In vitro pulmonary models

Porcine alveolar epithelial primary cells and Calu-3 cell monolayer to study pulmonary absorption

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Introduction

The respiratory tract is currently considered as an alternative to gastrointestinal or dermal drug delivery systems. Determination of drug permeability across the lung epithelial barrier generally speeds up the drug development process, and models are needed for this purpose.

Comparison of the biological properties of the different lung areas (see figure 1) clearly shows that each space (bronchi and alveoli) will respond differently to drug administration, and that drug absorption might be differently affected. Upper airways and deep lung have e.g. different absorption areas. The airway epithelium is covered by a thick viscous mucus gel layer in contrast to the thin film of alveolar surfactant, which results in different final volumes of dissolution. Cellular thickness in both tissues and cellular population are dissimilar (Steimer et al., 2005). Mucus and cilia are responsible for particle clearance in the bronchi, while macrophages have a very active role in this process in the deep lung. Drug metabolism and drug-drug interactions should also be considered, as well as efflux transporters; for instance, functional P-glycoprotein (P-gp) has been detected in bronchi and in human alveolar type I cells, which largely constitute the absorptive surface in the deep lung, whereas alveolar type II cells were negative for this protein (Campbell et al., Lehmann et al., 2001).

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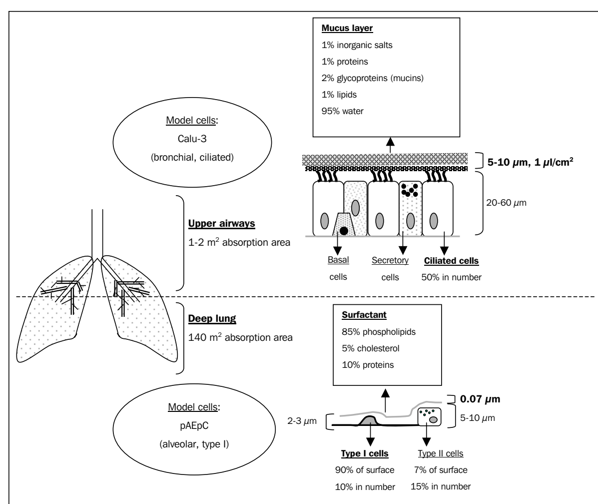

Figure 1: Comparison of the upper airways and the deep lung. Differences are found in the absorption area, cellular variety and height, and lining (mucus vs. surfactant) composition, thickness and total volume.

A reliable model for the upper airways the epithelial cell line Calu-3 is used, which is a human sub-bronchial cell line derived from a 25-year-old Caucasian male adenocarcinoma. After screening for airway secretory proteins and mRNA, it was found to be the only one out of 12 cell lines derived from human lung cancers, with the mRNA and protein content characteristic of the native epithelium. Cells feature epithelial morphology, adherent polarized monolayer growth, and tight junctions under liquid-covered conditions; additionally they secrete mucus if cultured under air-interfaced conditions. They express a plethora of efflux proteins which will probably have a high impact on drug absorption, such as MDR1 (P-gp), MRP1, 2, 3 and 5, Novel Organic Cation Transporter (OCTN2) and also LRP, Peptide Transporter 1 (PEPT1) or Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The ability of this cell line to form tight junctions and polarized monolayers is critical to their use as an in vitro drug absorption model. Indeed these cells are able to discriminate compounds of different apparent permeabilities (P_{app}). Across Barriers GmbH has already used this model in several studies, e.g to characterize the telomerase Inhibitor BRACO19 (Taetz et al., 2006) or different Cyclosporine A (CsA) formulations in respect of their pulmonary permeability (Trammer et al., 2008). Epithelial cell cultures are a good alternative to animal testing, but to date, no cell line is available to model the deep lung. Primary cell cultures are the only available alternative. Human Alveolar Epithelial cells as described in literature (Bur et al., 2007) have been used before (Elbert et al., 1999), but porcine cells are an alternative that can help overcome ethical issues (Boekema et al., 2003). The pig is in fact anatomically, physiologically, and biochemically very similar to man, and therefore comparability is expected.

