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Evaluation of layers of the rat airway epithelial cell line RL-65 for permeability screening of inhaled drug candidates

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ABSTRACT

A rat respiratory epithelial cell culture system for *in vitro* prediction of drug pulmonary absorption is currently lacking. Such a model may however enhance the understanding of interspecies differences in inhaled drug pharmacokinetics by filling the gap between human *in vitro* and rat *in/ex vivo* drug permeability screens. The rat airway epithelial cell line RL-65 was cultured on Transwell® inserts for up to 21 days at an air–liquid (AL) interface and cell layers were evaluated for their suitability as a drug permeability measurement tool. These layers were found to be morphologically representative of the bronchial/bronchiolar epithelium when cultured for 8 days in a defined serum-free medium. In addition, RL-65 layers developed epithelial barrier properties with a transepithelial electrical resistance (TEER) >300 Ω cm^2 and apparent ^{14}C-mannitol permeability (P_{app}) values between 0.5–3.0 × 10^{-6} cm/s; i.e., in the same range as established *in vitro* human bronchial epithelial absorption models. Expression of P-glycoprotein was confirmed by gene analysis and immunohistochemistry. Nevertheless, no vectorial transport of the established substrates ^{3}H-digoxin and Rhodamine123 was observed across the layers. Although preliminary, this study shows RL-65 cell layers have the potential to become a useful *in vitro* screening tool in the pre-clinical development of inhaled drug candidates.

1. Introduction

The inhaled route for drug delivery has been exploited for direct targeting of locally acting drugs since the 1950s (Barnes, 2009). More recently, the lung has also become an attractive alternative route for systemic delivery of compounds with poor oral bioavailability (Ehrhardt et al., 2008). While the human colonic Caco-2 cell line has been approved by the Food and Drug Administration (FDA) for permeability screening of orally administered drugs, an economical, ethical and high throughput model for absorption prediction of candidate inhaled drugs has yet to emerge.

In vitro models that have been employed for studying drug permeability, metabolism and toxicity in the bronchial epithelium include the Caco-2 cell line (Tronde et al., 2003), and the human bronchial epithelial cell lines Calu-3 (Meaney et al., 1999; Foster et al., 2000; Grainger et al., 2006), 16HBE14o- (Ehrhardt et al., 2002; Forbes et al., 2003) and BEAS-2B (Sporty et al., 2008). Additionally, commercially available normal human bronchial epithelial (NHBE) cells have been assessed for permeability modelling (Lin et al., 2007) and toxicity screening (Balharry et al., 2008).

Whilst interspecies variations in drug handling, pharmacokinetic and safety profiles are well recognised, *in vivo* animal data are required for regulatory approval of inhaled drugs, with the rat being the most commonly used species due to size and ethical justifications (Sakagami, 2006). Correlations between Caco-2 (Tronde et al., 2003), Calu-3 (Mathias et al., 2002) or 16HBE14o- (Manford et al., 2005) permeability data and absorption parameters in rat *in vivo* or isolated perfused lung (IPL) have been established for a limited number of drug compounds. However, instances where drug permeability in human respiratory cell culture systems failed to model rat *in/ex vivo* pulmonary absorption have been reported (Manford et al., 2005; Madlova et al., 2009).

The role of transmembrane transporters in drug disposition in the intestine, liver, kidney or brain is becoming increasingly recognised (Ayrton and Morgan, 2008). Amongst transporters present in the lungs (Bleasby et al., 2006), P-glycoprotein (P-gp, MDR1) and the organic cation/carnitine transporters (OCT and OCTN) have been detected in the human bronchial epithelium (Bosquillon, 2010). Although the influence of lung transporters on drug

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pharmacokinetic profiles remain largely unknown, OCT/OCTN-mediated transport of inhaled therapeutic compounds in bronchial epithelial cell culture models has been suggested (Ehrhardt et al., 2005; Nakamura et al., 2010; Mukherjee et al., 2012).

On the other hand, there is considerable debate regarding the impact of P-gp on drug disposition in the lungs. Functional studies in rat models have demonstrated negligible transporter-mediated absorption of P-gp substrates either *ex vivo* (Tronde et al., 2003; Madlova et al., 2009) or *in vivo* (Manford et al., 2005). In contrast, Francombe and colleagues have reported an increase in Rhodamine123 (Rh123) absorption from rat IPL in the presence of the P-gp potent inhibitor GF120918 in both the instillate and perfusate solutions (Francombe et al., 2008). Similarly, studies that have investigated the functionality of P-gp in human bronchial epithelial cell layers are conflicting (Bosquillon, 2010). Due to possible variations in substrate affinity for the human or rat transporters, a reliable assessment of P-gp involvement in pulmonary drug absorption might only be achieved through a combination of *in/ex vivo* data in rats and *in vitro* permeability measurements in both human and rat airway epithelial cell layers.

An *in vitro* model of the rat respiratory epithelium would assist in the evaluation of the role of transporters as well as interspecies discrepancies in inhaled drug permeability. Importantly, bias in *in vitro/in vivo* absorption correlations resulting from transporter heterology, variable substrate specificity and different pulmonary expression patterns in humans and rats would be minimised. This could improve the reliability of *in vitro* prediction and thus, guide the selection of drug candidates that progress to the late stages of pre-clinical development. Although a rat airway cell culture model is unlikely to replace drug testing in animals in the short term, it may nevertheless help reduce and refine the experimentation required.

