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Research paper

Monolayers of porcine alveolar epithelial cells in primary culture as an *in vitro* model for drug absorption studies

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Abstract

Filter-grown monolayers of porcine alveolar epithelial cells (pAEpC) in primary culture have been characterized as an *in vitro* model for pulmonary absorption screening of xenobiotics, including substrates of efflux systems.

Experimental conditions and a protocol for transport experiments were optimized using transepithelial electrical resistances (TEER) and permeability of marker compounds as acceptance criteria. Since new drugs often feature poor water solubility, monolayer integrity in the presence of a solubilizer (dimethyl sulfoxide) was tested. Transport studies were carried out with budesonide and triamcinolone acetonide, i.e., two drugs commonly administered to the lungs. Furthermore, expression of P-glycoprotein (P-gp) was assessed by immunofluorescence microscopy and transport studies employing the substrates rhodamine 123 and digoxin.

Hydrocortisone-supplemented (0.5 μg/ml) small airway basal medium as transport buffer and a maximal solubilizer concentration of 1.5% dimethyl sulfoxide were found to provide suitable conditions for drug transport studies across pAEpC, as reflected, e.g., by a minimum TEER of 600 $\Omega\,cm^2$. Permeation of marker compounds was reproducible throughout several cell preparations and proved the model successful in distinguishing between low- and high-permeable drugs. P-gp expression was confirmed by immunocytochemistry, even though transport studies revealed no polarity in transepithelial marker transport.

In conclusion, our results demonstrate that filter-grown monolayers of pAEpC can be used to study drug transport across alveolar epithelial barrier and thus, may represent a suitable *in vitro* model for pulmonary drug absorption and delivery.

Keywords: Primary cell culture; P-glycoprotein; Porcine lung; Transepithelial permeability; Pulmonary drug absorption

1. Introduction

Today, several *in vitro* models of pulmonary epithelia are available, including primary cultures and cell lines. However, with respect to the alveolar epithelium of the distal lung, the number of established models is still limited [1–3]. Isolation of porcine alveolar epithelial cells (pAEpC) and their primary culture to polarized monolayers has been described previously [4,5]. Hence, this subsequent study focuses on the suitability of the pAEpC model with regard to drug absorption studies. Appropriate experimental conditions are necessary to allow the comparability of results obtained from different cell isolations. Parameters have to be developed to prove and secure the reproducibility of the experimental system. In this context, drug development, solubilizers (e.g., DMSO or alcohols) are often used to overcome solubility problems of test compounds, but are simultaneously known to affect cultured cells.

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Therefore, it is important to assess the effect of solubilizing agents on the integrity of a cell monolayer. Furthermore, analytical quantification of drug compounds is facilitated, when a less complex matrix than featured by most of the cell culture media is used. Thus, in drug absorption studies, the latter are often replaced by simple, chemically defined, transport buffers, e.g., Krebs–Ringer buffer (KRB). However, the choice of a transport buffer might again prove crucial in terms of monolayer integrity, cell morphology, and expression/induction of transporter proteins. Other points of interest are the robustness of such a model in experimental handling, as well as presence and function of active drug transport proteins, in particular efflux systems.

Efflux systems are often responsible for the inability of drugs or other xenobiotics to overcome epithelial or endothelial barriers. Numerous studies have reported the presence of transporter proteins in various *in vitro* models, such as the intestinal Caco-2 cell line, models of the blood–brain barrier, MDCK, the alveolar cell line A549, and the bronchial cell lines Calu-3 and 16HBE14o- cells [6–13]. The multidrug resistance (MDR) gene encoded P-glycoprotein (P-gp) is the most prominent drug efflux transporter and belongs to the family of ABC transporters (ABC = ATP-binding cassette). In order to evaluate *in vitro* models with respect to their suitability for functional studies, it is relevant to know about the presence of such transporter systems.

The aims of this study were to: (i) optimize culture conditions and an experimental protocol for the new pAEpC model to be used in drug transport studies, (ii) to characterize this model with respect to the expression of efflux systems, and (iii) to apply this model by assessing the permeability of two drugs, budesonide and triamcinolone acetonide, which are typical for pulmonary administration. This should allow us to compare such data with those from other *in vitro* models, such as human bronchiolar cell lines (Calu-3, 16HBE14o-), primary rat or human alveolar epithelial cell monolayers, the human intestinal cell line Caco-2, and primary cultures of porcine brain endothelial cells (PBEC).

2. Materials and methods

2.1. Cell isolation and culture conditions

The methods of isolation and culture of porcine alveolar epithelial cells (pAEpC) were described in a preceding report [5]. The other cell types used in this study (e.g., Caco-2, Calu-3, 16HBE14o-, primary porcine brain endothelial cells (PBEC) and human alveolar epithelial cells (hAEpC)) were cultivated according to established protocols [14–18].

2.2. Transport studies

SAGM (small airway growth medium, Cambrex, Verviers, Belgium), supplemented with gentamycin, ampicillin (50 μg/ml each), and penicillin G (200 units/ml), served as transport buffer in initial transport experiments. In this context, sodium fluorescein (Flu-Na) at a final concentration of 10 μg/ml was chosen as marker for paracellular diffusion [5]. Due to the wide use of Flu-Na for this purpose, resulting data enable us to compare different *in vitro* models (e.g., primary cell cultures vs. cell lines) modeling different levels of the respiratory tract. Subsequent routine transport studies included the determination of permeability coefficients of the lipophilic propranolol as marker for high permeability [19] and rhodamine 123 as marker for P-gp-mediated efflux.

