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Review article

Human respiratory epithelial cell culture for drug delivery applications☆

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Abstract

Recent developments in delivering drugs to the lung are driving the need for in vitro methods to evaluate the fate of inhaled medicines. Constraints on experimentation using animals have promoted the use of human respiratory epithelial cell cultures to model the absorption barrier of the lung; with two airway cell lines, 16HBE14o- and Calu-3, and primary cultured human alveolar type I-like cells (hAEpC) gaining prominence. These in vitro models develop permeability properties which are comparable to those reported for native lung epithelia. This is in contrast to the high permeability of the A549 human alveolar cell line, which is unsuitable for use in drug permeability experiments. Tabulation of apparent permeability coefficients (P_{app}) of compounds measured in 'absorptive' and 'secretory' directions reveals that fewer compounds (<15) have been evaluated in 16HBE14o- cells and hAEpC compared to Calu-3 cells (>50). Vectorial (asymmetric) transport of compounds is reported in the three cell types with P-glycoprotein, the most studied transport mechanism, being reported in all. Progress is being made towards in vitro–in vivo-correlation for pulmonary absorption and in the use of cultured respiratory cells to evaluate drug metabolism, toxicity and targeting strategies. In summary, methods for the culture of human respiratory epithelial cell layers have been established and data regarding their permeability characteristics and suitability to model the lung is becoming available. Discerning the circumstances under which the use of human respiratory cell models will be essential, or offers advantages over non-organ, non-species specific cell models, is the next challenge.

Keywords: Bronchial epithelial cells; Alveolar epithelial cells; Pulmonary drug delivery; In vitro models

1. Introduction

1.1. Drug delivery to the lung

Inhalation of medicinal aerosols to the lung for the delivery of drugs to the systemic circulation has developed into one of the most promising alternatives to oral or invasive routes of administration. Although few modern medicines are delivered by inhalation for non-pulmonary therapy, this looks set to change as interest in using the lung as a route of drug delivery gains impetus [1,2]. The reasons for the attention that pulmonary delivery is now receiving are 3-fold; (i) the increasing numbers of therapeutic peptide and protein pharmaceuticals being developed with their associated difficulties in conventional oral delivery, (ii) recognition that the rate of absorption from the lung can provide improved pharmacokinetic profiles, and (iii) continued interest in respiratory targeting of drugs for lung disorders.

Advances in pharmaceutical technology have played an important role in enabling the delivery of 'pharmaceutically-difficult' molecules by inhalation. The development of safe and effective medicines for administration via the lung, however, will require not only the optimisation of device and formulation, but also an understanding of respiratory biopharmaceutics and the interaction of inhaled medicines with the lung (Fig. 1). While the technology for delivering drugs to the lung has improved, our understanding of how

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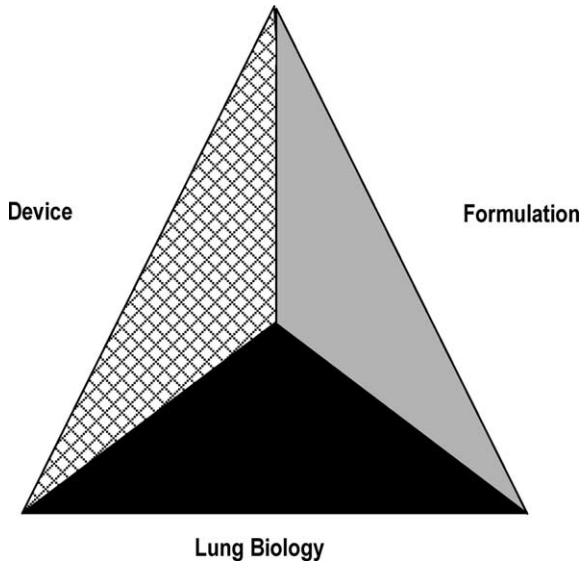

Fig. 1. Illustration of the inter-dependency in pulmonary drug delivery of delivery device, drug formulation and the biology of the lung. Knowledge of all three aspects is required for the rational design of an inhaled medicine, but recent advances in device design and formulation strategies are outstripping our understanding of how inhaled medicines interact with the lung.

inhaled medicines interact with the lung remains basic. Thus, sophisticated formulations are being developed and drugs are being designed with the aim of avoiding or promoting interaction with lung (for reviews, see Refs. [3–5]) but methods for evaluating or optimising these delivery strategies are lacking [5].

1.2. Evaluating the biopharmaceutics of inhaled medicines

In vitro techniques to evaluate drug and formulation interaction with the lung are required which can measure drug binding, uptake, transport (including the rate, mechanism and extent of absorption) and metabolism in the lung. Methods are also required to detect any adverse consequences of utilising the lung as a portal for delivery of medicines. The use of epithelial cell culture in research and development to investigate many of these aspects of drug delivery is well-established [6–9], although all new bioassays must be validated by reference to in vivo techniques.

Traditional reliance on the use of animals and animal tissue preparations to evaluate the fate of inhaled substances [10–12] is declining. In 1959, Russell and Burch published 'The Principles of Humane Experimental Technique'. They proposed that if animals were to be used in experiments, every effort should be made to *R*eplace them with non-sentient alternatives, to *R*educe to a minimum the number of animals used, and to *R*efine experiments which used animals so that they caused the minimum pain and distress. These guiding principles, the '3 *R*s' of animal research, were initially given little attention. Gradually, however, they have become established as essential considerations [13].

The acceptance of cell culture methods by the pharmaceutical industry has been facilitated by the advantages offered by the predictive ability and the higher and quicker throughput offered by these systems compared to in vivo tests employing animals [14]. These are important considerations as ever-greater numbers of new chemical entities require evaluation, often with limited compound supply and condensed time frames for drug development. The introduction of the biopharmaceutics classification system (BCS) into the guidelines of the Food and Drug Administration was another major step forward, with the biopharmaceutical properties of drug substances being classified, in part, according to their permeability measured in cell culture systems [15]. However, the greater complexity of the lung compared to the gastrointestinal tract in terms of access to the lumen, anatomical diversity, difficulty in drug administration and dosimetry makes the evaluation of respiratory biopharmaceutics a particular challenge. Furthermore, the number of medicines licensed for delivery by the inhaled route is many times lower than those for the oral route (26 compounds in Europe, excluding inhaled anaesthetics in 2001; from Ref. [16]), resulting in a paucity of human pharmacokinetic data with which to establish in vitro–in vivo-correlation.

