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ORIGINAL ARTICLE

Time- and passage-dependent characteristics of a Calu-3 respiratory epithelial cell model

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Abstract

Background: Although standard protocols for the study of drug delivery in the upper airways using the sub-bronchial epithelial cell line Calu-3 model, particularly that of the air-liquid interface configuration, are readily available, the model remains un-validated with respect to culture conditions, barrier integrity, mucous secretion, and transporter function. With respect to the latter, the significance of functional P-glycoprotein (P-gp) activity in Calu-3 cells has recently been questioned, despite previous reports demonstrating a significant contribution by the same transporter in limiting drug uptake across the pulmonary epithelium. Therefore, the aim of this study was the standardization of this model as a tool for drug discovery. *Methods*: Calu-3 cells were grown using air-interfaced condition (AIC) on polyester cell culture supports. Monolayers were evaluated for transepithelial electrical resistance (TEER), permeability to the paracellular marker fluorescein sodium (flu-Na), surface P-gp expression, and functionality. Mucous secretion was also identified by alcian blue staining. *Results*: TEER and permeability values obtained for Calu-3 monolayers were shown to plateau between day 5 and day 21 in culture with values reaching $474 \pm 44\ \Omega cm^2$ and $2.33 \pm 0.36 \times 10^{-7}$ cm/s, respectively, irrespective of the passage number examined. $32.7 \pm 1.49\%$ of Calu-3 cells cultured under these conditions detected positive for cell surface P-gp expression from day 7 onwards. Functional cell surface expression was established by rhodamine 123 drug extrusion assays. *Conclusion*: This study establishes a clear dependence on culture time and passage number for optimal barrier integrity, mucous secretion, and cell-surface P-gp expression and function in Calu-3 cells. Furthermore it provides initial guidelines for the optimization of this model for high throughput screening applications.

Key words: *Calu-3; culture conditions; P-glycoprotein; P-gp; respiratory epithelium*

Introduction

Recent advances in delivering drugs to the respiratory tract, both locally and systemically, have played an important role in the development of novel formulations and optimized delivery devices[1,2]. Contrary to the well-studied gastrointestinal tract, the respiratory system displays greater complexity with respect to accessibility, anatomy, dose delivery, and consistency[3]. In addition to this, the interactions of inhaled pharmaceutical preparations with the lung epithelium have not been investigated in sufficient depth.

The major site of deposition in the lung from current pharmaceutical aerosol devices is the airways, where the epithelium is the principal barrier to drug absorption. To effectively predict the fate of compounds delivered to the lung during the formulation process, a model of the airway epithelium reflecting the extent of drug permeability in vivo is required. This model should accurately represent the barrier properties of the bronchial epithelium in vivo, for both passively absorbed and actively transported compounds, capable of measuring drug transport rates across the respiratory epithelium and predicting bioavailability at the level of the lung.

Cell culture models provide a rapid, predictive, and cost-effective approach to assess bioavailability across pharmacological barriers. Various pulmonary epithelial in vitro models have been established to study drug delivery at the level of the upper airways and alveolar region[4–6]. These models differ in their capacity to mimic the in vivo state with respect to the expression of essential carrier

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proteins, the ability to form tight junctions, and the capacity for mucous secretion[7].

A reliable model for the study of drug delivery in the upper airways is the sub-bronchial epithelial cell line, Calu-3[8]. This cell line displays an mRNA and protein profile, typical of the lung epithelium[9]. Barrier models using these cells have been established using two culture configurations, namely culturing under liquid covered (LCC) or under air-interface conditions (AIC)[10]. Both models display a polarized epithelium, express essential drug transporter proteins, and form functional tight junctions[11]. Additionally, when grown under AIC conditions, Calu-3 cells form a pseudo-stratified layer of columnar cells, display enhanced ciliogenesis, secrete a thick mucous gel layer on the epithelium surface, and possess a superior barrier integrity typical of the upper airway epithelium[12].

Transporter proteins serve an important role in maintaining the integrity of pharmacological barriers throughout the body. The predominant drug transporter, P-glycoprotein (P-gp) is a plasma membrane, Mg^{2+}-dependent ATPase, which acts to extrude a wide range of structurally and functionally unrelated drugs out of cells in which it is expressed[13]. P-gp is found physiologically expressed at sites such as the luminal membranes of endothelial cells of the blood brain barrier, the blood testis barrier, epithelial apical membranes of the intestines and proximal tubules of the kidney, the biliary canalicular membranes of hepatocytes, and on the surface of pulmonary epithelial cells[14,15]. This tissue-specific localization coupled with its vectoral transport capacity serves to protect both vital organs and the organism as a whole from potentially harmful xenobiotic exposure.

