Expert Opinion

Pulmonary cell culture models to study the safety and efficacy of innovative aerosol medicines

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Background: Pulmonary cell culture models for the development of new aerosol medicines are attracting increasing interest. Ease of handling, ethical acceptance and high explanatory power are the main advantages of cell culture systems in pulmonary drug research. *Objective*: Pulmonary cell culture models are described and evaluated regarding their suitability for the biopharmaceutical characterisation of innovative aerosol medicines. *Methods*: The review focuses on the peculiarities of the pulmonary cell culture models arising from the specialised pulmonary epithelia, the clearance systems in the lung and the limited but functionalised lung fluid layers. Additional aerosol deposition systems suitable for the close to *in vivo* simulation of aerosol delivery on pulmonary cell cultures are described. *Results*: Suitable cell culture models of the cellular part of the human air–blood barrier are established and well characterised. However, the physical barriers on top of the cellular barriers – surfactant, mucus and so on – and their influence on the safety and efficacy of aerosol medicines, are still underestimated.

Keywords: 16HBE14o-, A549, Calu-3, hAEpC, lung lining fluid, mucus, surfactant

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1. Introduction

Cell culture models to investigate the safety and efficacy of medicines have been widely used in the last 25 years. *In vitro* models for almost all epithelial barriers of the human body are described in the literature [1]. In contrast to most of the other epithelial frontiers of the body, the human lung posses a sophisticated fluid system, resulting in a slightly moisturised surface. Furthermore, the lung, as a major port of entry, has developed a system to prevent the invasion of unwanted airborne particles from the environment into the body. Airway geometry, humidity, mucociliary clearance and alveolar macrophages play a vital role in maintaining the sterility of the lung and consequently are obstacles for aerosol medicines and in particular for the development of pulmonary controlled release formulations.

2. Epithelial barriers of the respiratory tract

To assess the value and the limitations of the models described below, it is necessary to elucidate the anatomy and functionality of the human air–blood barrier, the barrier we simulate with cell culture.

2.1 Epithelium of the deep, peripheral lung

The air–blood barrier in the gas exchange area is composed of alveolar epithelial cells (surface area 140 m^2) on one side and the capillary bed (surface area 130 m^2)

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on the other side of a thin basement membrane. The extensive surface area of the air–blood barrier in combination with its extreme thinness (0.1 – 0.5 μm) permits rapid gas exchange by passive diffusion. In the alveolar region, mainly two cell types are present: epithelial type I and II cells. The squamous type I cell covers approximately 96% of the alveolar surface area and has an average cell thickness of 0.26 μm. Characteristically the alveolar type I cell has a large cytoplasmic volume and displays only a few cellular organelles. These morphometric features are favourable for gas exchange and for drug transport. About 3% of the alveolar surface is covered by much smaller cuboidal type II cells, synthesising and secreting surface active materials [2].

2.2 Epithelium of the conducting airways

In contrast, the much smaller (1 – 2 m^2) airway epithelium provides a tight ciliated barrier that clears the airways of deposits in the airway mucus, prevents indiscriminate leakage of water and solutes into the airways, secretes components for the airway lining fluid and mucus layers and modulates the response of inflammatory cells, vessels and smooth muscle. The bronchial epithelium is composed of seven different cell types: goblet cells, basal cells, ciliated cells, brush cells, serous cells, Clara cells and neuroendocrine cells [3]. The epithelium lining the terminal bronchioles is columnar or cuboidal and is composed of ciliated cells and Clara cells.

The apical membranes of both the bronchial and alveolar epithelial cells are joined by tight junctions (TJs) dividing the cell membranes into functionally distinct apical and basolateral domains. Tight junctions represent the primary apical cell–cell contacts in epithelial and many endothelial cell sheets and are essential for their barrier function [4-6]. In addition, TJs act as a kind of fence maintaining the specific lipid and protein composition of apical and basolateral membrane domains in polarised epithelia. The association with different signalling molecules indicates that TJs are more than simple rigid barriers separating compositionally distinct environments [7]. The multi-protein complexes of the TJs are linked to several transmembrane proteins and peripheral proteins. Some of the tight junctional proteins are linked directly to the cytoskeleton [8], while others are involved in signal transduction [9,10].

Tight junctions are also sensitive against growth factors (EGF, VEGF), cytokines (TNF-α, various interleukins), hormones (glucocorticoids, oestrogen), drugs and nutrients [9-12]. The pathways to how these factors influence TJ permeability are diverse. Mechanisms that include endocytosis of TJ proteins, insertion of newly synthesised junctional proteins or altered protein–protein interactions are all under discussion. Recent studies have shown that ion channels and ion pumps like Na, K-ATPase are also involved in the regulation of TJ structure and function [13]. As a consequence of the existence of TJs we will find a higher transepithelial electrical resistance (TEER) in an epithelium with a high density of TJs than in a leaky epithelium. Less is known of the *in vivo* tightness of the human air–blood barrier, but from transport experiments with hydrophilic low molecular weight model drugs like Na-fluorescein, for cell cultures with a TEER of more than 500 Ω* cm^2 it is imperative to ensure that the observed transport processes are exclusively controlled by the cell monolayer and not by leaky or missing TJs [14].