In vitro models

pAEpC cultures

The method for pAEpC isolation has already been published by our group (Steimer et al., 2005, 2006, 2007). Porcine lungs are processed to eliminate visible bronchi and blood vessels, and after enzymatic digestion a cell suspension is obtained. After removal of contaminating macrophages and erythrocytes, cells are seeded onto fibronectin/collagen coated Transwell filters, where they attach and spread until confluence is reached.

Cells have been morphologically and biochemically characterized, and the epithelial pneumocytic phenotype has been confirmed:

- Cells express epithelial cell marker proteins such as cytokeratin.
- Other epithelial markers indicate that a mixture of type I and type II pneumocytes is obtained in culture. For instance, surfactant protein C and caveolin have been detected, which are markers of type II and type I pneumocytes.
- Scanning electron microscopy has shown the presence of multilamellar bodies in round-cells (figures 2 C and 2 E), characteristic of type II pneumocytes and also flat, big cells with caveoli, which correspond to a type I phenotype (figure 2F).

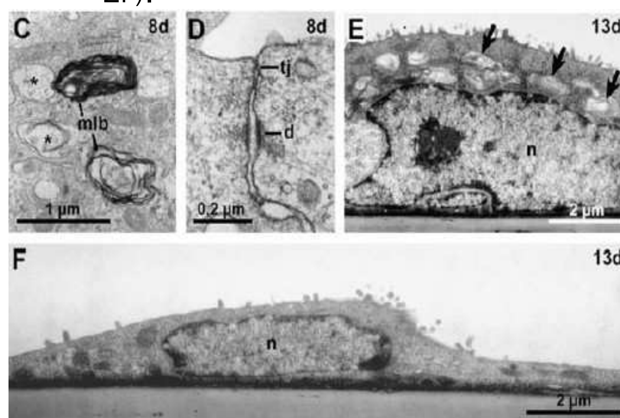

Figure 2: Scanning electron microscopy of cultivated pAEpC at different days post-plating. mlb (multi-lamellar bodies), tj (tight junction), d (desmosome), n (nucleus). Image from Steimer et al. (2006).

Cells are able to create tight monolayers, which is characteristic of epithelial barriers. This is supported by the tight junctions and desmosomes observed by electron microscopy (figure 2 D) and by the high Transepithelial Electrical Resistance (TEER) measured from cells in culture (figure 3). As shown in this representative case, TEER increases after cell seeding until values above 750 Ohm x cm^2 are reached. Then cells are suitable for performing transport experiments.

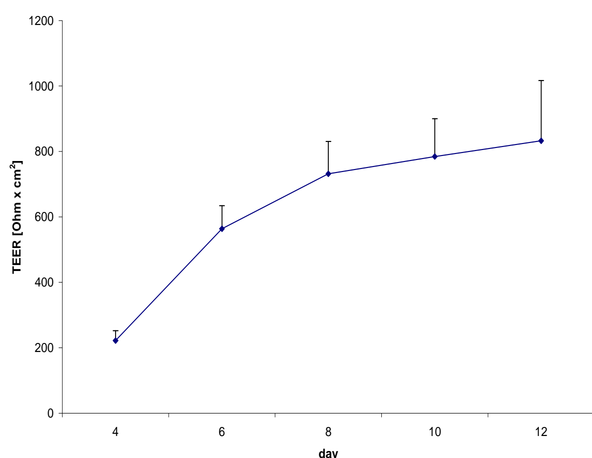

Figure 3: TEER value evolution during pAEpC culture. Data represent mean values ± SD (n = 12).

Calu-3 cultures

Two different methods are employed to culture Calu-3 cells on Transwell filters: under liquid-covered culture (LCC) conditions (Trammer et al., 2008) where growth medium is added to both the apical and basolateral wells of the Transwell filters, whereas in the air-interfaced culture (AIC) condition (Grainger et al., 2006), growth medium is only added to the basolateral side of the Transwell filters. The apical side is exposed to air. Under both conditions the cells form a polarized epithelial barrier with functional tight junctions within ten days.

Calu-3 cells grown in AIC condition resemble the native epithelium of the upper respiratory tract to a greater extent than the cells grown under LCC conditions not only because of the culture conditions itself, but also because of its phenotypic appearance. Calu-3 cells, for instance, form a pseudostratified layer of columnar cells under AIC conditions, whereas under LCC conditions they form a simple monolayer (Grainger et al., 2006).

