OCT2 was mostly located in apical membranes of ciliated cells in the upper airways.⁴⁸ In the case of OCT3, positive signals were localized to plasma membranes of basal cells and

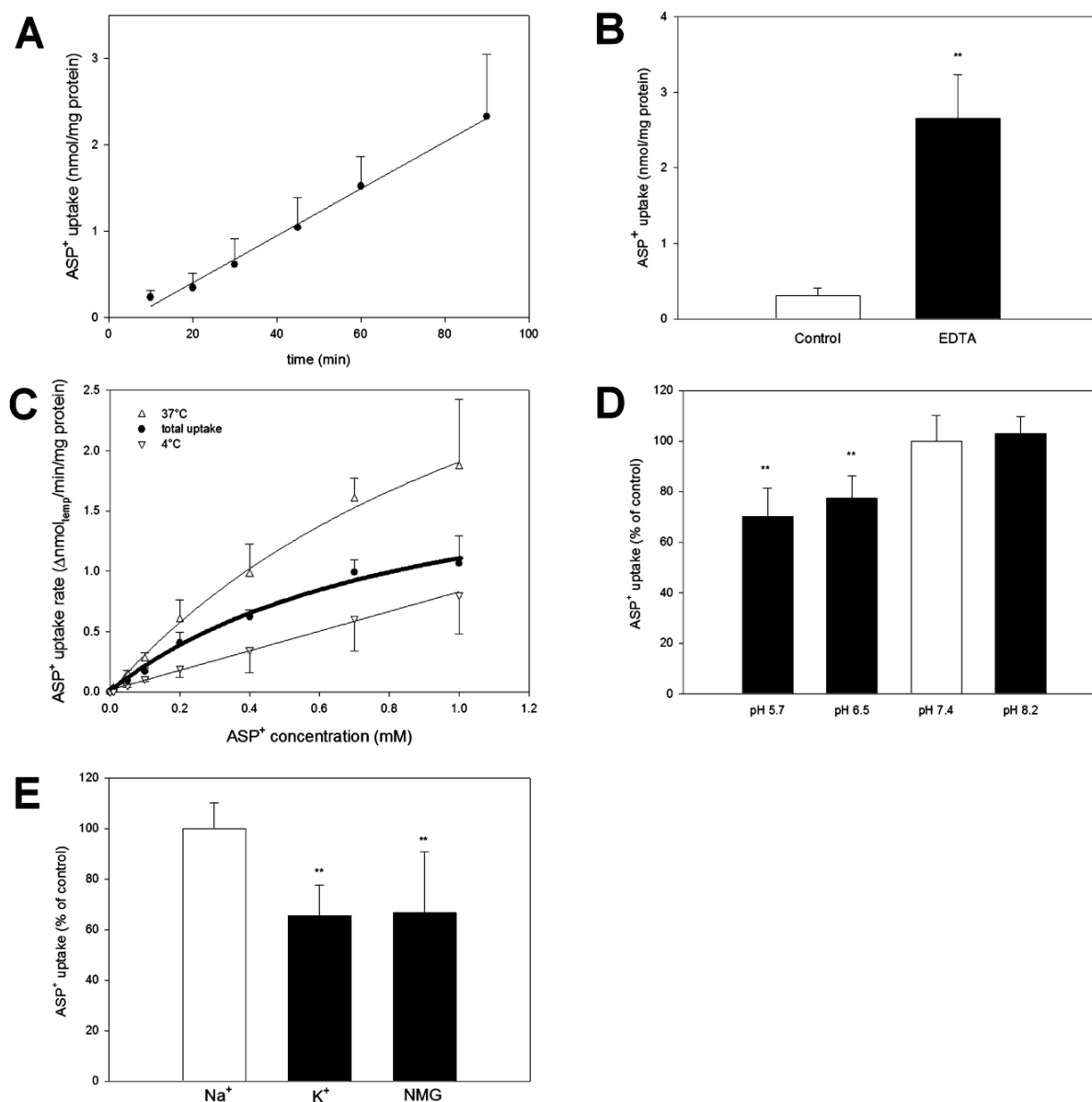


Figure 5. (A) Time-dependent uptake of ASP⁺ into NCI-H441 cell monolayers. Cell layers were grown in 24-well plates for 8 days and then incubated with ASP⁺ (10 μ M). Uptake values are corrected for the uptake at 4 $^{\circ}$ C. (B) Effect of tight junctional barrier on ASP⁺ uptake (10 μ M) was investigated. NCI-H441 cell monolayers were incubated with ASP⁺ in the presence and absence of 5 mM EDTA. (C) Concentration-dependent uptake of ASP⁺ into NCI-H441 human distal lung epithelial cells. Cell layers were incubated at pH 7.4 and 37 and 4 $^{\circ}$ C for 20 min. Total uptake rates (●) were calculated as the difference in ASP⁺ uptake between 37 $^{\circ}$ C (Δ) and 4 $^{\circ}$ C (▽). (D) Effect of extracellular pH on uptake of ASP⁺ into NCI-H441 cells. NCI-H441 cells were incubated with ASP⁺ (10 μ M) at pH 5.7–8.2, 37 $^{\circ}$ C for 20 min. (E) Effect of extracellular sodium concentration on ASP⁺ uptake by NCI-H441 cells. ASP⁺ uptake was measured at pH 7.4, 37 $^{\circ}$ C for 20 min. Na⁺ was replaced with K⁺ or N-methyl-D-glucamine (NMG). Data are represented as means $\pm$ SD, n = 9. * P < 0.05, ** P < 0.01 versus control.

basolateral membranes of intermediate cells.⁴⁸ The localization of OCTs in the distal lung, however, is still unknown. Horvath et al. showed evidence for an apical membrane localization of OCTN1 and OCTN2 in human airway and alveolar epithelium *in situ* and freshly isolated airway epithelial cells in primary culture.⁴⁹ NCI-H441 cells express OCT2 and OCTNs, but these transporters do not appear to be particularly active, while basally localized OCT1 facilitates the bulk of organic cation

uptake. This hypothesis, however, does not consider the possible involvement of other ASP⁺ recognizing transporters (e.g., MATE1) that have not been identified in this cell line to date.²³

CONCLUSIONS

This study confirms that NCI-H441 cells form electrically tight, polarized cell monolayers. Moreover, NCI-H441 cells express a