In order to find a suitable transport buffer for the pAEpC model, the following alternatives were tested: Krebs–Ringer buffer plus hydrocortisone (KRB + HC), SABM (small airway basal medium, Cambrex) and SABM + HC. The cell culture medium used for pAEpC, SAGM, which is obtained by adding a supplement kit of various growth factors, hormones and also hydrocortisone, to the basal medium, SABM, served as control. At days 5–8 of culture, cell culture medium was replaced by the respective transport buffer; pAEpC monolayers were then transferred into new cluster plates. TEER was monitored over time periods of up to 48 h, and experiments were completed by a transport study using Flu-Na.

Prior to transport experiments, the culture medium was replaced by SABM supplemented with 0.5 μg/ml hydrocortisone. Experiments were conducted on days 5–8 of culture, when cell monolayers reached plateau values in their TEER, as measured with an epithelial voltohmmeter (EVOM, WPI, Berlin, Germany) equipped with STX-2 "chopstick" electrodes. Subsequent to this exchange, cells were allowed to recover for at least 16 h (overnight). After equilibration, a minimum TEER value of 600 $\Omega\,cm^2$ was considered sufficiently high for drug transport studies.

To initialize a transport experiment, half the volume of the donor compartment (i.e., 250 μl for transport direction apical-to-basolateral (ab), and 750 μl for basolateral-to-apical (ba)) was replaced with the respective transport buffer, containing the test compound. The initial concentration of test molecules in the donor fluid was assayed by drawing 20-μl samples immediately after $t = 0$. Receiver samples (100 μl) were drawn serially from the respective downstream fluids at $t = 30$, 60, 90, and 120 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 a transport experiment, a 20-μl sample was

Abbreviations: (ab), transport direction from apical to basolateral; (ba), transport direction from basolateral to apical; Flu-Na, sodium fluorescein; hAEpC, human alveolar epithelial cells in primary culture; IQR, interquartial range; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; pAEpC, porcine alveolar epithelial cells in primary culture; P_{app}, apparent permeability coefficient; PBEC, porcine brain endothelial cells; P-gp, P-glycoprotein; Pro, propranolol; Rh123, rhodamine 123; SABM, small airway basal medium; SAGM, small airway growth medium; TEER, transepithelial electrical resistance.

drawn from the donor fluids. During the study, monolayers were incubated in a water-saturated 5% CO_2 atmosphere at 37 °C. Each transport experiment was performed using three monolayers (obtained from 2 to 10 different cell preparations) for flux measurements in either the (ab) or (ba) direction. In order to assess the integrity of monolayers during the flux studies, TEER was measured before and after each transport experiment.

Unidirectional fluxes (J) were determined from steady-state appearance rates of each compound accumulating in the receiver fluid. The apparent permeability coefficient, P_{app}, was calculated according to the equation

$$P_{app} = J/(A \times C_i), \quad (1)$$

where C_i is the initial concentration of the substance under investigation in the donor fluid and A is the nominal surface area of cell monolayers (1.13 cm^2) utilized in this study.

2.3. Quantification of epithelial cell height and surface coverage

To study the effect of transport buffer on cell morphology and barrier function, pAEpC monolayers were transferred from SAGM to SABM + HC. Control filters were kept in SAGM, which was refreshed in parallel. After an incubation time of 24 h, TEER was measured and monolayers were fixed and prepared for electron microscopy as described previously [5]. Specimens were compared with regard to their morphological ultrastructure, confluence, and height of cells.

Height of cell monolayers was measured in images taken from the block face of embedded filters after sectioning using a scanning electron microscope (ESEM XL30 FEG, FEI Co., The Netherlands) [20]. The advantage of this technique is that the block face does not show mechanical distortions or artifacts, which usually occur in mechanical sections through cells on filter supports. Thus block face imaging allows undisturbed quantification. Height measurements were performed on the screen using the operating software of the microscope. Two different methods were used to measure *monolayer thickness*. Method 1: A grid (25 μm mesh size) was placed on a section profile of the monolayer. Height of the cells or cellular processes was measured perpendicular to the filter surface at all positions where the grid crosses the filter (monolayer side). This procedure allows equidistant cell height measurements. Free filter area was defined as zero in height, but ignored for median calculation. Method 2: Height measurements were only taken at all cell nuclei visible in a section. The region of the cell nucleus represents usually the maximal height of a cell.

2.4. Tolerance to dimethyl sulfoxide (DMSO)

As described above, pAEpC monolayers were switched from culture medium to transport buffer and allowed to equilibrate for at least 16 h. Subsequently, DMSO was applied at final concentrations of 0, 0.25, 0.5, 1, 2, and 3% to the apical or basolateral sides of the monolayers. Flu-Na, administered in parallel (at 10 μg/ml), served as marker to test monolayer integrity. Permeability was analyzed for (ab) and (ba) transport, to distinguish DMSO impact on both aspects of the epithelium. Receiver samples were drawn serially from the respective downstream fluids at $t = 30$, 60, 90, and 120 min. After each sampling, fresh transport buffer of an equal volume was returned to the receiver side to maintain a constant volume. Each transport experiment was performed using three monolayers (obtained from four cell preparations). In order to assess the integrity of monolayers during the flux studies, TEER was measured before and after each transport experiment. Changes in TEER were expressed as [%] of the initial value.