2.3 Endothelial barrier

The lung is unique among tissues in that about 40% of total cellular composition is capillary endothelium, which is the largest capillary endothelial surface in the body. The alveolar–capillary endothelium has specialised organelle-free domains to provide a particularly thin (from 200 nm down to 30 – 35 nm) barrier for gas exchange [15]. Furthermore, the endothelial cells have a relatively large number of endocytic vesicles [16]. To date the endothelial barrier for drug absorption has not been sufficiently investigated, but it can be assumed that the endothelial structures also contribute to the tight barrier for drug absorption.

2.4 Cellular connective tissue

The interstitium of the lung, the extracellular and extravascular space between cells in the tissue, contains a variety of cells (fibroblasts, myofibroblasts, pericytes, monocytes, lymphocytes, plasma cells), collagen, elastic fibres and interstitial fluid. Their main role is to separate and bind together the specific cell layers in the tissue. The main drainage pathways for the interstitial fluid are the lymphatic vessels. The outer border of the interstitium is defined by the epithelial and endothelial basal membranes. The basement membrane modulates the movement of fluid, molecules, particles and cells from the air space and blood into the interstitium. However, plasma proteins and most solutes – including drugs – are thought to diffuse relatively unhindered through it [17]. **Figure 1** provides a qualitative view of the dimensions of the air–blood barrier in the different regions of the human lung.

3. Non-epithelial pulmonary barrier

In addition to the epithelial compounds of the barrier, the lung also possesses significant fluid barriers and sophisticated clearance systems.

3.1 Surfactant

Solid drug particles delivered to the respiratory tract need to be wetted and dissolved before they can be absorbed and exert their therapeutic activity. Although the humidity in the lung is near 100%, the volume of the epithelial lining fluid is small [18] The thickness of the lining fluid in the airways is estimated to be 5 – 10 μm and gradually decreases along the airway tree until the alveoli, where the thickness is

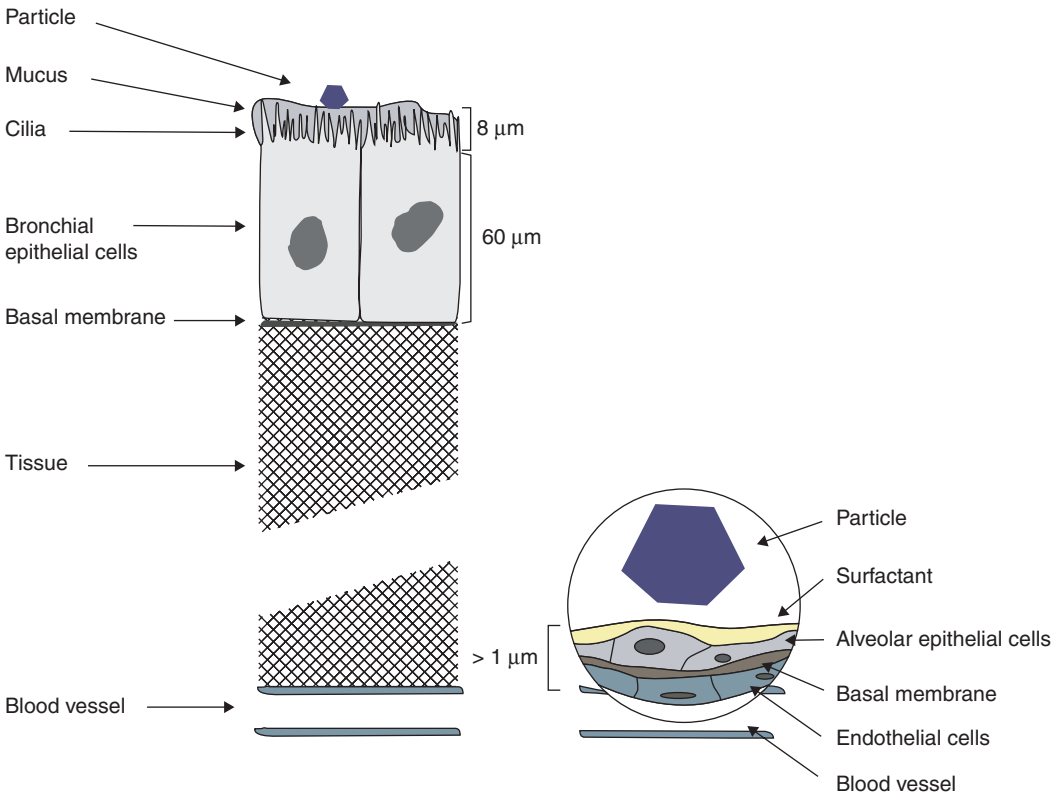

Figure 1. Schematic drawing of the bronchial and alveolar physical absorption barrier.