RL-65 is a rat airway (bronchial/bronchiolar) epithelial cell line that was isolated from 5 day old Sprague–Dawley rats (Roberts et al., 1990). This has been exploited to investigate cell-signalling pathways (Van Putten et al., 2001; Blaine et al., 2001; Wick et al., 2005; Bren-Mattison et al., 2005; Nemenoff et al., 2008) or the epithelial–mesenchymal transition (Wang et al., 2009; Felton et al., 2011) in airway epithelial cells preferentially to other cell lines due its non-cancerous origin and spontaneous immortalisation. However, to date, the potential of RL-65 layers as an *in vitro* model for pre-clinical drug screening has not been assessed. In addition, although the cell line has recently been successfully grown on Transwell® cell culture inserts (Wang et al., 2009), its ability to form layers morphologically similar to the native upper airway epithelium at an air–liquid (AL) interface, as described for Calu-3 (Grainger et al., 2006) and NHBE (Lin et al., 2007) cells, has not yet been demonstrated.

Here, we report the optimisation of RL-65 cell culture conditions on Transwell® inserts at an AL interface. The morphology and barrier properties of cell layers grown in two different media were characterised. Additionally, expression of selected drug transporters was quantified and P-gp functionality investigated in the model. This study provides an initial appraisal of the suitability of AL interfaced RL-65 layers for filling the current gap between rat *ex/in vivo* and human *in vitro* absorption models in pre-clinical drug development.

2. Materials and methods

2.1. Cell culture

The RL-65 cell line was obtained from the ATCC (Rockville, MD, USA) and used for experiments between passage numbers 3 and 17 from purchase. Cells were cultured in 75 cm^2 flasks using a serum-free medium composed of Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (DMEM/Ham F12) 1:1, supplemented with 85 nM selenium, 2.5 μg/ml bovine insulin, 5.4 μg/ml human transferrin, 30 μM ethanolamine, 100 μM phosphoethanolamine, 500 nM hydrocortisone, 5 μM forskolin, 50 nM retinoic acid and 0.15 mg/ml bovine pituitary extract (Sigma–Aldrich, Poole, UK). Medium was exchanged thrice weekly and cells were passaged when 90% confluent using a 1:20 split ratio. Calu-3 cells were purchased from the ATCC, used between passages 25–30 and cultured as outlined previously by Madlova et al. (2009). Normal human primary bronchial epithelial (NHBE) cells were purchased from Lonza (Slough, Berkshire, UK) and cultured (passage 2) using the Lonza proprietary B-ALI® kit according to the manufacturer's instructions.

RL-65 cells were seeded at a density of 1×10^5 cells/cm^2 on 0.4 μm pore size, 1.13 cm^2 polyester Transwell® cell culture supports (Corning Costar, High Wycombe, UK) and cultured in submerged (LL) conditions or raised at an air–liquid (AL) interface after 24 h. The cell culture medium was either that outlined above with the addition of 100 IU/ml penicillin and 100 μg/ml streptomycin antibiotic solution (herein referred to as serum free medium (SFM)) or an alternative serum containing medium (SCM) comprising DMEM/Ham F12 (1:1) supplemented with 10% v/v fetal bovine serum (non-USA origin, Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin antibiotic solution, 2 mM L-glutamine and 1% v/v non-essential amino acids (all from Sigma). For LL culture, the apical and basolateral compartments of the Transwell® contained 0.5 ml or 1.5 ml of medium, respectively. For AL culture, 0.5 ml of medium was added to the basolateral chamber only. The medium was subsequently replaced in respective compartments on alternate days.

NHBE cells were harvested once they reached ~90% confluence using the supplier's subculture reagents (Lonza) and seeded onto 0.4 μm pore size, 0.33 cm^2 polyester Transwell® inserts previously coated with rat tail collagen type I (BD Biosciences, Oxford, Oxfordshire, UK) at a density of 1.5×10^5 cells/cm^2. After 72 h, they were raised to an AL interface and cultured in the supplier's differentiation medium (Lonza) for 21 days. Thereafter, the medium was changed every 2–3 days.

2.2. Trans-epithelial electrical resistance (TEER) measurements

The TEER was recorded using an EVOM volt–ohm–meter with STX-2 chopstick electrodes (World Precision Instruments, Stevenage, UK). Measurements on cells in LL culture were taken immediately before the medium was exchanged. For cells cultured at the AL interface, 0.5 ml and 1.0 ml of medium was added to the apical and basolateral chambers, respectively. Cells were returned to the incubator to equilibrate for at least 20 min before TEER was measured. TEER values reported were corrected for the resistance and surface area of the Transwell® filters.

2.3. Histological preparation and staining

Cells were fixed on the Transwell® membrane using 3.7% w/v paraformaldehyde in PBS for 15 min at room temperature. The fixing solution was removed and cell layers were stored submerged in PBS at 4 °C until processed. For histology preparation, filters were excised from the inserts and sandwiched between two biopsy foam pads inside a histology cassette. Samples were subjected to 5 min incubations in increasing concentrations of ethanol in dH_2O (25, 50, 75, 90, 95, 100% v/v), followed by two 5 min exposures to xylene and a 30 min treatment in paraffin wax. Dehydrated samples were embedded in wax and 6 μm thick cross-sections cut using a RM 2165 rotary microtome (Leica, Milton Keynes, UK) before being mounted on poly-L-lysine coated histology slides. Cellular cross-

sections were incubated twice in xylene for 2 min and rehydrated in decreasing concentrations of ethanol in dH_2O (100, 95, 90, 75, 50, 25% v/v) for 2 min each. Slides were then immersed in 100% dH_2O before histological staining. All incubation steps for histological staining were performed at room temperature.