2.5. Expression and functionality of P-glycoprotein

2.5.1. Immunofluorescence microscopy

The anti-P-glycoprotein antibody (clone F4, Sigma, Deisenhofen, Germany) was diluted 1:100 in PBS containing 1% (w/v) bovine serum albumin (BSA). Mouse IgG1 was used as isotypic control. Transwell-grown pAEpC monolayers were stained on days 5 and 6 after cell plating. Cells were fixed for 10 min with 2% (w/v) paraformaldehyde and blocked for 10 min in 50 mM NH_4Cl, followed by permeabilization for 8 min with 0.1% (w/v) Triton X-100. After a 60-min incubation with 100 μl of the diluted primary antibody, the cell monolayers were washed three times with PBS, before incubation with 100 μl of a 1:100 dilution of an FITC-labeled goat anti-mouse $F(ab')_2$ fragment (Dako, Hamburg, Germany) in PBS containing 1% (w/v) BSA. Propidium iodide (1 μg/ml) was then added to counterstain cell nuclei. After 30-min incubation, the specimens were washed three times with PBS and embedded in FluorSave anti-fade medium (Calbiochem, Bad Soden, Germany). Images were obtained with a confocal laser scanning microscope (MRC-1024, Bio-Rad, Hemel, Hempstead, UK) with the instrument settings adjusted, so that no positive signal was observed in the channel corresponding to the green fluorescence of the isotypic control.

2.5.2. Transport of efflux markers

A MDR (multidrug resistance) phenomenon reveals itself as a polarized transport of substrates across a biological barrier; for apically localized efflux systems the (ab)-permeation is exceeded by the (ba)-permeation. Addition of specific inhibitors will compensate this effect and may help to identify such efflux systems. The quotient of permeability coefficients for both transport directions P_{app}(ba)/P_{app}(ab) is referred to as the transport ratio [21]. In absence of any active transport or efflux, this ratio is typically observed between 1 and 2, whereas in case of the presence of an efflux system, a higher ratio is found.

Functionality of the efflux pump, P-glycoprotein (P-gp/MDR1), has been tested using the substrates, rhodamine 123 [22] and [^{3}H]digoxin [23]. Verapamil (at 40 μM) and ritonavir (at 7 μM) are frequently used as inhibitors of P-gp function [6,24]. Inhibitors were present in both, donor and receiver solutions, to guarantee continued inhibition.

In addition, bi-directional fluxes of further substances across pAEpC monolayers were investigated: doxorubicin (a more selective P-gp-substrate than rhodamine 123) [8], sulforhodamine 101 (a MRP [multidrug resistance-related protein] substrate) [25], and the efflux pump substrate, furosemide [26,27].

2.6. Compound analytics

Fluorescence of samples was analyzed in 96-well plates using a fluorescence plate reader (Victor^2, Wallac Perkin Elmer, Rodgau, Germany) at excitation and emission wavelengths of 485 and 553 nm, respectively. Radiolabeled compounds were measured by liquid scintillation counting (Wallac 1450 Microbeta, Wallac Perkin-Elmer). All other compounds were analyzed by HPLC (Waters, Eschborn, Germany) applying validated protocols. If necessary, samples were diluted with the respective transport buffer.

2.7. Statistics

Results are expressed as means ± SD, except from Fig. 2, where SEM was used instead. Significance ($p < 0.05$) of differences in the group mean values for TEER and P_{app} was determined by two-way analysis of variance (ANOVA), followed by Student–Newman–Keuls post hoc tests.

Electron microscopy results for surface coverage and epithelial cell height are expressed as median and corresponding IQR (interquartial range). Significance ($p < 0.01$ for analysis of surface coverage and $p < 0.05$ in case of monolayer thickness) of differences in the total populations for the respective distributions was determined by test for homogeneity according to Kolmogorov and Smirnov.

3. Results

3.1. Integrity of pAEpC monolayers

An inverse correlation between the permeability coefficient P_{app} of Flu-Na (10 μg/ml) across pAEpC monolayers and the TEER value has been described previously [5]: No further significant decline in permeability of this hydrophilic marker was detected for monolayers with TEER values >600 Ω cm^2. Therefore, this value was defined as a minimal acceptance criterion for monolayer integrity in drug absorption experiments using the pAEpC model.

Flu-Na was chosen, because of the vast amount of data available from transport studies using the substance across other *in vitro* cell culture models of the respiratory tract; bronchial cell lines (Calu-3, 16HBE14o-), primary human alveolar epithelial cells (hAEpC), and the intestinal cell line Caco-2. The average permeability coefficient P_{app} for pAEpC was calculated as $3.49 \pm 1.66 \times 10^{-7}$ cm/s ($n = 39$ filters, obtained from 14 independent cell preparations, transport buffer SAGM) [5]. Fig. 1 compares permeability values across these barrier-forming models (data either generated at Saarland University (*) or Across Barriers GmbH). Permeability coefficients for Flu-Na across all models ranged between 1.04 and 8.50×10^{-7} cm/s. Considering variations reported in the literature for cell cultures in different laboratories (e.g., Caco-2 [5,28,29]), the paracellular tightness of pAEpC monolayers was similar to the other models, indicating comparable barrier properties.

3.2. Selection of suitable transport buffer

An ideal transport buffer should be complex enough to maintain the epithelial barrier function of cell monolayers, yet simple enough to enable analysis by HPLC. Krebs–Ringer buffer plus hydrocortisone (KRB + HC), SABM, and SABM + HC were tested in this regard. The influence of selected transport buffers on the TEER is illustrated in Fig. 2.