At the cellular level, a number of drugs routinely used in the management of respiratory as well as for other pathophysiological disease states have been shown to interact with P-gp[7]. Despite this, however, there has not been an extensive phenotyping of the extent of involvement by this drug transporter in cell culture models predictive of lung disposition, including the Calu-3 model. Previous attempts to assess the extent of P-gp-mediated drug extrusion have yielded contradictory findings regarding the role of P-gp in pulmonary drug disposition[16]. Variability in the extent of transporter involvement together with observed inconsistency in drug permeability between laboratories significantly limits the application of this model for drug discovery applications. In this regard, more research is required into the standardization of the Calu-3 model, particularly with respect to transporter expression and function. Earlier contributions in standardizing the model have demonstrated a correlation between cell density and the time with which confluence and maximal barrier integrity is achieved, with recommended densities of 5×10^5 cells/cm^2 being accepted in the literature[17]. Likewise assessments of the effects of Transwell filter coatings have demonstrated that Calu-3 cells adequately form tight barriers regardless of the presence of filter coating[6,18]. Parameters including passage number and culture period, although have been shown to significantly impact drug permeability[12], have not been examined with respect to effects on P-gp cell surface expression and transport function in the Calu-3 cell model. This element of the model is missing and furthering our understanding at this level may provide a basis for explaining the observed discrepancy in the importance of this transporter in pulmonary drug disposition.

The aim of this study was to systematically examine the effect of typical culture conditions, including cell passage number and culture period on barrier integrity, mucous secretion, and cell-surface P-gp expression and function.

For the first time, standardized conditions, which unequivocally show time dependence to these parameters and provide initial guidelines for the optimization of this model for high throughput screening applications, have been reported. The AIC model was chosen for this purpose as it has been shown to be superior with respect to epithelial morphology and function when compared to the LCC model[8,10,19]. The AIC models also allows for the direct exposure of epithelial cells to aerosol droplets or powders.

Materials and methods

Materials

Minimum essential medium (MEM) nonessential amino acids solution (×100), rhodamine123 (Rh123), trypan blue solution (0.4%, w/v), paraformaldehyde, propidium iodide (PI), dimethyl sulfoxide (DMSO), and verapamil hydrochloride were obtained from Sigma-Aldrich (Sydney, Australia). Fluorescein-sodium (flu-Na) was purchased from May & Baker Ltd. (Dagenham, England). Fetal bovine serum (FBS), glutamine (200 mM), Hank's balanced salt solution (HBSS), TrypLE Express, and phosphate buffered saline (PBS) were from Gibco, Invitrogen (Sydney, Australia). FITC-anti P-gp (clone17F9, BD Pharmingen) and FITC-isotype control mAb were from BD Pharmingen (San Jose, CA, USA). Alcian blue 1% (pH 2.5) in 3% acetic acid was purchased from Fronine laboratory (Sydney, Australia). Entellan New was obtained from ProSciTech (Thuringowa, Australia), Transwell cell culture inserts (0.33 cm^2 polyester, 0.4 mm pore size) and black 96-well plates were from Corning Costar (Lowell, MA, USA). All other culture plastics were from Sarstedt (Adelaide, Australia). All chemicals and reagents were of the highest analytical grade.

Cell culture

The Calu-3 cell line (HTB-55) was purchased from the American Type Cell Culture Collection (ATCC, Rockville, IN, USA). Cells between passages (33–40) were grown in 75 cm^2 flasks in complete Dulbecco's Modified Eagle's medium: F-12 containing 10% (v/v) fetal calf serum, 1% (v/v) nonessential amino acid solution, and 1% (v/v) L-glutamine solution and maintained in a humidified atmosphere of 95%air/5%CO_2 at 37°C. Cells were propagated and subcultured according to ATCC recommendations. To establish the air-liquid interface model, cells were seeded onto Transwell polyester inserts at a density of 5×10^5 cells/cm^2 in 100 μL apical and 500 μL basolateral medium. The apical medium was removed 24 hours after seeding and cells were fed every alternate day with fresh basolateral medium only. The monolayers were allowed to differentiate under air-interface feeding conditions over 2–21 days using cells at passages 24–40. This passage range was selected to encompass the range routinely used for this cell line[6,16,18,19].

Transepithelial electrical resistance of cell layers

The transepithelial electrical resistance (TEER) of Calu-3 monolayers was measured over time using an EVOM Voltohmmeter (World Precision Instruments, Sarasota, FL, USA) with STX-2 chopstick electrodes. Pre-warmed medium was added to the apical and basolateral sides of the Calu-3 monolayer. The monolayer was equilibrated for 30 minutes in a humidified atmosphere of 95%air/5%CO_2 at 37°C prior to resistance measurements. TEER was calculated by subtracting the resistance of a blank insert and corrected for the surface area of the Transwell polyester cell support. Three wells were assigned for measurement and the resistance of the cell monolayers in each of the three wells was measured 10 times between days 2 and 21 of culture at each passage examined.

Flu-Na uptake experiments

Flu-Na (MW 0.367 kDa) was used to assess paracellular drug transport as a measure of barrier integrity at each passage over 2–21 days in culture. To ensure that the integrity of the monolayer was maintained during the course of the experiment, TEER was measured before and after these studies. Before each experiment the culture medium was removed from each compartment and the monolayer was washed twice with warm HBSS (37°C). In the basolateral compartment, 600 μL of pre-warmed HBSS was placed and the cells were returned to the incubator at 37°C for 1–2 hours to equilibrate. To the apical compartment, 215 μL of a 2.5 mg/mL flu-Na solution was added. The cells were incubated at 37°C whilst constantly rotating on a shaking platform at 100 rpm under humidified condition. Twelve samples of 100 μL were taken from the basal compartment of each well over 4 hours, with each volume being replaced with equal amount of fresh warm buffer. The fluorescence of flu-Na was measured in black, 96-well plates using a fluorescence plate reader (POLARstar Optima; BMG Labtech, Offenburg, Germany), using excitation and emission wavelengths of 485 and 520 nm, respectively.

