Pulmonary epithelial cells as model to investigate *in vivo* drug absorption across the human air-blood barrier

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Contents

1 Short summary

The lung is more and more of interest for local as well as for systemic administration of drugs. Nevertheless, the development of modern inhalable medicines is moving forward only slowly. Especially the lack of safety and efficacy data combined with unsatisfactory *in vitro* models for the investigation of the complex processes on the air-blood barrier decelerates the development of new aerosol medicines.

In the first part of this thesis the transport of peptides with different molecular weight across submersed human alveolar epithelial cells has been investigated. The measured absorption / secretion rates allow quantifying of permeability and the identification of active or passive transports, but the influence of formulation parameters like size or charge disappears after preparing solutions and adding these in the fluid filled apical compartment of submersed cell culture. However, with the aid of a relatively simple insufflator syringe and air interface cultivated cells, a deposition more close to the *in vivo* situation was possible. It was found that air interface deposition yielded higher absorption rates and that differences in particle size significantly influenced the absorption rates only after air interface deposition but not after liquid interface deposition. Even if the application with the insufflator syringe offers the opportunity to deposit dry particles on the air interface of cell monolayers the method wasn't able to simulate *in vivo* relevant impaction processes. Especially in case of dry powder aerosols composed of large carrier lactose particles and adherent micronized drug crystals, impaction processes during aerosolisation normally accomplish separation of the drug from the carrier. Only the sufficiently small (< 5 µm) drug crystals are deposited in the deeper regions of the lung. As the insufflator fails to separate the drug crystals from the carrier lactose, and as the particle size of the carrier particles significantly influences the dissolution and absorption behaviour, a cell compatible aerosol impingement system was designed. A commercial available MSLI was modified to incorporate cell culture inserts in the relevant stages. Complex powder formulations could be size fractionated and the size fractions which are able to reach *in vivo* the deep lung were deposited on cell monolayersSignificantly changed absorption behaviour could be detected in dependency of cell culture fluid volume, particle size and deposition mode.

2 Kurzzusammenfassung

Im ersten Teil der Dissertation wurde die Permeation von gelösten Peptiden mit verschiedenem Molekulargewicht durch Monolayer humaner alveolarer Epithelzellen untersucht. Auch wenn das Arbeiten mit Lösungen für die Untersuchung intestinaler Absorptionsvorgänge die *in vivo* Situation ausreichend genau wiedergibt, stellt diese Methode keine realistische Applikationsart für Aerosole dar, da die menschliche Luft-Blut Schranke beim gesunden Patienten nur mit einem ausgesprochen dünnen Flüssigkeitsfilm bedeckt ist, der nur den hundertsten Teil der Dicke üblicher Flüssigkeitsschichten in submersen Zellkulturen ausmacht. Realitätsnah lassen sich jedoch Calu-3 Zellen als Modelle des Bronchialepithels, und primäre humane alveolare Epithelzellen als Modell der alveolaren Bereiche der Lunge, ohne flüssigkeitsgefülltes apikales Kompartiment kultivieren

Im zweiten Teil der Arbeit wurde mittels solcher an der Luft-Grenzschicht kultivierter zellulärer Modelle untersucht inwiefern die Applikation als Lösung oder in Form eines trockenen Pulveraerosols den Transport von Arzneistoffen beeinflusst. Nach Deposition trockener Aerosolformulierungen auf Luft-Grenzschicht kultivierte Zellen konnten signifikant schnellere Resorptionsvorgänge gemessen werden Obwohl die angewandte Applikation mittels einer Insufflator Spritze an sich schon eine sinnvolle Verbesserung von Transportexperimente an Modellen der Luft-Blutschranke darstellt, berücksichtigt die Insufflator Spritze nicht alle Aerosol Charakteristika. Vor allem im Falle von Aerosolen mit Laktose Partikeln als Wirkstoffträger war die Insufflator Spritze nicht in der Lage die Separation der mikronisierten Arzneistoffkristalle von den wesentlich größeren Laktose Trägern zu bewerkstelligen. Um auch diese Prozesse wirklichkeitsnah zu simulieren wurde im dritten Abschnitt der Arbeit ein zellkompatibler Kaskaden-Impaktor entwickelt. In diesem war es möglich sowohl eine realistische Auftrennung der Aerosole nach der Partikelgröße als auch die Deposition der einzelnen Partikelfraktionen auf Luft-Grenzschicht kultivierte Zellmonolayer nach zu ahmen.

3 Abbreviations

AIC	**Air interface culture**
AID	**Air interface deposition**
CF	**Cystic fibrosis**
EVOM	**Epithelial Volt Ohm Meter**
GLP-1	**Glucagon-like-peptide 1**
hAEpC	**Human alveolar epithelial cells**
HSA	**Human serum albumin**
HGH	**Human growth hormone**
HL (number)	**Human lung (number of the isolation)**
HPLC	**High pressure liquid chromatography**
IgG	**Immunglobuline G**
IL-8	**Interleukine-8**
IPL	**Isolated perfused lung**
LIC	**Liquid interface culture**
LID	**Liquid interface deposition**
LRP	**Lung resistance protein**
MDR	**Multi drug resistance**
MMAD	**Mass median aerodynamic diameter**
MSLI	**Multi stage liquid impinger**
pAEpC	**Porcine alveolar epithelial cells**
PBS	**Phosphate buffered saline**
PTH	**Parathyroide hormone**
rAEpC	**Rat alveolar epithelial cells**
TEER	**Transepithelial electrical resistance**
TF	**Transferrin**
TJ	**Tight-junctions**

4 Definitions

In the following chapters four abbreviations are frequently used. The terms “air and liquid interface culture” describe two different methods of cell cultivation, whereas the similar terms “air and liquid interface deposition” refer to two different methods to bring drugs in contact with the surface of epithelial cell culture models for the purpose of epithelial transport experiments.

Air interface culture - AIC

Only few cells of epithelial origin are growing *in vivo* on an air interface. In case of models of intestine, colon, vagina or other epithelia which are usually in contact with aqueous environment the submersed culture reflects the *in vivo* situation. However, for modelling of epithelia which are normally in contact with the air submersed culture doesn’t mimic the *in vivo* situation. Therefore it was tried to cultivate some cell types on semipermeable membranes without a fluid filled apical compartment. In this culture model only the basolateral compartment is filled with cell culture medium. To avoid hydrostatic pressure the fluid level in the basolateral compartment must be lower than the cell monolayer. Control of the cell growth by light microscopy as well as by electrical resistance measurement is limited. Apical secreted substances like mucus or surfactant are accumulated and not washed away during feeding.

Liquid interface culture - LIC

Liquid interface culture describes the cultivation of cells under fluid layers. The most cells need a fluid filled apical compartment. Furthermore culture as well as experimental handling is easier if a fluid filled apical compartment is available. In dependency of the used filter insert system fluid layers with a thickness between 400 and 600 µm are covering the cells. All secreted substances are distributed in the apical liquid compartment and mucosubstances are removed by changing of the medium.

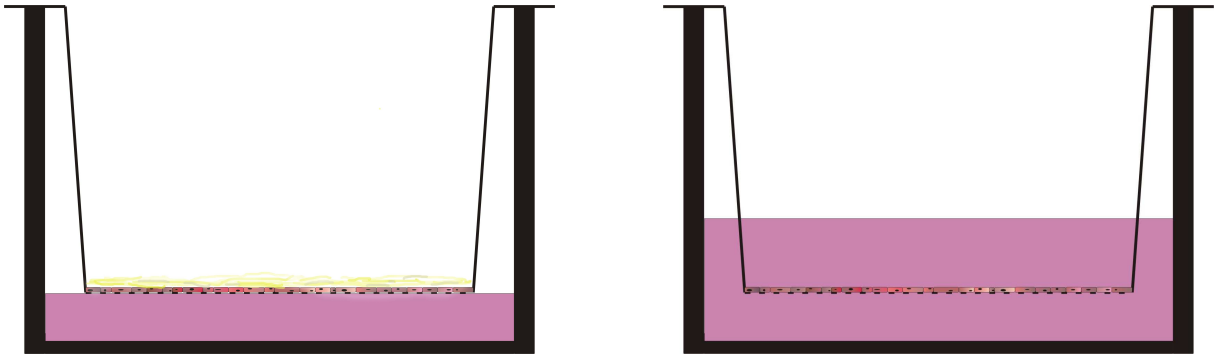

Figure 4-1 Schematic representation of AIC and LIC on semipermeable filtermembranes. In the AIC (left) the cells grow on the air, in the LIC (right) the cells are covered with cell culture medium.

Air interface deposition - AID

During air interface deposition dry aerosol particles are deposited on the surface of cell monolayers. The deposition can be done by a spatula, an insufflator syringe, or with the aid of impingement systems. Air interface deposition is widely used in environmental toxicology but more or less unknown in pharmaceutical aerosol research. Air interface deposition is the sole possibility to bring cells in contact with original particles without dispersing them first in a liquid medium. Properties like radicals on the surface, zeta potentials, diameter or surface structure are conserved and may be significantly different compared to the same particle dispersed in aqueous medium.

Liquid interface deposition - LID

In case of liquid interface deposition solutions or suspensions are applied in the apical compartment of a cell culture insert. Liquid interface deposition is possible on air interface cultivated cells as well as on liquid interface cultivated cells. It is important to distinguish between cultivation and deposition.

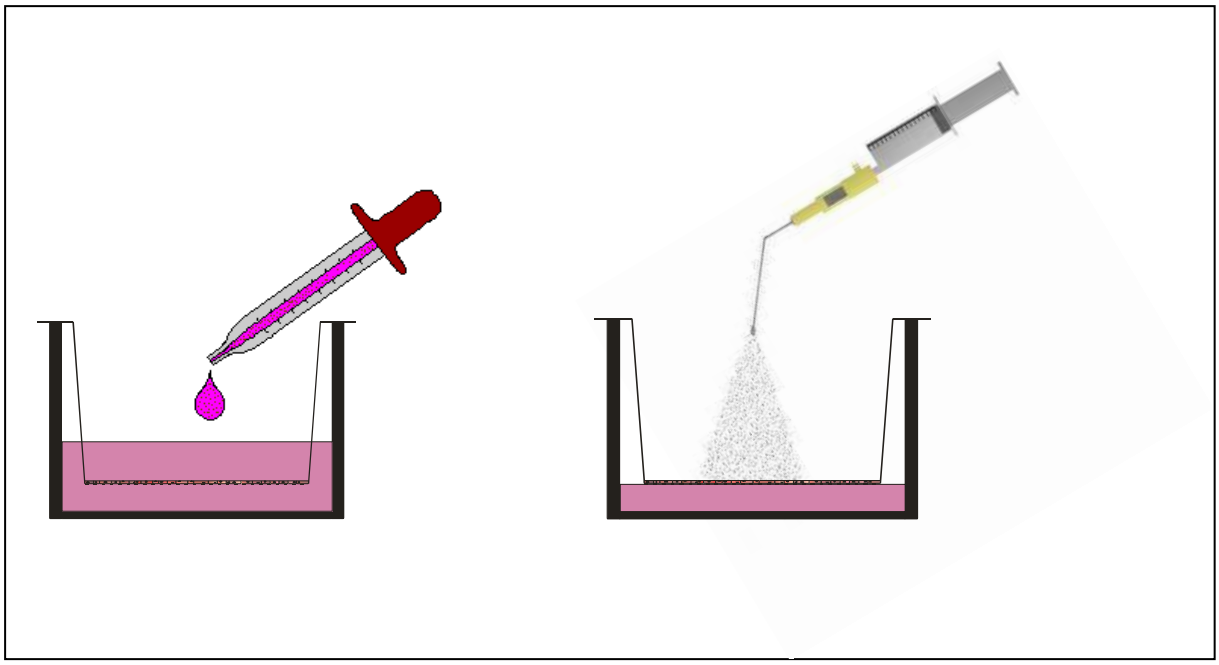

Figure 4-2 AID and LID in comparison. AID is accomplished with a Penncentury® insufflator syringe.

In summary four different combinations of cultivation and deposition techniques are possible:

- ❖ LIC / LID: The widely used submersed transport experiment, dissolved or suspended drug are applied in submersed cell culture systems.
- ❖ LIC / AID: Dry particles in the solid state are deposited on the surface of the fluid filled apical compartment. Wetting, dissolution and/ or sedimentation occur after deposition. In contrast to LIC / LID some particle properties can be conserved and influence especially dissolution velocity.
- ❖ AIC / LID: Cells which were cultivated without a fluid filled apical compartment were blanket with a drug solution or suspension. Typical features of the air interface cultivated cells like presence of mucus are conserved in parts.
- ❖ AIC / AID: Dry particles in the solid state are deposited on a dry cellular surface. All properties of the particle formulations are conserved. The contact between the particles and the cell is as close as possible. AIC / AID seems to reflect the *in vivo* situation after powder inhalation in the most realistic manner.

5 General introduction

Parts of the introduction have previously been published in:

Bur M, Henning A, Lehr C-M, Alveolar epithelial cell culture – a useful tool in aerosol drug delivery research, Respiratory Drug delivery X; Biological, Pharmaceutical, Clinical, and Regulatory Issues Relating to Optimized drug Delivery by Aerosol; Boca Raton Resort and Club, Florida, April 23-27, 2006.

Bur M, Bock U, Haltner-Ukomadu E and Lehr CM; In vitro Models for Pulmonary Drug Absorption", in K. Bechtold-Peters; H. Luessen (ed.) "Pulmonary Drug Delivery", Editio Cantor Verlag, Aulendorf, (2007), pp.58-81.

5.1 Background

The year 2006 marks two important events for the pulmonary drug delivery research. At first metered dose inhalers celebrate their 50th birthday, and secondly Exubera® – the inhalable insulin – came onto the market. But nevertheless, inhalation is still an underutilized route of drug delivery. Even if medicine application via the lung has been in clinical use since the earliest days of medical history and aerosolized medications were particularly popular at the end of the 19th century (especially Asthma cigarettes, containing stramonium leaves with atropine-like effects) only less than 50 different drugs are permitted as inhalable formulation at the moment. Even though inhalation drug therapy is applied since thousands of years, in most of the cases the pure drugs and no formulations are permitted for application in the lung. Solely lactose, water and sodium chloride are auxiliary materials which can be used for the formulation of medical aerosols. Sustained release, which is widely used in oral drug delivery, is not realized yet in inhalation therapy.

However in recent years, the lung has been studied more and more as a possible route of administration for the treatment of systemic diseases, such as diabetes mellitus. The lung provides direct access, thin resorption barrier, enormous surface area, and relatively low enzymatic activity, which all together are in favour of systemic absorption of drugs. In a foreseeable future, we will see not only more efficacious inhaled therapies for respiratory diseases, but also the introduction onto the market of aerosols for gene therapy and the treatment of systemic diseases. Especially missing knowledge about the processes on the air-blood barrier and the lack of safety data are constraining the development of new innovative inhalative medicines. 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.