estimated to be in the range of 0.01 – 0.08 µm [17,19]. The volume and composition of the epithelial lining fluid is controlled by active ion transport and passive water permeability of the respiratory epithelium. Like the gastric mucosa, the airway mucosa is coated with a layer of phospholipids, which, in association with mucins, lubricate and protect the epithelium from offending agents. In the alveolar region, the surface fluid consists of a thin biphasic layer of plasma filtrates overlaid by a layer of pulmonary surfactant [17]. The lung surfactant is synthesised and secreted by the alveolar type II cells and comprises a unique mixture of phospholipids and surfactant-specific proteins [2]. The characteristic lamellar bodies in the type II cells serve as a storage depot for the surfactant before it is secreted onto the alveolar surface [20,21]. The surfactant forms an insoluble film at the surface of the alveolar lining fluid and decreases the surface tension in the alveoli. Thereby the extensive alveolar air–liquid interface is stabilised, which promotes lung expansion on inspiration and prevents lung collapse on expiration. The lung surfactant has also been found to enhance local pulmonary host defence mechanisms by serving as a barrier against adhesion of microorganisms and to enhance phagocytosis by alveolar macrophages [22]. The lung surfactant undergoes a constant dynamic process of turnover and metabolism, including removal by the mucociliary escalator, phagocytosis and recycling. In drug development research it should be considered that complex interactions between drugs and lung surfactant may occur. Balakrishnan *et al.* [23] found, for example, a 107-fold increased solubility of griseofulvin in the presence of surfactant. However, the role of surfactant in pulmonary drug delivery is not yet completely understood. For an overview of the function and molecular composition of pulmonary surfactant, the work of Goerke *et al.* [24] can be recommended.

3.2 Alveolar macrophages

The alveolar macrophages are found on the alveolar surface. These phagocytic cells play important roles in the defence mechanism against inhaled bacteria and particles that have reached the alveoli. Macrophages arrive to alveoli via the capillaries after production within bone marrow from monocytes. Particles deposited on the surface of the lung of rats have been demonstrated to be phagocytised by alveolar macrophages within a few hours [25]. The macrophages are cleared from the alveoli to the bronchioles by the lining fluid, and then from the airways by the mucociliary escalator.

Since the harvesting of these cells by bronchoalveolar lavage was first described in 1961, alveolar macrophages have been extensively investigated. This population is the predominant cell type within the alveolus, and undoubtedly serves as the first-line of host defence against inhaled

organisms and soluble and particulate molecules. Early studies focussed on this endocytic role and delineated the cells' phagocytic and microbicidal capacities. More recent investigations demonstrated an extensive synthetic and secretory repertoire including lysozyme, neutral proteases, acid hydrolases and O_2 metabolites [26]. In addition, the complex immunoregulatory role of the macrophage has also been appreciated. These cells have been shown to produce a wide variety of pro- and anti-inflammatory agents including arachidonic acid metabolites of the cyclooxygenase and lipoxygenase pathways, cytokines that modulate lymphocyte function and factors that promote fibroblast migration and replication [27,28].

Of special interest are cocultures of epithelial cells and macrophages to investigate the interaction of two key figures in pulmonary drug absorption and drug clearance.

3.3 Mucociliary clearance

Mucociliary clearance is probably the most important mechanical defence in the lung. The lung is continually at risk of exposure to noxious environmental agents and respiratory pathogens. A sophisticated series of defence mechanisms have been developed to protect the airways from these insults, keeping the lungs clean and allowing gas exchange to occur. The conducting airways are protected by local mucociliary defence mechanisms that involve the integration of ciliated epithelium, periciliary fluid and mucus. Mucus acts as a physical and chemical barrier onto which particles and organisms adhere. Cilia lining the respiratory tract beat in a regular, coordinated manner, propelling overlying mucus from the airways to the oropharynx, where it is either swallowed or expectorated. Seiler *et al.* [29] measured the velocity of the mucociliary escalator at approximately 80 µm/s in healthy patients. The surface liquids of the ciliated airways are composed of two phases: one aqueous periciliary phase of epithelial lining fluid close to the cell surface, in which the cilia beat, and one gel phase of mucus on top of the aqueous phase. A phospholipid layer between the phases lowers the surface tension between them. Mucus is secreted primarily from the serous cells of submucosal glands and from goblet cells, and is composed of water (95%), glycoproteins (mucins) (2%), proteins (1%), inorganic salts (1%) and lipids (1%) [30]. Regulation of the water content is of significant importance to maintain the optimal viscoelastic properties of the mucus. The first barrier to the absorption of drugs is the airway surface liquid, including mucus. The thickness of this layer will determine the concentration of the drug in solution, and therefore its rate of entry into the tissue. The ability of the drug to penetrate the mucus barrier depends on particle charge, solubility, lipophilicity and size.

After this condensed summary of the anatomy and biology of the human air–blood barrier, we compare the existing *in vitro* models regarding their proximity to the *in vivo* situation and their possible fields of application.