Flow cytometric detection of cell surface P-gp

The Calu-3 monolayers, were harvested from the Transwell inserts by trypsinization. Cells were washed twice in PBS and labeled using either 20 μL FITC-anti P-gp (clone17F9) or FITC-isotype control mAb, following incubation for 30 minutes at room temperature in the dark. The cells were washed twice in PBS and resuspended in 200 μL PBS, spiked with (1 μg/mL) propidium iodide (PI) and analyzed by flow cytometer (FCM) using the FACS CALIBUR (BD, Franklin Lakes, NJ, USA) and BD CellQuest software (Becton Dickinson Biosciences, San Jose, CA, USA).

Transport experiments

The permeability of the typical fluorescent P-gp drug substrate Rh123 across Calu-3 monolayers at passage 40 and at day 21 in culture was used to validate functional P-gp expression in the established model. Permeability was measured in the presence and absence of the classic P-gp inhibitor verapamil (10 μM) so as to confirm a P-gp-mediated specific transport event. In brief, Calu-3 monolayers were established as described above, following which Rh123 transport was measured in the apical to basolateral (A–B) and basolateral to apical (B–A) directions, so as to establish directional flux. Once again the TEER was measured before and after these studies to ensure integrity of the monolayer during the course of the experiment. One μM Rh123 test solution was added in the apical (for A–B transport) or basolateral (for B–A transport) compartments. To provide a constant concentration in both compartments for the samples containing verapamil, HBSS was aspirated from both compartments and 200 or 500 μL of the test solution, comprised verapamil 10 μM in 0.2% (v/v) ethanol, to apical and basal chambers respectively, was added and pre-equilibrated for 1 hour.

Upon initiation of the transport experiments, a 10 μL sample was taken from the donor solution to determine the initial donor concentrations (C_0). Every 20 minutes, 100 μL samples from basal (A–B transport) or 100 μL

samples from apical (B–A transport) were removed and transferred to a black 96-well plate and immediately replaced with pre-warmed HBSS to maintain a constant volume. At the final time point (4 hours), 10 µL was removed from each donor solution to establish donor concentration. The fluorescence of Rh123 was measured with a fluorescence plate reader (POLARstar Optima; BMG Labtech, Offenburg, Germany) using excitation and emission wavelengths of 500 and 540 nm, respectively.

Apparent permeability data analysis

The apparent permeability coefficient of flu-Na and Rh123 across the Calu-3 monolayer (P_{app}) was calculated according to Equation (1), where V is the volume of the receiver chamber, A is the surface area of the Transwell membrane (cm^2), C_0 is the initial concentration in donor compartment (µg/mL), and (dC/dt) is the flux of the test agent.

$$P_{app} = (V / AC_0)(dC / dt). \quad (1)$$

Histological cell surface staining for mucous detection

Alcian blue was used to stain mucous secretion on the surface of monolayers. The monolayer cultures were washed twice with 100 µL PBS and fixed using 4% (v/v) paraformaldehyde for 20 minutes. The cells were washed again with PBS and stained using 100 µL alcian blue stain (1% $_{(w/v)}$ alcian blue in 3% $_{(v/v)}$ acetic acid/water at pH 2.5) for 15 minutes. The cells were rinsed multiple times with PBS. The inserts were air-dried and the filter membrane cut with a sharp point scalpel and mounted on glass slides using Entellan new mounting medium and sealed. Images were obtained using a CX41 microscope (Olympus, Tokyo, Japan) equipped with a Leica DFC280 digital camera (Heerbrugg, Germany). The images were post processed using Apple Automater (v 2.0.4 Apple Inc., Cupertino, California, USA) to center crop 400 × 300 pixel JPEG images. Each image was analyzed using Image J (v1.42q, NIH) with Color Profile (Dimiter Prodanov; Leiden University Medical Center, Leiden, Netherlands) and Colour Inspector 3D v2.0 (Kai Uwe Barthel; Internationale, Medieninformatik, Berlin, Germany) plug-ins. A three-dimensional color space was produced representing the 8-bit red-green-blue (RGB) value of each image. The ratio of blue (RGB_B ratio) was calculated by dividing the mean RGB_B by the sum of the RGB values for each image (RGB_R + RGB_G + RGB_B). The mean ratio of five images was used as an indication of the degree of mucous production at each time point, for each passage.

Statistical analysis

Data were analyzed by using SPSS® 17.0 statistical software package (SPSS Inc., Chicago, IL, USA). ANOVA one-way analysis (with Turkey's post-hoc analysis) was utilized to test for significance. Significant difference was based on $P < 0.05$. Data represent the mean ± SD of at least three independent experiments.