5.2 Structure and function of the lung

In this chapter the anatomy of the human lung as place of drug administration is elucidated. Mainly anatomical features which are of relevance for drug absorption are described in more detail. As one of the primary interfaces between the body and the environment, the respiratory system is constantly exposed to airborne particles, potential pathogens and toxic gases in the inspired air. As a result, a sophisticated respiratory host defence system, present from the nostrils to the alveoli, has evolved to clear offending agents. The system comprises mechanical (i.e. air filtration, cough, sneezing, epithelial tightness and mucociliary clearance), chemical (antioxidants, antiproteases and surfactant lipids) and immunological defence mechanisms. From a drug delivery perspective, the components of the defence system represent barriers that must be overcome to ensure efficient drug deposition and absorption from the respiratory tract.

5.2.1 Anatomy of the human respiratory organ

The fundamental function of the lung is the distribution of the inspired air and the expulsion of the gaseous waste product CO_2 from the body. Atmospheric air is pumped in and out regularly through a system of pipes, called conducting airways, connecting the alveolar gas exchange region with the outside of the body. About 85% of the total lung volume consist of gas exchange parenchyma (alveolar sacs, alveoli, and alveolar capillary network), and about 6-10% of conducting airways (bronchi and bronchioles). The remaining part of the lung consists of nervous and vascular tissue [1]. The conducting airways, which are composed of the nasal cavity and associated sinuses, the pharynx, larynx, trachea, bronchi and bronchioles, have to filter, heat and humidify the inspired air. Below the larynx the trachea, a pipe about 10 to 12 cm long and 2 cm wide is located. Its wall is fortified by cartilage rings. At its lower end, the trachea divides in an inverted Y into the two main bronchi, one each for the left and the right lung. From the end of the trachea to the periphery of the airway tree, the airways repeatedly branch dichotomously into two daughter branches with smaller diameters and shorter length than the parent branch [2]. For each new generation of airways, the number of branches is doubled and the cross-sectional area is exponentially increased. The conducting region of the airways generally constitutes generation 0 (trachea) to 16 (terminal bronchioles).

The respiratory region, where gas exchange takes place, generally constitutes generation 17-23 and is composed of respiratory bronchioles, the alveolar ducts, and the alveolar sacs (see Figure 5-1).

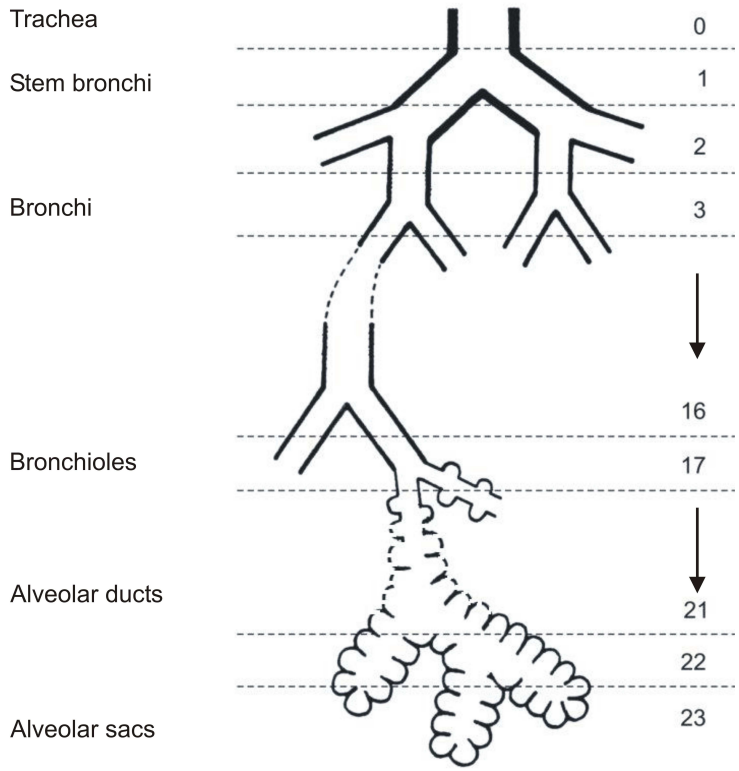

Figure 5-1 Model of human airway system assigned to generations of symmetric branching from trachea (generation 0) to the alveolar region (generations 15–23), ending in alveolar sacs. Modified after [2]

5.2.2 Major components of the lung – barriers for drug absorption

The lung epithelium can be divided in two parts according to function and localisation.

5.2.2.1 Epithelium

The air-blood barrier of the gas exchange area is composed of the alveolar epithelial cells (surface area 140 m^2) on one side and the capillary bed (surface area 130 m^2) 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. The airway epithelium provides a tight ciliated

barrier that clears the airways from deposits in the airway mucus, prevents indiscriminant 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, i.e. 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. In the alveolar region, mainly two cell types are present: the 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 few cellular organells. These morphometric features are favorable for gas exchange and for drug transport. About 3% of the alveolar surface is covered by the much smaller cuboidal type II cells, which synthesize and secrete surface active materials [4]. The apical membranes of the epithelial cells are joined by tight junctions that divide the cell membranes into the functionally distinct apical and basolateral domains. Tight junctions (TJ) represent the most apical cell-cell contacts in epithelial and many endothelial cell sheets and are important for their barrier function [5]. In addition, tight junctions act as a kind of fence maintaining the specific lipid and protein composition of apical and basolateral membrane domains in polarized epithelia. In freeze-fracture electron micrographs tight junctions appear as branched beaded strands of particles that fuse the outer leafs of the plasma membranes of opposing cells. Occludin [6] and claudins [7] were identified as the major integral membrane proteins forming the continuous tight junction strands. The junctional adhesion molecule, a member of the Ig superfamily of single transmembrane domain proteins, was found associated with tight junction strands [8]. Both, occludin and claudins have four transmembrane domains and their N- and C-terminal ends are located in the cytoplasm. This topology generates two extracellular loops that are supposed to provide the intercellular interaction sites [9]. The C-terminal domain of occludin and claudins serves as a binding site for a complex set of proteins including a number of PDZ-domain proteins (ZO-1, ZO-2, ZO-3), kinases and phosphatases [10]. This association with different signalling molecules indicates that tight junctions are more than simple barriers separating compositionally distinct environments. In contrast, they appear to be involved in the reception and conversion

of signals from and to the cell interior [11]. As a consequence of the existence of tight junctions we will find a higher transepithelial electrical resistance (TEER) in an epithelium with a high density of tight junctions then in a leaky epithelium. The body knows example for both cases. On the one hand the mammalian proximal tubule with less than 10 Ohm*cm^2, on the other hand the mammalian urinary bladder with an electrical resistance from more then 10,000 Ohm*cm^2. The human lung epithelium is also a tight epithelium (≈ 3,000 Ohm*cm^2), because it has to resist the loss of water from the body in the airspace.

5.2.2.2 Endothelium

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 specialized organell-free domains to provide a particularly thin (from 200 nm down to 30-35 nm) barrier for gas exchange [12]. Furthermore, the endothelial cells have a relatively large number of endocytotic vesicles [13]. Until now the endothelial barrier for the drug absorption is not sufficient investigated.

5.2.2.3 Interstitium and basement membrane

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 fibers, and interstitial fluid. Its main role is to separate and bind together the specific cell layers in the tissue. The main drainage pathway for the interstitial fluid is the lymphatic vessels. The outer border of the interstitium is defined by the epithelial and endothelial basement 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 are thought to diffuse relatively unhindered through it [14]. The light microscopic pictures Figure 5-2 to Figure 5-6 give an impression about the filigree structure of the human lung.

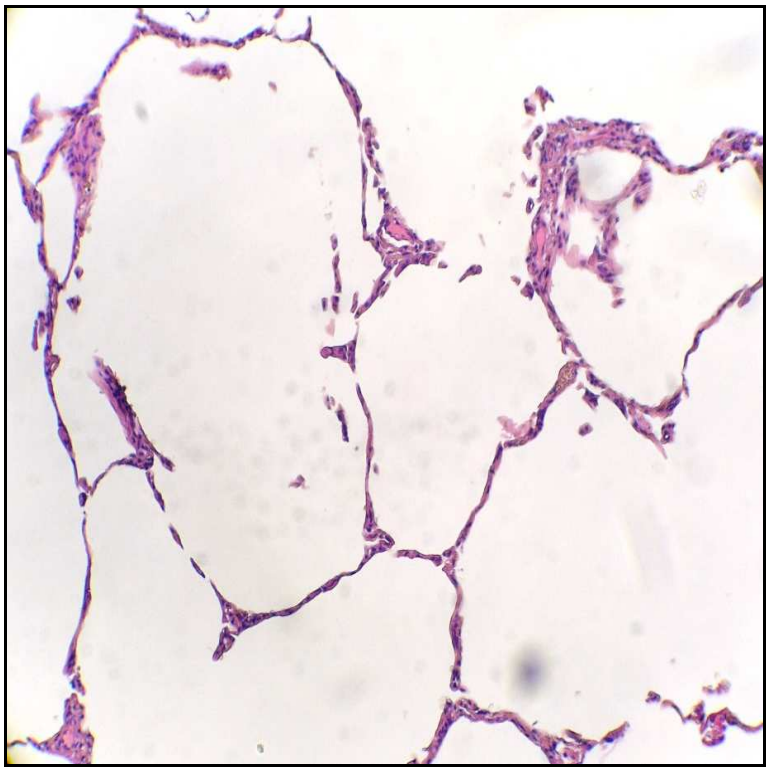

Figure 5-2 Cross-section through alveolar sacs in the deep lung. The flat cell bodies of the type I cells are dominating.

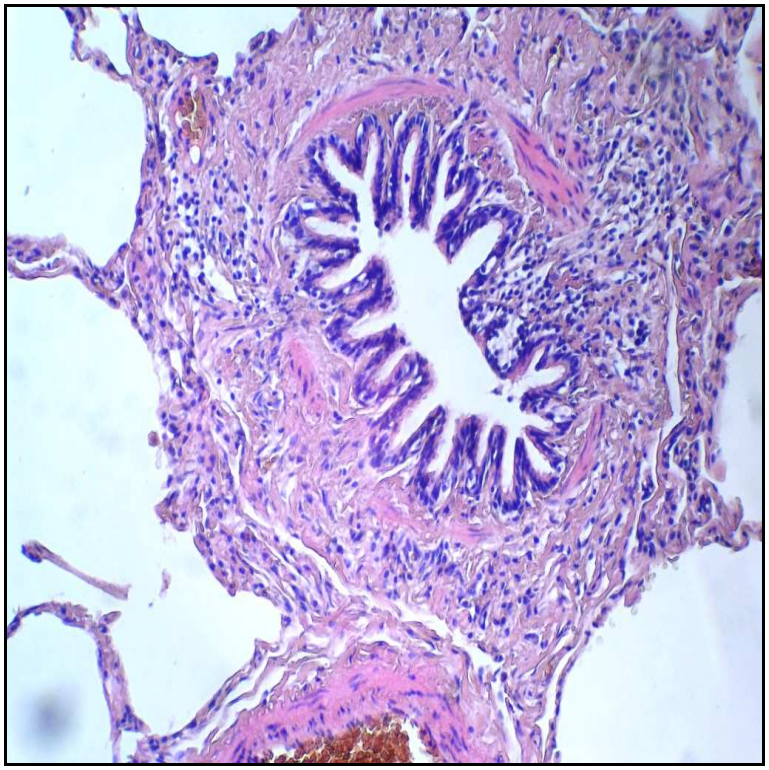

Figure 5-3 Overview picture of a bronchioles. Muscle cells and tissue circumvent the highly folded bronchial epithelium.

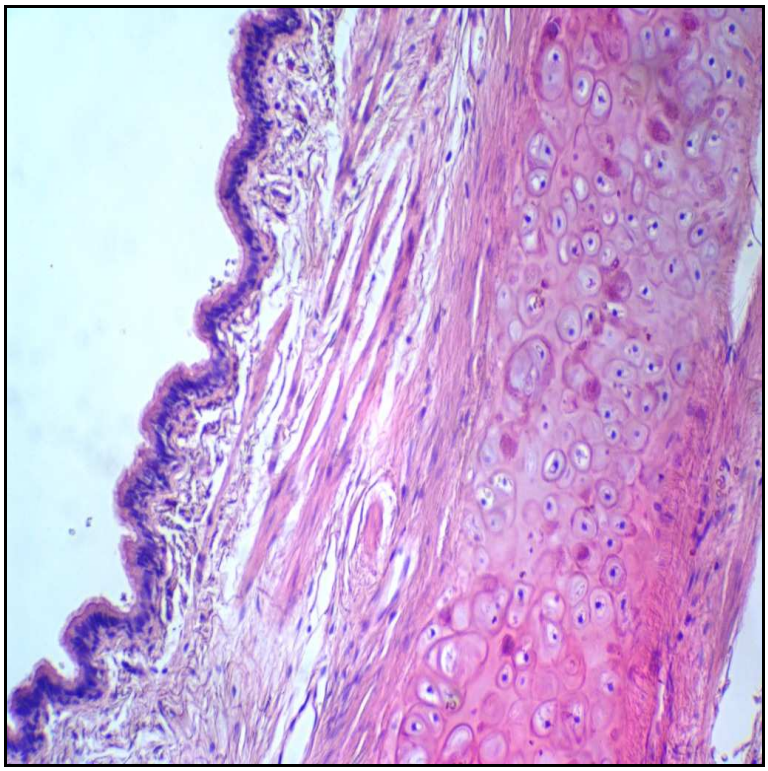

Figure 5-4 Section through bronchial tissue of the upper airways. Connective tissue and cartilage provide a basis for the ciliated bronchial epithelium.

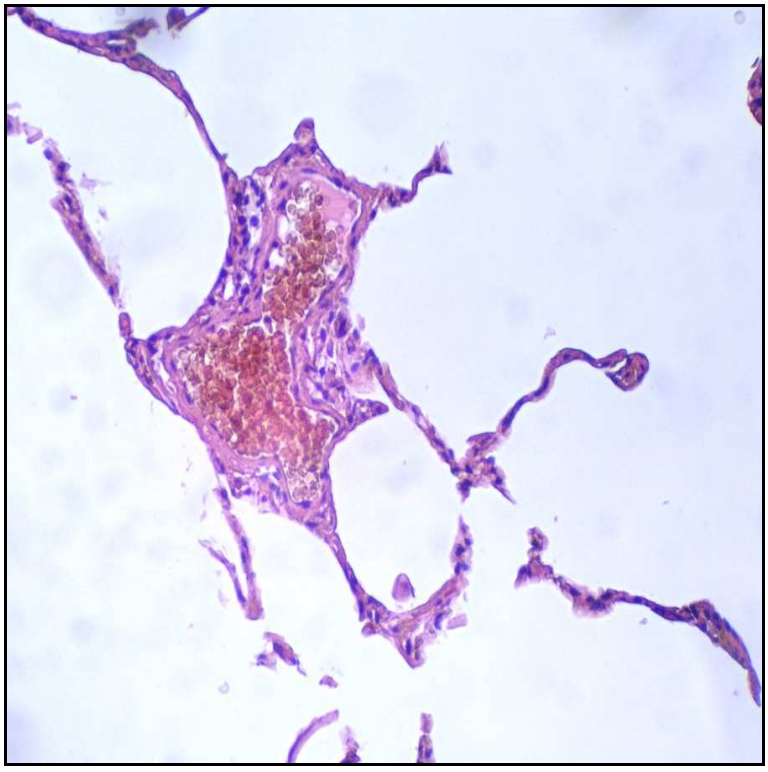

Figure 5-5 Cross-section through a blood vessel in the deep lung. The diffusion distance for gas and drug molecules is very short.