Furthermore, they show enhanced ciliogenesis and increased mucus secretion than cells grown in LCC conditions. In opposite to LCCs, AICs can be directly exposed to airborne factors. In addition, compounds such as nanoparticles or powders can be directly applied to the apical surface of the cells, without being diluted. Furthermore, the interrelation of drugs with factors naturally lining the epithelial surfaces of the respiratory tract such as mucus or surfactant and its impact in permeability and toxicity of drugs can be examined using AICs.

Results

Transport experiments - monolayer qualification

pAEpC

The method for the performance of transport experiments across pAEpC monolayers has been published by our group (Steimer et al., 2005, 2006, 2007). The analysis of compounds of known permeability (figure 4) shows how monolayers are able to differentiate substances of low and high permeability (Fluorescein and Propranolol, respectively). It can also be observed that transport of Rhodamine, a P-gp efflux transporter substrate has no directionality (equivalent apical to basolateral and basolateral to apical transport). This indicates that no such activity is present in our primary cell cultures. It must be considered that the model contains both type I and II alveolar cells, and that P-gp activity has been detected by others only in type I cells (Campbell et al., 2003). This supports our observations.

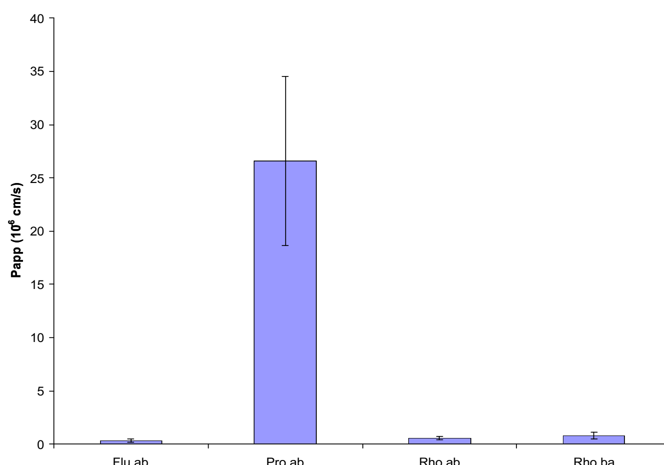

Figure 4: Permeability of marker compounds across pAEpC monolayers. Data represent mean values ± SD of 10 different isolations with 3 replicates each.

Calu-3

For both Calu-3 culture conditions (AIC and LCC) could be shown that Calu-3 monolayers are able to differentiate substances of low and high permeability (Mannitol and Propranolol, respectively). It can also be observed that transport of Rhodamine, a P-gp efflux transporter substrate has a directionality (higher apical to basolateral and basolateral to apical transport, figure 5).

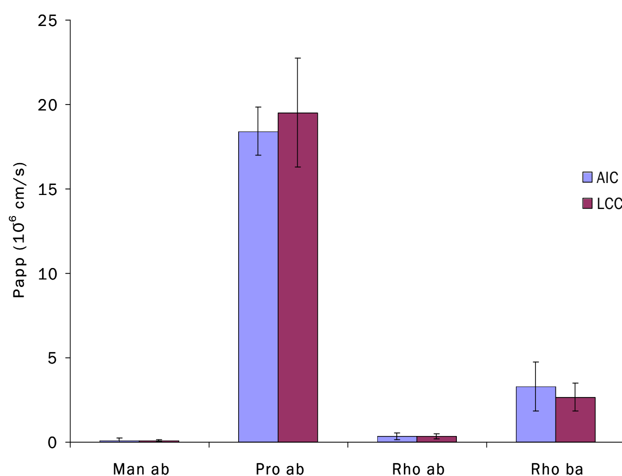

Figure 5: Permeability of marker compounds across Calu-3 cell monolayer under liquid-covered culture (LCC) conditions and air-interfaced culture (AIC) condition. Data represent mean values ± SD of 6 different experiments with 3 replicates under each condition.