Results

Transepithelial electrical resistance measurements

Calu-3 cells grew to confluence within 5 days, irrespective of passage number. When seeded on 0.4 µM membrane inserts these cells form tight epithelial monolayers over 21 days in culture. As a measure of monolayer integrity, TEER measurements were taken of Calu-3 monolayers in the air-liquid interface configuration at various intervals in culture between passages 30–40. Monolayer resistance measurements indicate that Calu-3 cells, when seeded at 5 × 10^5 cells/cm^2, generate a measurable TEER from day 2 onward, irrespective of passage number. There was a significant difference in barrier integrity between day 2 (301.52 ± 63.12 ?cm^2) and all other days examined (474.22 ± 44.76 ?cm^2), which was passage-independent. No significant difference was observed in the TEER from day 5 onward, with resistance values reaching a plateau for all passages examined (Figure 1).

Epithelial cell layer permeability

The transport of flu-Na across the cell monolayer was performed under the conditions described above, over a 4-hour period. The permeability of the paracellular marker across the membrane inserts alone (in the

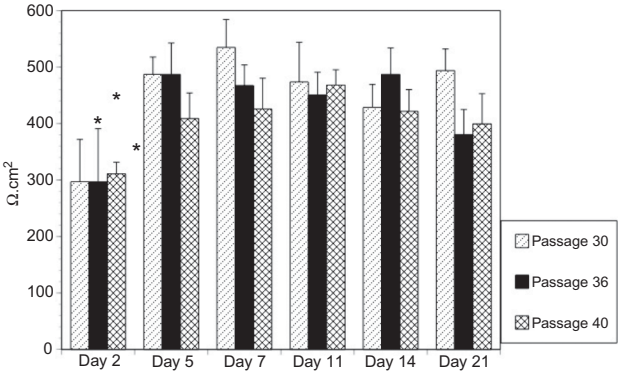

Figure 1. Transepithelial electrical resistance of Calu-3 cells seeded at a density of 5 × 10^5 cells/cm^2, under AIC condition on 0.33-cm^2 clear inserts plotted as a function of time (Mean ± SD, n = 10).

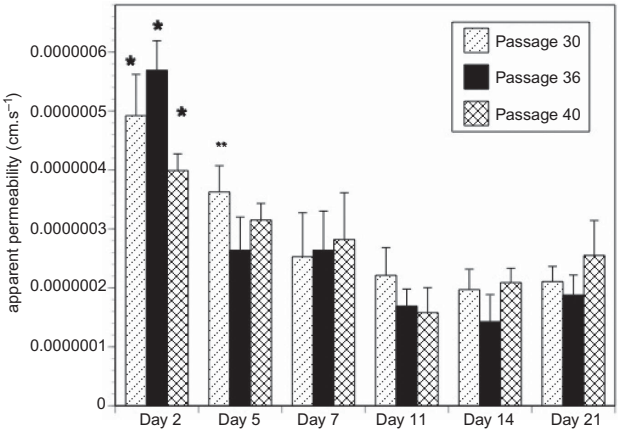

Figure 2. Transport of flu-Na across different passages of Calu-3 cell layers seeded at a density of 5×10^5 cells/cm^2, under AIC condition on 0.33-cm^2 clear inserts plotted over 21 days in culture (Mean ± SD, $n = 6$).

absence of cells) confirm that the membrane filter was not rate-limiting with respect to flu-Na flux (data not shown). Monolayers grown under AIC conditions demonstrated a significant decrease in permeability from day 2 onward, irrespective of passage number (Figure 2). This is consistent with the TEER measurements described above. There was a significant difference between the permeability of flu-Na at day 5 for passage 30 and all other days and passages examined. There was no significant difference in flu-Na permeability over time for passages 36–40 however, with flu-Na permeability reaching plateau. The apparent permeability (P_{app}) values obtained for these passages from day 5 onward were $2.33 \pm 0.368 \times 10^{-7}$ cm/s for flu-Na flux and were comparable to the permeability obtained in other works[10,12,18,19].

These results indicate a consistency between the TEER measurements and the permeability of flu-Na across the monolayer. The flux of the paracellular marker flu-Na changes very little after the monolayer reaches resistance readings of 474.22 ± 44.76 Ωcm^2 and above. These results also demonstrate that a confluent monolayer can be achieved as early as day 5, with membrane integrity stabilizing over time for passage 36–40 (Figure 2).

Cell surface P-gp expression and function

Calu-3 cells were analyzed for their surface P-gp expression by flow cytometry following direct immunolabeling. When compared to the isotype control, cell surface P-gp expression on Calu-3 cells was time dependent, with cell surface expression reaching a plateau of 32.7 ± 1.49% at day 11 onward for all passages examined (Figure 3A). There was no significant difference ($P > 0.05$) in the level of cell surface P-gp expression between passages from day 7 onward (Figure 3B), suggesting that P-gp expression was independent of the passages examined. As the isotype peaks overlayed and no difference was observed in different passages (data not shown), only one peak was included. To confirm that the detected surface P-gp was functional, Rh123 transport was assessed for directional flux in the presence and absence of the classic P-gp inhibitor verapamil at day 21, passage 40.