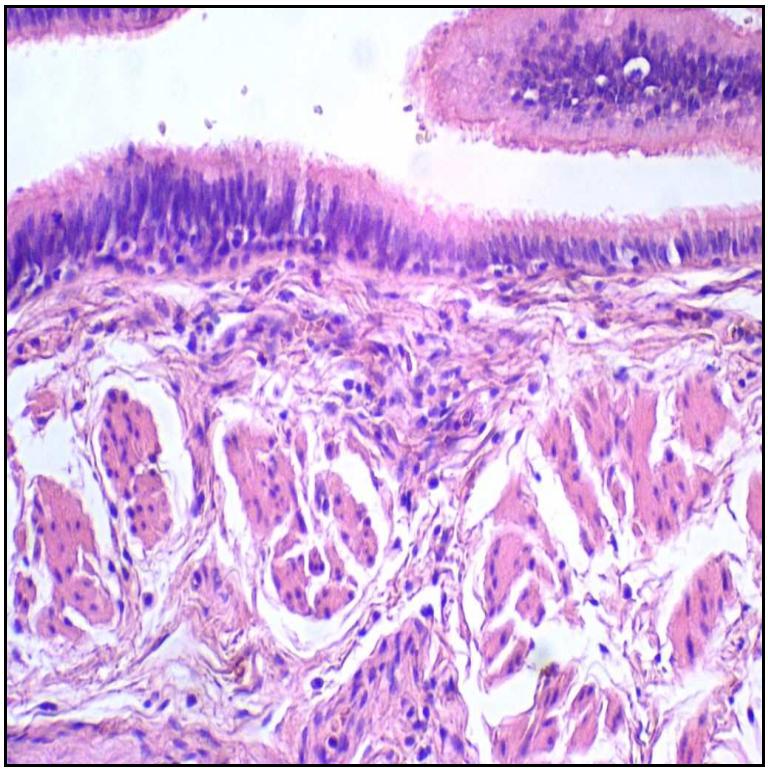

Figure 5-6 Columnar ciliated cells as a part of the mucociliary escalator in the upper airways.

5.2.2.4 Epithelial lining fluids

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 [15]. The thickness of the lining fluid in the airways is estimated to 5-10 μm and is gradually decreased along the airway tree until the alveoli, where the thickness is estimated to be about 0.01-0.08 μm [14]. 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 monolayer of pulmonary surfactant [14]. The lung surfactant is synthesized and secreted by the alveolar type II cells and comprises a unique mixture of phospholipids and surfactant-specific proteins [4]. The characteristic lamellar bodies in the type II cells serve as storage depot for the surfactant before this is secreted onto the alveolar surface [16]. 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 stabilized, which promotes lung expansion on inspiration and prevents lung collapse on expiration. The lung surfactant has also been found to enhance local pulmonary host defense mechanisms by serving as a barrier against adhesion of microorganisms and to enhance phagocytosis by alveolar macrophages [17]. The lung surfactant undergoes a constant dynamic process of turnover and metabolism, including removal by the mucociliary escalator, phagocytosis and recycling. In drug development it should be considered that complex interactions between drugs and lung surfactant are described. Balakrishnan et al. [18] found for example a 107-fold increased solubility of griseofulvin in presence of surfactant. The role of surfactant in pulmonary drug delivery is not yet completely understood.

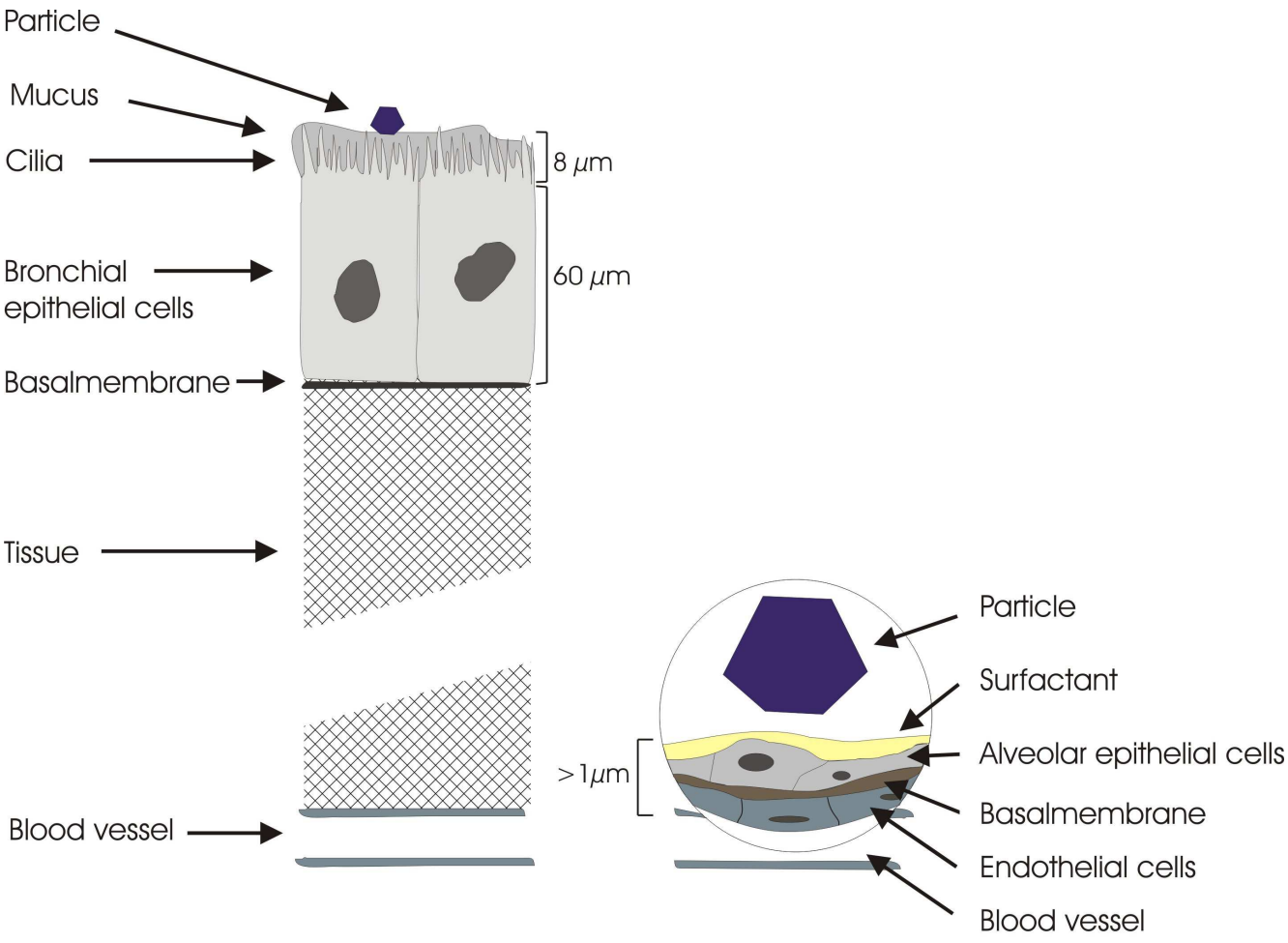

Figure 5-7 Schematic drawing of the bronchial and alveolar physical absorption barrier.

5.2.2.5 Alveolar macrophages

The alveolar macrophages are found on the alveolar surface. These phagocytic cells play important roles in the defence mechanisms 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 in the lung of rats have been demonstrated to be phagocytized by alveolar macrophages within a few hours [19]. The macrophages are cleared from the alveoli to the bronchioles by the lining fluid, and then from the airways by the mucociliary escalator.

Alveolar macrophages can be on the one hand trapped within the connective lung tissue of the alveolar walls and on the other hand mobile and scavenge for particles which are trapped within the surfactant layer. However, the lung tissue macrophages and alveolar macrophages share several functions: defense against pathogens, ingestion and destruction of potential allergens, clearing of particulates, and presentation of antigens to T cells.

The alveolar macrophage can leave the lungs by ascending in the layer of mucus on the 'mucociliary escalator' to the larynx or by passing into alveolar lymphatics.

Since 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 defense 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. 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 which modulate lymphocyte function and factors which promote fibroblast migration and replication.

5.2.2.6 Mucociliary clearance

The 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 on to 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.* [20] measured for the mucociliary escalator a velocity of approximately 80 µm/s in healthy patients. But also regulation of periciliary fluid is essential to optimize mucociliary clearance and to provide a milieu in which airway antimicrobial agents are effective. Disruption of the interplay between ciliated epithelium, periciliary fluid and mucus may occur in diseases such as cystic fibrosis and asthma.

The thickness of the mucus layer varies along the conducting airways, being about 8 µm in the trachea and about 2 µm in the bronchioles [21]. The mucus layer is continuous in the larger human bronchial airways, but consists of discontinuous spots in the smaller bronchi and bronchioles. 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%) [22]. Regulation of the water content is of significant importance to maintain the optimal viscoelastic properties of the mucus. The first barrier to 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.

5.2.2.7 Metabolic processes in the lung

The lung serves as a primary site for xenobiotic metabolism. Alveolar macrophages and bronchial epithelial cells are part of over 40 different cell types in lung tissue with different levels of metabolic competence [23, 24]. Compared to the liver and intestine, the lung is thought to play a minor role in the metabolism of drug compounds. Although a number of CYP isoforms have been identified in human lung tissue, a comprehensive survey of most human pulmonary xenobiotic metabolizing enzymes in different human lung tissue compartments has not been performed and the characterization of drug metabolizing functions of the different cell types is a complex task. Generally, all metabolizing enzymes found in the liver are also present in the lung, although in lesser amounts. Especially the proteolytic activity in the epithelial lining fluid and interstitial fluid is of major interest when aiming at pulmonary delivery of therapeutic peptides [25]. Even if the lung is thought to have a lower proteolytic activity than many other organs relatively high activity of exopeptidases (e.g. aminopeptidases) have been found in rat bronchoalveolar lavage fluid, on the surfaces of the cells lining the respiratory tract and in the pulmonary circulation [26]. However, the lung is the only organ through which the entire cardiac output passes; and in consequence of this high pulmonary blood flow, the metabolic capacity of the lung should not be ignored.

5.2.3 Mechanisms of drug transport across the air-blood barrier

Deposition of particles of a size >0.5 mm mass median aerodynamic diameter (MMAD) takes place via inertial impaction and gravitational sedimentation, with smaller particles being deposited due to Brownian diffusion. The site of deposition of an aerosol in the bronchial tree depends on the inspiratory manoeuvre and the characteristics of the aerosol. The depth of the deposition is inversely related to the inspiratory flow rate with high inspiratory flow increasing central deposition. Whole lung deposition also increases when a breath-holding pause after inspiration is included. The particles are deposited after inhalation on an epithelial barrier.

5.2.3.1 Small molecule drug absorption

The function of an epithelium is the control of resorption or secretion of substances. Some substances like water can diffuse through the epithelium by using the intercellular space, but most substances need an energy dependent transport.

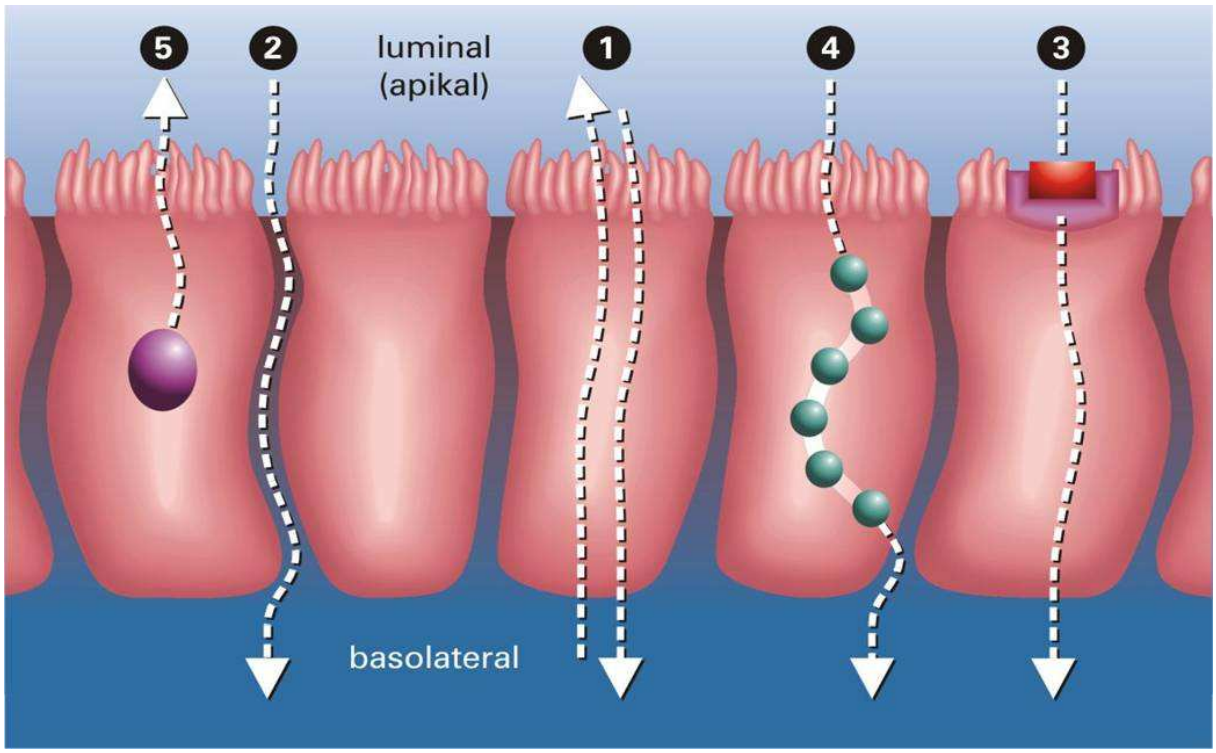

Figure 5-8 The five main transport routes for drug transport across an epithelium. Adopted from [27]

Five main transport pathways (Figure 5-8) are distinct: 1) transcellular diffusion; 2) paracellular diffusion; 3) carrier-mediated uptake at the apical domain followed by passive diffusion across the basolateral membrane 4) transcytosis 5) active secretion or efflux. Paracellular passive diffusion means the transport between two cell bodies, without any participation of cellular structures. This pathway is only possible for water itself and small water soluble molecules. Transcellular diffusion is also a passive, concentration driven process, but in this case the molecule has to penetrate and to diffuse along the membrane of the cell. Carrier mediated uptake at the apical domain followed by passive diffusion across the basolateral membrane is an important case for many physiological compounds but also for some drugs. With the aid of pumps, carriers and so one, molecules can be transported against a concentration gradient. Transcytosis is the preferred pathway for large molecules and is mediated by the cellmembran which includes the molecules in a cave and transports this cave through the cell body. Finally active secretion, also referred to as efflux, is an ATP dependent process, which results in a net inhibition of the passive, concentration driven absorptive transport of some drugs.