The culture of epithelial cell layers to model the epithelia of the airway and alveolar regions of the lung has undergone significant development in the last 5 years. The aim of this review is to update earlier commentaries on in vitro modelling of the respiratory epithelium for pharmaceutical purposes [17,18]. As differences in the protein expression exist between cells from different species [19–21], and the majority of medicine is developed to cure human beings, this review will concentrate exclusively on human respiratory cell models and will describe the use of these models for drug delivery applications.

2. Cell culture models of the respiratory epithelium

The structure and function of the lung and respiratory epithelium have been reviewed extensively from a biopharmaceutical perspective [22–30]. The airway and alveolar epithelium of the lung provide the principal physical barrier to drug absorption and are composed of different cell types and have distinct properties. The general rationale in modelling the epithelium has been to develop monocultures based on the cell type contributing the majority of the epithelial surface area. This corresponds to columnar cells in the airway and the alveolar type I cell (ATI) in the alveolar region (Fig. 2a). The selection of cell type to model the epithelium is restricted by the desirability of using human cells and the requirement that the cells form functional tight junctions in culture. The convenience and practical benefits of using cell lines, if suitable cell lines are available, must be balanced against any inherent differences to native cell phenotype.

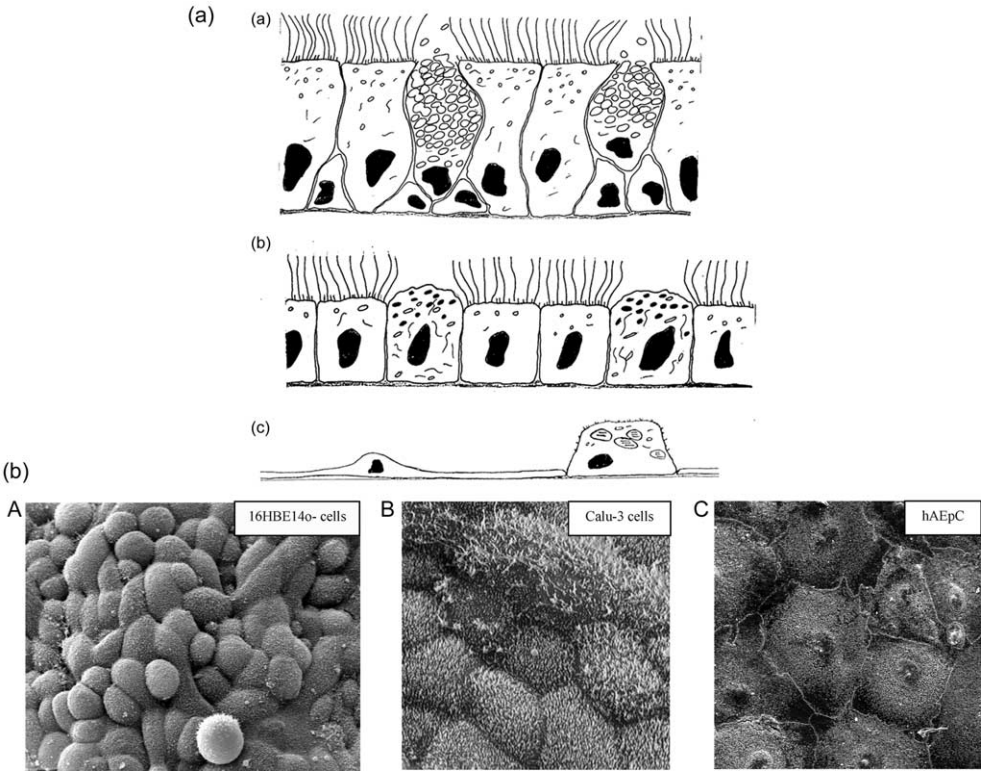

Fig. 2. (a) Illustration of the epithelium of the lung in the upper airways (a), lower airways (b), and alveoli (c). (b) (A) 16HBE14o- cells (1000×): scanning electron micrograph of cells cultured for 1 week at a density of 10^5 cells cm^{-2} on Transwell Clear inserts. The 16HBE14o- cell layer is comprised of cuboidal-shaped cells. (B) Calu-3 cells (2600×): scanning electron micrograph of cells cultured for 2 weeks at a density of 2×10^5 cells cm^{-2} on Transwell Clear inserts. The Calu-3 monolayer is comprised of columnar-shaped cells. (C) hAEpC monolayer (1000×): scanning electron micrograph of cells cultured for 1 week at a density of 6×10^5 cells cm^{-2} on Transwell Clear inserts. The hAEpC exhibit a morphology similar to type I alveolar epithelial cells.

Two human-derived cell lines, 16HBE14o- and Calu-3, are recognised to exhibit suitable respiratory bronchial epithelial cell-like phenotype and to acquire suitable barrier properties in culture [17,18]. For the alveolar region, no suitable cell lines are available and isolation and culture of alveolar type II (ATII) cells to ATI-like human alveolar epithelial cell (hAEpC) monolayers is necessary [31]. The morphology of 16HBE14o- cells, Calu-3 cells and hAEpC in culture (Fig. 2b) generally corresponds to that of the native epithelium, with Calu-3 cells appearing to be of a columnar phenotype, 16HBE14o- cells being cuboidal in shape and hAEpC cells being much flatter and laterally extended. The following sections describe the culture and characterisation of the human respiratory cell culture models.

2.1. Airway epithelial cells

The surface of the airways is lined by a pseudostratified epithelium. The air-interfaced cells are predominately ciliated columnar cells interspersed with goblet cells in the upper airways and cuboidal ciliated cells interspersed with Clara cells in the lower airways. Normal human bronchial epithelial cell culture can provide a mixed ciliated and goblet cell culture model of the airway, which has been reported to provide a permeability barrier which would be suitable for drug transport studies [32–34]. Pre-cultured human tracheo-bronchial cell layers composed of well-differentiated ciliated and goblet cells are also commercially available as the Epiairway™ system from the Mattek Corporation [35]. Although the Epiairway™ is marketed and used for drug delivery purposes, the commercial nature of these applications means that little data has been reported [35]. In general, primary culture is less convenient and economic than the use of cell lines and the primary co-culture cell layers have tended not to be used for biopharmaceutical purposes.