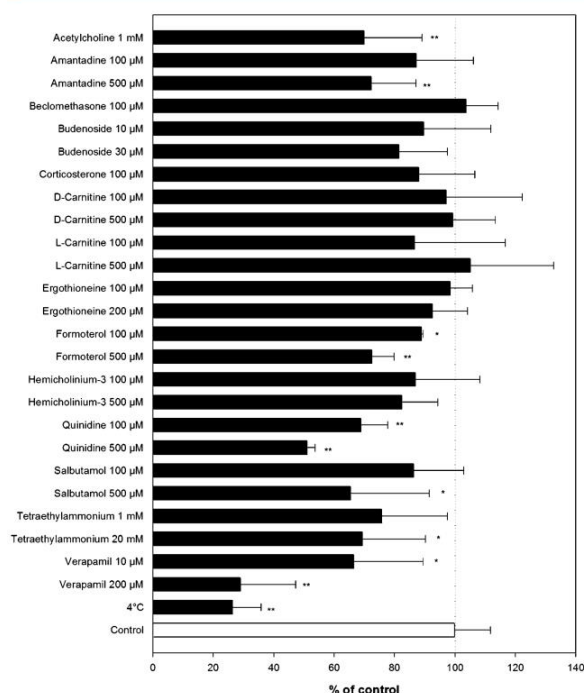


Figure 6. Inhibitory effects of modulators of organic cation transporter function and pulmonary administered drugs on ASP⁺ uptake into NCI-H441 cells. ASP⁺ uptake (at 10 μM) was measured in NCI-H441 monolayers grown in 24-well plates for 8 days at pH 7.4, 37 °C for 20 min in the presence of the indicated concentration of the respective compound. Data are represented as means ± SD, $n = 9$. * $P < 0.05$, ** $P < 0.01$ versus control.

number of key drug transporters (i.e., P-glycoprotein and SLC22 organic cation transporters) very similarly to human alveolar epithelial cells in primary culture. P-gp function, as determined by rhodamine 123 transport, was comparable between NCI-H441 cells and human primary ATI-like pneumocytes. These data taken together, NCI-H441 cells can be considered as an improvement to currently available cell lines of the human distal lung epithelial barrier. Particularly in cases where the presence of a polarized cell layer is important (e.g., in transport studies), the use of NCI-H441 can be of advantage.

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Author Contributions

JJS and CE designed the experiments. JJS, VEM, JCG and ES performed the experiments. JJS and JCG analyzed the data. HH carried out thoracic surgery for lung tissue specimens. ND and CML isolated and purified human ATII cells. CE and JJS wrote the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AIC, air-interface culture; ASP⁺, 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide; ATI, alveolar type I; ATII, alveolar type II; BCA, bichinchonic acid; BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HC-3, hemicholinium 3; HRP, horseradish peroxidase; ICC, immunocytochemistry; ICS, inhaled corticosteroid; ITS, insulin, transferrin, sodium selenite; KRB, Krebs-Ringer buffer; LCC, liquid-covered culture; MDCK, Madin-Darby canine kidney; OCT, organic cation transporter; OCTN, novel organic cation transporter; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; RAGE, receptor for advanced glycation end-products; Rh123, rhodamine 123; RPMI, Roswell Park Memorial Institute; SAGM, small airway epithelial cell growth medium; SP(-A, -B, -C, -D), surfactant protein (A, B, C, D); TEA, tetraethylammonium; TEER, transepithelial electrical resistance; ZO-1, zonula occludens protein 1

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1.10. Zusammenfassung

Das Fehlen eines genau charakterisierten, kontinuierlich wachsenden *in vitro* Modells des menschlichen distalen Lungenepithels stellt besonders im Rahmen von transepithelialen Transportstudien eine erhebliche Einschränkung der Forschung der inhalativen Biopharmazie dar. Das Interesse des Forschungsprojektes galt der Untersuchung, ob die NCI-H441-Zelllinie, die sich von einem humanen pulmonalen Adenokarzinom ableitet, das Potential birgt, als *in vitro* Modell für das humane distale Lungenepithel zu fungieren. Die Ausbildung von Zellbarrieren wurde durch immunozytochemische Färbung von Proteinen, die mit der Ausbildung von Tight Junctions assoziiert sind, nämlich dem Zonula Occludens Protein 1 (ZO-1) und dem Adhäsionsprotein E-Cadherin und durch Messung des transepithelialen elektrischen Widerstandes (TEER) untersucht. Darüber hinaus wurden Transportstudien mit Fluorescein-Natrium- und Fluorescein-Isothiocyanat-markierten Dextranen durchgeführt, wobei die verwendeten Substanzen als Marker für parazelluläre Transportprozesse dienten. Die Expressionsprofile von P-Glykoprotein (P-gp; ABCB1) und organischen Kationentransportern (OCT/Ns; SLC22A1-A5) wurden über Westernblot und Confokale Laser-Scanning Mikroskopie (CLSM) erstellt. Die Funktion von P-gp wurde anhand von Freisetzungs- und bidirektionalen Transportstudien an Zellmonolayern unter Verwendung von Rhodamin 123 (Rh123) und der beiden Inhibitoren Verapamil und LY335979 beurteilt. Die Aufnahme von 4-(4-dimethylamino)styryl)-N-methylpyridiniumiodid (ASP⁺) in NCI-H441-Zellen wurde gemessen, um die Funktion der organischen Kationentransporter *in vitro* zu bestimmen. Des Weiteren wurde das inhibitorische Potential verschiedener organischer Kationen auf die Aufnahme von ASP⁺ untersucht.

Als Ergebnis der Forschungsarbeit konnten NCI-H441 Zellen, die unter liquid-covered conditions (LCC) kultiviert wurden, nach 8-12 Tagen in Kultur konfluente, elektrisch dichte Monolayer mit TEER-Höchstwerten von circa 1000 $\Omega\cdot\text{cm}^2$ formen. Die gebildeten Monolayer waren in der Lage, parazellulär transportierte Marker in Abhängigkeit von deren Molekulargewicht zu unterscheiden. Die Expression von P-gp, OCT1, OCT2, OCT3, OCTN1 und OCTN2 wurde anhand von Westernblot und CLSM nachgewiesen und wies deutliche Ähnlichkeit zu entsprechenden Daten von frisch isolierten humanen Alveolarepithelzellen in Primärkultur auf. Eine zeitabhängige Freisetzung von Rh123 aus NCI-H441-Monolayern zeigte eine geringe, obgleich signifikante Abschwächung durch beide Inhibitoren. In Transportstudien wies Rh123 eine Nettosekretion auf, die wiederum durch *bona fide* P-gp–