4. Cell culture models of the human air–blood barrier

4.1 Immortalised cell cultures (cell lines)

High-throughput screening (HTS) is the most important process for testing a large number of diverse chemical structures on safety and efficacy [31]. However, a major limiting factor in the useful application of HTS is that the quality of the information generated is totally dependent on the quality of the cell system used. Ideally, freshly isolated human tissue samples should be used for such studies. In the case of cell types present in the blood this is relatively easy to obtain, but for other tissues biopsy material is required. There are obvious problems in routinely obtaining human tissues for experimental purposes, and even when it is possible, it is often difficult to obtain tissue from properly matched groups of individuals. This is further compounded by the fact that the exact composition of cell types is likely to vary between different biopsies, even if they are ostensibly derived in the same manner. Such differences will have a significant impact on the results of the analyses. Immortalised human cell cultures have been used in an attempt to overcome the problems of tissue availability.

Because of the different anatomy in the trachea and the deep lung, different cell systems simulating more closely the alveolar or the bronchial region have been established.

4.1.1 Bronchial cell cultures

4.1.1.1 Calu-3

Calu-3 is an adenocarcinoma cell line derived from a 25-year old Caucasian male. It was suggested to express tight barrier properties on the basis of electrophysiological studies. The presence of TJ proteins was confirmed by immunoblotting and functional properties of the monolayers were studied by measurements of transepithelial electrical resistance and mannitol permeability [32]. Calu-3 cells have been the subject of a relatively large number of investigations. After a few investigations about the characteristics of the cells with ion channels or receptors, the cell line was rapidly used as a tool for transport studies. Mathias *et al.* [33] studied the permeability characteristics of Calu-3 to passive and actively transported drugs and they correlated the data with other *in vitro* models and rat lung absorption *in vivo*. Air interface cultured Calu-3 cells grown on collagen-coated permeable filter supports formed 'tightly' polarised and well-differentiated cell monolayers with apical microvilli and tight-junctional complexes. Calu-3 cells actively transported amino acids, nucleosides and dipeptide analogs. Beyond the use as a transport model, Calu-3 cells can be also employed for the investigation of metabolic processes. Borchard *et al.* [34] cultivated Calu-3 cells on microporous filters at an air interface for 16 – 18 days, incubated the cells with glucocorticosteroid budesonide and, with the aid of mass spectrometry of cell extracts, fatty acid conjugates of budesonide were detected. These fatty esters of budesonide form an

intracellular depot within the cells, from which budesonide is slowly released over a time period of 10 h, with a significantly higher amount being released to the apical side of the cell monolayer. Glucosteroids were also employed for the investigation of the efflux system *P*-glycoprotein in Calu-3 cells [35]. The P-gp modulation efficacy of glucosteroids was determined by their ability to increase the accumulation of the P-gp substrate rhodamine 123 in the cells. Because of the high degree of tightness and the easy cultivation conditions, Calu-3 cells are widely used for transport studies. Although Calu-3 is a bronchial (i.e., not alveolar) epithelial cell line, it is also often used as a model of the pulmonary epithelium in general. To increase the simulation of the *in vivo* conditions, the cultivation of the cell lines under air interface culture conditions (AIC) was tried. The zonula occludens-1 protein (ZO-1), as an indicator for the TJs, was found in cells grown in both AIC and liquid culture conditions (LCC). However, only LCC-grown cells exhibited protein ZO-1 localised as a zonula occludens-like regular belt connecting neighbouring cells. The presence of typical TJs has been confirmed by electron microscopy. Immunostaining for occludin, claudin-1, connexin 43 and E-cadherin has demonstrated intercellular junction structures only in the cells in LCC. These morphological findings have been paralleled by higher transepithelial electrical resistance values and similar fluxes of hydrophilic permeability markers under LCC compared with AIC conditions [14]. Cell morphology as well as mucus production can be influenced by the culture conditions. Air interface culture yields a higher production of mucosubstances. Furthermore, air interface cultivated cells show a more bronchial-like structure. Columnar cell bodies and mucus secreting cells characterise the AIC culture. In contrast, liquid interface culture causes real monolayers with flattened cells and without mucus. Although AIC is technically sophisticated it should be favoured over the liquid interface culture because of a closer correspondence to the *in vivo* situation. Calu-3 cells are the most commonly used cell system if a tight cell monolayer for permeability studies is required.