For morphological staining, slides were immersed in Mayer's haematoxylin stain for 10 min and excess stain removed by rinsing for 2 min in dH_2O. Samples were then submerged for 2 min in Scott's tap water (3.5 g sodium bicarbonate, 20 g magnesium sulphate in 1 L dH_2O) before incubation in 1% v/v eosin in dH_2O for 5 min. For mucus staining, samples were submerged in a 1% w/v alcian blue in 3% v/v acetic acid pH 2.5 for 5 min. Excess stain was removed with a 2 min dH_2O wash before incubation in neutral fast red for 5 min. For both types of staining, the samples were rinsed in dH_2O until the colour ran clear and finally, mounted with glycerol on cover slips for imaging.

2.4. Scanning electron microscopy

Cells were fixed in a 1:1 mixture of medium and fixing solution (2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) which was added to both apical and basolateral chambers of the Transwell®. After 5 min, the solution was removed and replaced with 2.5% v/v gluteraldehyde fixing solution and samples were stored at 4 °C for up to 2 weeks.

For scanning electron microscopy (SEM) processing, all fixing solution was aspirated from both chambers of the Transwell® and 1% w/v osmium tetroxide in PBS added to both compartments. After 90 min, the solution was removed and rinsed five times with PBS before dehydration in progressively increasing concentrations of ethanol in dH_2O (25%, 50%, 75%, 95% and 100%). Samples were critically point dried with CO_2 using an EM CPD030 (Leica, Milton Keynes, UK) and filters were removed and mounted on aluminium stubs with adhesive carbon tape. The samples were gold coated for 5 min using a sputter coater SCD030 unit (Balzers Union, Milton Keynes, UK) under an argon atmosphere and analysed with a SEM 6060LV unit (JOEL, Welwyn, UK) at an accelerating voltage of 30 kV and stage height of 10 mm.

2.5. Immunofluorescence microscopy

All medium was aspirated from the Transwell® and cells were washed twice with PBS at pH 7.4. Samples were fixed for 15 min using 500 μl of 3.7% w/v paraformaldehyde in the apical chamber. After the elapsed time, paraformaldehyde was removed and PBS added to both chambers. Fixed samples were stored up to 14 days at 2–8 °C prior to analysis.

Fixed cell layers were permeabilized with 0.1% v/v Triton X-100 in PBS for 5 min and rinsed in PBS. Samples were blocked for 30 min with 1% w/v bovine serum albumin (BSA) in PBS and incubated with 10 μg/ml mouse anti-zonula occludens (zo-1) monoclonal antibody (Invitrogen, Paisley, UK) or 20 μg/ml UIC2 mouse anti-mdr1 (Enzo Life Science, Exeter, UK) monoclonal antibody or a mouse anti-β-tubulin IV monoclonal antibody (Sigma) at a 1:500 dilution for 60 min at 37 °C. Cells were washed in 1% w/v BSA in PBS before incubation with FITC-labelled goat anti-mouse IgG (1:64) (Sigma) in PBS for a further 30 min at room temperature. Cell nuclei were counter-stained with propidium iodide (PI) 1 μg/ml in PBS for 30 s. Inserts were washed with PBS and the filter was excised and mounted on a slide using DABCO anti-fade mounting media (all from Sigma). Samples were imaged by a Meta 510 confocal microscope (Zeiss, Welwyn Garden City, UK) with excitation at a wavelength of 488 nm and 543 nm and emission observed at 519 nm and 617 nm for FITC and PI, respectively.

2.6. Gene expression analysis

RL-65 cells were harvested from Transwell® inserts on the day functional experiments were performed. Cells were washed once with PBS, filters were excised and snap frozen in liquid nitrogen before transferral to −80 °C storage until processing. For mRNA isolation, 1.2 ml RNA STAT-60 (Tel-test, Friendswood, TX) was added to 12 excised filters and the samples were processed according to the manufacturer's protocol. RNA preparations were assessed for quantity and purity using a Nanodrop ND-1000-UV–Vis spectrophotometer (Nanodrop Technologies, Wilmington, USA). Integrity of the RNA preparation was assessed by the presence of distinct 18S and 28S bands on a 1% w/v agarose gel using a Tris borate-EDTA buffer (0.09 M Tris borate, 2 mM EDTA, pH 7.8) at 90 mV for 60 min.

cDNA was prepared from 2 μg of total RNA using the Superscript™ First-Strand Synthesis System (Invitrogen, Paisley, UK) with random hexamer primers according to the manufacturer's protocol. Samples were incubated at 65 °C for 5 min then held on ice for 1 min before the addition of Superscript III reverse transcriptase. Samples were then incubated at 25 °C for 10 min followed by reverse transcription at 50 °C for 50 min. The reaction was terminated by heating to 85 °C for 5 min to inactivate the enzyme.

Quantitative PCR was carried out on a 7500 Real Time PCR Sequence Detection System (Applied Biosystems, Foster City, CA). TaqMan analysis was performed in a 25 μl reaction mixture containing 30 ng cDNA, TaqMan Universal PCR Master Mix (comprising AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimised buffer) and Assay-on-demand™ gene expression assay mixes containing specific primers and probes (all from Applied Biosystems). The PCR conditions comprised a 2 min incubation at 50 °C followed by a 10 min polymerase activation at 95 °C. This was followed by 40 cycles alternating between 95 °C for 15 sections and 60 °C for 1 min each.

Amplification curves were analysed using the SDS version 3.2 software (Applied Biosystems, Foster City, CA). The baseline and threshold values were set and the Ct values extracted for each gene of interest. Relative quantification was calculated using the geometric mean of two selected house-keeping genes, gapdh and mvp. Relative gene expression levels were calculated using the equation $2^{-\Delta Ct}$. An arbitrary classification system was applied to the data quantifying relative expression levels as 'high' >0.5, 'moderate' between 0.02 and 0.5, 'low' between 0.001–0.02 and 'negligible' <0.001.