Modulatoren inhibierbar war. Die Aufnahme von ASP^+ war zeit- und temperaturabhängig mit $K_m = 881,2 \pm 195,3 \mu M$ und $V_{max} = 2,07 \pm 0,26 \text{ nmol/min/mg Protein}$. Tetraethylammonium, Amantadin, Chinidin und Verapamil blockierten die Aufnahme von ASP^+ signifikant, wohingegen der Effekt der beiden OCTN Substrate, D- beziehungsweise L-Carnitin und L-Ergothionein weniger ausgeprägt war. NCI-H441 Zellen sind die erste Zelllinie mit Ursprung im humanen distalen Lungenepithel, die in der Lage ist, Monolayer mit nennenswerten Barriereeigenschaften auszubilden. Darüber hinaus war die Expression und Aktivität von Arzneistofftransportern in NCI-H441 Zellen konsistent mit Berichten über ebenjene Eigenschaften in humanen Alveolarepithelzellen in Primärkultur. (Deutsche Übersetzung des Abstracts aus Salomon *et al.*, 2014)

2. Chapter II

Expression of multidrug and toxin extrusion 1 transporter (MATE1/SLC47A1) in human respiratory epithelial cells

2.1. Introduction

During the last years, investigations on the role of multidrug and toxin extrusion (MATE) proteins primarily focused on their influence on transport, excretion and accumulation of exogenous and endogenous substrates in kidney and liver, these organs being the main sites of drug metabolism and clearance. The expression of MATE1 that shows a broader tissue distribution than the kidney-specific MATE2/K, has hardly been studied in the respiratory epithelium and hence, neither the physiological role of MATE1 in the lung nor its role in the disposition of pulmonary administered drugs are known.

In 1998, Morita *et al.* discovered a novel multidrug efflux protein in cells of *Vibrio parahaemolyticus*, NorM and its homologue in *Escherichia coli*, YdhE. NorM was shown to be responsible for norfloxacin efflux in cells of *Vibrio parahaemolyticus*, resulting in increased resistance to several antimicrobial agents, not only norfloxacin but also ciprofloxacin, kanamycin and streptomycin (Morita *et al.*, 1998). In 1999, bacterial NorM was identified as prototype of a newly established family of multidrug efflux transporters termed MATE (multidrug and toxin extrusion) family (Brown *et al.*, 1999), being assigned as the fifth family of multidrug efflux transporters in addition to well-known families, i.e. the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family and the ATP-dependent multidrug transporters (Brown *et al.*, 1999; Putman *et al.*, 2000). Otsuka *et al.* found two human orthologues of bacterial MATE, designated human MATE1 and MATE2, with encoding genes located in tandem on chromosome 17 (Otsuka *et al.*, 2005). Interestingly, the expression of MATE1 and MATE2 differs between body tissues: MATE1 is found in adrenal gland, kidney, liver, testis, skeletal muscle (Otsuka *et al.*, 2005; Masuda *et al.*, 2006) whereas

MATE2-K, a functional alternative splice variant of MATE2, seems to be a kidney-specific antiporter. Both kidney-expressed MATE1 and MATE2-K are located in brush border membranes of the proximal tubules. Furthermore, it has been shown that the protein sequence of MATE2-K shares 52% identity with MATE1 (Masuda *et al.*, 2006). Studies illustrated that MATE1 was mainly localised to the apical membrane in renal tubule cells and bile canaliculi (Otsuka *et al.*, 2005). Investigation of the secondary protein structure of MATE has shown that both mammalian MATE1 and MATE2 consist of 13 transmembrane helices (TMHs) with an intracellular NH₂ terminus and an extracellular COOH terminus (Zhang and Wright, 2009). Therefore, the external COOH terminus of MATE is accessible for anti-MATE antibodies aligning to the C-terminal region of the protein in non-permeabilised cells. The presence of the 13th TMH is not essential for the transport function but seems to affect turnover characteristics of the transporter and thus, deletion of terminal TMH resulted in a decrease in turnover number (Zhang and Wright, 2009). Matsumoto *et al.* assumed in 2007, that certain glutamate residues, i.e. Glu273, Glu278, Glu300 and Glu389, which are located in the substrate binding pocket of MATE1, are essentially involved in substrate recognition, as these acidic residues can potentially interact with organic cations (OCs) as substrates (Matsumoto *et al.*, 2007). In 2006, Tsuda and colleagues discovered that MATE facilitates the electroneutral antiport of H⁺ and tetraethylammonium (TEA), a prototypic organic cation, by using an oppositely directed H⁺ gradient as driving force for exchange (Tsuda *et al.*, 2006). Studies on the substrate specificity of MATE1 and MATE2-K conducted by Tanihara *et al.* showed that both proteins not only act as transporter for OCs, e.g. TEA, 1-methyl-4-phenylpyridinium (MPP⁺), cimetidine, metformin, guanidine, procainamide and topotecan, but also facilitate transport of several anionic compounds, e.g. oestrone sulphate, acyclovir and ganciclovir. Both cationic and anionic substrates showed similar affinity for MATE1 and MATE2-K. The zwitterionic cephalosporine antibiotics cephalexin and cephadrine were reported to be specific substrates of MATE1, but not of MATE2-K, whereas quinolone antibiotics were demonstrated to inhibit transport activity of both MATE1 and MATE2-K (Tanihara *et al.*, 2007).

The aim of this work was to investigate, whether MATE1 is present in human lung epithelium using freshly isolated alveolar epithelial cells (hAEPc) as well as cell lines of bronchial (Calu-3), bronchiolar (NCI-H441) and alveolar (A549) origin as models. These investigations employed Western blot and CLSM.

Secondly, knockdown studies of MATE1 in A549 epithelial cells were conducted to optimise transfection conditions for future studies on the functional characterisation of MATE1 in lung epithelium.

2.2. Experimental section

2.2.1. Cell culture

A549 cells (American Type Culture Collection, ATCC CCL-185) were purchased from LGC Promochem (Teddington, UK). Cells of passage numbers 73, 82, 87, 88, 90 and 95 were grown at 40,000 cells/cm² and cultivated in an 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F-12) supplemented with 5% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich, Dublin, Ireland).

Calu-3 cells (ATCC, HTB-55) were obtained from LGC Promochem (Teddington, UK). Cells of passage number 48 were seeded at a density of 75,000 cells/cm² using minimum essential medium (MEM) supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acid solution, 1% sodium pyruvate solution and 0.5% glucose solution (all from Sigma-Aldrich).

“NCI-H441 (ATCC, HTB-174) cells were obtained from LGC Standards (Teddington, UK).” Cells of passage number 66 were cultured *“in Gibco RPMI (Roswell Park Memorial Institute)-1640 medium (Biosciences, Dun Laoghaire, Ireland) supplemented with 5% FBS, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich).”* Cells were seeded at a density of 75,000 cells/cm² in 24-well plates (Greiner BioOne, Frickenhausen, Germany). *“Twenty-four hours post-seeding, the medium was replaced with RPMI-1640 medium, which in addition contained dexamethasone (200 nM, Sigma-Aldrich) and insulin-transferrin-sodium selenite (ITS) supplement (Roche Diagnostics Limited, West Sussex, UK).”* (Salomon *et al.*, 2014)

Human alveolar epithelial cell isolation and culture

See Chapter I, Experimental section, human alveolar epithelial cell isolation and culture, page 2.