5.2.3.2 Transport proteins

For some transport processes, the epithelial cell needs specialized transport proteins. These transport proteins can be divided in two groups:
Carriers which generate their energy by antiport or by cotransport of a second molecule, and pumps which need a separate energy source.
Carriers are further subdivided in symporter and in antiporter. Mainly in epithelia with a high resorption or secretion power like the epithelium of the Henley loop a lot of different transport proteins are expressed. In the human lung, exchanger for sodium, potassium, and calcium were detected. ß- Receptors as well as cardiac glycoside sensitive transporters were investigated. In the alveolar region of the human lung the function of these transporters is the maintenance of an osmotical gradient, which is the source for the water equilibrium in the lung [28].

5.2.3.3 Efflux systems

Multidrug resistance (MDR)- phenomena, caused by cellular efflux systems have been identified as an important element of various biological delivery barriers, such as the intestinal mucosa or the capillary endothelium of the brain and their relevance for the efficacy of drug therapy is meanwhile widely accepted [29]. It may therefore also be expected that the presence of some membrane associated transporter molecules, such as P-glycoprotein (P-gp) and the multidrug resistance protein associated protein-1 (MRP1), also play a role in limiting drug absorption through the pulmonary epithelium. To date, the exact role of the lung resistance related protein (LRP) in MDR is unclear. Delivered drugs may be without any effect if they are transported out of the cells after a very short period of time. Vice versa, inhibition of efflux systems, such as P-gp, is discussed as a way to increase drug absorption and efficacy. In the human lung there is so far no direct evidence for P-gp-mediated drug efflux, but in human lung cell culture the P-gp has already been found. Therefore, one might infer that P-gp-like efflux systems are also existent in the human lung *in vivo*.

5.2.3.4 Blood circulation

After overcoming all these drug absorption barriers the drug molecules reach the blood and can be transported in the different regions of the human body. Two different circulatory systems, the bronchial and the pulmonary, supply the lungs with blood. The bronchial circulation is a part of the systemic circulation and is therefore under high pressure. It receives only small parts of the cardiac output and supplies the airways (from the trachea to the terminal bronchioles), pulmonary blood vessels and lymph nodes with oxygenated blood and nutrients. In addition, it seems to be important for the distribution of systemically administered drugs to the airways and for the absorption of inhaled drugs from the airways. The pulmonary circulation comprises an extensive low-pressure vascular bed, which receives the entire cardiac output. It perfuses the alveolar capillaries to secure efficient gas exchange and supplies nutrients to the alveolar walls. Alveolar deposited drugs are transported via the pulmonary blood circulations very fast in the different regions of the body.

5.3 Biological models for assessment of pulmonary drug absorption

In order to respond to the flood of new active ingredients currently being generated by combinatorial chemistry or molecular biological synthesis, selection procedures able to filter out rapidly and economically those drug candidates with the highest development potential are required. This necessitates the measurement of fundamental biopharmaceutical parameters very early in the drug development process. Any pharmaceutically active agent must be able to overcome the body's natural protective mechanisms. A broad variety of biological barriers can be simulated in the laboratory by cell monolayer models. Apart from ethical aspects, the advantage of these *in vitro* test systems is that permeability studies can be performed at high throughput rates under controlled and reproducible conditions. The validity of such a model is ultimately reflected in its ability to accurately predict the behaviour of an active ingredient at the corresponding *in vivo* barrier.

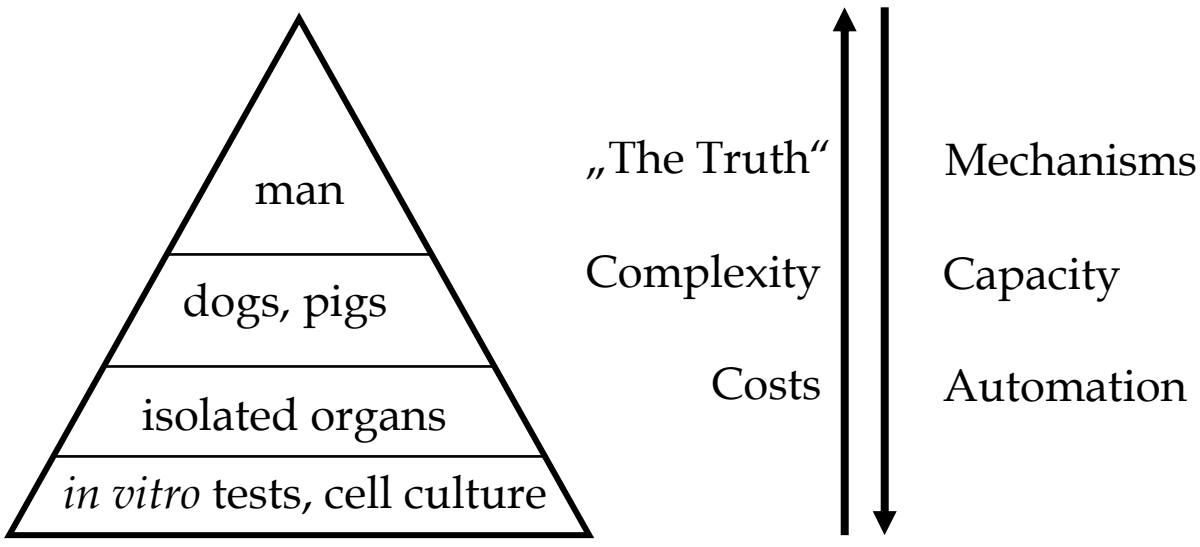

Figure 5-9 In vitro test systems – the golden mean between manageability and explanatory power.

5.3.1 Isolated perfused organs

Experiments utilising a whole isolated perfused lung (IPL) for the examination of various hemodynamic parameters such as pulmonary blood flow and arterial pressure-flow relationships have been reported for over 80 years. Experimental conditions such as temperature, pH, hydrostatic and osmotic pressures within the pulmonary perfusate can be readily controlled. The most common species used in IPL models is the rat. If the lung is totally isolated from the animal then the vascular system that remains intact is that which serves only the pulmonary region, and hence airway to perfusate transfer of solute will reflect predominantly the pulmonary transport properties and less that of the tracheo-bronchial tree. IPL was used for a lot of resorption studies, Byron and Patton [30] investigated the kinetic of pulmonary drug absorption of synthetic polypeptides and Manford [31] showed a good correlation between the drug permeability in the isolated perfused rat lung and in 16HBE14o- cells. However, the functional enzymatic equipment of the whole isolated perfused lung is advantage and disadvantage at the same time. Only in this ex vivo system we can simulate with highest touch to reality deposition and absorption of pharmaceutical relevant aerosols. The most important disadvantage of the IPL model is the relatively short tissue viability of two or three hours. After this space of time Saldias et al. [32] observed a functional breakdown of the epithelium.

5.3.2 Cell culture systems

5.3.2.1 Immortalized cell cultures

High-throughput screening (HTS) is the most important process of testing a large number of diverse chemical structures against disease targets to identify 'hits'. HTS is characterized by its simplicity, rapidness, low cost, and high efficacy. As a multidisciplinary field, HTS involves automated operation-platforms, highly sensitive testing systems, specific cell based screening models (*in vitro*), abundant components libraries and data acquisition and processing systems [33]. However, a major limiting factor in 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 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 where 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. Immortalized human cell cultures have been used in an attempt to overcome the problems of tissue availability.

5.3.2.1.1 Bronchial cell cultures

Calu-3

Calu-3 is an adenocarcinoma cell line derived from a 25-year old Caucasian male. It has been suggested to express tight barrier properties on the basis of electrophysiological studies. The presence of tight-junctions (TJ) proteins was confirmed by immunoblotting and functional properties of the monolayers were studied by measurements of transepithelial electrical resistance and mannitol permeability [34]. Calu-3 cells have been the subject of relatively many investigations. After a few investigations about the equipment of the cells with ion channels or receptors, the cell line was relatively fast used as tool for transport studies. Mathia et al. [35] 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 "tight" polarized and well differentiated cell monolayers with apical microvilli and tight-junctional complexes. Solute permeability was dependent on lipophilicity and inversely related to molecular size. Calu-3 cells actively transported amino acids, nucleosides and dipeptide analogs. The permeability characteristics of Calu-3 cells correlated well with primary cultured rabbit tracheal epithelial cells *in vitro*, and the rate of drug absorption from the rat lung *in vivo*. Also the transport pathway of zinc insulin across the Calu-3 cell monolayer was elucidated. Transport of zinc insulin was found to be higher in the absorptive direction, than in the secretory direction [36]. Beyond the use as transport model Calu-3 cells can be also employed for the investigation of metabolic processes. Borchard et al. [37] cultivated Calu-3 cells on microporous filters at an air interface for 16-18 days, and incubated the cells with the glucocorticosteroid budesonide. With the

aid of mass spectrometry of cell extracts fatty acid conjugates of budesonide were detected. It seems that Calu-3 cells are able to store budesonide by intracellular conjugation. Therefore, it was suggest using of the Calu-3 cell model as a tool for examination of local pharmacokinetics and metabolism of glucocorticosteroids at the bronchial epithelium. Glucosteroids were also employed for a study about the efflux system P-glycoprotein in Calu-3 cells [38]. The P-gp modulation efficacy of glucosteroids was determined by its ability to increase the accumulation of the P-gp substrate rhodamine 123 in the cells. Because of the high 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 often used also 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 was tried. ZO-1, as indicator for the tight- junctions, was found in cells grown in both AIC and LCC (liquid culture conditions). However, only LCC-grown cells exhibit protein ZO-1 localized as a zonula-occludens-like regular belt connecting neighbouring cells. The presence of typical tight-junctions 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 the hydrophilic permeability marker fluorescein-Na under LCC compared with AIC conditions [39]. Also the expression of mucus could be observed only after AIC culture. To visualize the presence of mucus on the apical surface of air interface cultivated Calu-3 cells acid mucosubstances were stained with alcian blue. Slices of cell monolayers were fixed in a formaldehyde solution (4% in PBS) at room temperature for 30 min. Samples were then dehydrated through a graded series of ethanol at 70%, 96%, and 100% at room temperature and kept in xylene until embedding. For mounting, samples were soaked in paraffin wax at 60℃ over night and embedded the next day. Embed ded samples were cut in four-micrometer thin sections with a microtome (Leica Microsystems, Nussloch, Germany) and mounted on glass slides at room temperature. Samples were dewaxed with xylene and were rehydrated by a graded series of ethanol at 100%, 96%, and 70%, for 10 min each, at room temperature. The sections were washed with de-ionized water and stained with Alcian Blu (3% 30 min, room temperature). After washing the sections with de-ionized water, the sections were counterstained with fast nuclear red

(5 min, room temperature) and dehydrated through a graded series of ethanol at 70%, 96% and 100% and xylene. Sections were stored in xylene until mounting with cover slips using Roti-Histokitt. The sections were examined with an Axiovert XY light microscope (Carl Zeiss, Jena, Germany) at 400x magnification. Strongly acidic mucosubstances stain blue, nuclei pink to red, and the cytoplasm pale pink.

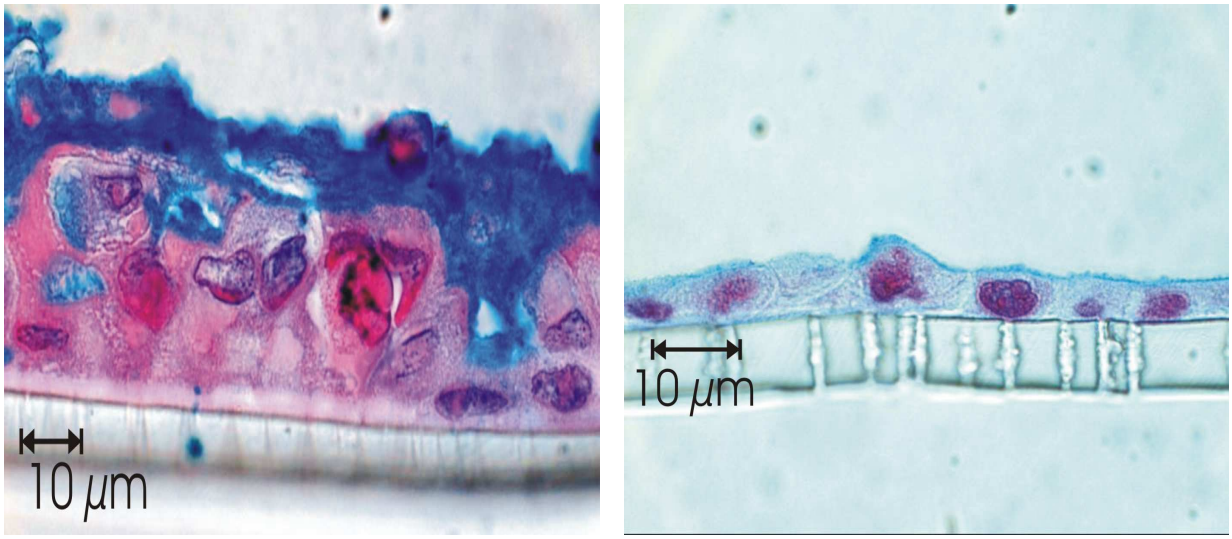

Figure 5-10 Light microscopic picture of Alcian blue staining of Calu-3 cells after 8 days air interface culture (left side) or liquid interface culture (right side).

Cell morphology as well as mucus production can be influenced by the culture conditions. Air interface culture yields higher production of mucosubstances. Furthermore air interface cultivated cells show a more bronchial-like structure. Columnar cell bodies and mucus secreting cells characterize the AIC culture. In contrast, liquid interface culture causes real monolayers with flattened cells. Mucus seems to be washed away.

16HBE14o-

Another human bronchial epithelial cell line 16HBE14o-, immortalized by virus transformation, shows also significant transepithelial resistance and can be used for transport studies. In comparison to the Calu-3 cells the 16HBE14o- cell line seems to express more P-glycoprotein, lung resistance-related protein (LRP) and caveolin-1.

Immunocytochemical staining showed expression of P-gp localized at the apical membrane of 16HBE14o- cell layers. The flux of rhodamine 123 across cell layers exhibited a greater appearance 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* [40].