Transfer of pAEpC monolayers from culture medium to any other liquid, or exchange of culture medium, always caused a decline in TEER of about 50% (measured approx. 2 h after replacement), which was followed by a slow recovery. Therefore, an equilibration time of 16 h was chosen after each medium change prior to a later transport experiment. Two-way analysis of variance revealed a statistically significantly ($p \leqslant 0.001$) lower TEER value for KRB compared to the other test solutions. Permeability coefficients

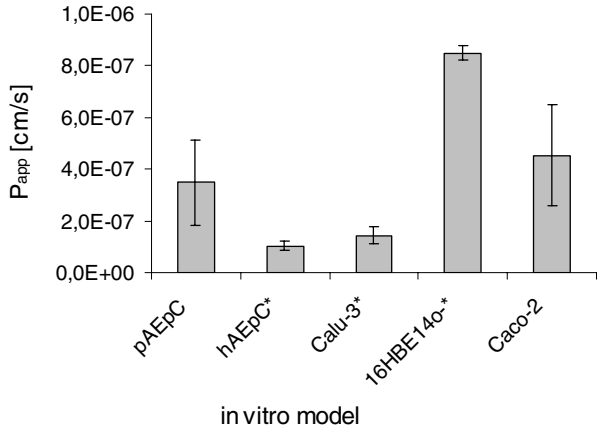

Fig. 1. Comparison of epithelial barrier properties quantified for different *in vitro* models of the respiratory tract, including cell lines and primary cells. Absorptive permeability coefficients (mean ± SD) were determined for the hydrophilic marker compound Flu-Na. * (Asterisk) indicates data from Saarland University ($n = 12$ except for pAEpC: $n = 39$, and for Caco-2: $n = 57$) [5,13]. Despite the differences in epithelial cell type and donor species, the passive paracellular diffusion across the monolayers was within the same order of magnitude (×10^{-7} cm/s).

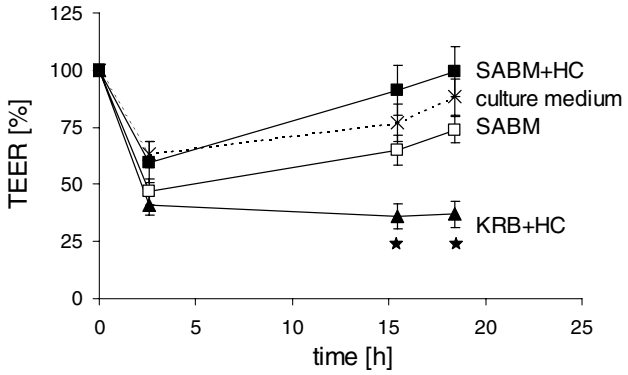

Fig. 2. Influence of different transport buffers on the transepithelial electrical resistance (TEER) of pAEpC monolayers. TEER values prior to replacement of culture medium were assumed as 100%. TEER was monitored over 25 h. Changes were expressed as decline [%] ±SEM from $n = 12$ monolayers obtained from four different cell isolations. Asterisks (*) mark statistical significance to other test buffers (culture medium SAGM = control).

for Flu-Na were also determined and gave similar results (data not shown). According to these data, KRB is not a suitable transport buffer for pAEpC monolayers, whereas SABM and SABM + HC seemed appropriate and comparable.

3.2.1. Cell morphology

After 24 h of incubation with SAGM (culture medium) or SABM + HC (transport buffer), the morphology of pAEpC monolayers differed only slightly. At day 6, most of the filter surface was covered by a confluent monolayer [5], which was dominated by flat cells with broad cytoplasmic extensions and interspersed by cells with protruding nuclei. Fig. 3A and B visualize the ultrastructure of these two cell morphologies. Functional cellular junctions were already present, as confirmed by TEER measurements preceding the fixation for electron microscopy and which revealed comparable resistance values for both treatments. The fraction of flat, extended cells seemed higher in case of the hydrocortisone containing SABM (Fig. 4A and B). This observation was supported by the result of epithelial height measurements.

3.2.2. Height of monolayers

A slight impact on epithelial height was observed between cell monolayers incubated in SAGM or SABM + HC (Table 1). Because of the high IQR of the height values, the relative difference between culture medium and transport buffer indicates just a trend: Cells in the nuclear region, as well as peripheral extensions, are more flat (16–17%) when grown in SABM + HC, in comparison to cells kept in SAGM. However, apart from the monolayer height, growth and development of cells was similar for the compared test liquids within an observation time of 24 h.

Concluding from these results, the hydrocortisone-supplemented basal medium was chosen as routine transport buffer, because of its close resemblance to the culture medium and at the same time lower complexity. Moreover, pAEpC monolayers transferred into SABM + HC flatten out faster.

3.3. Tolerance to DMSO

Effects of the solubility enhancer DMSO on the integrity of pAEpC monolayers were compared by monitoring TEER and the permeation of Flu-Na. Fig. 5 illustrates changes in TEER (A) and in flux of Flu-Na (B) caused by the tested DMSO concentrations. The solvent was applied to either side of pAEpC monolayers.

Only at the highest concentration of DMSO (3%), the monitored parameters differed significantly from values obtained without DMSO. Concentrations up to 1.5% of DMSO did not affect the integrity of cultured pAEpC monolayers. Nevertheless, effects of DMSO on active transport processes were not tested and thus, cannot be completely excluded.