All cells types were routinely grown in T-75 flasks (Greiner BioOne) at 37°C in 5% CO₂ atmosphere and sub-cultured when approximately 80% confluence was reached. The culture medium was exchanged every 48 h. Cells were grown to confluent monolayers on 24-well tissue culture plates (Greiner BioOne) for 5 (A549), 8 (hAEpC/ATI-like, NCI-H441) and 12 (Calu-3) days at 37°C in 5% CO₂ atmosphere before being used in studies described below.

2.2.2. Western blot analysis

Total cell protein extracts were generated from A549, Calu-3, NCI-H441 and hAEpC monolayers grown on 24-well tissue culture plates (Greiner BioOne) for 5 (A549), 8 (hAEpC, NCI-H441) and 12 (Calu-3) days. *“Cell lysis was performed on ice in cell extraction buffer (Invitrogen, Karlsruhe, Germany) containing protease inhibitors (Sigma-Aldrich). Cell samples were sonicated twice for 10 s and then the lysate was centrifuged (10,000g at 4°C) for 20 min. The resulting supernatants were collected and sample concentrations were determined using a standard protein assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer’s instructions. Protein was loaded at equal concentrations and separated by electrophoresis using 10% polyacrylamide gels. After electrophoresis, transfer onto immunoblot polyvinylidene fluoride membranes (Bio-Rad) was performed at 25V for 30 min. Membranes were then blocked in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 0.1% Tween 20 (pH 7.4) for at least 1 h at room temperature followed by incubation overnight at 4°C with the relevant primary antibody.”* Primary polyclonal rabbit anti-MATE1 antibody was purchased from Aviva (San Diego, CA) and used in a 1:500 dilution. *“Incubation with primary antibody was followed by three washings with PBS containing 0.1% Tween 20 for 10 min each and subsequent incubation with the HRP-conjugated secondary anti-rabbit antibody (Promega, Medical Supply Company, Dublin, Ireland) at a concentration of 1:12,500 at room temperature for 1 h. After additional three washings with PBS containing 0.1% Tween 20, peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland).”* After detection of relevant protein bands, membranes were stripped, blocked and incubated with a primary monoclonal mouse anti- β -actin antibody (Sigma-Aldrich) overnight at 4°C and a secondary anti-mouse antibody (Promega) for at least 1 h at room temperature. β -actin was used as an internal control to standardise the amount of protein. Relative levels of protein abundance were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system (Bio-Rad). (Salomon *et al.*, 2014)

2.2.3. Protein assay

Dilutions containing different concentrations of BSA in water (i.e. from 0 - 400 µg/ml) were prepared using Bio-Rad Protein Assay Standard II in order to create a standard curve to calculate the actual protein amount of each sample. Two microliter of each protein sample and 38 µl of distilled water were mixed by vortexing (dilution factor 1:20) and 10 µl of either protein sample dilution or standard solution were added to a 96-well plate (Greiner BioOne) in triplicate. Then, 200 µl dye was added to each well, using Bio-Rad Protein Assay Dye Reagent concentrate in a 1:5 dilution in water. Absorbance values of standards and samples were measured using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at a wavelength of 595 nm (Figure 1).

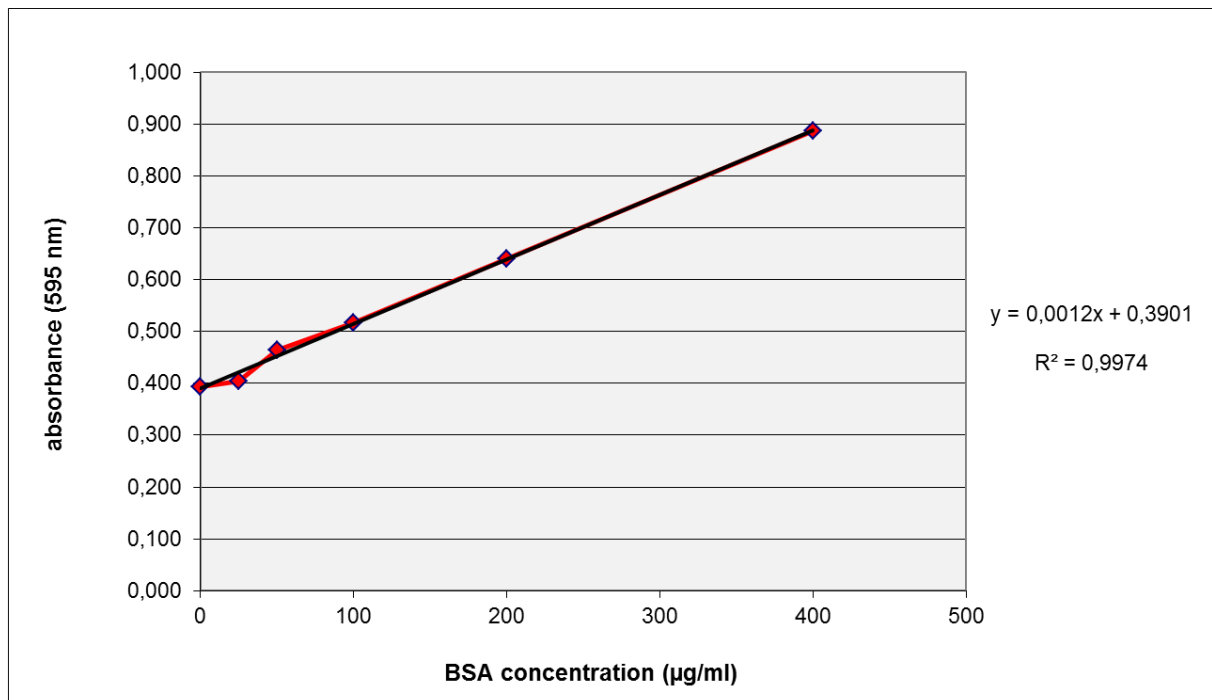


Figure 1. Absorbance values of various concentrations of BSA at 595 nm.

The resulting standard curve (red) and equation were used to calculate the actual protein amount of each sample. Each data point represents the mean ($n=3$). For BSA concentrations used, see text above. BSA, bovine serum albumin.

2.2.4. Sequence alignments of the anti-MATE1 antibody

Here, I analysed in how far SLC47A1 (MATE1) transporter protein and the correspondent anti-SLC47A1 antibody shared homology in common domains. In particular, the protein binding site of the antibody was identified using the BLAST[®] NCBI Data Base by detecting the positions of amino acids that define the length of the protein sequence (information provided by the manufacturer). FASTA sequences of the total transporter protein sequence and the detected antibody binding site were aligned using MultAlin software to graphically illustrate the position of the binding site (online available- <http://multalin.toulouse.inra.fr/multalin/>). If needed, UniProtKB software was used first to identify Swissprot ID, then FASTA-sequence of the protein was copied into BLAST.