CFBE41o-

The CFBE41o- cell line was generated by transformation of cystic fibrosis 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 [41]. 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 should be useful for studies in the scope of CF gene transfer or alternative treatment using small drug molecules and gathering further knowledge about the disease on the cellular level, without the need for primary culture.

5.3.2.1.2 Alveolar cell cultures

A549

The A549 cell line possesses 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 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 needed. 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 approached the characteristics of cell-free filters alone. The 'leaky' monolayers formed by the latter airway carcinoma cell lines failed to show significant immunostaining of the tight junction protein ZO-1. The leaky formation of tight junctions in A549 is also the cause for very low transepithelial electrical resistances by these monolayers. This suggests that the formation of peripheral rings of ZO-1 staining is related to the formation of tight junctions and that these junctions are the probable reason why these monolayers have low permeability to mannitol. A549 exhibited staining for desmoplakin, but no staining of E-cadherin. The functional tight junction 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 be a question of optimized culture conditions [42].

The use of immortalized cell lines is limited by the fact that in many cases cells loses 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.

5.3.2.2 Primary cell cultures

5.3.2.2.1 Animal origin

Rat cells

Because of the relatively easy isolation protocol and the trouble-free availability, rat alveolar epithelial cells 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. [43]. 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 Ohm*cm^2).

Porcine 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 more additional animals will have die for research purposes. Furthermore the morphology of porcine mucosa seems to be comparable with human epithelial cells especially with regard of electrophysiology and enzymatic equipment [44]. Steimer et al. [45] characterized 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 tight junctions. Maximum transepithelial electrical resistance of about 2,000 Ohm*cm ^{2} were 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 of alveolar specific cell markers like caveolin for type I and surfactant protein C for type II cells. First transport experiments with sodium fluorescein showed the qualification of the pAEpC model for pulmonary drug absorption studies.

5.3.2.2.2 Human origin

Lung alveolar epithelium *in vivo* is composed of two specialized epithelial cell types, the squamous alveolar epithelial type I cell, which constitutes approximately 93% of the alveolar epithelial surface area, 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 the type I cells *in vivo* [4, 46]. 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 Ohm*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 for different purposes. Although the isolation of primary human alveolar cells has been described before [47] 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 (hAEpC) and their primary culture was described by Elbert et al. [48], which results in confluent monolayers capable of generating tight-junctional complexes and high transepithelial electrical resistance. The morphological cell change from an type II phenotype to an type I - like cell phenotype over time of culture was described by Fuchs et al. [49]. 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 [50] including both the clearance of endogenous protein from the airspace and the absorption of inhaled therapeutic protein. Primary type II alveolar cells are isolated from human non-tumour lung tissue, which is obtained from patients undergoing lung resection. The isolation is performed according to a protocol described by Elbert et al.[48], for a detailed description of the isolation procedure please see chapter 6.2.2. Formation of functional tight-junctional complexes and generation of confluent monolayers after a few days in culture was routinely determined by measuring TEER using an electronic voltmeter. After reaching confluence the alveolar monolayers of hAEpC typically revealed TEER values of 1,000-2,000 $Ohm*cm^2$ on day 6-8 post seeding. The formation of tight junctions was also routinely monitored by immunofluorescent staining for zonula occludens protein-1 [49].

6 Human primary epithelial cells for investigation of transport processes of macromolecules over the pulmonary epithelium

Abstract:

In this study, we investigated bi-directional fluxes (i.e., in absorptive and secretive directions) of human serum proteins [albumin (HSA), transferrin (TF), and immunoglobulin G (IgG)] and peptides/proteins of potential therapeutic relevance [insulin, glucagonlike peptide-1 (GLP-1), growth hormone (GH), and parathyroid hormone (PTH)] across tight monolayers of human alveolar epithelial cells (hAEpC) in primary culture. Apparent permeability coefficients (P_{app}; $\times 10^{-7}$ cm/s, mean±S.D.) for GLP-1 (6.1 ± 0.9 (absorptive) versus 1.9 ± 0.5 (secretive)), HSA (2.4 ± 1.0 versus 0.2 ± 0.3), TF (0.9 ± 0.2 versus 0.3 ± 0.1), and IgG (0.4 ± 0.2 versus 0.2 ± 0.1) were all strongly direction-dependent, i.e., net absorptive, while PTH (2.2 ± 0.3 versus 1.8 ± 0.8), GH (8.3 ± 1.2 versus 9.0 ± 3.4), and insulin (0.8 ± 0.2 versus 0.7 ± 0.4) showed no directionality. Trichloroacetic acid precipitation analysis of tested molecules collected from donor and receiver fluids exhibited very little degradation. This is the first study on permeability data for a range of peptides and proteins across an *in vitro* model of the human alveolar epithelial barrier. These data indicate that there is no apparent size-dependent transport conforming to passive restricted diffusion for the tested substances across human alveolar barrier, in part confirming net absorptive transcytosis. The obtained data differ significantly from previously published reports utilising monolayers from different species. It can be concluded that the use of homologous tissue should be preferred to avoid species differences.

Parts of this chapter have been published in:

Bur M, Huwer H, Lehr CM, Hagen N, Guldbrandt M, Kim KJ, Ehrhardt C; Assessment of transport rates of proteins and peptides across primary human alveolar epithelial cell monolayers; European Journal of Pharmaceutical Sciences. 2006 Jun; 28(3): 196-203.

6.1 Introduction

Although immunocytochemical and biochemical approaches have been used to demonstrate the presence of serum proteins (e.g. albumin, transferrin and immunoglobulin G) on the epithelial surface of the distal air space and in bronchoalveolar lavage fluid, the ability of proteins to traverse the alveolar epithelium remains a subject of debate [28, 51]. Besides the discussion in the physiological arena, the lung is discussed as potential alternative route for the systemic delivery of proteins and peptides [30]. The goals of this part of the thesis were to assay the *in vitro* permeability characteristics of a series of drug compounds across monolayers of primary cultured human alveolar epithelial cells (hAEpC) grown on tissue culture-treated filter inserts. These monolayers comprise alveolar epithelial type I-like cells and develop high barrier resistance (>1,500 Ohm*cm^2) [48]. The drug compounds used for this study were proteins and peptides having different structural features with a molecular weight (MW) range from 3,300 to 150,000 Da. Permeability data of these proteins across hAEpC monolayers has not been published previously; however, some of the compounds have been used in transport experiments in other *in vitro* and/or *in situ* models [28, 51, 52]. For glucagon-like peptide-1 (7-37) (GLP-1) and parathyroid hormone (1-38) (PTH) no permeability studies are available at all, while reports on the permeability of insulin, growth hormone (GH), serum albumin (HSA), transferrin (TF) and immunoglobulin G (IgG) are available to a certain extent. Since all proteins under investigation are either endogenous compounds or derivatives thereof, it is likely that interactions with the corresponding receptors occur, which, in some cases, might result in internalisation and transcytosis. Therefore, a brief summary on receptor expression pattern, especially in lung tissues, has been added, where data was available.

6.2 Materials and methods

6.2.1 Proteins

Human insulin (I0259), fluorescein isothiocyanate (FITC)-labelled human serum albumin (A7016) and FITC-labelled immunoglobulin G (F9636) were obtained from Sigma (Deisenhofen, Germany). 125I-labelled parathyroid hormone (1-38) (T-055-12) and glucagon-like peptide-1 (7-37) (T-028-13) were purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany) and 125I-labelled growth hormone (NEX100050) and transferrin (NEX2120050) came from PerkinElmer (Rodgau, Germany).

6.2.2 Cell culture

Primary human type II alveolar epithelial cells (hAEpC) were isolated from nontumour lung tissue which was obtained from patients undergoing lung resection. The use of human material for isolation of primary cells was reviewed and approved by the respective local ethical committees (State Medical Board of Registration, Saarland). Isolation was performed according to a slightly modified protocol previously described by Elbert et al. [48]. Briefly, the chopped tissue was digested using a combination of 150 mg trypsin type I (T8003, Sigma) and 0.641 mg elastase (LS002279, CellSystems, St. Katharinen, Germany) in 30 ml BSS (balanced salt solution, 137 mM NaCl, 5.0 mM KCl, 0.7 mM Na_2HPO_4•7 H_2O, 10 mM HEPES (N-[2-hydroxy-ethyl]piperazine-N´-[2-ethanesulfonic acid]), 5.5 mM glucose, penicillin (100 units/ml) and streptomycin (100 µg/ml), pH 7.4) for 40 min at 37℃. The alveolar epithelial type II cell population was purified by a combination of differential cell attachment, percoll density gradient centrifugation and by magnetic cell sorting (Anti-HEA (EpCAM) MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated type II cells were then seeded at a cell density of 600,000 cells/cm^2 on collagen/fibronectin coated polyester filter inserts (Transwell® Clear 3470, 6.5 mm in diameter, 0.4 µm pore size, Corning, Wiesbaden, Germany) using SAGM medium (CC-3118, Cambrex Bio Science, Verviers, Belgium) containing penicillin (100 units/ml) and streptomycin (100 µg/ml) and with addition of low serum (1% foetal calf serum). Formation of functional tight-junctional complexes and generation of confluent monolayers was routinely determined by measuring transepithelial

electrical resistance using an epithelial voltohmmeter (EVOM, WPI, Berlin, Germany). After reaching confluence, hAEpC monolayers typically revealed TEER values of 1,500 – 3,500 Ohm*cm^2 on day 7-8 post seeding. Formation of tight junctions was also routinely monitored by immunolabelling for the tight junctional protein, occludin. The average yield of type II cells was 0.8 * 10^6 cells/g tissue (n = 19) with a purity of type II cells in the range of >90%, determined by staining for alkaline phosphatase.

6.2.3 Transport studies

Transport experiments were conducted using hAEpC monolayers from two different isolations on day 7 or 8, when TEER values peaked. Both sides of cell layers were washed twice with pre-equilibrated bicarbonated Krebs-Ringer buffer (KRB, 15 mM HEPES (N-[2-hydroxy-ethyl]piperazine-N´-[2-ethanesulfonic acid]), 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.55 mM glucose, pH 7.4). Transwell® Clear grown cell layers were then placed in new 24- well cluster plates containing 800 µl per well of KRB prewarmed to 37°C. After 60 min of equilibration, transport experiments were initiated (i.e., t = 0) by replacing the donor fluid with 220 µl (apical) or 820 µl (basolateral) of KRB containing the respective drugs. The initial concentration in the donor fluid was assayed by drawing 20 µl samples immediately after the initiation of flux measurements. Samples (100 µl) were drawn serially from the receiver compartment at t = 30, 60, 120, 180 and 240 min. After each sampling, fresh transport buffer of an equal volume was returned to the receiver side to maintain a constant volume. At the end of the transport experiment, again 20 µl samples were drawn from the donor fluid and assayed for its drug content. Each experiment was performed in duplicates using 4-6 cell layers in either apical-to-basolateral (AB) or basolateral-to-apical (BA) direction, using cells from two different isolations. In order to assess the integrity of cell layers during the flux experiment, TEER was measured before and after each transport experiment. Flux (J) was determined from steady-state appearance rates of each compound in receiver fluid. The apparent permeability coefficient, P_{app}, is calculated according to the equation

$$\mathbf{P_{app} = J / (A * C_i)}$$

where C_i is the initial concentration of the substance under investigation in the donor fluid and A the nominal surface area of cell layers (0.33 cm^2) utilised in this study.

6.2.4 Sample analysis

Radioactivity

Samples of 125I-labelled proteins (GLP-1, PTH, GH and TF) were collected in scintillation vials and 2 ml of Ultima Gold scintillation cocktail (PerkinElmer) were added. Activity of the samples was assessed on a Tri-Carb liquid scintillation counter (PerkinElmer).

Fluorescence

Fluorescence of samples of FITC-labelled HSA and IgG were analysed in 96-well plates using a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively.

ELISA

Samples containing insulin were analysed by an enzyme-linked immunosorbent assay (ELISA, Active Insulin, Diagnostic Systems Laboratories, Sinsheim, Germany) according to the manufacturers instructions.

Data analysis

Data are presented as mean ± standard deviation (n) where n is the number of observations. Differences among group means were determined by one-way analysis of variance followed by post-hoc Newman-Keuls procedures, $p < 0.05$ was taken as the level of significance.

Stability of the protein label

Binding efficiency of the label was assessed by trichloroacetic acid (TCA) protein precipitation. Briefly, an equal volume of 20% TCA was added to the respective protein sample and left for 30 min incubation on ice. Subsequent to centrifugation (15 min at 4℃), the supernatant was carefully removed, 300 µl cold acetone were added and the sample was centrifuged again (5 min at 4℃) . Then, supernatant and pellet were individually assessed for either radioactivity or fluorescence. In none of the investigated samples unbound label could be detected.

6.3 Results of peptide transport studies

6.3.1 Glucagon-like peptide 1

Glucagon and related peptides constitute a family included in the proglucagon molecule, which is identical in sequence in the pancreas, intestine and brain. In gut L cells the C-terminal portion of proglucagon is predominantly processed to glucagons-like peptide-1 (GLP-1) and GLP-2. Further processing of GLP-1 produces the truncated and amidated forms of the peptide; GLP-1(1-36) (MW 4,111 Da) amide, GLP-1(7-36) amide (MW 3,297 Da) and GLP-1(7-37) (MW 3,355 Da), which all retain biological activity. GLP-1 receptors have been reported to be located in pancreatic endocrine cells, gastric glands, and in adipocyte, lung and brain membranes [53]. Upon binding to its receptor, GLP-1 and -2 stimulate insulin secretion in a glucosedependent manner and have significant effects on gastrointestinal motility and secretion. In the lung, GLP-1 receptors have been determined in rat and human in submucosal glands of the trachea, the smooth muscle of pulmonary arteries and in cells considered to be type II pneumocytes [54-56], where they are responsible for mucus secretion, pulmonary smooth muscle relaxation and increased surfactant secretion [57, 58]. Here, for the first time bidirectional transport studies with GLP-1(7-37) were performed. Twelve monolayers of hAEpC for absorptive (i.e. apical to basolateral) transport experiments and 12 monolayers for secretive (i.e. basolateral to apical) transport experiments were used from isolations HL 193 and HL 194. Respective mean TEER values were $1{,}965 \pm 589$ Ohm*cm^2 (absorptive) and $1{,}966 \pm 411$ Ohm*cm^2 (secretive). GLP-1(7-37) showed a significant net absorption across the monolayers.