Drug permeability experiments

When pAEpC monolayers are compared with other cellular lung models, it can be observed that they behave similarly in terms of permeability, when only passive transport is present: for instance, see figure 6, budesonide transport is similar through Calu-3 cells (LCC, bronchial model) and through pAEpC cells. Nevertheless, differences are observed when active transport is involved. It can be seen in figure 7 how digoxin permeability (also a P-gp substrate) is different in both cases, as Calu-3 are active for the efflux transporter. Additionally, comparison with other non-pulmonary cell types (e.g. Caco-2, gastrointestinal model, see figures 6 and 7) clearly shows that lung epithelium has different properties, i.e. it is less permeable.

Considering all these results, it can be concluded that specific in vitro models are needed that represent the lung, and moreover, bronchial and deep lung regions need to be also separately modeled.

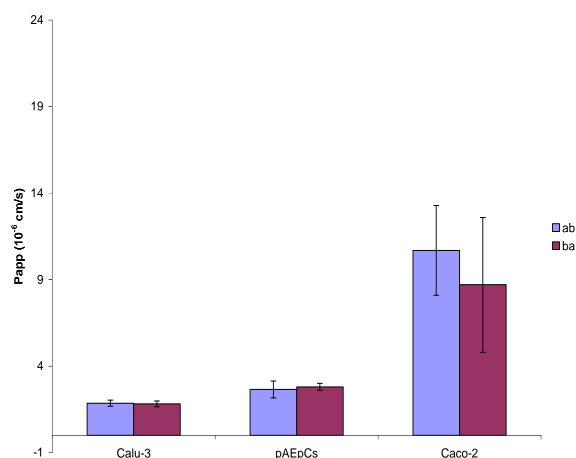

Figure 6: Permeability coefficients of budesonide for different cell types (Calu-3: LCC). Data represent mean values ± SD (n = 3).

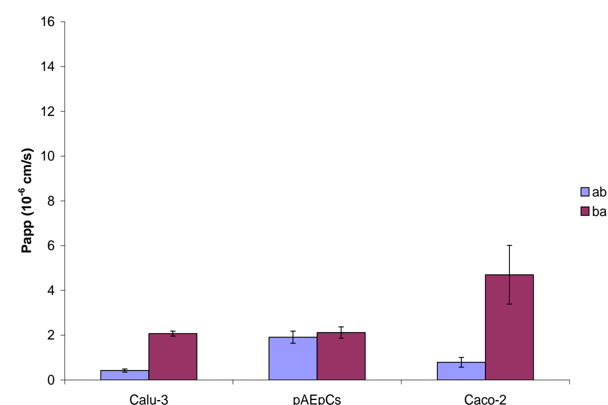

Figure 7: Permeability coefficients of digoxin for different cell types (Calu-3: LCC). Data represent mean values ± SD (n = 3).

Services of Across Barriers GmbH - when to use which model

With its knowledge and experience Across Barriers GmbH is your competent partner regarding all absorption questions during the life of your product.

Our pulmonary in vitro models are used to investigate and characterize the pulmonary permeability of compounds:

- Is your compound low permeable or high permeable?
- Does it show a dose linearity?
- Is your compound systemically or only locally available?
- Are there drug-drug interactions?

- Are active transport systems, e.g. P-gp or others, involved in the transport?
- Does your compound show toxic effects? There are different ways to investigate the toxicity of your compound: TEER, MTT, LDH etc.

Our model can be used for formulation optimization:

- You are developing a new inhalative formulation? You can use our model to compare your different formulations or excipients.
- Use different excipients to see the influence on the permeability of your compound and decide which one has the best effect.
- How your compound needs to be applicated? Test single and multiple dosing using our models.

As Calu-3 cells represent the upper part of the respiratory tract, they will be used e.g. to investigate nasal permeability or if your compound is intended to permeate on the bronchi. AIC will be used especially to investigate compounds without diluting them or to see the impact of mucus on the transport.

Is your compound intended to reach the deeper lung and to permeate across the alveolar epithelium, pAEpC will be the model to choose.

Using Across Barriers GmbH pulmonary in vitro models you minimize time consuming and expensive in vivo studies.

Across Barriers GmbH not only provides the in vitro model, we will also design the optimized in vitro study including preliminary tests and the analytics. Therefore preliminary characterizations of your compound in terms of physicochemical properties, e.g. solubility or stability parameters, and also the quantification of your compound via e.g. LC-UV or MS or scintillation counting in the generated samples are part of the service package Across Barriers GmbH is providing you!

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