Directional flux of Rh123 was observed with a significant 2.4-fold greater permeability in the B–A direction at passage 40, day 21. The observed P_{app} values were $5.45 \pm 0.49 \times 10^{-6}$ cm/s and $2.27 \pm 0.165 \times 10^{-6}$ cm/s in the B–A and A–B directions, respectively. The addition of 10 μM verapamil inhibited this directional flux by threefold, confirming that the transport of Rh123 was P-gp mediated (Figure 4).

Mucous secretion

Alcian blue allows for the discriminatory detection of cellular acidic mucous alterations through recognition of sialylated (deep blue staining) mucous[20]. A distinct film of acidic mucosubstances was observed on the apical surface of the Calu-3 epithelium. In general, mucous secretion (measured by the RGB_B ratio) increased with respect to culture time. However, a confluent mucous coat was not observed until day 11 for passages 36 and 40 and day 21 for passage 30. In addition, it is important to note, that while no statistical difference in RGB_B ratio was observed post-11 days, for passages 36 and 40, the plateau may be a limitation of the measurement technique, which is qualitative in nature. Representative microscope, images, data analysis, and a plot of the RGB_B ratio with respect to time and passage number are shown in Figure 5.

Discussion

The Calu-3 AIC model of upper airway epithelium provides an in vitro model predictive of the in vivo physiological state[6]. The application of this model however, is hampered by significant variability in drug permeability, raising questions regarding the exact involvement of drug transporters, specifically P-gp in governing drug permeability across the epithelium[16,21]. Variability in drug permeability across barrier models in general has been shown to result from aspects pertaining to the culture conditions. For instance, for the Caco-2 model for gastrointestinal permeability, conditions such as time in culture, membrane support, seeding density, and media supplements have been shown to influence morphology, barrier integrity, and the expression of transporter proteins[22].