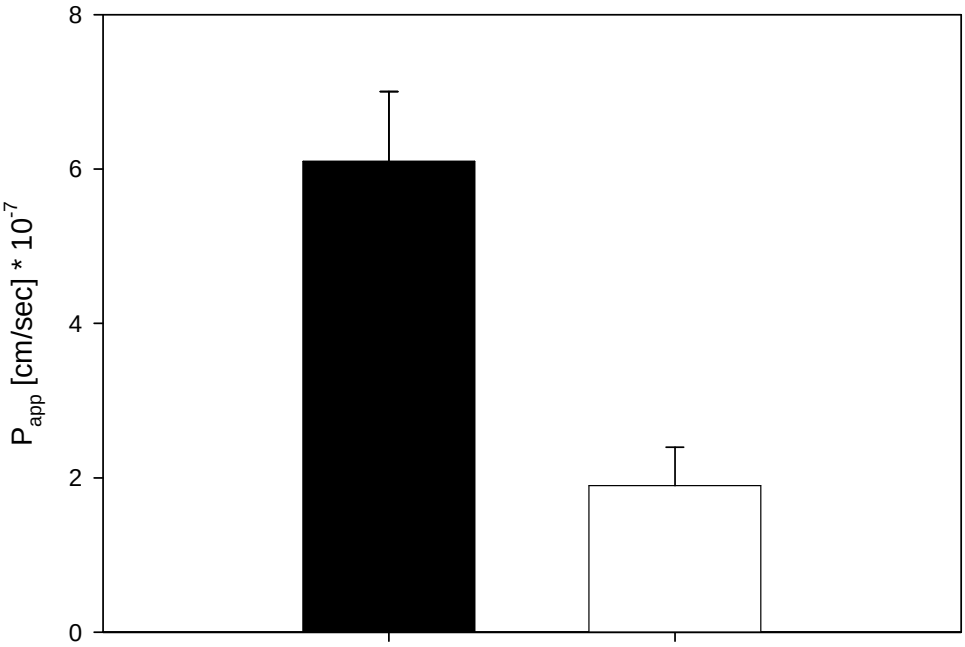

Figure 6-1 Transport of 125I GLP-1(7-37) across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* =12), the empty bar secretive direction (*n* = 12).

6.3.2 Parathyroid hormone

Parathyroid hormone (PTH) is secreted by the parathyroid glands and is a major mediator of calcium and phosphate metabolism through its interactions with receptors in kidney and bone. It has been purified extensively and appears to be a protein containing 84 amino-acid residues, a sequence of which about 33 to 35 are necessary for biological activity. PTH binds with high affinity to PTH1 and PTH2, members of the superfamily of G protein-coupled receptors. Northern blot analysis of normal human tissues revealed a limited tissue distribution in kidney, lung, placenta and liver [59]. In the rat, PTH receptor-1 transcripts are highly expressed in PTH target tissues, kidney and bone. Receptor transcripts, however, also are expressed in many other tissues, including aorta, adrenal gland, bladder, brain, cerebellum, breast, heart, ileum, liver, lung, skeletal muscle, ovary, placenta, skin, spleen, stomach, uterus and testes [60]. PTH2 receptor amino acid sequence is most similar to PTH1, but unlike the PTH1 receptor, it is activated by PTH and not by PTH-related peptide (PTHrH). PTH2 receptor messenger RNA is abundantly expressed in arterial and cardiac endothelium and at lower levels in vascular smooth muscle. It is also

abundant in the lung, both within bronchi and in the parenchyma, and is present within the exocrine pancreas [61]. Parathyroid hormone-related protein (PTHrP) is a growth inhibitor for rat alveolar type II cells and could be a regulatory factor for rat alveolar epithelial cell proliferation after lung injury [62]. PTHrP also suppressed cell proliferation to approximately 80% of the control level and increased surfactant protein A production in human H441 respiratory epithelial cells [63]. PTH(1-34) has shown an absolute bioavailability of ~34% in *in vivo* study in rats making it a promising candidate for pulmonary drug delivery [64, 65]. Here, for the first time bidirectional transport studies with PTH(1-38) (MW 4,458 Da) were performed. Ten monolayers of hAEpC for absorptive transport experiments and 11 monolayers for secretive transport experiments were used from isolations HL 186 and HL 189. Respective mean TEER values were 2,982 ± 1,310 Ohm*cm^2 (absorptive) and 2,938 ± 800 Ohm*cm^2 (secretive). PTH(1-38) showed no significant directionality.

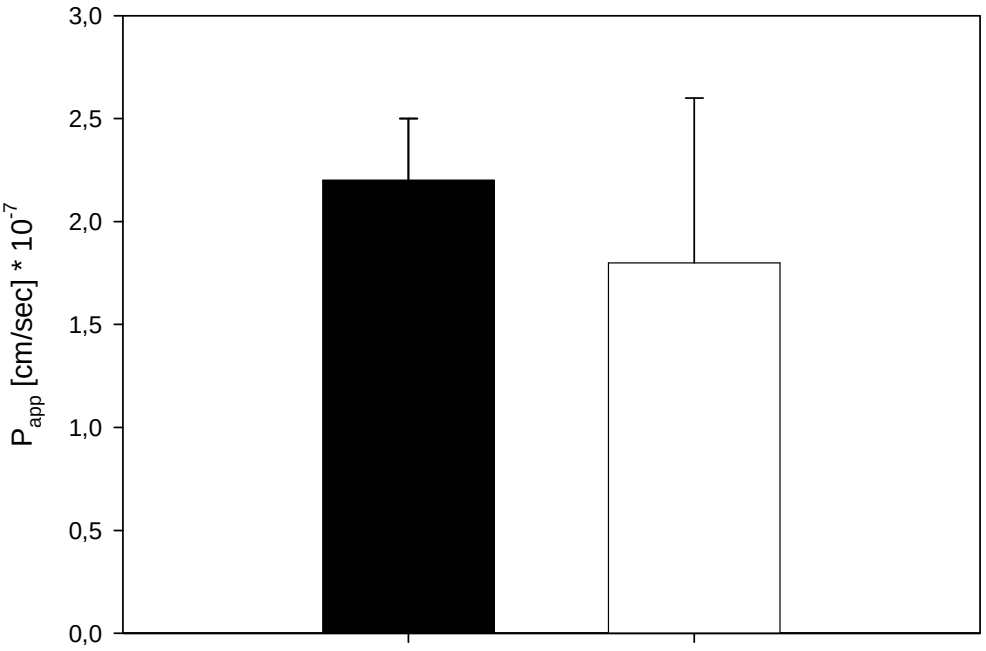

Figure 6-2 Transport of 125I PTH(1-38) across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* =10), the empty bar secretive direction (*n* = 11).

6.3.3 Insulin

A two-chain polypeptide hormone produced by the ß-cells of pancreatic islets. Its molecular weight is approximately 5,800 Da. The ∀- and ∃-chains are joined by two interchain disulfide bonds. The ∀-chain contains an intrachain disulfide bond. Insulin regulates the cellular uptake, utilisation, and storage of glucose, amino acids and fatty acids and inhibits the breakdown of glycogen, protein and fat. Because of the high potential of inhalational application, permeability characteristics of insulin have been widely investigated in different available *in vitro* models. The results of these investigations, however, are not always very consistent. Pezron and co-workers reported a net secretion across Calu-3 bronchial epithelial cells [36], while other groups, using 16HBE14o- cell layers, observed a mere paracellular transport without any directionality [66]. Insulin receptors are expressed in lung tissue, but do not seem to be involved in the processing of the protein [67-69]. The relatively low systemic bioavailability of inhaled insulin might be due to enzymatic degradation in the air space or phagocytosis by alveolar macrophages. Across hAEpC monolayers insulin did not exhibit a significant ($P < 0.05$) asymmetry in permeability. In addition, the observed P_{app} values were in the same order of magnitude as reported for FITC-labelled dextran (MW 4,000 Da) across hAEpC monolayers by Elbert et al. [48], indicating that no active transport process is involved. The P_{app} value in absorptive direction was $7.66 \pm 1.48 * 10^{-8}$ cm/s, in the secretive direction it was $7.23 \pm 3.55 * 10^{-8}$ cm/s. Cells from isolations HL 79 and HL 85 were used. Mean TEER values were 1,430 Ohm*cm^2 for the absorptive and 1,220 Ohm*cm^2 for the secretive direction. The comparably lower permeability values could be allegeable to a possible degradation of the protein.

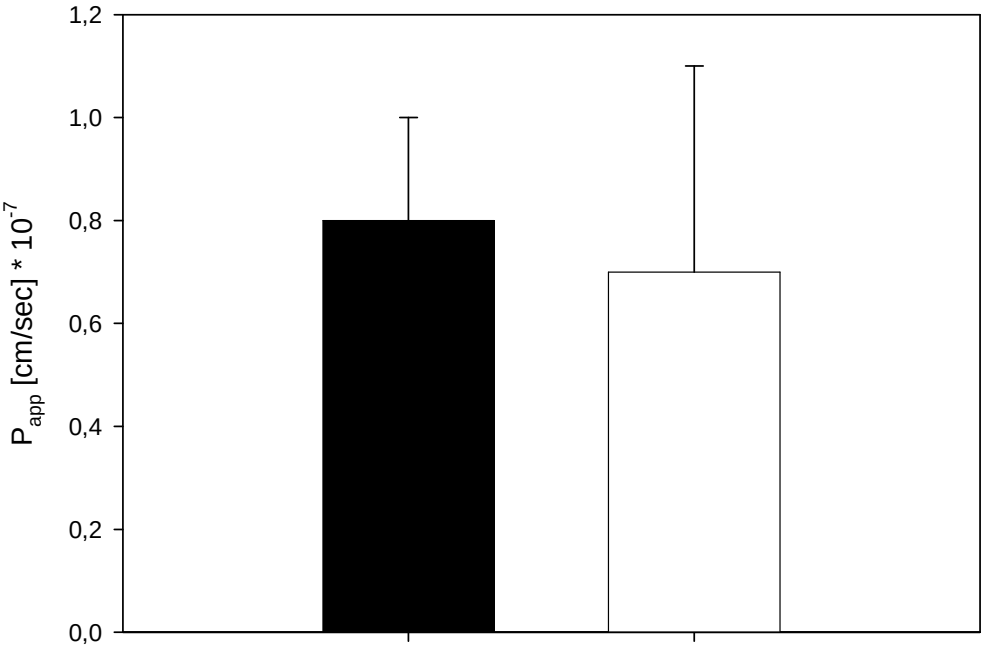

Figure 6-3 Transport of insulin across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* = 8), the empty bar secretive direction (*n* = 8).

6.3.4 Growth hormone

The protein encoded by this gene is a member of the somatotropin/prolactin family of hormones which play an important role in growth control. The gene, along with four other related genes, is located at the growth hormone locus on chromosome 17 where they are interspersed in the same transcriptional orientation; an arrangement which is thought to have evolved by a series of gene duplications. The five genes share a remarkably high degree of sequence identity. Alternative splicing generates additional isoforms of each of the five growth hormones, leading to further diversity and potential for specialisation. This particular family member is expressed in the pituitary but not in placental tissue as is the case for the other four genes in the growth hormone locus. Mutations in the gene or deletions of the gene lead to growth hormone deficiency and short stature. The isoform I has 191 amino acid residues and a molecular weight of 22,125 Da. Action of GH is regulated upon binding to the membrane-bound growth hormone receptor (GHR) and/or the soluble growth hormone binding protein (GHBP). The GHR has been found in adult rabbit lung [70], rat foetal and neonatal lung (together with GHBP) [71], inflamed normal human

airway cells [72], but not in foetal human lung [73] and adult and foetal murine lung [74]. The GHR has also been found in Caco-2 cells [75], for which previously an asymmetric, but P-glycoprotein inhibitor-dependent absorption of GH has been reported [76]. Since 15 years, considerably successful studies have been conducted to administer GH via the pulmonary route [77, 78]. Regional deposition as well as formulation had significant impact on the absolute bioavailability, which has been found to vary between 8% to 45% [79, 80]. In our bidirectional transport studies with GH eight monolayers of hAEpC were used for absorptive transport experiments and 12 monolayers for secretive transport experiments. The cells were cultured from isolations HL 192 and HL 193. Respective mean TEER values were 1,702 ± 598 Ohm*cm^2 (absorptive) and 2,243 ± 460 Ohm*cm^2 (secretive) HG showed no significant directionality.

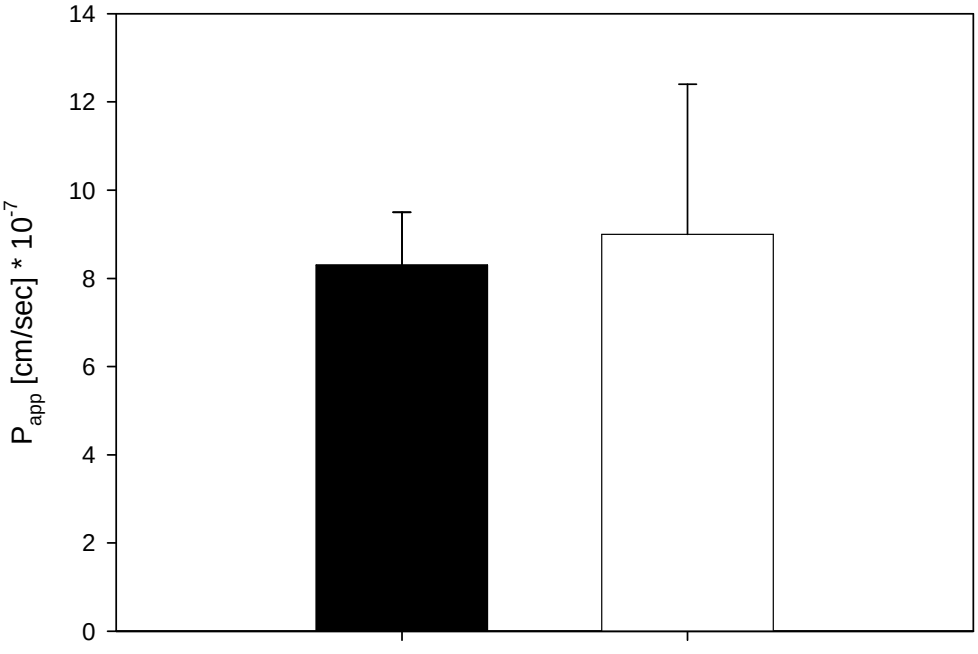

Figure 6-4 Transport of 125I growth hormone across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* =8), the empty bar secretive direction (*n* = 12).