16HBE14o- cells are transformed bronchial epithelial cells from a 1-year old heart lung transplant patient and form confluent layers with differentiated epithelial morphology and functions [36]. Calu-3 cells are derived from a bronchial adenocarcinoma of the airway and form confluent mixed cultures of ciliated and secretory cells [37]. Culture methods and properties of these cell lines have been described in reports of studies that aimed to establish the barrier properties of the cells to solute transport [38–40]. Detailed protocols for culture, maintenance, growth and permeability assessment of 16HBE14o- and Calu-3 cell have been published [41], but it should be noted that culture methods vary between laboratories (Table 1). The influence of air–liquid interface or submerged culture appears to be one of the major influences on the transepithelial electrical resistance (TEER)

Table 1
Culture conditions and barrier properties of human respiratory epithelial cells cultured for permeability studies

Respiratory cell layer	Passage number	Seeding (cells cm^{-2})	Coating of culture surface	Serum supplement	Day of use	TEER ($\Omega\ cm^2$)	Solute (F or M) flux P_{app} ($\times 10^{-6}$ cm s^{-1})	Reference
16HBE14o- (air–liquid culture)	47–78	1.0×10^5	None	Fetal calf serum	17	127	F 4.90	[38]
	15–48	2.5×10^5	Vitrogen	Ultroser G	6–7	247	M 2.36	[39]
	65–75	4.3×10^5	Vitrogen	Ultroser G	7	150–240	M 3.20	[17]
16HBE14o- (submerged culture)	47–78	1.0×10^5	None	Fetal calf serum	7–9	533–791	F 0.85	[38]
	nr	6.0×10^5	Matrigel	Fetal calf serum		272	M 0.31	[94]
	nr	5.0×10^5	Matrigel	Fetal calf serum	6–8	327	M 1.29	[124]
Calu-3 (air–liquid culture)	nr	1.0×10^5	None	Fetal calf serum	10	500	F 0.11	[125]
	38–56	1.0×10^5	None	Fetal calf serum	8	700	F 0.22	[45]
	20–40	5.0×10^5	Rat tail collagen	Fetal bovine serum	8–16	1056–1126	F 0.15, M 0.14	[40]
	28–44	1.0×10^5	Vitrogen	Fetal calf serum	16–18	350–400	M 0.09	[83]
Calu-3 (submerged culture)	nr	1.0×10^5	None	Fetal calf serum	16	1185	F 0.11	[125]
	38–56	1.0×10^5	none	Fetal calf serum	7	2300	F 0.23	[45]
	20–40	1.0×10^5	Collagen	Fetal bovine serum	8–10	1000	M 0.47	[43]
	20–40	1.7×10^5	Rat tail collagen/ laminin	Fetal bovine serum	10	900	M 0.39	[81]
	nr	5.0×10^5	Matrigel	Fetal calf serum	6–8	359	M 0.31	[124]
	nr	5.0×10^5	Matrigel	Fetal calf serum		301	M 0.78	[94]
hAEpC (submerged culture)	Primary culture	6.0×10^5	Fibronectin/collagen	Fetal calf serum	6–8	>2000	F 0.10	[122]

Transepithelial electrical resistance (TEER) and apparent permeability coefficients (P_{app}) of small hydrophilic fluid-phase (paracellular transport) markers, mannitol (M) and fluorescein (F), reported in 16HBE14o-, Calu-3 and hAEpC cell layers. nr, not reported.

and permeability to the common permeability markers, mannitol and fluorescein, in 16HBE14o- cells (Table 1). It has been reported that the use of submerged culture conditions may reduce the level of differentiated morphology expressed by Calu-3 cells [42], although other authors have claimed, this is not to be the case [43]. It does appear that the importance of using air–liquid interfaced conditions for Calu-3 cells is not as critical in promoting cellular differentiation as it is for primary cultures [32,44]. Mucus production by Calu-3 cells, the formation of cilia and robustness to media change/manipulation by both Calu-3 and 16HBE14o- cells may also be reduced under submerged culture conditions [45].

Other factors that might affect the properties of the cell layers include the passage number of the cells, the surface the cells are cultured on, cell seeding density, composition of the culture medium and any supplements and time in culture. The coating of the culture surface and media supplements used have been reported to affect the permeability of primary cultured airway cells [32] leading to the use of Vitrogen coating and Ultroser G by the originators of the 16HBE14o- cell line [36]. However, both Calu-3 and 16HBE14o- cells form adequate permeability barriers in the absence of filter coating and with the use of standard fetal calf/bovine serum and non-essential amino acids. Seeding densities of approximately 10^5 cells cm^{-2} have typically been used for both cell lines, although the main effect of using higher or lower seeding densities is to achieve confluence and the maximum permeability barrier earlier or later in culture [39,42].

Inter-laboratory variation in culture methods and the resultant differences in the cell barrier to drug transport must be considered when the permeability of solutes across cell layers are compared (Table 2). Not only can paracellular and transcellular permeability (Section 3.1) be affected by culture conditions, but culture conditions will also have an effect on the expression and function of other cellular properties such as the expression and activity of transport proteins or enzymes (Section 3.2). This has been noted in other cell culture models, such as Caco-2 cells, in which culture conditions have been shown to influence the permeability and expression of transporters [46,47].