2.7. Transport studies

All transport experiments were conducted in standard buffer solution (SBS) comprising Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cell layers were allowed to equilibrate in SBS for 60 min at 37 °C before TEER measurements were taken. Each condition was carried out in quadruplicate and only layers with a resistance >250 Ω cm^2 were accepted for experimentation. For transport studies with radiolabelled markers, donor compartments were filled with 0.51 ml (apical to basolateral (AB) transport) or 1.51 ml (basolateral to apical (BA) transport) of SBS containing 25 nM ^{3}H-digoxin and/or 6.55 μM ^{14}C-mannitol. Receiver compartments were filled with 1.5 ml (AB transport) or 0.5 ml (BA transport) of SBS. At the start and end of the experiment, 10 μl samples were taken from the donor compartments for determination of the initial and final concentration. Every 30 min over a 2 h period, 300 μl samples (AB transport) and 100 μl samples (BA transport) were taken from the respective receiver chambers and replaced with the same volume of SBS. Cells were maintained at 37 °C and rotated at 60 rpm on an orbital shaker throughout the

experiment. Two ml of OptiPhase HiSafe 2 scintillation fluid (Perkin Elmer, Cambridge, UK) was added to each sample and radioactivity determined in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

For permeability assessment of the fluorescent dye Rhodamine123 (Rh123), experiments were set up similarly to radioactive transport experiments outlined above with the donor solution comprising 5 μM Rh123 in SBS. Every 30 min for a 2 h period, 100 μl samples were taken from the receiver chambers and analysed neat. The 10 μl samples from the donor wells were diluted 1:99 with SBS and 100 μl of this used for analysis. All samples were transferred to a black 96 well plate and analysed at an excitation wavelength of 485 nm and emission wavelength of 538 nm using an Infinite® M200 PRO spectrophotometer (Tecan, Reading, UK). The Rh123 concentration in each sample was determined from a calibration curve.

Apparent permeability coefficients (P_{app}) were calculated using the following equation: $P_{app} = \frac{dQ/dt}{AC_0}$ where dQ/dt is the flux of the substrate across the cell layer, A is the surface area of the filter and C_0 is the initial concentration of the substrate in the donor solution.

2.8. Statistics

For all TEER and permeability data generated, results were expressed as mean ± SD. Datasets with $n \geqslant 5$ were assessed for normality and the data fitted a normal (Gaussian) distribution. Therefore normality was assumed for all datasets where $n < 5$ and each were compared using a two-tailed, unpaired Student's t-test with Welch correction applied (to consider unequal variance between datasets). Statistical significance was evaluated at a 99% confidence level ($p < 0.01$). All statistical tests were performed using GraphPad InStat® version 3.06.

3. Results

3.1. Barrier properties of RL-65 cell layers

The barrier properties of RL-65 cell layers were assessed by TEER measurements, expression of the tight junction protein zo-1 and permeability of the paracellular marker ^{14}C-mannitol. TEER was measurable from day 4 after seeding for RL-65 cells cultured in both media (Fig. 1). At passage 3, cells cultured in SFM either at an AL interface or under submerged conditions displayed a similar TEER profile with maximal TEER between days 8 to 10 in culture. Thereafter, this steadily declined to <100 Ω cm^2 at day 18 in culture, when cells had detached from the filters (Fig. 1A). At day 8 in SFM, cell layers cultured at the AL interface produced significantly higher ($p > 0.01$) TEER values (667 ± 65 Ω cm^2) compared with their submerged culture counterparts (503 ± 50 Ω cm^2). At later passages, (passages 6, 9 and 12) maximal TEER values after 8 days in culture were 200–400 Ω cm^2 (data not shown), in agreement with TEER values obtained by Wang and co-workers (Wang et al., 2009). The TEER profile for submerged RL-65 cell cultures maintained in SCM was similar to that in SFM. However, when cells were cultured at an AL interface, TEER values steadily increased to reach a plateau ~400–600 Ω cm^2 after 10 days in culture which was maintained until the end of the 22 day monitoring period (Fig. 1B). Based on these TEER values, RL-65 cell layers were further characterised at the AL interface after 8 days in SFM and 8 and 21 days in SCM.

Immunocytochemistry experiment on RL-65 layers cultured at the AL interface for 8 days in both media showed a positive staining for the zo-1 protein along the cell perimeter, in agreement with the location of tight junction proteins (Fig. 2).