3.4. Expression and functionality of P-glycoprotein

3.4.1. Immunofluorescence microscopy

A positive signal for P-glycoprotein/MDR1 was detected in pAEpC monolayers at days 5 and 6 of culture by immunocytochemistry, as illustrated in Fig. 6. The

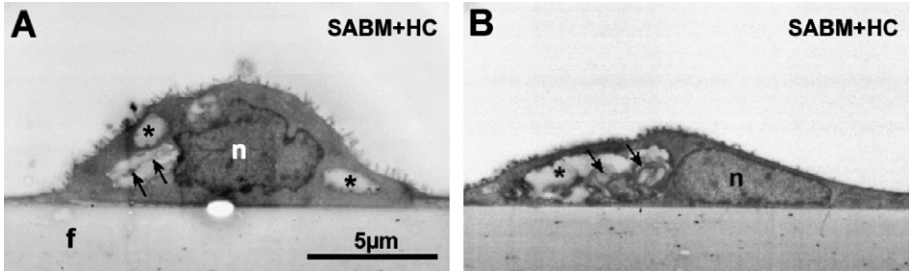

Fig. 3. Shape and internal ultrastructure of cells incubated in transport buffer SABM + HC by block-face imaging. Two representative cells, one with a more round sectioning profile (A) and one with a flatter profile (B), are shown. Both cells contain vacuole-like structures (*) filled with membranous material (arrows). n, nucleus.

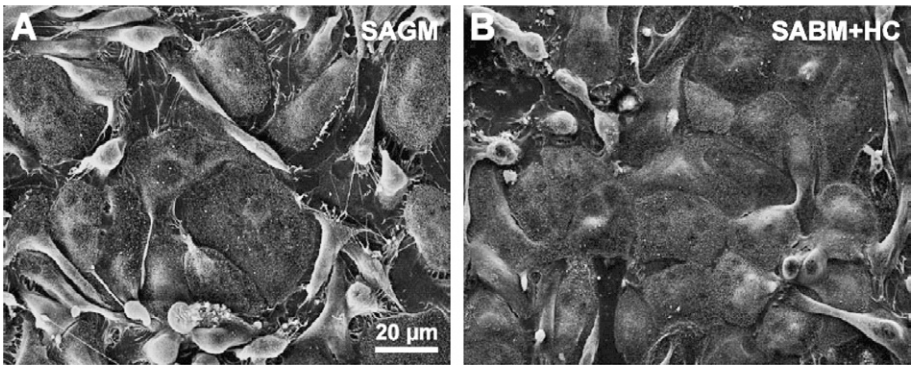

Fig. 4. Influence of transport buffers on cell morphology. Representative scanning electron microscopy of pAEpC monolayers (on day 6) incubated with culture medium SAGM (A) or transport buffer SABM + HC (B), respectively. When comparing the monolayer sections, a larger proportion of flat extended cells can be observed in case of incubation with SABM + HC. This situation was the same in several other sections as well.

Table 1
Survey of epithelial cell height in pAEpC monolayers quantified by two different methods

	SAGM			SABM + HC			Relative difference (%)
	Height[a] (µm)	IQR	n[b]	Height[a] (µm)	IQR	n[b]	
Method 1	**2.7**	5	123	**2.3**	2.6	147	**16**
Method 2	**7.8**	4.5	78	**6.45**	3.9	85	**17**

[a] Height is given as median value with corresponding interquartial range (IQR).
[b] n indicates the number of measurements.

functionality of the protein, to act as an active efflux system, was investigated in the following transport studies.

3.4.2. Transport of efflux markers

Several known substrates of drug transporters were studied in both, absorptive and secretory direction across monolayers of pAEpC. The determined permeability coefficients and transport ratios are summarized in Table 2.

The (ba)/(ab) transport ratio of the P-gp-substrates, rhodamine 123 and [^{3}H]digoxin, was calculated as approximately 1 (i.e., no net secretion was observed). The result did not change in presence of the P-gp-inhibitors, verapamil and ritonavir. A polarized P-gp-mediated transport across porcine alveolar epithelial cell monolayers could therefore be excluded *in vitro* (at the investigated time of culture, days 5–8). The transport ratios of other ABC-transporter substrates, sulforhodamine 101 and furosemide, also did not exhibit any transporter activity. Doxorubicin, another MDR substrate, proved cytotoxic at the used concentration and was therefore not further investigated.

3.5. Quality control and acceptance criteria

When using a cell culture system for the assessment of drug permeability, certain exclusion limits and acceptance criteria have to be introduced. For this purpose, Flu-Na ([ab]-direction) was chosen as low permeability marker and propranolol ((ab)-direction) as high permeability marker. Rhodamine 123 ((ab)- and (ba)-direction) served as marker for P-gp activity. P_{app} values of these selected compounds were routinely determined throughout several cell isolations.