2.2. Alveolar epithelial cells

ATII cells constitute about 60% of alveolar epithelial cells and about 15% of all lung parenchymal cells, while they cover less than 5% of the alveolar air spaces of adult human lungs [48]. It is well known that ATII cells play a major and multi-functional role in alveolar homeostasis, including proliferation and differentiation into ATI cells as a repair/replacement mechanism. The many functions of ATII cells were recently well reviewed [49]. By comparison, the ATI cell has received little attention. The general functions of ATI cells were relatively unexplored until recently when specific marker molecules that can be used for definitive identification of ATI cells were identified (for a review, see Ref. [50]). Putative functions of ATI cells include control of proliferation of peripheral lung cells, metabolism and/or degradation of peptides and peptide growth factors,

Table 2
Apparent permeability coefficient (P_{app}) of compounds measured in the apical to basolateral (Ap-Bl) absorptive and basolateral to apical (Bl-Ap) secretory directions across the human respiratory epithelial cell culture models, 16HBE14o- cells, Calu-3 cells and alveolar type I-like cells (hAEpC)

Compound	P_{app} ($\times 10^{-6}$ cm s^{-1})		
	Ap-Bl	Bl-Ap	Reference
16HBE14o- cells			
Air-interface culture			
Atenolol	7.18	–	[38]
	1.52	–	[39]
FITC-dextran 4000	1.53	–	[38]
Metoprolol	2.94	–	[39]
Propranolol	26.60	–	[38]
	28.60	–	[39]
Salbutamol	1.42	–	[39]
Submerged culture			
Atenolol	3.87	–	[38]
Budesonide	16.20	17.60	[126]
FITC-dextran 4000	0.42	–	[38]
Insulin	0.4[a]	–	[85]
Propranolol	15.93	–	[38]
Rhodamine 123	1.52	4.48	[80]
Salbutamol	2.77	0.42	[126]
Triamcinolon acetonide	15.40	16.90	[126]
Calu-3 cells			
Air-interface culture			
Antipyrine	29.3	–	[40]
Budesonide	5.33	4.11	[83]
Caffeine	21.8	–	[40]
Chromoglycate	0.025	–	[40]
Dexamethasone	7.4	–	[40]
FITC-dextran 4000	0.006	–	[40]
FITC-dextran 10,000	0.002	–	[40]
FITC-dextran 20,000	0.001	–	[40]
FITC-dextran 70,000	0.0002	–	[40]
Flunisolide	8.11	1.22	[86]
Inulin	0.066	–	[40]
Insulin	0.002	–	[40]
Leucine	21.1	–	[40]
Lucifer yellow	0.043	–	[40]
Para amino hippuric acid	0.258	–	[40]
Propranolol	14.4	–	[40]
Rhodamine 123	6.6	75.9	[76]
Salbutamol	0.137	–	[40]
Theophylline	9.5	–	[40]
Tetraethylammonium bromide	0.054	–	[40]
Uridine	0.93	–	[40]
(Cocaine[b], epinephrine, lucifer yellow, morphine, nicotine, salbutamol, sucrose)	$\propto \log D$[c]	–	[127]
(Texas red/BSA)	Low	–	[114]
Submerged culture			
AZT	27.0	39.0	[81]
Budesonide	15.00	14.90	[126]
Ciprofloxcin	6.92	≡Ap-Bl[d]	[82]
Diazepam	17.0	–	[81]
Flunisolide	8.05	1.25	[86]
IgA	Ap-Bl> Bl-Ap		[128]
Insulin	0.004	0.03	[43]
Monomeric insulin analogue	0.029	0.029	[43]
Polyethylene glycol	0.096	–	[43]
Ritonavir	0.316	5.83	[81]
Salbutamol	1.99	0.44	[126]
Saquinavir	0.502	9.07	[81]
Triamcinolon acetonide	23.10	21.10	[126]
(Cyclosporin A)	Ap-Bl< Bl-Ap		[81]
(Digoxin, verapamil)	~10[e]	≡Ap-Bl[d]	[82]
hAEpC			
Submerged culture			
Atenolol	0.516	0.494	[126]
Budesonide	13.30	15.10	[126]
FITC-dextran 4000	0.0171	0.0112	[31]
FITC-dextran 10,000	0.0105	–	[31]
FITC-dextran 20,000	0.0080	–	[31]
FITC-dextran 40,000	0.0029	–	[31]
FITC-dextran 70,000	0.0019	0.0010	[31]
Insulin	0.0766	0.0723	[126]
Propranolol	14.20	13.80	[126]
Rhodamine 123	1.41	4.35	[126]
Salbutamol	7.46	0.520	[126]
Triamcinolon acetonide	19.90	15.30	[126]

[a] Calculated from data in Ref. [85].
[b] Cocaine damaged the cell layer.
[c] P_{app} values not reported.
[d] Ap-Bl, Bl-Ap equivalence stated.
[e] Estimated from figure in Ref. [82].

generation of cyto/chemokines, regulation of alveolar fluid balance, and transcellular ion and water transport.

At present, monolayers of pneumocytes in primary culture are the only reliable model for studying alveolar transport processes [51]. The human cell line A549 has been utilised to study the physiology of the ATII cell and as a permeability screen for pulmonary administered substances [52,53]. However, considering the lack of functional tight junctions in this cell line, its use to study drug absorption is of limited value (Section 3.3). Protocols for the isolation and culture of ATII cells, which can be cultured to form tight monolayers of ATI-like cells, have been published for different species, e.g. rat [54–56], mouse [57], rabbit [58], pig [59], and human [31,60,61]. Human alveolar cell culture models are based on alveolar epithelial cells derived from human patients undergoing lung resection surgery [62]. Briefly, the tissue is chopped and subsequently digested using a combination of trypsin and elastase. The ATII cell population is then purified by a combination of differential cell attachment, percoll density gradient centrifugation and by magnetic cell sorting. When the ATII cells are grown on polystyrol membranes, they form monolayers of ATI-like hAEpC with typical TEER values of ~2500 Ω cm^2 on day 6–8 post seeding.

A number of approaches have been developed to distinguish between ATII and ATI cell phenotype in culture. These include simple morphological characterisation (e.g. presence of lamellar bodies, cuboidal shape), plus

modified Papanicolaou-staining, use of cell type-specific lectins [63] and immunohistochemical/immunocytochemical markers such as aquaporin-5 [64], caveolin-1 [61,65], and the receptor for advanced glycation endproducts (RAGE) [66,67]. The expression of markers, however, may be altered due to the specific culture conditions and the situation is further complicated by the transient appearance of an intermediate phenotype during differentiation, in which both ATI and ATII cell-like features may co-exist [68]. The difficulty in providing definitive evidence showing that ATII cells yield terminally differentiated ATI cells in culture necessitates the use of a term, 'ATI cell-like' phenotype.