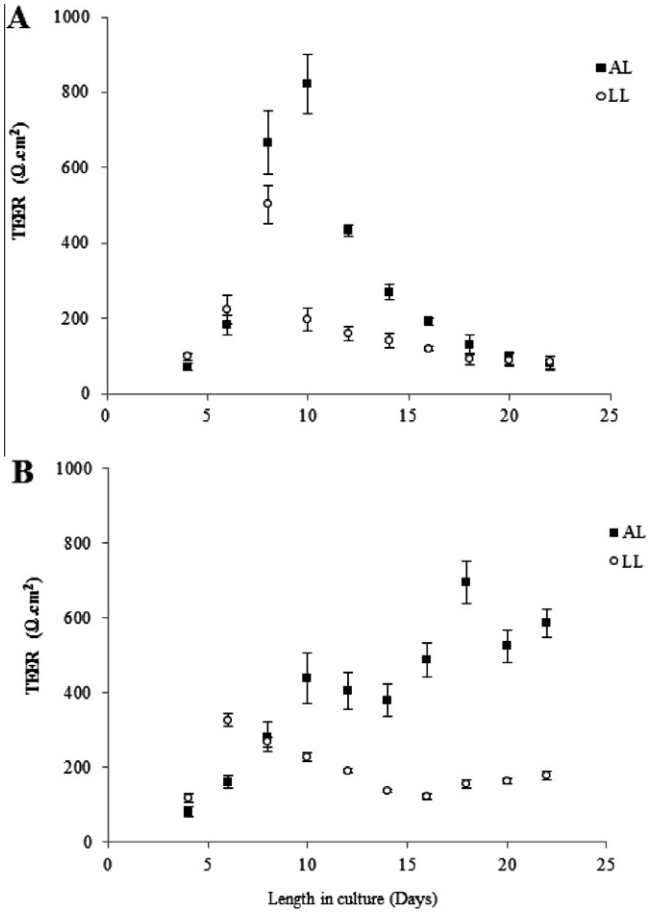

Fig. 1. Influence of the culture medium on TEER development in RL-65 layers maintained in (A) SFM and (B) SCM at an air–liquid interface (AL) or under submerged conditions (LL). Data are represented as mean ± SD (n = 4–6 cell layers).

^{14}C-mannitol permeability studies resulted in P_{app} values ranging from 0.54 ± 0.11 to 3.09 ± 0.36 × 10^{-6} cm/s, depending on the conditions and length in culture (Table 1). Those were in the same range as in-house and published P_{app} obtained in existing human bronchial epithelial cell culture models (Table 1). After 8 days at an AL interface, ^{14}C-mannitol P_{app} values were significantly lower in RL-65 layers grown in SCM than in layers maintained in SFM, in agreement with the higher TEER achieved in SCM. As previously reported for the Calu-3 and 16HBE14o- cell lines (Forbes et al., 2003; Sakagami, 2006), a strong inverse correlation (R = 0.9658) with power regression was indeed found between TEER and ^{14}C-mannitol P_{app} values in RL-65 layers (Fig. 3).

3.2. RL-65 cell layer morphology

The morphology of RL-65 layers was characterised using histological and SEM examinations. Cross-sections of RL-65 cell layers cultured in SFM for 8 days depicted 2–3 layers of cuboidal cells similar to that observed for sections of NHBE cells maintained at an AL interface for 21 days (Fig. 4A and D). In contrast, RL-65 cells cultured in SCM for 8 days formed a viable layer 1–3 cells thick adjacent to the filter underneath a ~5 μm thick layer of pink/purple eosin stained material containing no viable cells (Fig. 4B). After 21 days, the non-viable apical substance had extended to a ~30 μm thick stratum and viable RL-65 cells formed a flatter single layer adjacent to the filter (Fig. 4C). Alcian blue staining failed to show the presence of mucopolysaccharides at the surface of RL-65 cell layers while positive staining was observed apically in

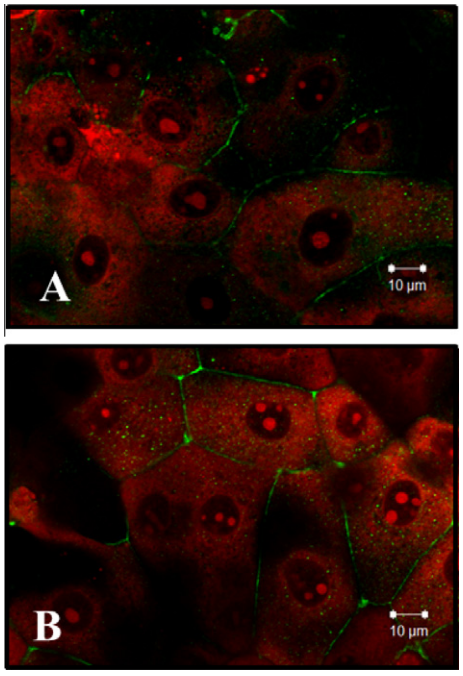

Fig. 2. Confocal laser scanning microscopy of RL-65 cell layers immunolabelled for the tight junction protein zo-1 (green). Cells were cultured in (A) SFM or (B) SCM for 8 days at an air–liquid interface and counterstained with propidium iodide (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of apparent permeability coefficients (P_{app}) for the paracellular marker ^{14}C-mannitol in different *in vitro* bronchial epithelial cell models cultured at the air–liquid interface. Data are represented as mean ± SD of 8 inserts for RL-65 cells and 20 inserts for Calu-3 and NHBE cell layers (in house data).

Cell type	Days in culture	^{14}C-Mannitol P_{app} (1×10^{-6} cm/s)
Calu-3	21	0.49 ± 0.23
NHBE	21	0.55 ± 0.13
16HBE14o-[a]	7	2.36 ± 0.66
RL65 (SFM)	8	3.09 ± 0.36
RL65 (SCM)	8	1.29 ± 0.16
RL65 (SCM)	21	0.54 ± 0.11

[a] Values were taken from the literature Forbes et al. (2003).

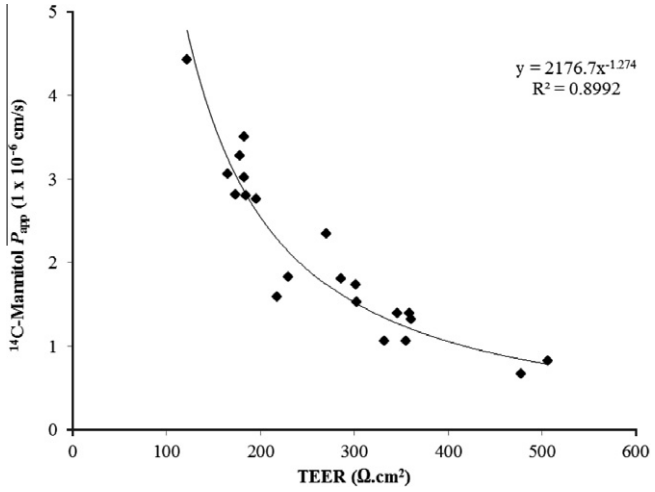

Fig. 3. Relationship between TEER value and ^{14}C-mannitol apparent permeability in RL-65 cell layers. Cells were cultured at the AL interface in SCM or SFM for either 8 or 21 days.