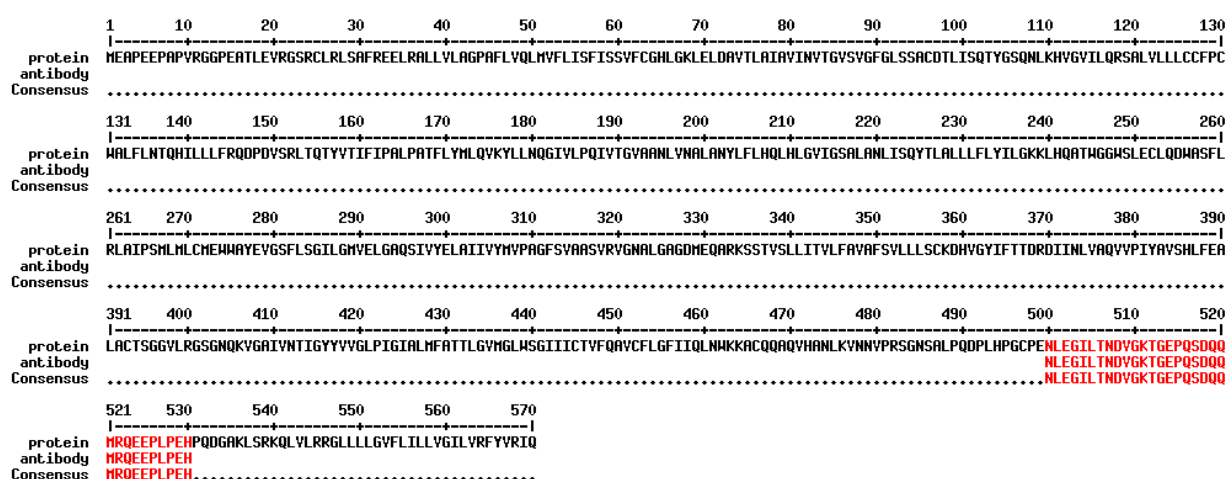


Figure 2. Peptide sequence of human MATE1 protein, consisting of 570 amino acid residues.

Protein binding site (red) of the anti-MATE1 antibody is located at the C-terminal region of the protein (amino acid positions 500 to 530).

2.2.5. Immunocytochemistry

“Lab-Tek chamber slides (Nunc, Roskilde, Denmark) were used to grow cell monolayers for immunocytochemistry.” Cells were washed twice with PBS, “fixed with 4% (w/v) paraformaldehyde solution for 10 min and then incubated for 10 min in PBS containing 50 mM NH₄Cl, followed by permeabilisation with 0.1% (w/v) Triton X-100 in PBS for 8 min.” Primary and secondary antibodies were diluted in PBS containing 1% (w/v) BSA. “After 60 min incubation with 200 µl of a 1:50 dilution” of a polyclonal rabbit anti-MATE1 antibody (Aviva), “cell monolayers were rinsed three times with PBS containing 1% (w/v) BSA, before incubation with 200 µl of a 1:300 dilution of the respective Alexa Fluor-labelled F(ab')₂ fragment (Biosciences) in PBS containing 1% (w/v) BSA was carried out. Propidium iodide (1 µg/ml in PBS) was used to counterstain cell nuclei. After 30 min of incubation, the specimens were again rinsed three times with PBS containing 1% (w/v) BSA and embedded in FluorSave anti-fade medium (Merck, Nottingham, UK). Images were obtained using a confocal laser scanning microscope (Zeiss LSM 510, Göttingen, Germany) with the instrument’s settings adjusted so that no positive signal was observed in the channel corresponding to the fluorescence of the relevant isotypic controls (data not shown).” (Salomon et al., 2014)

2.2.6. Transfection experiments

Twenty-four hours before transfection, A549 cells were seeded at a density of 40,000 cells/cm² on 24-well plates (Greiner BioOne) using a 1:1 mixture of DMEM/F-12 supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated for 24 h at 37°C in 5% CO₂ atmosphere and then, a 10 µM siRNA-stock solution was diluted in 50 µl serum-free culture medium per well, consisting of a 1:1 mixture of DMEM/F-12 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Relevant concentrations of transfection reagent, depending on the amount of siRNA used, were added to the siRNA dilution, mixed by vortexing and incubated for 10 min at room temperature to allow formation of transfection complexes. MATE1 siRNA, AllStars negative control siRNA and HiPerfect Transfection Reagent were obtained from Qiagen (West Sussex, UK). Prior to transfection, 250 µl of media per well were removed and then transfection complexes were added drop-wise onto the cells. After transfection, the plate was gently agitated to ensure uniform distribution of transfection complexes and cultured at 37°C in 5% CO₂ atmosphere. Culture medium was exchanged for serum-containing media 24 h after transfection. Each transfection was carried out in parallel using non-treated control cells, mock-transfected cells (treated with scrambled siRNA and transfection reagent) and transfected cells (treated with MATE1 siRNA and transfection reagent). Mock-transfected cells were used to indicate, whether the transfection process or transfection reagent caused any negative or cytotoxic effects on cell growth. Total cell protein was isolated 24 h (day 1), 48 h (day 2) and 72 h (day 3) after transfection according to a protein isolation protocol (see above). Expression of MATE1 remaining was quantified using densitometric analysis of the immunoblot with a ChemiDoc documentation system. Protein levels were normalised to corresponding β-actin levels.

2.3. Results

2.3.1. Expression of MATE1 in human respiratory epithelial cell lines and human primary alveolar epithelial cells

A semi-quantitative analysis of MATE1 in freshly isolated human alveolar epithelial cells (hAEpC) in primary culture and cell lines of bronchial (i.e. Calu-3), bronchiolar (i.e. NCI-H441) and human alveolar epithelial cell type (i.e. A549) was performed by Western blot, using a polyclonal anti-MATE1 antibody that was binding to the C-terminal region of MATE1 (Figure 2).

Protein expression levels of MATE1 revealed a clean and strong immunoactive protein band of approximately 62 kDa (Figure 3) in all cell types analysed.