3. Drug delivery applications of respiratory epithelial cell culture models

3.1. Drug permeability

When last reviewed [17], the transport data in human respiratory airway cell culture models was largely limited to the proceedings of conferences [69–71]. Subsequently, permeability data has appeared for a variety of compounds in both 16HBE14o- and Calu-3 cell lines (Table 2). The permeability of fewer compounds (several small organic molecules plus FITC-dextran 4000 and insulin) has been measured in 16HBE14o- cells compared to Calu-3 cells in which the permeability of over 50 compounds of a diverse physicochemical nature has been reported. Dependence of permeability on lipophilicity was found in both 16HBE14o- [39] and Calu-3 cells [40], as has been observed for passively absorbed compounds in cell culture models of other epithelia [72,73]. Size dependency has also been reported in Calu-3 cells [40]. Solute permeability in both cell lines has been compared to that found in normal rabbit tracheal epithelial cell cultures and found to be similar [39,40].

Size dependency was also observed in monolayers of human alveolar epithelial cells [31]. Lipophilicity can also be correlated to the permeability of substances across hAEpC monolayers (Fig. 3) and can, to a certain extent, predict their absorptive behaviour. Nonetheless, reports on the permeability of (drug) compounds across monolayers of human alveolar epithelial cells are still scarce (Table 2). This may be attributed to the restrictions pertaining to the use of human tissues in certain countries or a lack of availability of human tissue to many laboratories.

3.2. Drug absorption mechanisms

Studies of passive permeability of compounds across polarised cell monolayers generally find dependency on molecular size and lipophilicity. Thus, similar permeability trends are found in different cell lines with the result that permeability measured in cell lines of different organ and species origin can be used to predict drug absorption from alternative absorption sites. Examples of this are the use of

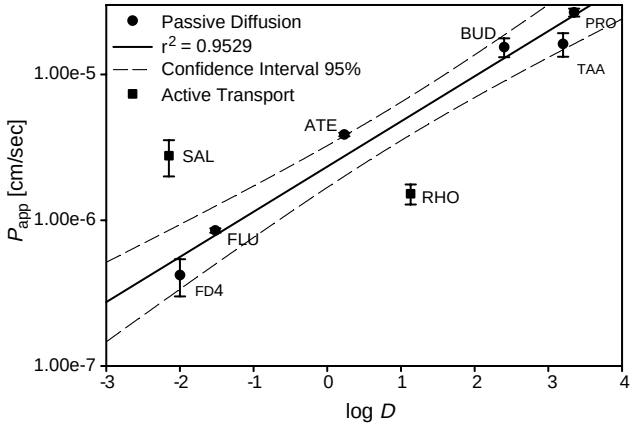

Fig. 3. Correlation between absorptive permeability across hAEpC monolayers and lipophilicity (log D at pH 7.4). The passively diffusing substances (●) show a very good correlation ($r^2 = 0.95$), while all substances that are outlyers of the confidence interval (95%) are known substrates of active transport processes (■). Abbreviations: ATE, atenolol; BUD, budesonide; FD4, FITC-labelled dextran 4000; FLU, fluorescein sodium; PRO, propranolol; RHO, rhodamine 123; SAL, salbutamol; TAA, triamcinolone acetonide.

canine kidney epithelial cells (MDCK) to predict human oral absorption [74] and human gastrointestinal cells (Caco-2) to predict absorption from the rat lung [75]. In contrast, active transport mechanisms by which compounds traverse the air–blood barriers of the human lung are more likely to provide new insights and improved strategies for pulmonary delivery of drugs into the systemic circulation and targeting drugs to lung parenchymal cells via specialised processes (e.g. receptor-mediated uptake). A particular strength of cell culture techniques is that they are amenable to study mechanisms of drug transport, although the study of active transepithelial transport processes requires a tight layer of polarised growing cells, because otherwise unrestricted diffusive processes may mask any directed active transport. Transport mechanisms that have been identified as being present in the lung include P-gp [76], organic ion transporters [77] and peptide transporters [78].

P-glycoprotein is the most studied transport mechanism in Calu-3 and 16HBE14o- cell lines. The expression of the MDR1 protein has been demonstrated by both Calu-3 [79] and 16HBE14o- [80] cell lines. Uptake and secretion of P-gp substrates by Calu-3 cells has been shown [79] and vectorial transport has been shown in both Calu-3 cells [76,81] and 16HBE14o- cells [80], although these are modest by comparison with the efflux seen in Caco-2 cells. The induction of P-gp in Calu-3 cells by supplementation of 1α,25di-hydroxy vitamin D_3 and basolateral to apical efflux of cyclosporin A, ritonavir and saquinavir has been reported [81]. Induction of P-gp reduced the apical to basolateral (absorptive) transport of saquinavir 3-fold, with no alteration of the transcellularly transported non-P-gp substrate marker diazepam. Interestingly, the absence of vectorial transport of P-gp substrates has also been reported in Calu-3 cells [82,83] and 16HBE14o- cells (unpublished

data; Forbes), implying that activity of the transport mechanism may depend on culture conditions. Calu-3 cells have also been used to screen inhaled drugs for susceptibility to P-gp transport [84]. However, the influence of P-gp on the absorption of inhaled substrates has not been shown in vivo.

Insulin transport has been studied in both 16HBE14o- [85] and Calu-3 [43] cell lines. Paracellular transport was the mechanism of insulin permeation in both cell lines, and lower permeability in Calu-3 cells reflected the tighter junctions in that cell line. Mechanistically, asymmetric transport was found in Calu-3 cells and attributed to aggregation of insulin monomers at the apical surface. Basolateral to apical transport was not measured in 16HBE14o- cells, but different absorption enhancing mechanisms were distinguished for two permeability enhancing agents. In contrast to insulin, the transport of budesonide was elucidated to be transcellular (the compound can be taken up by Calu-3 cells) and passive (concentration-dependent, non-vectorial) [83], despite budesonide being a P-gp substrate [84]. Budesonide, but not flunisolide, was conjugated to fatty acids intracellularly resulting in retention of the compound in the cells and apical release over a period of 10 h. It was not reported whether this mechanism is specific for respiratory epithelia or particular to Calu-3 cells [83]. Interestingly, vectorial fluticasone transport has been reported in the apical to basolateral direction [86] and this has been hypothesised to be caused by P-gp located at the basolateral surface [42,87].