6.3.5 Albumin

Albumin is a soluble, monomeric protein which comprises about one-half of the blood serum protein. Albumin functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a role in stabilising extracellular fluid volume. It is a globular unglycosylated serum protein of molecular weight 65,000 Da. The human albumin gene is 16,961 nucleotides long from the putative 'cap' site to the first poly(A) addition site. It is split into 15 exons which are symmetrically placed within the 3 domains that are thought to have arisen by triplication of a single primordial domain. Albumin is synthesised in the liver as preproalbumin which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum. The product, proalbumin, is in turn cleaved in the Golgi vesicles to produce the secreted albumin. Active processing of albumin has been reported for different species and tissues. Of particular interest are studies utilising monolayers of human intestinal epithelial Caco-2 cells [81], rat alveolar epithelial [43] and endothelial cells [82]. A concentration dependent absorption of albumin could also be observed from the intact lung [28]. Although the underlying mechanisms are still element of discussion, a series of hypothesises has been confirmed 1) albumin transport is dominated by the transcytosis pathway, 2) this mode of transport is saturable within physiological concentrations, 3) binding of albumin to a limited number of high-affinity sites on the epithelial and endothelial cell may activate albumin transport by the release of caveolae from the membrane, and 4) the bulk of the albumin within the transport vesicles is in the fluid phase [82-84]. It should be, however, noted that most experiments were conducted using serum albumin of bovine origin and not from the respective donor species of the tissue. In the presented study, transport studies of human serum albumin were conducted using 10 monolayers of hAEpC for absorptive transport experiments and 8 monolayers for secretive transport experiments. The monolayers were cultured from isolations HL 184 and HL 195. Respective mean TEER values were $4{,}278 \pm 1{,}779$ Ohm*cm^2 (absorptive) and $2{,}056 \pm 535$ Ohm*cm^2 (secretive). In Figure 6-5, the P_{app} values for albumin are depicted. The relatively high error might be ascribed to the low concentrations in the samples which were close to the resolution limit of the fluorescence reader. Nevertheless, a noted tendency to net absorption can be observed.

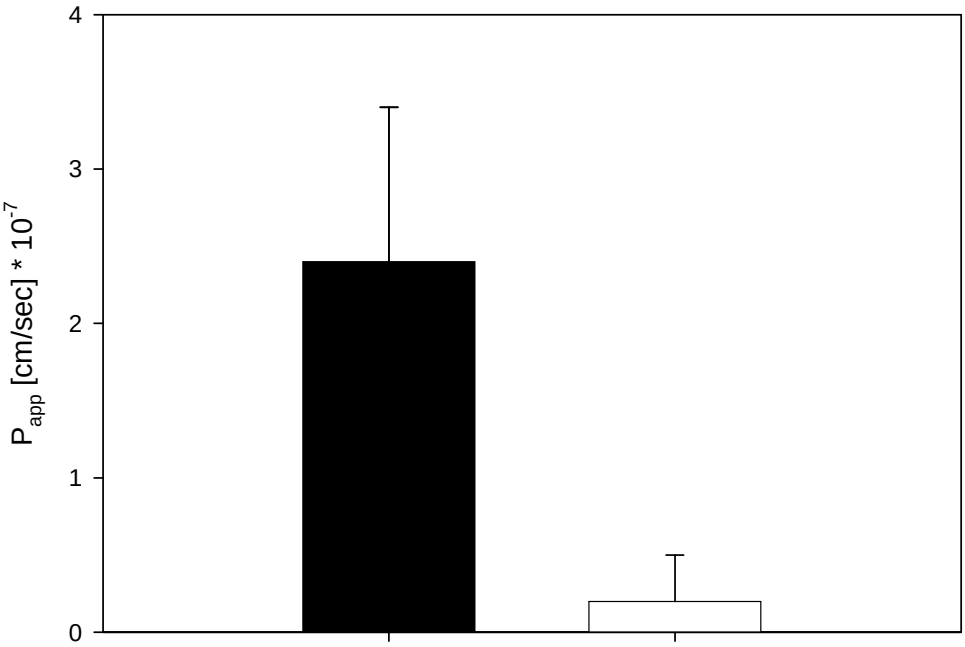

Figure 6-5 Transport of FITC albumin across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* =10), the empty bar secretive direction (*n* = 8).

6.3.6 Transferrin

Transferrin is a glycoprotein with an approximate molecular weight of 76,500 Da. It is thought to have been created as a result of an ancient gene duplication event that led to generation of homologous C- and N-terminal domains each of which binds 1 ion of ferric iron. The function of this encoded protein is to transport iron from the intestine, reticuloendothelial system and liver parenchymal cells to all proliferating cells in the body. In addition to its function in iron transport, this protein may also have a physiologic role as granulocyte/pollen-binding protein (GPBP) involved in the removal of certain organic matter/allergens from serum. Receptors for TF have been found on Caco-2 cells [85] and also in human bronchial epithelial cells [86] and rat alveolar type II epithelial cells [87]. In all cases, the receptor is mostly expressed on the basal aspect of the cells. In Calu-3 cells, TF was found to be secreted mostly from the apical site [88], while significant net absorption was observed in rat alveolar epithelial monolayers. Intriguingly, an enhanced absorption of TF-conjugates has been demonstrated, despite the basal localisation of the transferrin receptor [89, 90]. Eight monolayers of hAEpC for absorptive and 9 for secretive transport experiments were

used from isolations HL 186 and HL 189. Respective mean TEER values were 2,976 ± 1,257 Ohm*cm^2 (absorptive) and 3,336 ± 661 Ohm*cm^2 (secretive). Transferrin showed a significant net absorption across the monolayers.

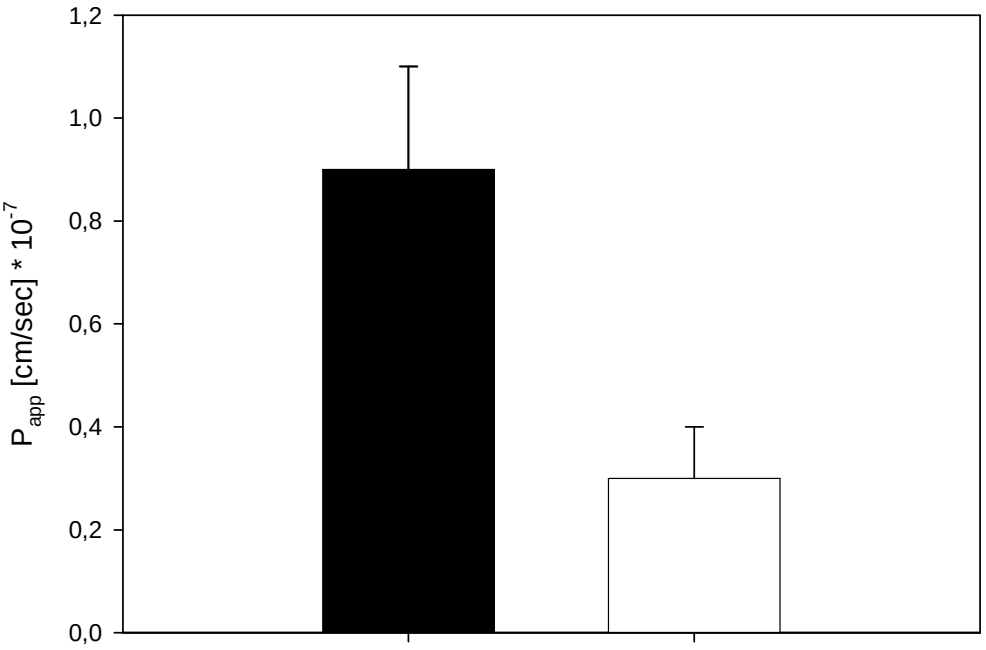

Figure 6-6 Transport of 125I transferrin across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* = 8), the empty bar secretive direction (*n* = 9).

6.3.7 Immunoglobulin G

IgG antibody molecules have biological properties such as transport across the maternal-foetal membranes, interaction with the classical complement system and fixation to heterologous tissues resided in the Fc fragment of IgG. IgG molecules have a molecular weight of 150,000 Da and a sedimentation coefficient of 7S. Four subclasses of IgG have been identified on the basis of antigenic and structural difference residing in the heavy chains designated r1, r2, r3 and r4. Exactly how IgG crosses epithelial barriers to function in host defence and mucosal immunity remains unknown [91, 92]. The MHC class I-related Fc receptor (FcRn) has been found on the epithelial surfaces of the intestine [93], bronchi [94] and alveoli [95] and might play an important role in IgG transcytosis. The transport of human IgG was conducted using 8 monolayers of hAEpC for each absorptive and secretive direction.

The monolayers were cultured from isolations HL 191 and HL 196. Respective mean TEER values were 1,523 ± 274 Ohm*cm^2 (absorptive) and 1,767 ± 463 Ohm*cm^2 (secretive) In Figure 6-7, P_{app} values for IgG transport are shown. The relatively high error might be ascribed to the low concentration in the samples which were very close to the resolution limit of the fluorescence reader. IgG showed a slight but not significant net absorption across hAEpC monolayers.

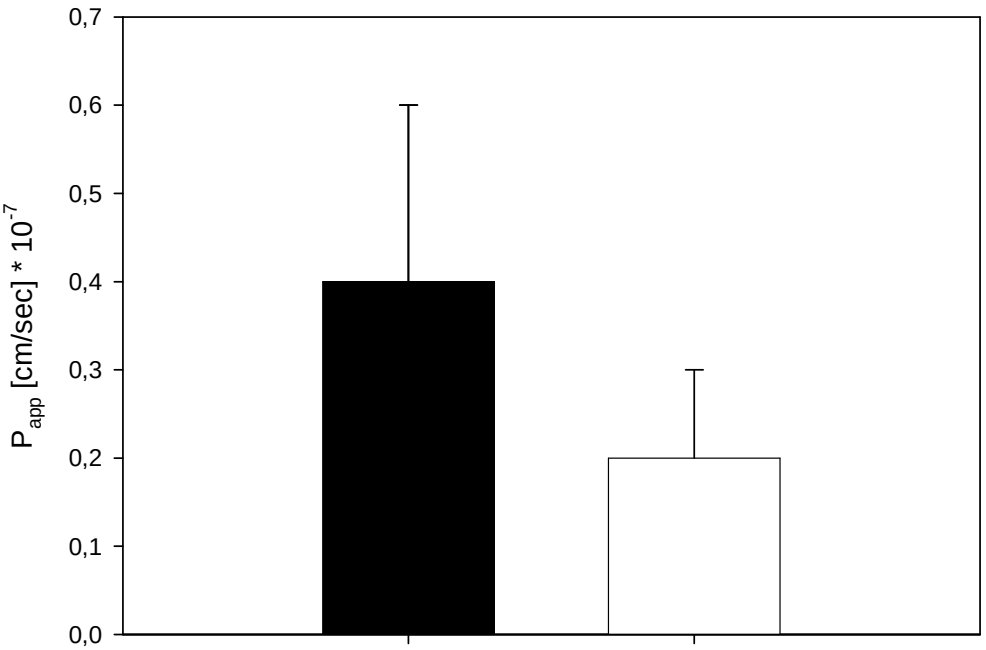

Figure 6-7 Transport of FITC IgG across hAEpC monolayers [cm/sec]. The filled bar represents absorptive (*n* = 8), the empty bar secretive direction (*n* = 8).

6.4 Summary

In this study, transport characteristics of a series of proteins and peptides across monolayers of polarised primary human alveolar epithelial cells (hAEpC) have been assessed. Permeability data of these molecules across hAEpC monolayers has not been reported to date; however, some of the compounds have been used in transport experiments in other *in vitro* and/or *in situ* models [28, 51, 52]. For glucagon-like peptide-1(7-37) (GLP-1) and parathyroid hormone (1-38) (PTH) no permeability studies are available at all, while reports on the permeability of insulin, growth hormone (GH), serum albumin (HSA), transferrin (TF) and immunoglobulin G (IgG) are available to a certain extent.

Of the investigated compounds, GLP-1, HSA, TF and IgG showed net absorptive transport behaviour, while PTH, GH and insulin exhibited no distinct directionality. None of the compounds revealed net secretion or any significant breakdown during the flux studies. Due to the high potential of inhalational application as opposed to injections required, permeability characteristics of insulin have been widely investigated in several respiratory *in vitro* models. The results of these investigations, however, are not always very consistent. Pezron and co-workers reported net insulin secretion across Calu-3 bronchial epithelial cells [36], while other groups, using 16HBE14o- cell layers, observed symmetric transport [66], suggesting paracellular diffusion of insulin.

As for GH transport, receptors for GH (GHR) have been reported in Caco-2 cells [75], where previously an asymmetric, but P-glycoprotein inhibitor-dependent, absorption of GH was shown [76]. Over the last 15 years, the absorption of GH via the pulmonary route has been reported with relatively good bioavailability (8 - 45%) [78]. Regional deposition as well as formulation appears to make significant impact on the absolute bioavailability of GH [79, 80].

Non-passive (i.e., transcytotic) transport of albumin across monolayers of human intestinal epithelial Caco-2 cells [81], rat alveolar epithelial cells [43], and endothelial cells [82] has been reported. A concentration-dependent absorption of albumin was also shown for the intact rat lung [28]. Although the underlying mechanisms are slow to emerge, it appears that albumin transport is most likely dominated by the transcytosis pathway, in that albumin transport saturates with physiological

concentrations and that binding of albumin to a limited number of high-affinity sites at the caveolae on the epithelial and endothelial cell may constitute albumin transport across lung air-blood barrier [82-84]. It should be noted that most experiments were conducted using serum albumin of bovine origin, and not homologous type as in our studies. Receptors for TF have been found in Caco-2 cells [85], human bronchial epithelial cells [86] and rat alveolar type II epithelial cells [90]. In all cases, the receptor is mostly expressed on the basal aspect of the cells. In Calu-3 cells, TF was found to be net secreted into apical fluid [88], while significant net absorption was observed in rat alveolar epithelial cell monolayers [52]. Intriguingly, an enhanced absorption of TF-conjugates has been demonstrated, despite the basal localisation of the transferrin receptor in Caco-2 and rat pneumocytes [87, 88].

Exactly how IgG crosses epithelial barriers to function in host defence and mucosal immunity remains unclear, although MHC class I-related Fc receptors (FcRn) are reported to be expressed at the epithelial barriers of the intestine [93], bronchi [94] and alveoli [95]. Transcytosis of IgG mediated by FcRn across rat alveolar epithelial cell monolayers and other barriers has been published recently[95].

When permeability data were compared with molecular weight values, no clear cut “inverse” relation can be found (Figure 6-8; Figure 6-9; Table 1).

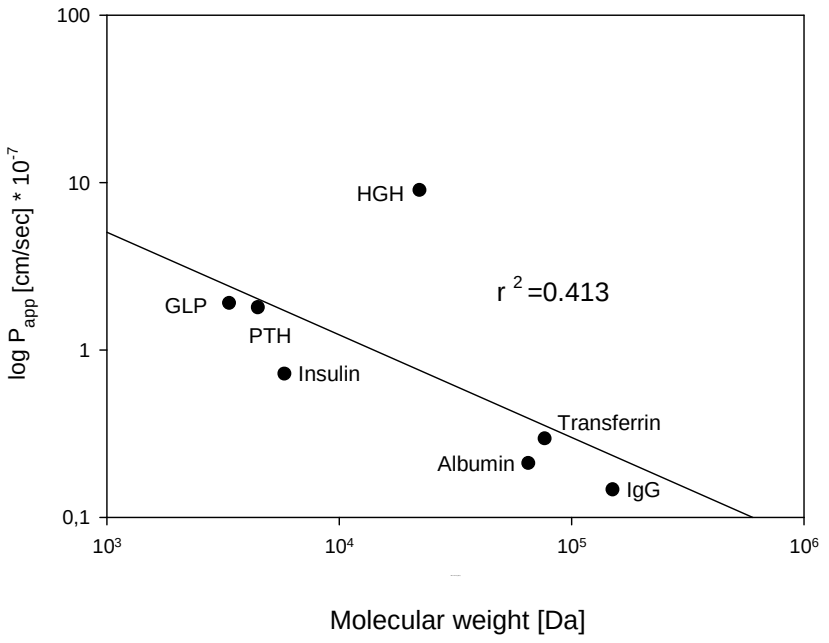

Figure 6-8 Correlation between molecular weight and rate of basolateral to apical transport.