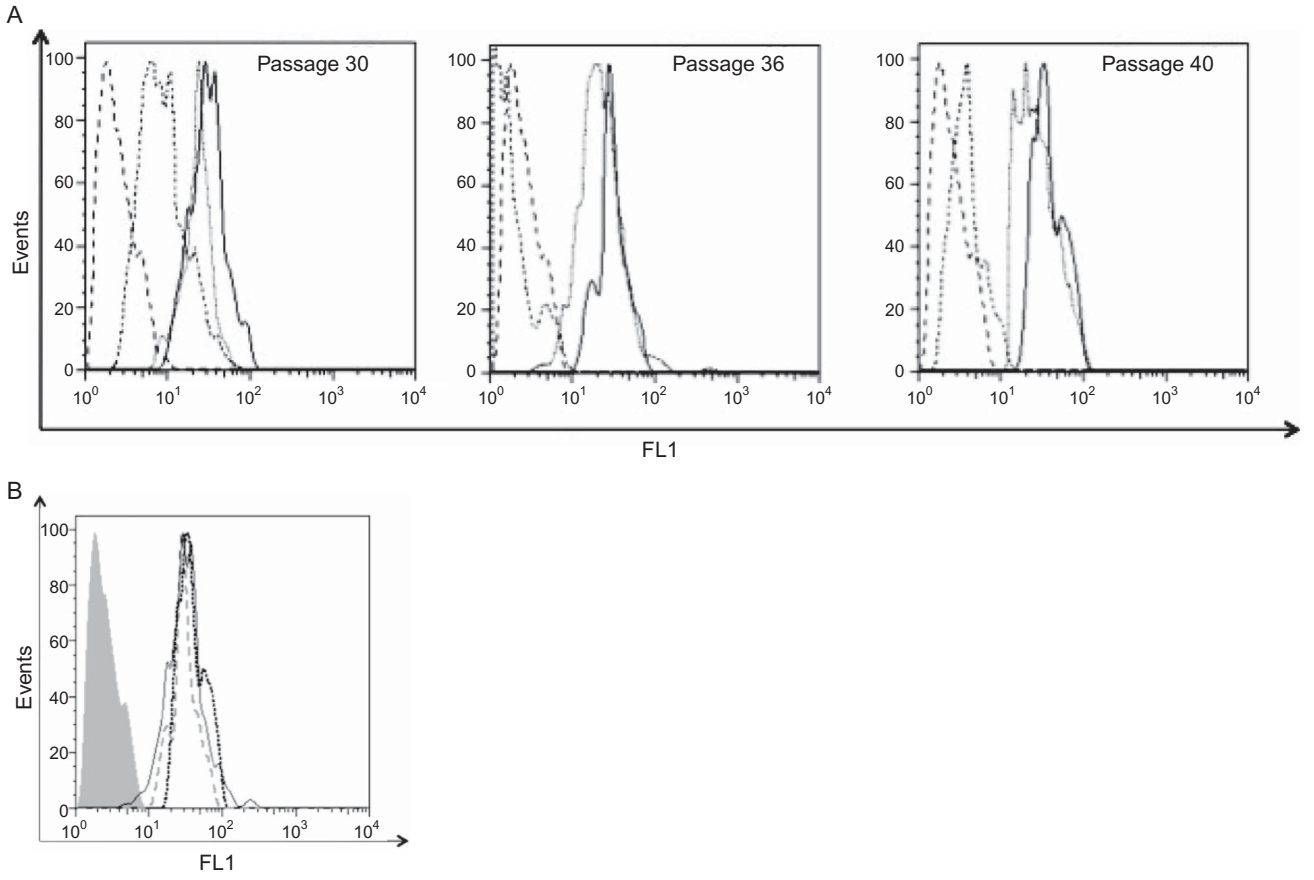

Figure 3. (A) Comparison of the cell surface P-gp expression in Calu-3 monolayers with increasing passage number. Dashes show isotype control, dots show day 2, gray and back lines show days 7 and 21, respectively. (B) Comparison of cell surface P-gp expression at day 21 for passages 30, 36, and 40 for Calu-3 cell monolayers. Shaded peak shows isotype control. The peak in gray line, the dashed peak, and the peak in black line show surface P-gp expression at day 21 for passages 30, 36, and 40, respectively (data representative of at least three independent experiments).

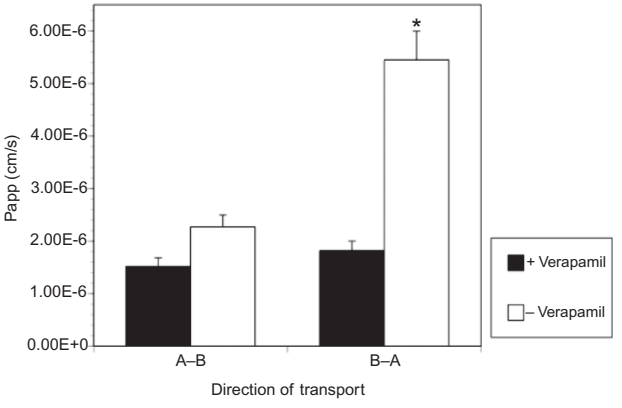

Figure 4. Apparent permeability coefficient of Rh123 across Calu-3 monolayer cultured under AIC condition at passage 40, 21 days in culture, in the presence (■) and in the absence of verapamil (□) (Mean ± SD, $n = 3$).

Since the Calu-3 AIC model has been shown to display suitable characteristics for the study of drug delivery across the upper airways, namely the presence of tight junctions, mucous secretion, and correct morphology, it is necessary to examine the effects of such conditions on aspects governing drug passage across the epithelium should it serve a purpose as a tool in predicting pulmonary drug permeability.

The effect of seeding density on growth characteristics and cell differentiation[5] are extensively known, with nominal cell-seeding densities for homogenous cell lines being in the vicinity of 50,000 cells per vessel[23–25]. For adherent cell monolayers with barrier function, including Caco-2 (intestinal model), MDCK (renal epithelium model), and the Calu-3 model, the effects of seeding density impact on time to reach confluency and hence maximal cell differentiation[7], as well as impacting on the tendency to the formation of monolayers as opposed to multilayers[22]. Nominal seeding densities have been employed by most researchers for the Calu-3 model[17]. This seeding density has been shown to result in a full-differentiated model over 21 days in culture.

Likewise, microporous membrane supports are a standard method for the culture and study of epithelial cell cultures. These membranes range in filter chemistry