In hAEpC active transport of two solutes has been identified, namely the secretory transport of rhodamine and the absorptive transport of salbutamol. These are outliers from the confidence interval (95%) of the relationship between lipophilicity and permeability for passively diffusing compounds (Fig. 3). Putative transporters for the active transport have been identified in hAEpC; P-gp and organic cation transporter [88,126]. ATI cells exhibit a number of transport features which might be unique for the cell type. It has long been known that ATI cells contain numerous, small, flask-shaped membrane invaginations, or caveolae, that open to the alveolar lumen or interstitial space. In addition to these caveolae at the cell membranes, numerous small vesicles are also noted in both ATI (but not ATII) cells and pulmonary vascular endothelial cells. Caveolin-1, a 21–24-kDa protein, is the major scaffolding protein that forms the vesicular skeleton of the caveolae which is selectively expressed in ATI rather than ATII cells and has been identified in hAEpC [61]. Investigations conducted in tight monolayers of rat alveolar epithelial cells showed that the clearance of a number of (endogenous) proteins (e.g. IgG, albumin, transferrin) occurred by active transport across these cells (for a review, see Ref. [89]).

3.3. In vitro–in vivo correlation

A basic consideration in evaluating the respiratory cell culture models is their permeability compared to that of native airway and alveolar lungs. Values are not available for human lungs, but TEER and mannitol permeability have been reported for animal respiratory epithelium. The TEER of rabbit airway epithelium has been reported to be 260–300 $\Omega\,cm^2$ [90,91], which is exceeded by the TEER of the 16HBE14o- and Calu-3 cell layers shown in Table 1. For comparison, TEER for well-differentiated primary cultured human tracheobronchial cells have been reported in the ranges 300–500 [34] and 450–650 $\Omega\,cm^2$ [35]. Encouragingly, where data is available for the permeability of airway tissue to mannitol (in dog and guinea pig; [12]) values are comparable to permeability in the 16HBE14o- and Calu-3 cell models, while the lower permeability of isolated rat lung, which is thought to reflect the alveolar region [12], is similar to permeability in hAEpC (Fig. 4).

The first in vitro–in vivo comparisons of permeability in airway cell models with absorption from the intact lung have been performed. The availability of in vivo data is limited, and the variation in methodology used in pulmonary absorption experiments (e.g. species, administration by aerosol or solution, measurement of blood levels or pulmonary retention), restrict the availability of coherent data sets. In vitro–in vivo correlation has been reported for Calu-3 cells [40] by comparing solute permeability in cell culture to data obtained mainly by Schanker and co-workers in the 1970–1980s using disappearance of intratracheally administered compounds in rats. The work of Tronde et al. [16,75] has recently provided in vivo and ex vivo data for absorption from rat lungs for several compounds. Permeability in the non-respiratory Caco-2 cell line provided a good correlation with absorption from the rat lung and these studies are being extended to measure the of permeability of the same compounds in 16HBE14o- cells.

For the hAEpC model in vitro–in vivo correlation has not been reported. Effros and Mason [92] showed an apparent inverse relationship between molecular weight and rate of lung clearance of molecules of different sizes in vivo. This relationship has been found in alveolar epithelial models, with similar data being reported in rat [93] and human [31] monolayers (Fig. 5a). By comparison, protein permeability [53] and FITC-dextran permeability [52] are over 2 orders of magnitude higher in A549 cells as highlighted in Fig. 5a. A further illustration of the ‘leakiness’ of A549 cells is that other epithelial cell models are highly restrictive to the transport of large molecules such as FITC-dextran 70,000 (Fig. 5b). The high permeability of A549 cells is a result of the cell line being functionally deficient in tight junctions [94] and the excessive ‘leakiness’ of A549 cell layers has been recognised previously [31,51]. It was the use of ‘tight’ monolayers that allowed the permeability of proteins across the rat ATI-like cells to be recognised as being higher than predicted by their molecular size [93]. These observations point to transcytosis involving caveolae and clathrin-coated pits being the likely route of alveolar epithelial protein transport and, intriguingly, these actively absorbed proteins

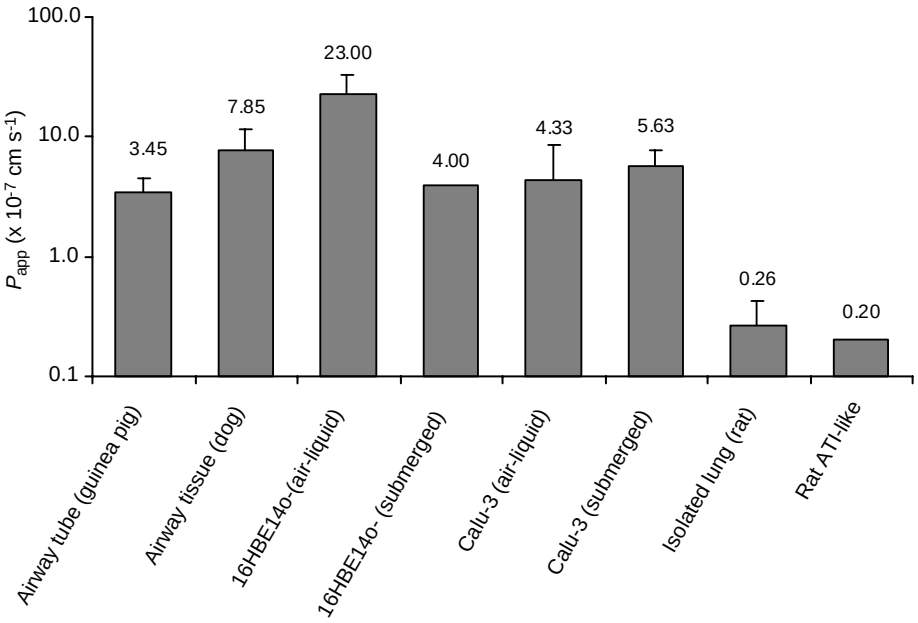

Fig. 4. The apparent permeability coefficient (P_{app}) of mannitol in respiratory tissues and cell culture models. All data represents mean ± SD for the permeability of mannitol reported in several studies. Airway tube ($n=3$) and diffusion chamber mounted airway epithelium ($n=6$) data are from studies reviewed by Widdicombe [12], isolated rat lung ($n=3$) data are estimated by Widdicombe [12], 16HBE14o-, Calu-3 and rat alveolar epithelial cell data are calculated using values in Table 1.

were processed relatively intact, whereas other proteins undergo significant degradation [93].