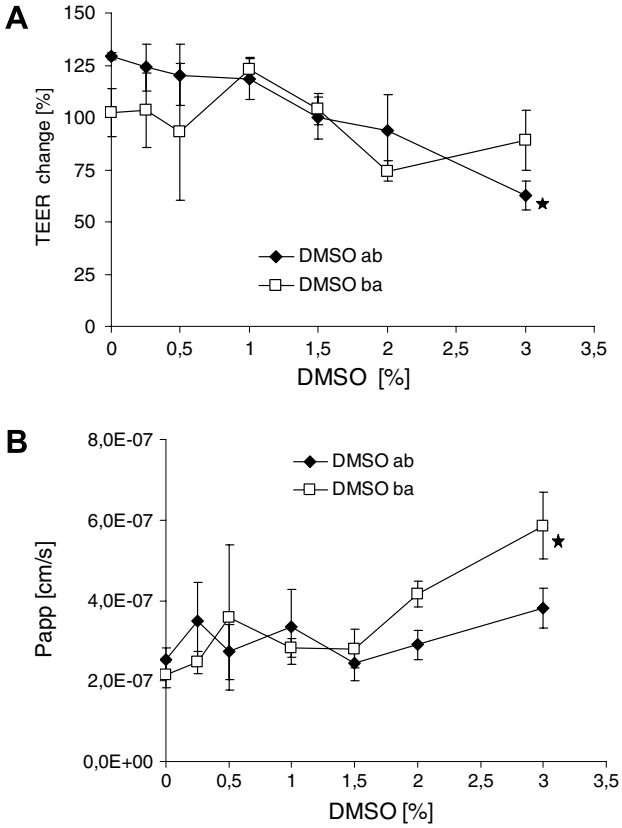

Fig. 5. Influence of the solubility enhancer DMSO on epithelial barrier properties of pAEpC monolayers (asterisks mark statistical significance of differences to other DMSO concentrations according to analysis of variance). (A) Effect of varied DMSO concentrations on the development of TEER (mean ± SD, $n = 6$). (B) Changes in monolayer integrity caused by different DMSO concentrations assessed by permeability of 10 µg/ml Flu-Na (mean ± SD, $n = 6$) for both transport directions (ab) and (ba). A statistically significant difference in TEER and in permeability was observed at the highest concentration of 3% DMSO.

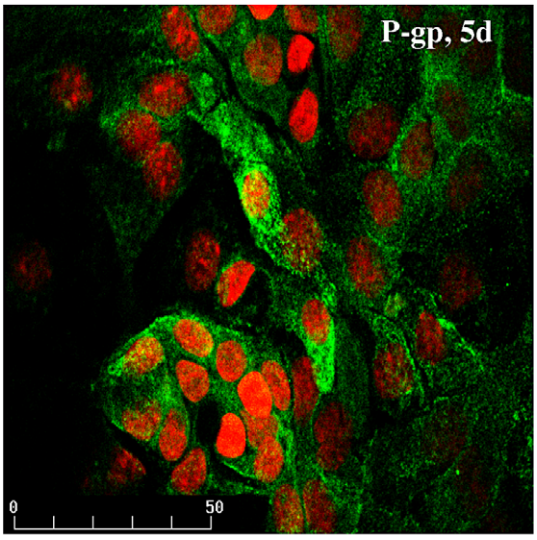

Fig. 6. Confocal laser scanning microscopy of Transwell-grown pAEpC monolayers immunolabeled for P-glycoprotein. Cell nuclei were counterstained with propidium iodide (red). At days 5 and 6 of culture, pAEpC displayed a positive signal for P-gp (green) at the apical aspects of the cell membranes, as well as P-gp positive spots in the cytoplasm. Scale bar represents micrometer (μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

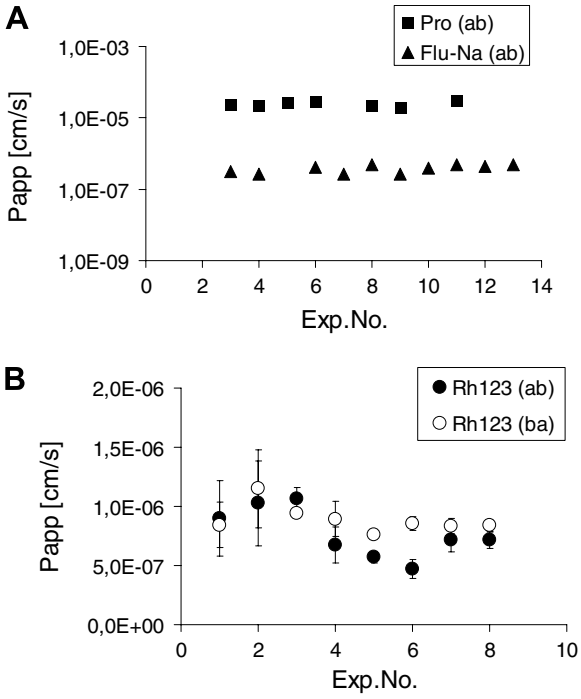

Fig. 7. Permeability coefficients (mean ± SD, $n = 3$) observed throughout a series of pAEpC isolations. (A) High permeability marker propranolol (■) and low permeability marker Flu-Na (▲) determined for transport direction (ab); log scale. Data confirm reproducible permeation characteristics for different cell isolations. (B) P_{app} values of the efflux marker rhodamine 123 (Rh123), determined for transport directions (ab) (●) and (ba) (○).

According to the data presented in Fig. 7A, permeability of both, high and low permeability markers, was reproducible across independent cell preparations. The mean permeability coefficients were: $0.38 \pm 0.11 \times 10^{-6}$ cm/s for Flu-Na and $25.31 \pm 5.03 \times 10^{-6}$ cm/s for propranolol. The number of tested cell isolations was 10 (Flu-Na) and 7 (pro), respectively (triplicates each, transport buffer SABM + HC). Bi-directional transport studies with rh123 also showed a consistency over several cell isolations (Fig. 7B), confirming the absence of a functional P-gp-mediated efflux.

Based on these results and considering the variations attributed to primary cell culture, the following acceptance criteria were defined: $P_{app}(\text{pro}) \geqslant 2.0 \times 10^{-5}$ cm/s and $P_{app}(\text{Flu-Na}) \leqslant 5.0 \times 10^{-7}$ cm/s. With respect to efflux systems, the calculated $P_{app}(\text{ba})/P_{app}(\text{ab})$ transport ratio should not exceed 1.5 for rh123. Cell monolayers, which did not meet these criteria, were excluded from use in subsequent transport studies.

3.6. Transepithelial permeability of drug substances relevant to pulmonary drug delivery

Budesonide and triamcinolone acetonide, which are commonly administered by oral inhalation for the treatment of acute and chronic airway diseases, were tested for their permeability across pAEpC monolayers. Permeability coefficients are summarized in Table 3.