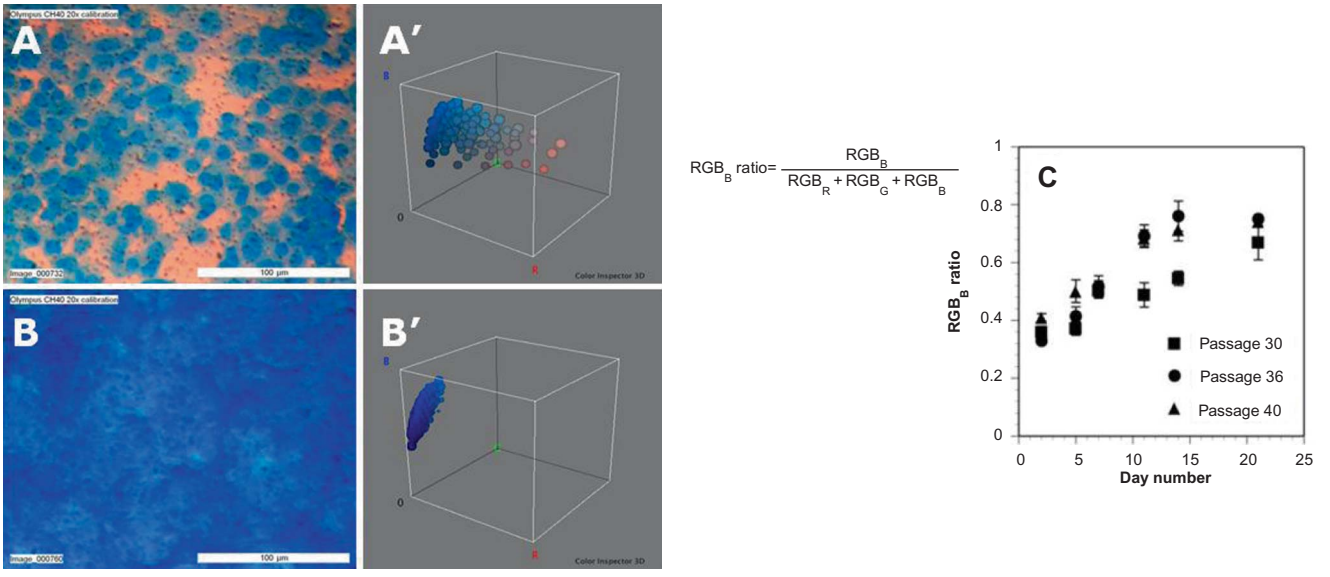

Figure 5. Mucous staining of passage 40 Calu-3 cells at: (A) day 2 and (B) day 21. (A′ and B′ show RGB color space analysis for A and B images). (C) Plot of RGB_B ratio as a function of passage and time in culture (Mean ± SD, $n = 5$).

and pore size and allow cells to uptake and secrete molecules in the basal to apical and apical to basal directions. These membrane supports facilitate cell differentiation and polarization and hence serve to produce in vitro models that mimic the in vivo state. The polyester Transwell membranes provide a transparent membrane with superior chemical resistance, making this membrane ideal for the study of different formulations across the barrier. Pores of 0.4 μM are recommended for the study of polarized transporter proteins and hence drug transport[26–29].

Aspects such as passage number and time in culture also significantly impact drug permeability[12]. However, the effects of these culture conditions, with respect to P-gp cell surface expression and consequently transport function, have not been examined. Fu and coworkers have previously established that functionally active P-gp is observed only when the transporter traffics and localizes at the level of the plasma membrane[30].

However, to date, most studies probing for functional expression of P-gp have used Western blot analysis and RT-PCR21. These methodologies, which rely on whole cell lysates, do not establish the extent of functional transporter expressed at the cell surface. Direct immunolabeling followed by flow cytometric detection provides a sensitive technique for establishing the effects of time and passage number on cell surface expression of antigens[31].

For Calu-3 cells, reported studies have typically employed the use of passages 20–56[7].

To our knowledge, there has been no systematic assessment of the functionality of the model over time within this range, particularly with respect to P-gp cell surface expression and mucous secretion. The authors report for the first time that at passages 30–40, barrier integrity (as assessed by both the TEER and flu-Na permeability) was comparable to that observed at higher passages, under similar conditions[12,18]. Similarly, a strong time dependence was observed for cell surface P-gp expression at these passages, indicative of differentiation and polarization of the model with functional transporter capacity under these conditions[30]. Confirmation of transporter functionality at the cell surface was established with the directional transport of the fluorescent P-gp drug substrate Rh123. Consequently, in the authors' opinion, the effect of time in culture significantly impacts the extent of P-gp involvement in the Calu-3 model and may explain the interlaboratory variability reported for this transporter, particularly with respect to in vitro cell based models[16,21].

Mucous secretion by the Calu-3 model, being a phenotype representative of physiological functionality, was likewise confirmed at these passage numbers and over time in culture.

These findings again confirm the presence of a differentiated and polarized phenotype in this specific model under these conditions.

Although the phenotype described herein may mature further with increasing passage, the potential for de-differentiation and altered cellular characteristics likewise increases. In the authors' opinion, a model of early passage, which displays the characteristics sought and allows for measurements of drug permeability, requiring short culture times is certainly advantageous. A time-dependent pattern for the maturation

of barrier integrity, transporter expression with peak expression of these characteristics was observed at day 7 for the Calu-3 cells described. This is of shorter duration compared to previous reports recommending 13–15 days in culture for the optimal study of drug permeability[7].