In general, the absorption of passively transported compounds can be predicted using non-lung cell culture models, with in vitro–in vivo relationships reflecting the 'tightness' of the respective intercellular routes which progressively restrict the transport of larger and more hydrophilic compounds. The use of respiratory cells will, however, be crucial for the study of lung-specific drug transport or metabolic pathways. As respiratory cells are the target for inhaled formulations or particles, it will also be important to use these cells to evaluate drug targeting strategies.

3.4. Drug metabolism and toxicology

Studies that utilise respiratory epithelial cells for drug metabolism and toxicological purposes are generally not dependent on the formation of functional tight junctions and an epithelial-like permeability barrier in culture. Human respiratory cell lines that do not form functional tight junctions and have not hereto been considered suitable can therefore, be used for such studies. This includes, in particular, the cell lines BEAS-2B (airways) and A549 (alveolar). As drug metabolising enzymes of the human airway and alveolar respiratory epithelia are ideally located for the presystemic metabolism of inhaled drugs, the identity and activity of phase I and phase II enzymes in cultured respiratory cells can be studied to provide an indication of the biotransformations that may occur in vivo. Enzyme expression and activity in human respiratory epithelial cell lines have been compared to that in the lung.

Cytochrome P450 (CYP) enzymes, the principal phase I xenobiotic-metabolising enzymes, have been studied in A549 cells [95–97], BEAS-2B cells [97–99], Calu-3 cells [99,100] and NHBE cells [98,99]. CYP activity in NHBE cells was reported to be slightly low and the expression pattern slightly altered. Despite the presence and/or activity of many CYPs of the human lung being identified in each of these respiratory cell types in vitro, published data is not sufficient to declare whether any cell line is a good model of the lung for drug metabolism. It remains unclear whether the spectra of phase I enzyme activities in particular cell types reflect accurately the airway or peripheral regions of the lung from which they are derived.

Phase II metabolising enzymes, including the major conjugating enzyme families, UDP-glucuronosyl transferases (UGT), glutathione S-transferases (GST) and *N*-acetyl transferases (NAT) have also been identified in lung tissue [101]. UGTs have been found in in NHBE, Calu-3 and BEAS-2B cells [99]. Of relevance to inhaled peptide and protein delivery, significant proteolytic activity has also been found in A549 cells [102] and BEAS-2B cells [103], and Calu-3 cells [104].

Human respiratory cell cultures have also found use for evaluating the toxicity and biocompatibility of inhaled medicines with the respiratory epithelium. A549 and BEAS-2B cells have been used extensively to investigate the effects of inhaled environmental pollutants such as dust [105], cigarette smoke [106], diesel exhausts [107] using

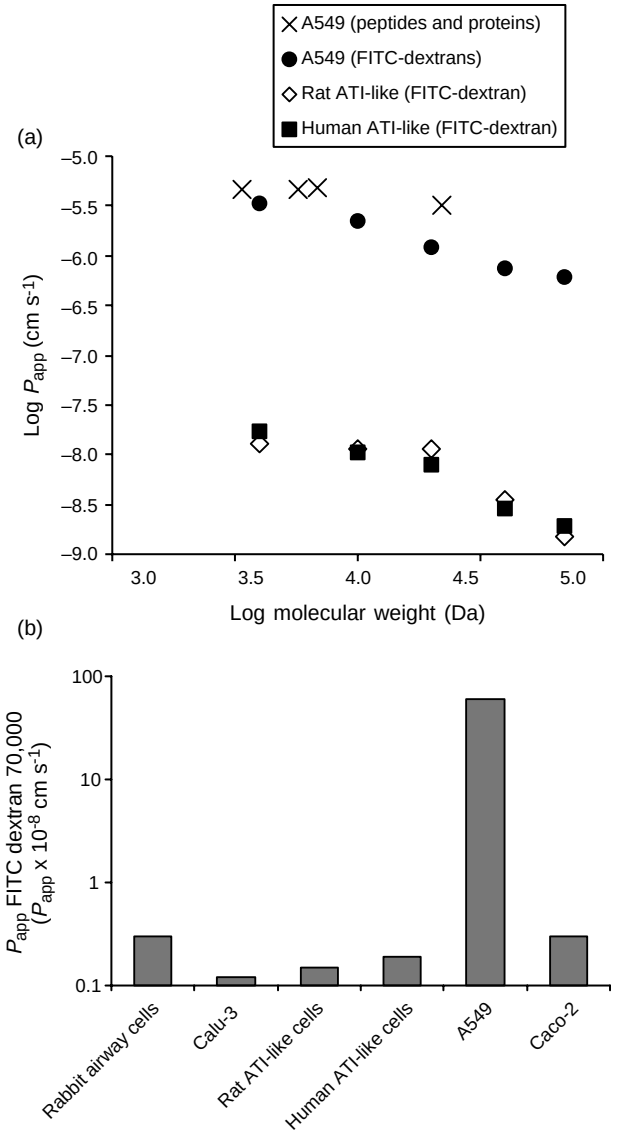

Fig. 5. (a) Rat [93] and human [31] alveolar type I (ATI)-like cell monolayer permeability to macromolecules compared with the permeability of A549 cells to dextrans [52] and peptides and proteins [53]. (b) The apparent permeability coefficient (P_{app}) of FITC-labelled-dextran 70,000 MW illustrating the leakiness of A549 cell monolayers compared to other cell culture models. In all other cell layers, the apparent permeability coefficient (P_{app}) of dextran 70,000 is $<10^{-8}$ cm s^{-1}. In A549 cell layers, P_{app} is over two orders of magnitude times greater. Data from rabbits [129], Calu-3 [40], rat and human ATI-like cells [31], A549 [52], Caco-2 cells [130].

standard toxicity assays such as cellular metabolic activity, membrane integrity and the release of proinflammatory cytokines. Similar techniques have been used to study the biocompatibility of inhaled drugs and formulations with these cell lines.