4.1.1.2 16HBE14o-

Another human bronchial epithelial cell line, 16HBE14o-, immortalised by virus transformation, also shows significant transepithelial resistance and can be used for transport studies. In comparison to the Calu-3 cells, the 16HBE14o- cell line expresses more P-glycoprotein, lung resistance-related protein (LRP) and caveolin-1. Immunocytochemical staining showed expression of P-gp localised at the apical membrane of 16HBE14o- cell layers. The flux of rhodamine 123 across cell layers exhibited a greater apparent permeability (P_{app}) value for the secretory direction. This asymmetry disappeared in the presence of verapamil, a P-gp inhibitor. The 16HBE14o- cell line may be a suitable candidate for an *in vitro* model for mechanistic studies of drug transport processes involved in the smaller airways because it shows drug transport systems that are also present in the human bronchus *in vivo* [36]. Further cell lines sometimes used are HAEo- cells [37] and BEAS-2B cells, both derived from normal human (bronchial) epithelial cells (obtained from autopsy of non-cancerous individuals) and immortalised using the adenovirus 12-simian virus 40 hybrid virus [38]. Whenever tight cell monolayers of bronchial origin are required, 16HBE14o- cells are a good choice.

4.1.1.3 CFBE41o-

Although the aforementioned cells are of cancerous origin, they originate from healthy bronchial cells and not diseased cells. However, the investigation of diseases can benefit from disease models.

Cystic fibrosis (CF) is a lethal genetic disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), which mainly functions as a chloride channel. The main clinical symptoms are chronic obstructive lung disease with excessive inflammation and chronic infection, which is responsible for most of the morbidity and mortality associated with CF, and pancreatic insufficiency. The CFBE41o- cell line was generated by transformation of CF tracheo-bronchial cells with SV40 and is homozygous for ΔF508-CFTR over multiple passages in culture, and expresses a number of proteins relevant in the context of pulmonary drug absorption, for example P-gp, LRP and caveolin-1 [39]. The CFBE41o- cell line should be useful for studies on CF gene transfer, alternative treatments using small drug molecules and gathering further knowledge about the disease on the cellular level without the need for primary culture.

4.1.2 Alveolar cell cultures

The number of available cell cultures representing the alveolar region is much smaller than the number of bronchial cell models.

The A549 cell line possesses a type II cell phenotype and has been widely used as a system to study the regulation of pulmonary surfactant synthesis. However, cultured A549 cells do not undergo a transition to form a phenotype similar to that of a type I cell. Furthermore, although the A549 cell has received some attention as a monolayer culture for the study of solute transport, its cell architecture and barrier properties are quite distinct from that of a type I cell monolayer. Thus, an *in vitro* cell model of the human alveolar epithelium possessing the relevant qualities of the alveolar epithelium *in situ* is definitely required. The A549 line is a human lung adenocarcinoma derived by explant culture from the peripheral airways of a Caucasian male with lung cancer. A549 cells show a very high mannitol permeability coefficient and approach the characteristics of cell-free filters alone. The leaky formation of TJs in A549 is also the cause for very low transepithelial electrical resistances by these monolayers. The functional TJ deficits of the A549 cell line seem to preclude its use in permeability studies.

Nevertheless, some authors have also reported comparatively high TEER values and permeability rates for filter-grown A549 cells, which might indicate the requirement for optimised culture conditions [40].

The use of immortalised cell lines is limited by the fact that frequently cells lose their characteristics during *in vitro* culture and will senesce after a certain number of cell divisions. Immortal cell lines from primary cultures are not a perfect representation of the original cells in primary culture. Because of these problems, a great majority of researchers resort to the use of primary non-cancer cell lines.

4.2 Primary cell cultures

4.2.1 Rat alveolar epithelial cells

Because of the relatively easy isolation protocol and the trouble-free availability, rat alveolar epithelial cells (rAEpC) are the most commonly used primary animal cell model for pulmonary research. Protein transport across alveolar epithelial cells in rat primary culture has been investigated with special regard to the transport mechanisms and the underlying pathways by Kim *et al.* [41]. Primary cultured rat pneumocyte monolayers grown on tissue culture-treated polycarbonate filters were used for this study. These monolayers comprise alveolar epithelial type I-like cells and develop high barrier resistance (> 2,000 Ω^* cm^2).

4.2.2 Porcine alveolar epithelial cells

The advantage of porcine-based cell culture lies in its ease of availability, because animals intended for slaughter can be used. In other words, the source material is no problem and no additional animals have to die for research purposes. Furthermore, the morphology of porcine mucosa seems to be comparable to human epithelial cells, especially with regard to electrophysiology and enzymatic equipment [42]. Steimer *et al.* [43] characterised porcine alveolar epithelial cells (pAEpC) in primary culture in consideration of morphology, bioelectrical and biochemical properties. pAEpC were shown to grow in confluent monolayers with functional TJs. Maximum transepithelial electrical resistance of about 2,000 Ω^* cm^2 was observed and the presence of tight junctional proteins could be proven. The differentiation from type II cells to type I-like cells could be monitored by immunostaining using alveolar-specific cell markers like caveolin for type I and surfactant protein C for type II cells. The first transport experiments with sodium fluorescein showed the qualification of the pAEpC model for pulmonary drug absorption studies.