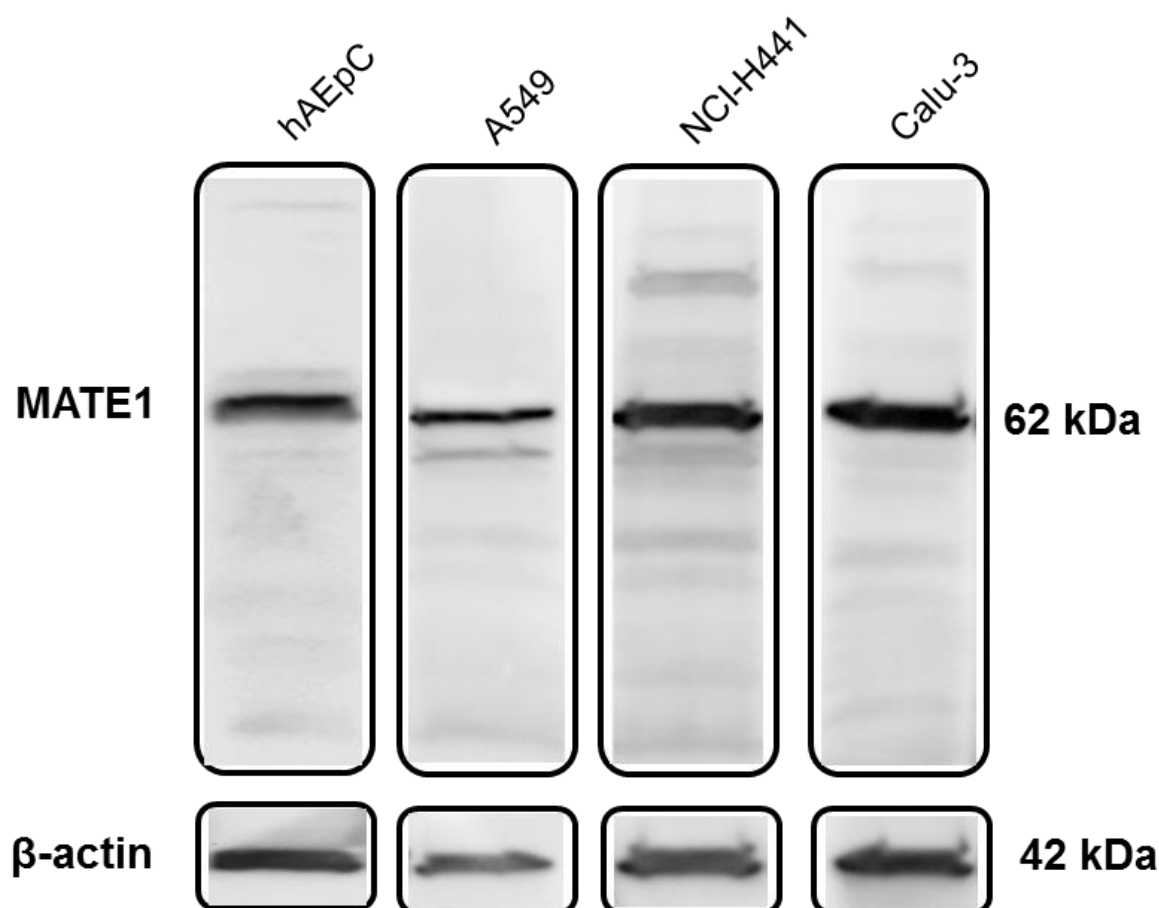


Figure 3. Western blot analysis of MATE1 expression in human respiratory epithelial cells.

Total cell protein samples were extracted from cells grown on 24-well plates on day 5 (A549, p73), day 8 (hAEpC/ATI-like and NCI-H441, p66) or day 12 (Calu-3, p48) of cultivation. According to manufacturer's information this is consistent with the molecular weight of MATE1 protein (62 kDa). p, passage number.

Localisation of MATE1 expression in monolayers of hAEpC, A549, Calu-3 and NCI-H441 cells was assessed by immunocytochemistry (Figure 4). Confocal laser scanning micrographs clearly showed a signal for MATE1 in plasma membranes and along cell-cell contacts of hAEpC, Calu-3 and NCI-H441 cells, whereas the signal was less defined in A549 cells, probably because this cell line does not form polarised cell layers (Winton *et al.*, 1998).

These data correspond well to observations made by Otsuka *et al.* in renal tubule cells and bile canaliculi (2005).

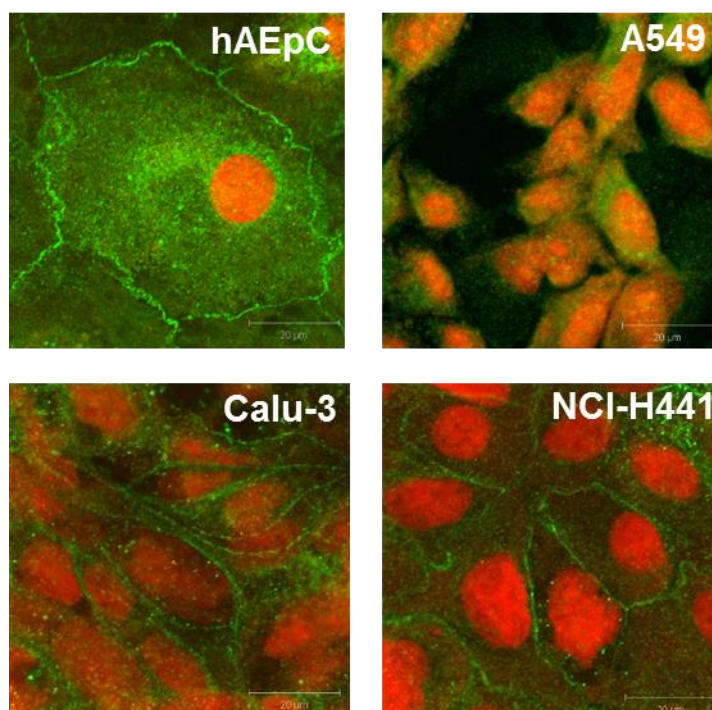


Figure 4. Immunostaining of MATE1 (green) in human respiratory epithelial cells.

Cells were grown on non-coated chamber slides and stained on day 5 (A549), day 8 (hAEpC/ATI-like and NCI-H441) or day 12 (Calu-3) of cultivation. Cell nuclei were counterstained using propidium iodide (red). Bar represents 20 µm.

2.3.2. Optimisation of MATE1 siRNA transfection conditions in A549 cells

Here, I tried to develop and optimise a siRNA transfection protocol for A549 cells to be able to examine the effect of decreased MATE1 levels on drug transport process in future studies. A549 cells were used as this cell line is unable to form functional tight junctions (Winton *et al.*, 1998) making it easier for transfection complexes to penetrate cell barriers in order to knockdown MATE1 expression.