Conclusions

A functional Calu-3 cells air-interface model for the study of drug delivery in the upper airways that allows for excellent barrier integrity, optimal cell surface functional P-gp expression, and mucous secretion has been described. The novel model is physiologically relevant with respect to maintaining early passage characteristics and is well suited for application for high throughput capacity given its dependence on short culture times.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Gonda I. (2000). The ascent of pulmonary drug delivery. J Pharm Sci, 89:940–5.
2. Gonda I. (2006). Systemic delivery of drugs to humans via inhalation. J Aerosol Med, 19:47–53.
3. Sullivan VJ, Mikszta JA, Laurent P, Huang J, Ford B. (2006). Noninvasive delivery technologies: Respiratory delivery of vaccines. Expert Opin Drug Deliv, 3:87–95.
4. Elbert KJ, Schäfer UF, Schäfers H-J, Kim, K-J, Lee VHL, Lehr C-M. (1999). Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies. Pharm Res, 16:601–8.
5. Forbes B, Shah A, Martin GP, Lansley AB, Forbes B, Shah A, et al. (2003). The human bronchial epithelial cell line 16HBE14o– as a model system of the airways for studying drug transport. Int J Pharm, 257:161–7.
6. Mathias NR, Timoszyk J, Stetsko PI, Megill JR, Smith RL, Wall DA, et al. (2002). Permeability characteristics of Calu-3 human bronchial epithelial cells: In vitro-in vivo correlation to predict lung absorption in rats. J Drug Target, 10:31–40.
7. Forbes B, Ehrhardt C, Forbes B, Ehrhardt C. (2005). Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm, 60:193–205.
8. Foster KA, Avery ML, Yazdanian M, Audus KL. (2000). Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. Int J Pharm, 208:1–11.
9. Hamilton KO, Topp E, Makagiansar I, Siahaan T, Yazdanian M, Audus KL. (2001). Multidrug resistance-associated protein-1 functional activity in Calu-3 cells. J Pharmacol Exp Ther, 298:1199–205.
10. Ehrhardt C, Fiegel J, Fuchs S, Abu-Dahab R, Schaefer UF, Hanes J, et al. (2002). Drug absorption by the respiratory mucosa: Cell culture models and particulate drug carriers. J Aerosol Med, 15:131–9.
11. Ramesh Babu PB, Chidekel A, Utidjian L, Shaffer TH. (2004). Regulation of apical surface fluid and protein secretion in human airway epithelial cell line Calu-3. Biochem Biophys Res Commun, 319:1132–7.
12. Grainger CI, Greenwell LL, Lockley DJ, Martin GP, Forbes B. (2006). Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. Pharm Res, 23:1482–90.
13. Bebawy M, Morris MB, Roufogalis BD. (2001). Selective modulation of P-glycoprotein-mediated drug resistance. Br J Cancer, 85:1998–2003.
14. Fenneteau F, Turgeon J, Couture L, Michaud V, Li J, Nekka F. (2009). Assessing drug distribution in tissues expressing P-glycoprotein through physiologically based pharmacokinetic modeling: Model structure and parameters determination. Theor Biol Med Model, 6:2.
15. Fromm MF. (2003). Importance of P-glycoprotein for drug disposition in humans. Eur J Clin Invest, 33:6–9.
16. Madlova M, Bosquillon C, Asker D, Dolezal P, Forbes B. (2009). In-vitro respiratory drug absorption models possess nominal functional P-glycoprotein activity. J Pharm Pharmacol, 61:293–301.
17. Florea BI, Cassara ML, Junginger HE, Borchard G, Florea BI, Cassara ML, et al. (2003). Drug transport and metabolism characteristics of the human airway epithelial cell line Calu-3. J Control Release, 87:131–8.
18. Borchard G, Cassara ML, Roemele PE, Florea BI, Junginger HE, Borchard G, et al. (2002). Transport and local metabolism of budesonide and fluticasone propionate in a human bronchial epithelial cell line (Calu-3). J Pharm Sci, 91:1561–7.
19. Fiegel J, Ehrhardt C, Schaefer UF, Lehr CM, Hanes J, Fiegel J, et al. (2003). Large porous particle impingement on lung epithelial cell monolayers—toward improved particle characterization in the lung. Pharm Res, 20:788–96.
20. Inglis SK, Corboz MR, Ballard ST. (1998). Effect of anion secretion inhibitors on mucin content of airway submucosal gland ducts. Am J Physiol Lung Cell Mol Physiol, 274:L762–6.
21. Hamilton KO, Backstrom G, Yazdanian MA, Audus KL (2001) P-glycoprotein efflux pump expression and activity in Calu-3 cells. J Pharm Sci, 90:647–58.
22. Behrens I, Kissel T. (2003). Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 cell monolayers? Eur J Pharm Sci, 19:433–42.
23. Butler M, Thilly W. (1982). MDCK microcarrier cultures: Seeding density effects and amino acid utilization. In Vitro Cell Dev Biol Plant, 18:213–19.
24. Chen X-M, Kitts DD. (2008). Determining conditions for nitric oxide synthesis in Caco-2 cells using taguchi and factorial experimental designs. Anal Biochem, 381:185–92.
25. Ng Y, Chase H. (2008). Novel bioreactors for the culture and expansion of aggregative neural stem cells. Bioprocess Biosyst Eng, 31:393–400.
26. Grobstein C. (1953). Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. Nature, 172:869–71.
27. Toropainen E, Ranta V-P, Talvitie A, Suhonen P, Urtti A. (2001). Culture model of human corneal epithelium for prediction of ocular drug absorption. Invest Ophthalmol Vis Sci, 42:2942–8.
28. Turowski P, Adamson P, Sathia J, Zhang JJ, Moss SE, Aylward GW, et al. (2004). Basement membrane-dependent modification of phenotype and gene expression in human retinal pigment epithelial ARPE-19 cells. Invest Ophthalmol Vis Sci, 45:2786–94.
29. Widdicombe J, Sachs L, Finkbeiner W. (2003). Effects of growth surface on differentiation of cultures of human tracheal epithelium. In Vitro Cell Dev Biol Animal, 39:51–5.
30. Fu D, Bebawy M, Kable EP, Roufogalis BD, Fu D, Bebawy M, et al. (2004). Dynamic and intracellular trafficking of P-glycoprotein-EGFP fusion protein: Implications in multidrug resistance in cancer. Int J Cancer, 109:174–81.
31. Crivellato E, Travan L, Candussio L, Klugmann F, Decorti G. (1997). Identification of P-glycoprotein at the membrane of mast cell secretory granules. An immunofluorescence and protein A-gold electron microscopical investigation. Histochem J, 29:193–8.