4.2.3 Human alveolar epithelial cells (hAEpC)

Lung alveolar epithelium *in vivo* is composed of two specialised epithelial cell types: the squamous alveolar epithelial type I cell and the surfactant-producing cuboidal alveolar epithelial type II cell. Current evidence supports the hypothesis that type II cells serve as the sole progenitor for type I cells *in vivo* [44,45]. Accordingly, isolated type II cells in culture lose their characteristic phenotype and acquire over a 5- to 10-day period morphological and biochemical markers characteristic of type I cells. Morphological changes during differentiation include the generation of monolayers with high transepithelial electrical resistance (> 1,000 Ω^* cm^2) and a loss of microvilli, an increase in the cell surface area and the development of thin cytoplasmic attenuations extending away from a protruding nucleus. The isolation of type II cells predominantly from rat and rabbit lung tissue and their culture over time, leading to a primary culture of type I-like cells is now an established technique used for multiple purposes. Although the isolation of primary human alveolar cells has been described before [46], human primary cells are not commonly used as an *in vitro* model for the air–blood barrier. The isolation of human alveolar type II epithelial cells and their primary culture, which results in confluent monolayers capable of generating tight junctional complexes and high transepithelial electrical resistance, was described first by Elbert *et al.* [47]. The morphological cell change from a type II phenotype to a type I-like cell phenotype over time of culture was described by Fuchs *et al.* [48]. Moreover, the formation of characteristic plasma membrane structures termed caveolae and the synthesis of their major structural protein, caveolin-1, was observed in these cells. The caveolae membrane system is of interest because of its potentially important role in macromolecule transport across the air–blood barrier of the lung, including both the clearance of endogenous protein from the airspace and the absorption of inhaled therapeutic protein [49]. Primary type II alveolar cells can be isolated from human non-tumour lung tissue, which can be obtained from patients undergoing lung resection. The isolation can be performed according to a protocol described by Elbert *et al.* [47].

Alveolar epithelial type II (ATII) cells can also be derived from human embryonic stem (hES) cells. The transfection and culture procedure, which facilitates via genetic selection the differentiation of hES cells into an essentially pure (> 99%) population of ATII cells, was described by Wang *et al.* [50].

Furthermore, the type II cell phenotype can be protected in the type II state under special culture conditions. In the presence of keratinocyte growth factor, isobutylmethylxanthine, 8-bromo-cyclicAMP and dexamethasone, isolated type II cells do not differentiate to type I cells. The preserved phenotype was confirmed by morphologic appearance and measurement of the expression of surfactant protein SP-A, SP-B, SP-C as a type II marker. The cells contain lamellar inclusion bodies and have apical microvilli [51]. Albeit an established and validated model, the widespread use of human alveolar cells is limited by lack of material and a complex, time-consuming and cost-intensive isolation procedure. For a tabular overview of the most relevant pulmonary cell cultures, see **Table 1**.

Table 1. Overview of frequently used pulmonary cell systems in the field of safety and efficacy studies of innovative aerosol medicines.

Pulmonary cell	Barrier properties	Culture conditions	Transport and efflux systems	Field of application	Ref.
Calu-3	350 – 1200 Ω* cm^2	AIC + LIC	Pgp, LRP	Transport and metabolism	[70-72]
16HBE14o-	120 – 800 Ω* cm^2	AIC + LIC	Pgp, LRP	Transport	[14,73,74]
CFBE41o-	1150 Ω* cm^2	AIC + LIC	Caveolin, Pgp, LRP	Diseases model for CF	[75]
A549	0 – 140 Ω* cm^2	LIC	Not investigated	Toxicology and cellular uptake	[76,67]
hAEpC	> 2000 Ω* cm^2	AIC + LIC	Caveolin, Pgp, LRP	Transport and toxicology	[62,77]

5. Determination of drug permeability with the aid of pulmonary cell culture

It would go beyond the scope of this review to report detailed protocols for the cell culture-based determination of efficacy and safety of pulmonary drug formulations. Drug permeability, cellular binding and efflux effects on the one hand, and metabolic activity, cell membrane integrity and inflammatory response on the other hand can be investigated for pulmonary cells with the same protocols and test assays as for oral administered drugs. The majority of passively transported drugs evaluated in Caco-2 cells as a model for the intestine yield the same permeability as when evaluated in the pulmonary Calu-3 cells. However, in cases of active transport processes or the involvement of efflux systems, the transport rate can differ from cell line to cell line. For example, Hamilton *et al.* [52] showed that Calu-3 cells express lower levels of Pgp than Caco-2 cells and therefore reported in Calu-3 cells an accumulation of the Pgp substrates rhodamine 123. All qualities of pulmonary administered drugs are dependent upon the physico-chemistry properties of the drug molecule, which can be determined using traditional methods established in oral drug research. In the case of the pulmonary drug application, however, the formulation properties, including aerodynamic diameter and surface chemistry, influence significantly the therapeutic efficacy and safety.