Calu-3 and NHBE cell layers (data not shown). SEM images of the RL-65 apical surface revealed a heterogeneous cell population (Fig. 5A). At closer magnification, small cylindrical appendages, ~2 µm in length and <0.5 µm in diameter were observed protruding from the apical cell surface of RL-65 cells cultured in SFM, suggesting the presence of microvilli or immature cilia (Fig. 5B). This assumption was supported by a localised positive immunohistochemical staining for the cilia marker β-tubulin at the surface of the layers (Fig. 5C).

3.3. Expression and functionality of drug transporters in RL-65 layers

Gene expression analysis of selected transporters revealed similar relative mRNA levels in RL-65 cells cultured for 8 days in either SFM or SCM. Expression levels were negligible (<0.001) for abcb1a (mdr1a), abcc2 (mrp2), slc22a1-3 (oct1-3) whilst a low (0.001–0.02) or moderate (0.02–0.5) expression was observed for abcb1b (mdr1b) and slca5 (octn2), respectively (Table 2).

The presence and functionality of P-gp (mdr1) proteins were probed respectively by immunocytochemistry and bi-directional permeability studies with the two established substrates, ^{3}H-digoxin and Rh123. A positive immunocytochemical signal was obtained on the apical surface of RL-65 cell layers cultured in both media for 8 days while no green fluorescence was detected when cells were only incubated with the FITC-labelled secondary antibody (Fig. 6). However, no statistical difference ($p > 0.05$) between AB and BA transport across 8-day old RL-65 layers was observed for any of the two P-gp substrates investigated (Fig. 7), suggesting negligible transporter-mediated drug trafficking in the cell culture model.

4. Discussion

In vivo and *ex vivo* absorption studies are frequently conducted in rats to predict the pharmacokinetics of inhaled drug candidates in humans (Tronde et al., 2003). However, variations in drug disposition in human and rat lungs have yet to be fully appraised. A rat respiratory epithelial cell culture model suitable for permeability screening would aid better understanding of interspecies differences in pulmonary drug absorption, including the role of drug transporters, in addition to providing an ethical alternative to animal testing.

This study evaluates the potential of layers of the bronchial/bronchiolar epithelial rat cell line, RL-65, as an *in vitro* permeability screening tool. It demonstrates that RL-65 cells cultured at an AL interface on Transwell® supports formed layers morphologically similar to the upper airway epithelium with a TEER and ^{14}C-mannitol paracellular permeability values in agreement with those in established human bronchial epithelial cell models. Expression of the drug transporters P-gp and octn2 was confirmed in the cell layers, although no vectorial transport of widely used P-gp probes was observed. This preliminary characterisation of air-interfaced RL-65 cell layers identifies a potentially useful tool for investigating differences in drug permeability between the human and rat airway epithelia.

Morphological analysis of RL-65 cells grown in presence of serum revealed multilayered cultures with an uppermost layer of non-viable cells (Fig. 4), thus providing a poor representation of the native epithelium. This indicated that a serum containing medium is unsuitable for the development of RL-65 cells into polarised layers mimicking the airway epithelium. Likewise, sub-optimal growth of the cell line had previously been described in presence of serum (Roberts et al., 1990). Our study also demonstrated that the sole consideration of markers of epithelial barrier formation such as TEER and paracellular permeability values is potentially

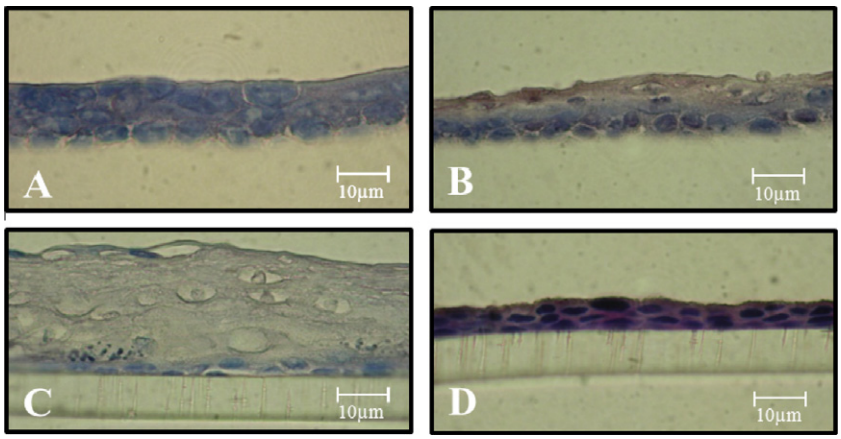

Fig. 4. Haematoxylin and eosin histological staining of 6 µm cross-sections of (A–C) RL-65 and (D) 21 day old air-interfaced NHBE cell layers. RL-65 cells were cultured at an AL interface in (A) SFM for 8 days or SCM for (B) 8 days or (C) 21 days. All images are orientated with the apical cell surface closest to the top of the image.