For transfection conditions see Table 1. Concentrations of siRNA and transfection reagent were studied in the range of 100-150 nM and 2-2.5 μ l, respectively. Expression of MATE1 protein remaining was quantified using densitometric analysis of the Western blot. Although concentrations of both MATE1 siRNA and transfection reagent differed between passage numbers, cells of passage numbers 82, 87 and 95 showed similarly low knockdown of MATE1 72 h after transfection (i.e. 3.1%, 0.2% and 2.7%, respectively) with high protein expression levels remaining (i.e. 96.9%, 99.8% and 97.3%). Cells of passage numbers 88 and 90, however, exhibited higher percentage of knockdown (i.e. 49.9 and 41.2, respectively) resulting in lower MATE1 levels (i.e. 50.1% and 58.8%, respectively) compared to cells of passage numbers 82, 87 and 95 (Figure 5). These preliminary results suggest a possible optimal final concentration of 100-150 nM siRNA, whereupon a combination of several various siRNA types seems to be more effective than use of a single siRNA type. This observation needs to be confirmed in independent studies.

Table 1. MATE1/SLC47A1 siRNA transfection protocol in A549 cells.

siRNA concentrations and volumes are shown per well of a 24-well plate. Cells were seeded at a density of 40,000 cells/cm² on 24-well plates 24 h prior to transfection. Transfection experiments were carried out in triplicates. p, passage number.

		p, A549 cells				
MATE1 siRNA type		82	87	88	90	95
SLC47A1_1		50	50	33.33	50	50
SLC47A1_2		/	50	33.33	50	50
SLC47A1_5		/	/	33.33	50	50
final siRNA conc	(nM)	50	100	99.99	150	150
Transfection Reagent	(μ l)	2	2	2	2.5	2.5
Expression of MATE1*	(%)	96.9	99.8	50.1	58.8	97.3
Knockdown of MATE1*	(%)	3.1	0.2	49.9	41.2	2.7

* 72 h post transfection with MATE1 siRNA

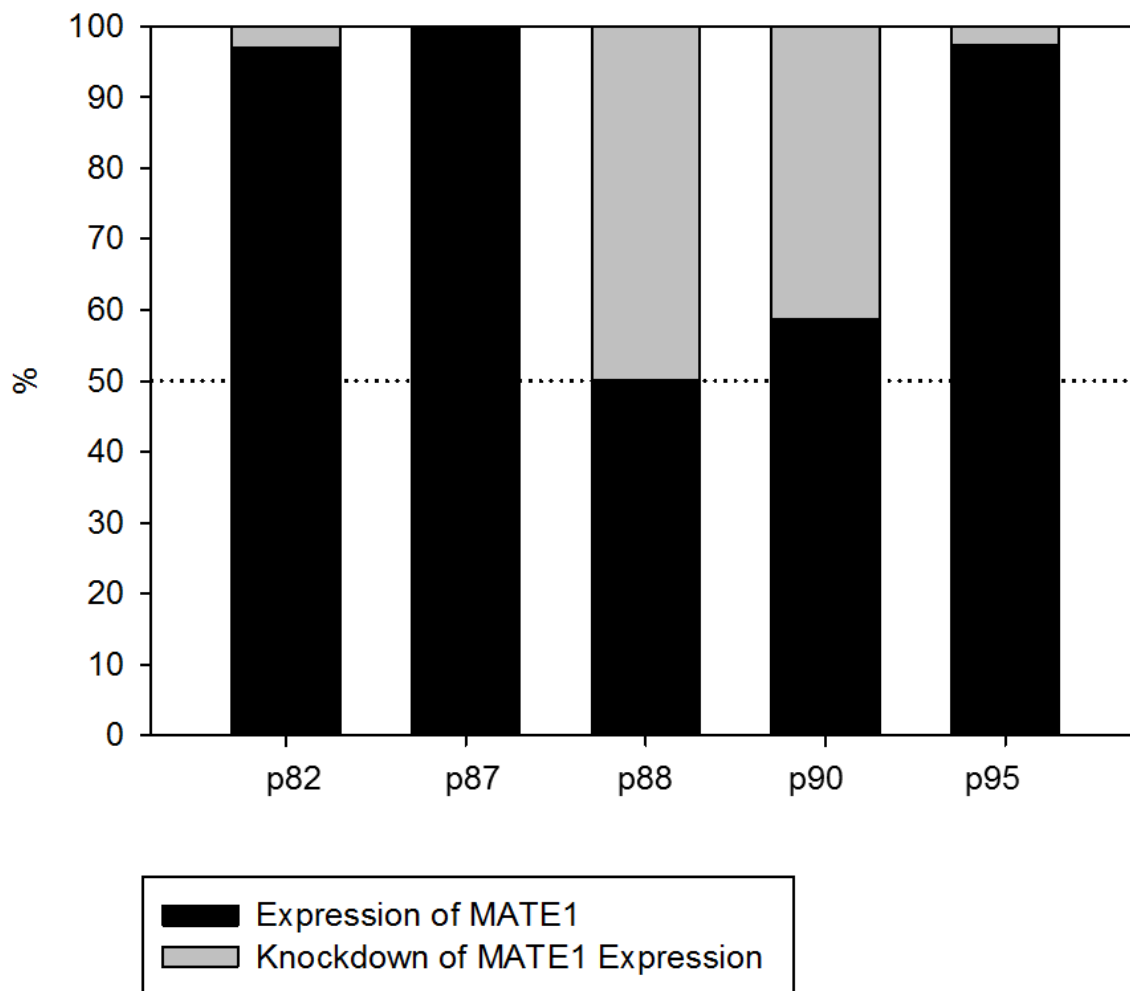


Figure 5. Expression and knockdown of MATE1 in A549 cells 72 h post transfection. Each transfection experiment was carried out in triplicates. Protein levels were quantified by densitometric analysis of the Western blots. MATE1 expression/knockdown levels are shown in relation to corresponding β -actin levels. p, passage number.

2.4. Discussion

The current knowledge of MATE1 expression and function in the lung still paints a rather patchy picture. During the last years, research has been mainly focused on MATE1's biological function in liver and kidney. Recently, however, the transporter was reported to be highly expressed on mRNA level in A549 and BEAS-2B cell culture models by Courcot *et al.* (2012). MATE1 was not detected in human bronchial epithelial primary cells, but at moderate levels in various lung tissues (Courcot *et al.*, 2012). Here, first evidence is provided that MATE1 is expressed on protein level in the human lung.

The expression of MATE1 was confirmed in freshly isolated alveolar epithelial cells in primary culture as well as in the continuously growing respiratory cell culture models, A549, Calu-3 and NCI-H441 cells. The Western blot data were in good agreement with results obtained by immunocytochemistry. CLSM studies showed a localisation to the luminal membranes in monolayers of Calu-3, NCI-H441 and hAEPc.