The results found across pAEpC monolayers were well comparable to data obtained from other *in vitro* models of respiratory epithelia, such as human alveolar epithelial primary cells (hAEpC) and two human bronchial epithelial cell lines Calu-3 and 16HBE14o- (Fig. 8). In detail, pAEpC monolayers showed a slightly higher permeability for

Table 2
Permeability coefficients and calculated transport ratios of MDR substrates across pAEpC monolayers

Substrate	Concentration	n[a]	P_{app}(ab)[b]	P_{app}(ba)[b]	Ratio (ba)/(ab)
Rhodamine 123	10 μM	8	0.77 ± 0.27	0.89 ± 0.19	**1.2**
[^{3}H]Digoxin	2.5 μCi/ml	2	1.91 ± 0.27	2.12 ± 0.25	**1.1**
[^{3}H]Digoxin + 7 μM ritonavir	2.6 μCi/ml	2	1.90 ± 0.27	1.83 ± 0.42	**1.0**
Sulforhodamine 101	100 μM	2	0.39 ± 0.13	0.45 ± 0.15	**1.2**
Doxorubicin	100 μM	2	Toxicity problems		–
Furosemide	200 μM	3	0.66 ± 0.11	0.83 ± 0.19	**1.3**

The figures in bold represent the ratio (ba)/(ab).
[a] n represents the number of tested cell isolations (triplicates each).
[b] Mean P_{app} ($\times 10^{-6}$ cm/s) values ± SD for bi-directional transport.

Table 3
Permeability coefficients and calculated transport ratios of pulmonary administered drug substances across pAEpC monolayers

Compound	Concentration (μM)	P_{app}(ab)[a]	P_{app}(ba)[a]	Ratio (ba)/(ab)
Budesonide	30	2.65 ± 0.49	2.80 ± 0.20	**1.1**
Triamcinolone acetonide	60	1.97 ± 0.63	1.49 ± 0.01	**0.8**

The figures in bold represent the ratio (ba)/(ab).
[a] Mean P_{app} ($\times 10^{-5}$ cm/s) values ± SD for bi-directional transport.

budesonide if compared to the other three *in vitro* models, whereas permeation in case of triamcinolone acetonide was similar. No net directionality was observed for any substance (neither budesonide nor triamcinolone acetonide) across cell monolayers of any *in vitro* model (see Table 3, Fig. 8).

4. Discussion

In the presented study, a robust and easily accessible model for *in vitro* drug transport across the alveolar epithelial barrier was established. Porcine alveolar epithelial cells (pAEpC) were grown to confluent monolayers with functional cell junctions. Experimental conditions were defined to use pAEpC monolayers for the assessment of drug permeability. We found that hydrocortisone-supplemented SABM is the optimal transport buffer of the tested alternatives; a maximum final concentration of 1.5% DMSO can be tolerated; 16 h of equilibration time after transfer of cells to transport buffer is necessary; a minimum TEER of 600 Ω cm^2 ensures a tight monolayer. No functionally active MDR-related efflux systems were detected, although P-gp was found to be expressed along the apical aspect of cultured monolayers by immunofluorescence microscopy. Electron microscopy revealed that cell differentiation was not complete at day 6 of culture. Despite the characteristic type I and type II morphology, the inner structure of cells (e.g., vacuoles filled with membranous material) indicated an intermediate mixture of pneumocyte features. Nevertheless, the plateau phase in TEER development (culture days 5–8) [5] unified the presence of characteristic alveolar cell markers and appropriate barrier properties for drug transport studies as described previously [ref. 5].

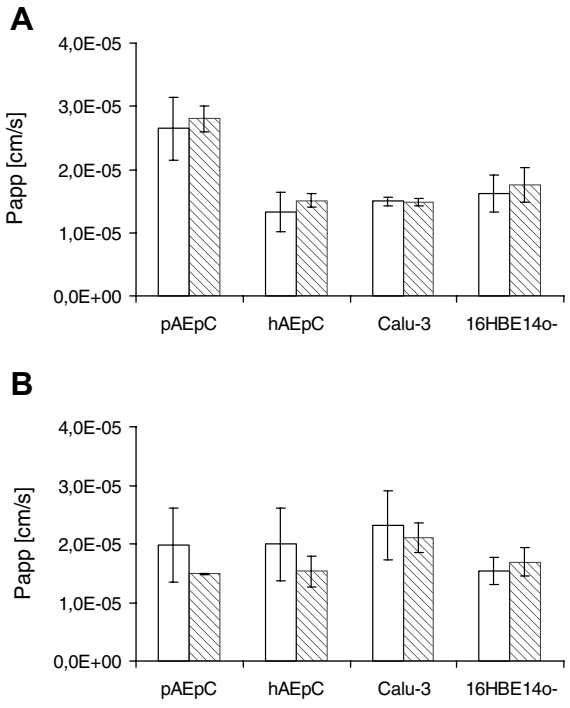

Fig. 8. Permeability coefficients (mean ± SD, $n = 8$; $n = 3$ for pAEpC) of pulmonary delivered drug compounds, budesonide (A) and triamcinolone acetonide (B), were compared in bi-directional transport studies across pAEpC and hAEpC in primary culture, as well as the bronchial cell lines Calu-3 and 16HBE14o-. Open columns represent (ab)-, hatched columns indicate (ba)-transport direction.

Table 4
In vitro cell culture models and their reported transport ratios for the P-gp-substrate rhodamine 123

Model	Ratio P_{app}(ba)/P_{app}(ab) for rhodamine 123
pAEpC	1.2
hAEpC	3.1*
Calu-3	4.9
16HBE14o-	2.9*
Caco-2	13.2
PBEC	2.4

Unpublished data of Across Barriers GmbH; except * = Saarland University.