Adding a drug solution to the apical compartment of a cell culture, the standard method in oral drug research, does not mimic the *in vivo* situation in the lung in which an aerosol particle or droplet deposits on a slightly moistened surface. The aerosol deposition directly on the cells leads to enormous differences compared to liquid interface deposition (LID). There are huge differences for both the aerosol particles as well as for the cells. Regarding the fact that predominantly particles with diameters of 1 – 5 µm can be deposited in the alveolar region, where a fluid layer of only 7 – 70 nm deep covers the cells, following particle deposition on the lung surface, the majority of the surface area of the particles would not be submerged in the liquid layer. In this hypothetical scenario, erosion from the minor liquid layer on the underneath and absorption of liquid to the unsubmerged surface would be observed. An immediate coating of deposited particles is only described for nanoparticles [53] and not for the inhalation-relevant microparticles. Although nobody knows the processes occurring in reality after particle deposition on the air–blood barrier, the application of particles excessively modified or even solvated by the dispersion in large amounts of aqueous buffer where surface properties like zeta potential and roughness are changed, seems to be quite far away from reality. Furthermore, for droplet aerosols, a much higher concentration gradient after the application of aerosol droplets compared to a diluted drug solution can be expected. Fluid layers with a thickness of several micrometres – typically for Transwell experiments – are not compatible with the gas exchange function of the human lung. Consequently, droplet aerosols should not be diluted in *in vitro* experiments by using LID models. As addressed above, the application of drugs in a physiologically relevant manner should be conducted via aerosol deposition on air interface cell culture inserts. Regarding techniques to deposit aerosols on cell surfaces, pharmaceutical science may profit from the experience in environmental toxicology. However, while toxicological studies on aerosols are typically focusing on the exposure of xenobiotic compounds at certain, mostly low, concentrations over a given period of time, pharmaceutically relevant aerosols are administered usually as a metered, single bolus dose. For an overview of cell compatible aerosol deposition devices, we recommend [54] and **Table 2**.

The higher concentration gradient after air interface deposition results in faster drug absorption compared to liquid interface deposition. This so-called ultrafast absorption of drugs has been described by several authors [55,56] and explained due to a large resorption surface combined with a steep concentration gradient. A second difference between adding solution or dry powder aerosol to the donor compartment is given by the different contact area. If solutions are added

Table 2. Cell compatible aerosol deposition.

System	Deposition mechanism	Deposition efficacy	Particle spectrum	Cell stress	Field of application	Ref.
Cultex®	Sedimentation	Low	Nano- and microparticles	Low	Toxicology	[78-80]
Stagnation point flow deposition on cell membranes	Brownian diffusion	Low	Nanoparticles	Low	Toxicology	[83,84]
Modified multi-stage impingers	Impaction	High	Microparticles	High	Toxicology and drug permeation	[70,85,86]

to the donor compartment, a maximal contact area between substance and cell surface will be caused. By the application of single drug particles, only parts of the cell surface or the lining fluid layer will be in contact with the applied substance. Both steep concentration gradients and high local drug amounts limit the use of Ficks law because of missing sink conditions in the donor compartment and no linearity. The resulting transported amount/time curve is characterised by a steep initial 'burst' transport and a subsequent slowly absorbed part. The amount/time curve can be interpreted by the calculation of the mean permeation time (MPT) according to the equation:

$$[(C_{max} \times t_{C_{max}}) - AUC]/C_{max} = MPT \quad (1)$$

whereby AUC is the area under the curve formed by plotting the transported drug amount versus the time. The calculated mean permeation time can be correlated with the total transported amount. As a consequence a flux with the dimension [ng/s] can be calculated.

6. Investigation of drug safety with the aid of pulmonary cell culture

Proven safety and high bioavailability of a drug or excipient after oral administration does not guarantee safety and efficacy after inhalation. Regarding the evaluation of pulmonary safety, expertise from environmental toxicology can be used, with the limitation that pharmaceutical biocompatibility measurements are focused on bolus inhalation and not on long time/low dose inhalation. Unfortunately, cell culture models were not considered in the development of the document 39B, the official draft guidance document on acute inhalation toxicity measurement [57] However, studies have reported little correlation between the relative toxicity of particles when comparing lung toxicity rankings following *in vivo* animal instillation versus *in vitro* cell culture exposures. The cited study [58] was designed to assess the capacity of *in vitro* screening studies to predict *in vivo* pulmonary toxicity of fine or nanoscale particles in rats. For the *in vivo* experiments, the lungs of exposed rats were lavaged and inflammation (neutrophil recruitment) and cytotoxicity end points (bronchoalveolar lavage fluid lactate dehydrogenase values) were measured at defined time points. For the *in vitro* component of the study, three different culture conditions were utilised. Cultures of rat lung epithelial cells, primary alveolar macrophages, as well as cocultures of epithelial cells and macrophages were incubated with particles, and the culture fluids were evaluated for cytotoxicity as well as inflammatory end points [58].

As regards safety experiments it can be affirmed that the deposition conditions influence the results of the experiments. From Kendall *et al.* it was shown that when nanoparticles are collected directly into normal lung lining liquid, the particles aggregate into larger (5 µm) dense structures compared with samples collected in air or into saline. The control showed that the agglomeration effects were not due to drying *per se*, but were specifically associated with the protein-rich solution of the lung lining fluid [59,60].