These results suggest that MATE1 might play a role in membrane transport and efflux of pulmonary applied drugs. Further experiments need to be done in the future to illuminate the role of MATE1 facilitating transport in the lung and also its interplay with other drug transporter proteins, e.g. P-glycoprotein (P-gp) and organic cation transporters (OCTs), especially in terms of organic cation transport. To definitely determine, whether a single transporter is involved in absorption of a compound, e.g. a drug molecule, at a certain location in the lung or the interplay of various membrane transporters is required for uptake, selective inhibitors are needed. The difficulty lies in the nature of the transporters themselves, as both OCTs and MATE1 are polyspecific transporters, which means they are able to facilitate transport of substrates with different sizes and molecular structure but they exhibit large variations regarding turnover characteristics and substrate affinity for different compounds (Koepsell *et al.*, 2007). The transporters' potential to recognise a broad range of various substrates makes them capable of taking up, distributing and excreting structurally diverse endogenous and exogenous cations as well as cationic drugs. Due to their ability for polyspecific substrate recognition, OCTs and MATE1 share overlapping substrate specificity in many cases, which makes it difficult to determine the actual contribution of the individual transporter, if there are no transporter-specific inhibitors available. The close interplay between OCT2 and MATE1 became particularly obvious in the kidney where OCs are taken

up in proximal tubule cells by basolaterally located OCT2 (Koepsell, 2004; Motohashi *et al.*, 2002) and then they are excreted at the brush-border membrane through a co-operation between MATE1 and P-gp (Otsuka *et al.*, 2005).

Due to the absence of appropriate, specific inhibitors, I carried out first knockdown experiments using MATE1 siRNA, as the use of RNAi and knockout techniques is another important tool to investigate transporter contribution to drug absorption process.

In terms of knockdown experiments, siRNA studies in A549 cells so far did not consistently obtain the desired efficiency. In contrast to the first part of experimental work, i.e. the confirmation of MATE1 expression in human respiratory epithelial cells, knockdown experiments in A549 cells did not result in a consistent reduction of MATE1 expression. Knockdown of protein expression *in vitro* is depending on various factors influencing the success of transfection process, e.g. seeding density and time of transfection. If cell density at transfection time is too high already, this fact will make penetration of cell barriers much more difficult for transfection complexes and concentration of transfection complexes might be too low for cell count. In addition to that, the ideal concentration of transfection reagent has to be found in order to achieve the best result, but to avoid cytotoxic effects on cell growth and therefore cell death. Another factor is the ideal ratio of siRNA and transfection reagent to form functional and stable transfection complexes. Furthermore, there are parameters, we cannot influence at all: the quality and functionality of both siRNA and transfection reagent provided by the manufacturer and location and structure of the targeted gene, MATE1. According to manufacturer's information, use of a combination of various siRNA types provides a better knockdown result than application of a single siRNA. Any of these factors affecting the outcome of a transfection experiment serve as potential sources of error, making it difficult to determine ideal transfection conditions. Results of transfection studies were inconsistent and the reproducibility was unsatisfactory, e.g. cells of passage numbers 90 and 95 were exposed to equal transfection conditions, but MATE1 knockdown and expression data differed by almost 40%. Further investigations are hence necessary to optimise MATE1 siRNA transfection conditions in A549 cells being this only a first step for further studies.

In summary, there are still many unanswered questions regarding the expression of membrane drug transporters at the pulmonary epithelium. However, the expression of MATE1 in A549, Calu-3, hAEPc and NCI-H441 cells could be confirmed and with the available techniques, it shall be possible to close the gap between the lung and other organs in future studies.

2.5. References

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2.6. Zusammenfassung

Bereits 1998 wurde mit NorM aus *Vibrio parahämolyticus* ein neues, bakterielles Effluxprotein entdeckt (Morita *et al.*, 1998), welches später als Prototyp einer neuen Klasse von Multidrug Efflux Transportern, der MATE (multidrug and toxin extrusion) Familie zugeordnet wurde (Brown *et al.*, 1999). Im Jahre 2005 gelang es Otsuka *et al.*, das humane Ortholog des bakteriellen NorM zu identifizieren. MATE Proteine, bei denen man zwischen MATE1 und MATE2 differenziert, zeigen je nach Isotyp eine unterschiedliche Gewebeverteilung (Otsuka *et al.*, 2005; Masuda *et al.*, 2006) und spielen bei der Sekretion von kationischen und zum Teil auch anionischen und zwitterionischen endogenen und exogenen Substanzen eine entscheidende Rolle (Tanihara *et al.*, 2007). Die bisherigen Untersuchungen über Expression und Gewebeverteilung von MATE konzentrierten sich bis jetzt in erster Linie auf Organe, die für den Metabolismus und die Entgiftung von endogenen Substanzen und Xenobiotika verantwortlich sind, nämlich Leber und Niere, wobei in der Leber nur MATE1 exprimiert wird während im Nierengewebe sowohl MATE1 als auch das ausschließlich nierenspezifisch exprimierte MATE2-K, eine Splicing Variante von MATE2 (Masuda *et al.*, 2006), identifiziert wurde. Aus diesem Grund konzentrierte sich diese Arbeit auf die Expression von MATE1 in humanen respiratorischen Epithelzellen, da auch inhalativ applizierte Arzneistoffe je nach Molekülgröße und Molekülladung potentielle Substrate von MATE1 darstellen.

Es konnte mithilfe von Westernblot gezeigt werden, dass das Effluxprotein MATE1 sowohl in humanen primären Alveolarepithelzellen (hAEPc) als auch in einigen Zelllinien (A549, Calu-3 und NCI-H441), die als *in vitro* Modelle für die morphologisch und funktionell verschiedenen Bereiche des Lungengewebes dienen, exprimiert wird. Immunzytochemische Analysen der Zellmonolayer haben gezeigt, dass MATE1 überwiegend im Bereich der Plasmamembranen und auch vereinzelt intrazellulär in den Zellorganellen lokalisiert ist.

Des Weiteren wurden Transfektionsstudien in A549 Zellen durchgeführt, um die Transfektionsbedingungen im Hinblick auf zukünftige Untersuchungen über die Rolle und Funktion von MATE1 in humanen respiratorischen Epithelzellen zu optimieren um einen reproduzierbaren Knockdown der MATE1 Expression zu erreichen. Erste Ergebnisse der Transfektionsstudien waren nicht konsistent und die Resultate schwankten trotz gleicher Transfektionsbedingungen um bis zu 40%. Aus diesem Grunde bedarf es einer weiteren

Optimierung der Transfektionsbedingungen und damit auch des Transfektionsprozesses. Wichtig für die Zukunft sind weiterführende Untersuchungen der Funktionalität und Substratspezifität von MATE1 im Lungengewebe.

3. Curriculum vitae

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