Currently available cell lines, such as A549 (human), L2 (rat), and AK-D (cat), lack the ability to form tight monolayers, due to their missing or incomplete expression of functional tight junctions. Nevertheless, as they show alveolar epithelial type II cell morphology, these cell lines might represent appropriate models for studies of metabolism or toxicology. However, the use of cell culture models for drug transport studies requires the formation of tight polarized monolayers. Thus, with regard to the alveolar epithelium of the distal lung, one still has to rely on primary cultured pneumocyte monolayers. Although exhibiting some phenotypic features of alveolar epithelial cells, continuous cell lines generally appear not capable to imitate permeation properties of intact alveolar epithelium. Due to this confined imitation of barrier properties, these cell lines should not be used for *in vitro* drug absorption studies [30]. Instead, primary culture of isolated pneumocytes seems to be the only reasonable alternative for research on alveolar epithelial transport processes and cell biology. A human system [20,31,32] is certainly preferable, but due to the lack of availability of human tissue and

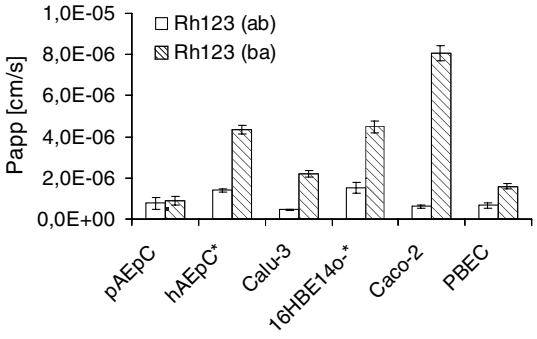

Fig. 9. Comparison of permeability coefficients (mean ± SD, $n = 12$; $n = 24$ for pAEpC) for the P-gp-substrate, rhodamine 123, determined in bi-directional transport studies across different *in vitro* models: porcine (pAEpC) and human (hAEpC) alveolar epithelial cells, the bronchial cell lines Calu-3 and 16HBE14o-, the intestinal cell line Caco-2 and porcine primary brain endothelial cells (PBEC). The most apparent efflux was observed across Caco-2 monolayers (data generated at Across Barriers GmbH, or at * = Saarland University).

ethical issues pertaining to use of human tissues in certain countries, most studies are based on isolation and culture of cells from the lungs of small laboratory animals including mouse [33,34], rat [35,36], and rabbit [37,38]. The similarity in size and weight speaks in favor of the establishment of a porcine-derived model [4,5]. An interspecies comparison of characteristics of cells from the alveolar regions of normal lungs showed relatively constant numbers of cell types in the alveolar region and in their average thickness, size, and surface areas across several species [39]. These results might justify the use of animal derived *in vitro* models.

Several efflux transporters, including the well-described P-gp, have been discovered in various *in vitro* models, as well as in native tissues. Focusing on the human respiratory tract, a study comparing expression and distribution of P-gp in normal and tumor human tissues detected the protein in normal human bronchial epithelium, less clearly in alveolar macrophages, but not at all in pneumocytes [40]. This result was confirmed by Western blot analysis using the alveolar epithelial cell line A549, where no P-gp or multidrug resistance-related protein (MRP) was found [11]. During examination of bronchial epithelium derived from lung carcinoma patients, the MDR1 glycoprotein was detected: (i) at the apical surface of ciliated epithelial cells (from surface epithelium) or ciliated collecting ducts, (ii) on apical and lateral surfaces of serous cells from bronchial glands and (iii) at the luminal surface of endothelial cells in bronchial capillaries. In contrast, no staining of mucus secreting cells was observed [41]. In case of MRP minimal or no expression was found in normal alveolar type I and type II cells [42]. But the Calu-3 cell line, which is representative for bronchial airway epithelium, was suggested to possess MRP1 functional activity that is subordinate to P-gp efflux [12].

The fact that most data generated in pAEpC monolayers are comparable to those reported for the human hAEpC model recommends the porcine model as an interesting alternative. The differences regarding functionality of MDR proteins between the two models are compensated by the easy availability of tissue material, without ethical approval.

In comparing different *in vitro* models for the respiratory tract, the lowest (ba)/(ab) transport ratio of 1.2 or rhodamine 123 was found in pAEpC monolayers (Fig. 9, Table 4). Nevertheless hAEpC monolayers, as the next closely related approach to pAEpC, also showed a comparatively low transport ratio of 3.1. More distinct efflux phenomena could be detected in the Caco-2 model featuring a ratio of 13.2.

With regard to morphological characterization and transepithelial permeability, results obtained with pAEpC were similar to those reported for related cell culture models and the native tissue *in vivo* [2,4,13,20,22,32,35,39,43,44]. In total there was a good correspondence to other *in vitro* models and to the human alveolar model in particular. Nevertheless, in studies on the absorption of human proteins (e.g., insulin, calcitonin, and growth hormone) species differences could become relevant. Likewise, active transport processes, which may be of importance for the pulmonary application of low molecular weight compounds, are not considered so far.

5. Conclusions

Porcine alveolar epithelial cells in primary culture are well suited as an *in vitro* model for drug absorption studies relevant to the distal lungs. The cells have the ability to form polarized monolayers of characteristic alveolar epithelial type-I-like phenotype, which are able to distinguish between high and low permeability markers. In this study, the baseline parameters have been put down to use the pAEpC model as a screen in the routine assessment of drug permeability.

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