The use of the barrier-forming 16HBE14o- and Calu-3 cells is necessary when altered permeability is used as an indicator of toxicity, or when the efficacy–toxicity relationship of absorption enhancers is studied. TEER reduction has been used as an indication of adverse effects of model toxicants [108] and inhalation delivery vehicles [109] in 16HBE14o- cells and corresponded well with standard toxicological tests. 16HBE14o- and Calu-3 cells have recently proved useful for the study of absorbance enhancers [110] and inhalable formulations or excipients [111].

The metabolic characteristics and toxicological sensitivity of cell lines used for the above studies should mimic those of their respective bronchial or alveolar origins. All cell lines carry the caveat that phenotypic differences between the transformed cells and normal human cells in vivo may exist and differences in biological responses may be observed in vitro compared to in vivo.

3.5. Drug targeting

Drug targeting to the lung may be considered to include delivery of aerosols directly to the respiratory epithelium by inhalation, engineering of inhaled particles to promote uptake by respiratory epithelial cells (and macrophages) and the design of molecules or formulations to promote retention or clearance from the lung. In vitro methods which mimic the conditions that are encountered in the lung following inhalation are required to guide formulation design from a biopharmaceutical perspective. Conventional ‘complete immersion’ methods used to characterise particle water uptake rates, polymer degradation kinetics, drug diffusion rates, and particle dissolution rates may not be relevant for particles designed for inhalation due to the extremely thin aqueous layers found in the lungs [112,113]. These aqueous layers range in depth from approximately 8 μm in the bronchial region to less than 0.2 μm in the alveolar region [112], which is one order of magnitude smaller than a typical aerosol particle, and two orders of magnitude smaller than a large porous particle.

Particle interactions with human respiratory cell layers have been reported using aqueous suspensions of respirable particles [114–117]. More recently, methods for delivering aerosols directly to the surface of air-interface epithelial cell layers have been developed for 16HBE14o- [118] and Calu-3 cells [45]. The advantage of this approach is that mechanistic processes that cannot be studied directly in vivo can be investigated in vitro, as illustrated by the use of Calu-3 cells to study the mechanism of absorption of inhaled heparin and low molecular weight heparin [119]. Other targeting strategies that have been evaluated in human respiratory cell layers include the absorption enhancing effects of cyclodextrins [120], chitosan and hyaluronic acid [121], identification of internalising peptides [122] and lipid carriers for gene delivery [123]. In addition, Calu-3 cells showed the ability to perform fatty acid esterification of budesonide [83]. In preclinical studies, this esterification is correlated to a prolonged local tissue binding and efficacy, which is not found when the esterification is inhibited by an esterification blocker.

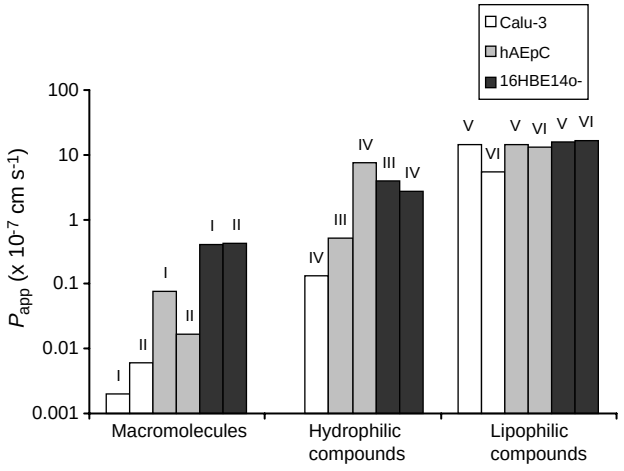

Fig. 6. Comparison of the absorptive apparent permeability (P_{app}) of macromolecules, hydrophilic compounds and lipophilic compounds in the Calu-3, hAEpC and 16HBE14o- respiratory epithelial permeability models. Abbreviations: I, insulin; II, FITC-dextran 4000; III, atenolol; IV, salbutamol; V, propranolol; VI, budesonide.

4. Conclusion and future developments

Work in each of the drug delivery applications described in this review is ongoing in academic and industrial laboratories worldwide. Methods for the culture of human respiratory epithelial cell layers are established and data regarding their permeability characteristics and suitability to model the lung is becoming available. A crude analysis, using permeability data that is available for compounds evaluated in each of the 16HBE14o-, Calu-3 and hAEpC models, indicates that large differences in absolute permeabilities in each cell line are found for macromolecules and to a lesser extent for hydrophilic compounds (Fig. 6). Each model, however, is able to discriminate clearly between the permeabilities of macromolecules, hydrophilic and lipophilic compounds (Fig. 6) as occurs for pulmonary absorption in vivo. At present, there is little to indicate that any model provides an advantage in predicting the absorption of any particular class of molecule from the lung.

Discerning under what circumstances the use of these cell models offer advantages over non-organ, non-species specific cell models is the next challenge. The nature of this challenge and the need for validated robust in vitro methods means that there is enormous scope for collaboration towards a better understanding of respiratory biopharmaceutics and how these can be studied. The importance of this to the pharmaceutical industry was recently highlighted in a review by Edwards and Dunbar, 'aerosol design is limited by partial knowledge of the lungs' physiological environment and, driven largely at this stage by market forces, relies on a mixture of new and old science, pharmaceutical science intuition and a degree of biological-impact empiricism that speaks to the importance of an increased level of academic involvement' [5]. The benefit of cell cultures is that they provide a system in which to study the underlying mechanisms by which inhaled medicines interact with the lung.

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