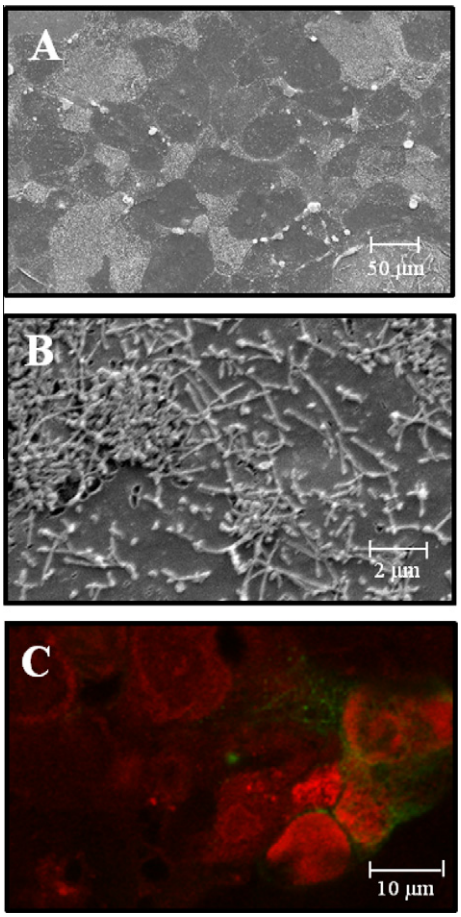

Fig. 5. Characterisation of the apical cell surface of RL-65 cell layers cultured at an AL interface for 8 days in SFM. (A and B) Scanning electron microscopy (SEM) images at 230× or 4000× magnification, respectively. (C) Immunohistological staining for the cilia marker β-tubulin (green). Cell nuclei were counterstained with propidium iodide (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

misleading for a reliable assessment of cell-based absorption screens, and highlights the importance of morphological examinations in the characterisation of those models.

In contrast, RL-65 cells maintained in a defined serum-free medium for 8 days produced cultures displaying 1–3 layers of cuboidal cells, similar to normal human bronchial cells (Fig. 4).

Table 2
Gene expression of selected ATP binding cassette (ABC) and solute-linked carrier (SLC) transporters in RL-65 cell layers cultured at an air–liquid interface for 8 days in either SFM or SCM. Relative gene expression levels are described as – : <0.001 (negligible), +: 0.001–0.02 (low), ++: 0.02–0.5 (moderate), +++: > 0.5 (high), relative to gapdh and mvp. N/A: data not available.

Gene	Protein	SFM	SCM	Assay ID	Sequence Accession ID
mvp	lrp	+++	+++	Rn00575634_m1	NM_022715
gapdh	gapdh	+++	+++	Rn01775763_m1	NM_017008
abcb1a	mdr1a	–	–	Rn01639253_m1	NM_133401
abcb1b	mdr1b	+	+	Rn00561753_m1	NM_012623
abcc2	mrp2	–	–	Rn00563231_m1	NM_012833
slc22a1	oct1	–	–	Rn00562250_m1	NM_012697
slc22a2	oct2	–	–	Rn00580893_m1	NM_031584
slc22a3	oct3	–	–	Rn00570264_m1	NM_062183
slc22a4	octn1	N/A	N/A	–	–
slc22a5	octn2	++	++	Rn00570533_m1	NM_019269

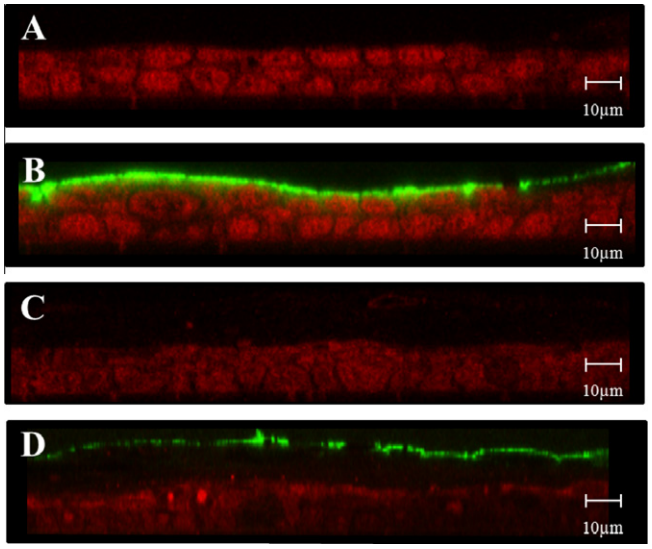

Fig. 6. Confocal scanning microscopy of RL-65 cell layers immunolabelled for P-glycoprotein (green). Cells were cultured for 8 days at an air–liquid interface in either (A and B) SFM or (C and D) SCM. Figures (A and C) show the secondary antibody controls. Cell nuclei were counterstained with propidium iodide (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, the possible presence of ciliated cells in absence of detectable mucus secretions might suggest a bronchiolar origin

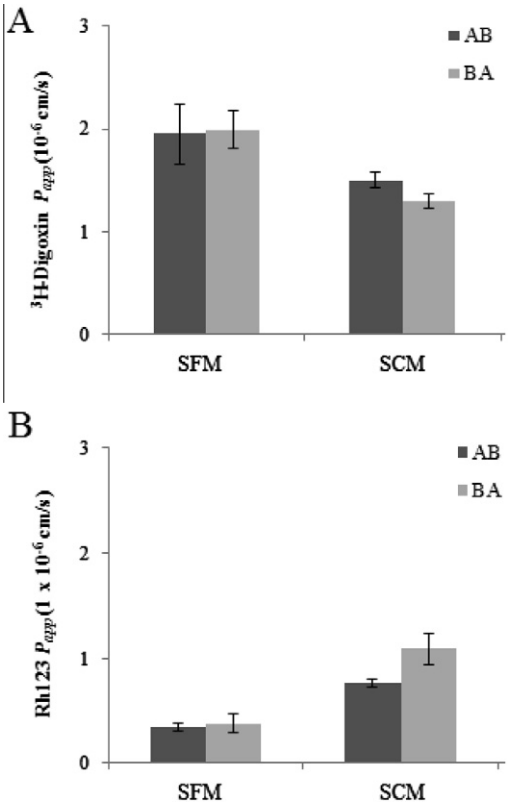

Fig. 7. Apical to basolateral (AB) and BA transport of (A) ^{3}H-digoxin and (B) Rh123 across 8 day old RL-65 cell layers grown in SFM or SCM. Data are represented as mean ± SD (n = 4–6 cell layers).