The lack of safety data is generally regarded as the reason why at present relatively few drugs and excipients are approved for pulmonary application [61-66]. The non-optimised *in vitro* test systems especially decelerate the development of modern inhalable medicines.

7. Expert opinion

Various lung cell cultures have been described in the literature. Some cell lines such as Calu-3, 16HBE14o- and A549 are established models in biopharmaceutical research and very helpful to investigate the safety and efficacy of inhalative medicines in an early stage of development. Information about drug permeability can be obtained by the usage of tight monolayers building Calu-3 or 16HBE14o- cells. Also, toxicity can be estimated with the aid of A549 cells. However, a human alveolar epithelial cell line representing type I phenotype is still missing. In other words, the cell type forming 95% of the lung surface and therefore of special interest for drug delivery as well as inhalation

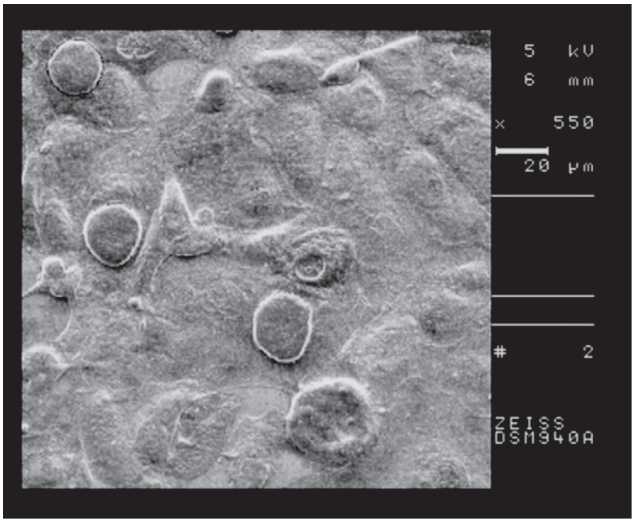

Figure 2. Coculture of immortalised mouse macrophages (MHS cells) on top of primary human alveolar epithelial cells.

toxicology is not available as an immortalised cell culture today. Fortunately the technique of cell transfection and immortalisation is fast-paced, so there is genuine hope that an adequate model of the air–blood barrier in the deep lung will be developed shortly.

It is also obvious that the trends are moving towards more complex cell culture systems. Combinations of endothelial, epithelial and dendritic cells are described [67], and cocultures of epithelial cells and macrophages are also published [68]. A coculture of immortalised alveolar mouse macrophages cultivated on top of primary human alveolar epithelial cells is shown in **Figure 2**. Even though the value of the existing models is limited by problems regarding interspecies incompatibility or by the combination of cancer and primary cells, they can act as role models for future optimised cell culture systems. Complex cell culture systems are necessary to answer the question of how innovative medicines like (nano)particles interact with and ultimately cross the human air–blood barrier, for example. After decades of drug screening focused on bioavailability prediction, more attention is being paid to mechanistic investigations. In a triple culture of epithelial cells, dendritic cells and macrophages, Gehr *et al.* [53] investigated the cellular interplay after particle deposition. Dendritic cells gained access to the apical side of the epithelium where they sampled particles and interacted with macrophages [69]. Surprisingly little is known about the communicative cellular network in the lung and, especially in the context of inhalation toxicology, the lack of suitable cell culture models and deposition techniques is manifest.

In the past few years the fluid lining layers have become in a focus of research. Mucus as well as surfactant are very complex systems interacting with particles deposited in the lung. There is no artificial surfactant on the market to simulate the interfacial and immunological properties of native surfactant. The isolation procedure by broncho-alveolar lavage changes the composition as well as the chemistry of the surfactant and mucus. Without a very close-to-reality surfactant phase in the cell culture models, studies on the interactions of solid particles with cellular structures are afflicted with errors. Besides particle aggregation or disaggregation, surfactant proteins influence the immunological response of the lung. No drug or particle can interact with the cellular barrier without a prior contact with surfactant.

Alveolar macrophages attracted by signals emitted by epithelial cells can change the alveolar resorption of deposited particulate drugs dramatically. Besides the alveolar macrophages, mucociliary clearance is a real obstacle for pulmonary drug delivery. Until now no retarded pulmonary drug application has been possible. The missing success of the first inhalable insulin product was caused in part by the impossibility to deliver retarded insulin. Bronchial mucociliary clearance and alveolar macrophage mediated clearance remove particles from the lung very quickly and effectively. Only with the knowledge of the mechanisms underlying the clearance processes can sustained drug release in the lung be possible. At present no cell model simulating mucociliary clearance is available. Although beating heart muscle cells have been developed, no one has generated bronchial cells able to produce mucus and to express beating cilia. With the aid of this model it would be possible to indentify particle properties influencing mucociliary clearance and consequently to elucidate ways to circumvent the mucociliary escalator.

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