European Journal of Pharmaceutical Sciences 47 (2012) 481–489

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

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

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ARTICLE INFO

Article history:
Received 30 March 2012
Received in revised form 6 June 2012
Accepted 8 July 2012
Available online 20 July 2012

Keywords:
In vitro models
Pulmonary delivery
Drug inhalation
Absorption prediction
Drug transporters
In vitro/in vivo correlation

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.

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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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http://dx.doi.org/10.1016/j.ejps.2012.07.007

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-

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