for RL-65 cells. These cell layers also exhibited TEER ~250–600 Ω cm^2 (Fig. 1), i.e., in the same range as Calu-3 (Borchard et al., 2002; Fiegel et al., 2003), 16HBE14o- (Forbes et al., 2003) and NHBE (Lin et al., 2007; Madlova et al., 2009) layers. ^{14}C-mannitol permeability across the layers was measured as ~3.0 × 10^{-6} cm/s (Table 1). Although higher than reported for Calu-3 (Forbes and Ehrhardt, 2005) and NHBE (Madlova et al., 2009) cell layers, this value is comparable to paracellular transport data published in 16HBE14o- layers (Ehrhardt et al., 2002; Forbes et al., 2003). RL-65 layers at an early passage (3–4) achieved higher TEER values than at a later passage (6–18), suggesting an alteration in barrier properties with increasing passage number. A similar trend has also been reported for NHBE cell layers which lose the ability to form a permeability barrier after 3–4 passages (Widdicombe et al., 2005). In comparison to NHBE cells, the RL-65 cell line nevertheless provides an extended passage window for use in drug permeability measurements.

Gene expression analysis of selected drug transporters revealed the presence of octn2 and mdr1b in RL-65 cell layers (Table 2). This is in agreement with the high expression of OCTN2 in the human bronchial epithelium (Horvath et al., 2007) and the higher levels of mdr1b as compared to mdr1a transcripts detected in rat lungs (Brown et al., 1993; Brady et al., 2002), respectively. Additionally, apical expression of P-gp was confirmed in RL-65 cell layers by immunocytochemistry (Fig. 6), in accordance with its localisation in rat bronchial epithelial tissue (Campbell et al., 2003). However, no apparent efflux of ^{3}H-digoxin and Rh123 was observed across the layers (Fig. 7). As both compounds are substrates for the two P-gp isoforms (mdr1a/b) found in rats (Schinkel et al., 1997; Takeuchi et al., 2006; Suzuyama et al., 2007), our data suggests the transporter was not functional in 8-day old RL-65 cell layers.

The presence of functional P-gp in human bronchial epithelial cell culture models remains controversial to date (Bosquillon, 2010). Several studies have concluded the transporter was responsible for the apparent efflux of various substrates in NHBE, 16HBE14o- or Calu-3 cell layers (Lin et al., 2007; Ehrhardt et al., 2003; Hamilton et al., 2001; Patel et al., 2002; Brillault et al., 2009) while others have reported an absence of P-gp in Calu-3 layers (Cavet et al., 1997) or a negligible impact on drug transport in the Calu-3 and NHBE models (Madlova et al., 2009; Hutter et al., 2011).

Although ^{3}H-digoxin is a recommended substrate probe for P-gp (Rautio et al., 2006; Huang et al., 2007), it has been shown to be transported by other human transporters as well, especially the Multidrug Resistance Proteins (MRP) and Organic Anion Transporting Polypeptides (OATP) (Kullak-Ublick et al., 2001; Mikkaichi et al., 2004; Yamaguchi et al., 2010; Taub et al., 2011). Similarly, Rh123 has been described as a substrate for MRP1 (Hamilton et al., 2001), the Breast Cancer Resistance Protein (BCRP) (Doyle et al., 1998) and OCT (Masereeuw et al., 1997; van der Sandt et al., 2000). The absence of vectorial transport of ^{3}H-digoxin and Rh123 in RL-65 cell layers also indicates these other transporters may not be expressed or functional in the model.

Transport studies were performed in RL-65 cell layers 8 days after seeding on Transwell® inserts. There is currently no standardised time in cultures prior to permeability measurements in human bronchial epithelial cell layers and these are commonly conducted in 8–21 day old cell layers. However, there are indications in the literature which suggest transporter levels in pulmonary *in vitro* absorption models may be affected by the length in culture, with an optimal expression and activity achieved after 21 days (Madlova et al., 2009; Haghi et al., 2010; Mukherjee et al., 2012). Therefore, 8 days in culture may not have been sufficient for expression of fully functional transporter systems in RL-65 cell layers. In the culture conditions tested, the layers could nevertheless not be used for drug transport studies after 9–10 days on Transwell® as the TEER decreased to <200 Ω cm^2 thereafter, before cells eventually detached from the filters. There is therefore a need to prolong the time these can be maintained at an AL interface. For instance, culture on different filter material or substrate coatings and optimisation of the medium composition may improve the usefulness of the model as a pre-clinical permeability screening tool.

5. Conclusion

The RL-65 cell line was successfully grown at an air–liquid interface in a defined serum-free medium for 8 days. RL-65 layers exhibited suitable absorption barrier properties including TEER and paracellular permeability in the same range as established human bronchial epithelial models. Furthermore, they expressed transporters present in the native epithelium, although their functional activity was not demonstrated. This initial study indicated that, following further optimisation of the culture conditions, RL-65 cell layers may offer a valuable *in vitro* model for permeability screening in rats and assist in the evaluation of interspecies differences in pulmonary drug absorption.

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