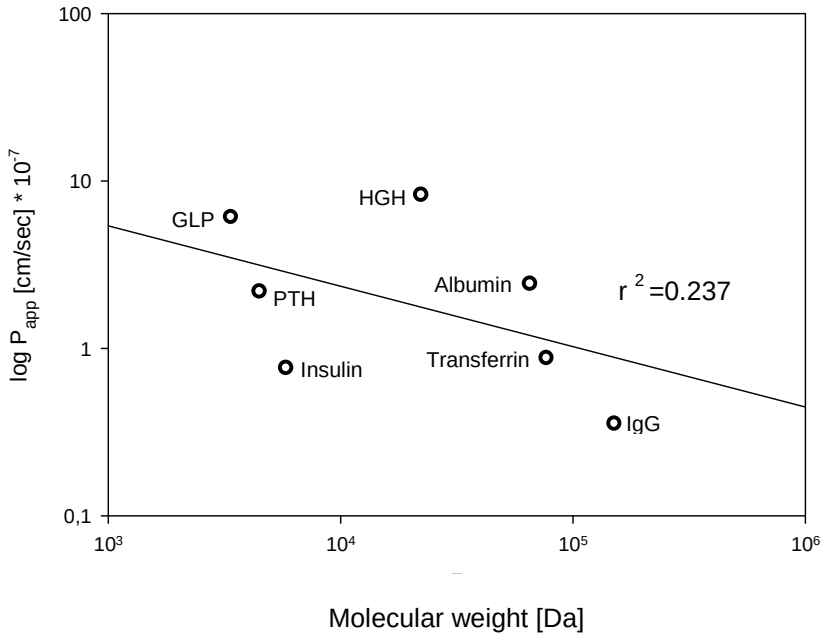

Figure 6-9 Correlation between molecular weight and rate of apical to basolateral transport.

This may be related to involvement of non-passive transport processes, at least for some of the macromolecules. In general, the observed permeability values of the peptides and proteins followed the same trend were and in the same order of magnitude as those observed in an *in vitro* model of rat pneumocyte monolayers (see Table 1). Apparent differences in the absorption profile (i.e., poor correlation between human vs. rat monolayer studies) might be attributable to the use of homologous proteins used in the present study, as opposed to non-homologous proteins (e.g., bovine serum albumin and human growth hormone) used in the rat monolayer studies.

	Molecular weigth	IEP	P_{app} A to B (cm/sec)x10^{-7}	P_{app} B to A (cm/sec)x10^{-7}	P_{app} A to B (cm/sec)x10^{-7} rat	P_{app} B to A (cm/sec)x10^{-7} rat
GLP-1 (7-37)	3,355	4.9	6.13±0.87	1.91±0.51	nd	nd
PTH (1-38)	4,458	8.6	2.20±0.30	1.80±0.77	nd	nd
Insulin	5,800	5.4	0.77±0.15	0.72±0.36	nd	nd
Growth hormone	22,125	5.0	8.33±1.24	9.02±3.43	nd	nd
Albumin	65,000	4.9	2.45±1.02	0.21±0.31	0.77±0.32	0.39±0.01
Transferrin	76,500	5.9	0.88±0.15	0.30±0.03	1.10±0.35	0.47±0.02
IgG	150,000	5.8 - 7.3	0.36±0.22	0.15±0.16	0.91±0.06	0.17±0.09

Table 1 Values for molecular weight, isoelectric point (IEP) and apparent permeability (*P*app) across monolayers of hAEpC. Comparative values across rAEpC monolayers (rat) are taken from Matsukawa *et al.* [52]

This study, for the first time, shows transport characteristics of a series of proteins and peptides across monolayers of polarised primary human alveolar epithelial cells. The obtained data differ significantly from previously published reports utilising monolayers from different species. It can be concluded that the use of homologous tissue should be preferred to avoid species-differences.

The liquid interface deposition on epithelial pulmonary cells doesn't reflect the *in vivo* situation in the healthy lung. In case of utilizing 3460 filter inserts with a surface of 1.13 cm^2 and an apical volume of 500 µl a fluid layer with a thickness of 442 µm covers the cells. If the lung *in vivo* was covered with similar thick fluid layers like in the LID experiments the human lung would be filled with 56 litres of fluid (140 m^2 * 442 µm). However, measurement of the fluid volume in the healthy lung figured out only 100 ml liquid in the complete lung. Consequently, only fluid layers in the range of 0.5 to 1 µm covers the lung surface. Thin fluid layers are essential to permit gas diffusion and therefore interferences in the fluid regulation in the human lung cause always perilous complications.

Also the resorption of administered pharmaceutical aerosols can be influenced by the fluid layers in the lung. Concentration gradients and dissolution rates are two parameters which are controlled directly by the fluid volume. In the following chapter the influence of the deposition conditions on the transport of drugs across our models of the human air-blood barrier were investigated.

7 Effect of air versus liquid interface deposition on transport of salbutamol sulphate and budesonide across pulmonary cell monolayers

Abstract:

Dry powder aerosol particles are deposited after inhalation on the air-blood barrier of the lung, represented by the bronchial and/or alveolar epithelium. Our aim was to study the influence of drug solubility, fluid volume, and particle size on transport rate across pulmonary cell monolayers in dependency of air or liquid interface aerosol deposition.

Different aerosol powder formulations containing budesonide and salbutamol sulphate, respectively, were used to represent each a poorly and a highly soluble drug. The aerosol powders were either administered in the solid state by an insufflator syringe or an impinger on air exposed monolayers of human pulmonary epithelial cells, or first dispersed in a physiological buffer and then administered with a pipette to the cells, covered by a larger apical liquid volume.

For the highly soluble salbutamol sulphate, the transport rate is mainly determined by the concentration gradient in the donor volume, i.e. smaller liquid volumes increases drug transport and drug absorption after air-interface deposition of dry powders is significantly higher than after administration of a drug solution. However, if the drug is adsorbed on large (> 100 µm) carrier particles absorption after air interface deposition was reduced, probably due to too thin liquid layer.

For the poorly soluble budesonide, the transport rate is mainly limited by solubility and wetting of the drug powder, i.e. the difference between air and liquid interface deposition is less pronounced, but drug absorption is still significantly improved, if the drug is deposited as a micronized powder (< 2 µm) in comparison to larger particles.

Air interface deposition yields higher transport rates and better reflects the situation in vivo than liquid interface deposition. When designing novel in vitro models for the simultaneous study of drug deposition and absorption using pulmonary epithelial cell cultures, also the aerodynamic properties /deposition behaviour of the particles must be adequately addressed.

Parts of this chapter have been submitted for publication in:

Bur M, Huwer H, Muys L, Lehr CM; Effect of air versus liquid interface deposition on transport of salbutamol sulphate and budesonide across pulmonary cell monolayers; Pharmaceutical Research

7.1 Introduction

Progress in powder technology as well as in device development nowadays enables effective deposition of medical aerosols in the different regions of the lung [96, 97]. An enormous surface, low enzymatic activity, thin resorption barrier, and high blood flow are the major advantages of the lung as site for local and systemic drug delivery [97]. In difference to the intestine, vagina, colon, or the endothelium, the human air-blood barrier is an air interface epithelium. But this does not mean that it is completely dry. Corresponding to its different functions, the lung epithelium is covered with a lining fluid of different function and dimensions. The conducting airways are covered with a mucus layer which can be divided further in three parts: an aqueous sol phase adjacent to the epithelial cells and embedding the beating cilia, an overlying viscous gel phase, and on the top of the mucus small amounts of surfactant. Special viscoelastic properties and sufficient big volumes of fluid allow the embedding and transport of particles to the pharynx. The thickness of the bronchial mucus layer has been reported to vary in the range of 20 to 60 µm [21].

The situation is however different in the respiratory area of the peripheral lung. There, a relatively thick mucus layer like in the bronchial region would inhibit the gas exchange. Only 7 to 70 nm of surfactant [15] – a mixture of phospholipids with special viscoelastic properties and low surface tension [98] – coats the epithelium in the deep lung. Mucus is not present in the deep lung but for small airways Geiser et al. [99] reported the simultaneously presence of mucus and surfactant. Simplistic, it can be assumed that the bronchial region is covered with a 10 to 100 µm thick highly viscous mucus layer and the alveolar region with a 10 to 100 nm low viscous liquid layer.

In consequence, particles deposited in the lung first interact with the lung surface liquid layer. This means that i.e. particles with a diameter of 3 µm can be submersed in the surface liquid layer in the bronchial region, but at most be wetted in alveolar region. In essence, this scenario is sketched in Figure 7-8. Erosion and dissolution, which typically control drug release from drugs administered in the solid state and maybe rate limiting for drug absorption are limited by the constricted fluid volume. Submersed cell culture systems as they are typically used in the pharmaceutical

science to study transport processes across e.g. the intestinal epithelium (e.g. Caco-2 cells) are only of limited relevance for the more or less dry surface of the lung epithelium. In this study the influence of air interface deposition (AID) of pharmaceutical aerosols on air interface cultured pulmonary cell monolayers on the absorption rate was addressed. To reflect the different epithelial structures in the bronchial and the alveolar region Calu-3 cells as model for the bronchial part of the lung and human primary alveolar epithelial cells for simulating of the air-blood barrier in the deep lung [100] were used. Different powder formulations of the highly water soluble salbutamol sulphate and the poorly water soluble budesonide were used to study the influence of the deposition conditions on the absorption rate across two different cell culture models of the upper and lower pulmonary epithelial barrier, respectively.

7.2 Materials and Methods

7.2.1 Aerosol powders

Salbutamol sulphate and budesonide in pharmaceutical quality were a kind gift of Boehringer Ingelheim (Ingelheim, Germany). As models for different dry powder formulations SalbuHEXAL® Easyhaler® (Hexal, Holzkirchen, Germany), Ventilastin® Novolizer® (Viatris, Bad Homburg, Germany), and Salbutamol Cyclocaps® (Jenapharm, Jena, Germany) were used. All three contain salbutamol sulphate and micronized lactose as carrier.

Easyhaler® BudiHEXAL (Hexal, Holzkirchen, Germany), Cyclocaps® Budesonide (Jenapharm, Jena, Germany) and the Autoinhaler® (CT Arzneimittel, Berlin, Germany) were selected as different budesonide formulations.

7.2.2 Aerosol application

7.2.2.1 Unfractionated powder application with insufflator syringe

The DP-4 Dry Powder Insufflator (Penn-Century®, Philadelphia, USA) was used to deposit amounts between 1 and 5 mg of powders on the surface of cell monolayers. The lactose – drug mixtures were taken out from the reservoirs (Easyhaler®, Novolizer®) or the capsules (Cyclocaps®), respectively, and transferred in the holding chamber of the DP-4. In case of the Autoinhaler® the ring tablet was pulverized in a mortar and the powder sieved through a 500 µm mesh sieve before aerosolisation. A

10 ml syringe was used for applying the necessary amount of air to produce small puffs of aerosolized powders.

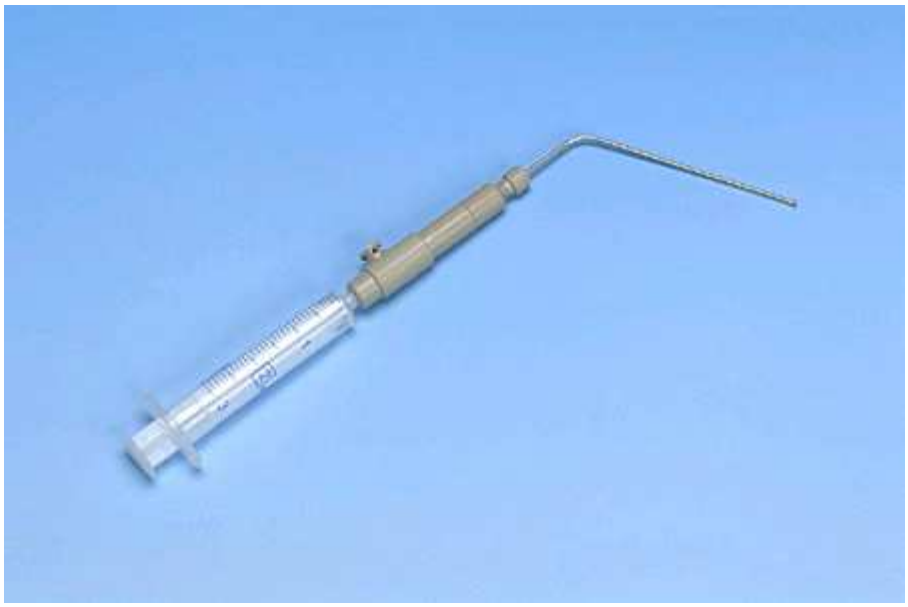

Figure 7-1 DP-4 with air syringe (www.penncentury.com)

7.2.2.2 Application of size fractionated powders with a multi stage liquid impinger

A multi stage liquid impinger (MSLI) with integrated cell monolayer was utilized to separate the carrier lactose from the adhesive drug particles and simultaneously deposition of the released drug particles on the cell surface. Filter inserts - containing monolayers of Calu-3 cells cultivated on the air interface - were placed directly under the fourth stage nozzle of the MSLI (Erweka, Heusenstamm, Germany). Details on the construction and validation of the modified impinger are described in chapter 8.3. Powder from the generic DPI´s were deposited onto the monolayers after aerosolisation for 30 s at 30 L/min from gelatine capsules (Eli Lilly, Indianapolis, IN, USA) using a Spinhaler device (Fisons, Bedford, MA, USA). The filter insert was then returned to a 12-well plate and placed back into an incubator at 37℃.

7.2.3 Cell culture

7.2.3.1 Human alveolar epithelial cells (hAEpC)

Isolation of primary human type II pneumocytes was performed according to a protocol modified from those of Elbert et al. [48] and Ehrhardt et al. [101]. For more detailed description of isolation procedure and culture see chapter 6.2.2. Integrity of cell monolayers was every second day determined by measuring transepithelial electrical resistance using an epithelial voltohmmeter (EVOM, WPI, Berlin, Germany).

7.2.3.2 Calu-3 cells

The human adenocarcinoma cell line Calu-3 was obtained from ATCC (Manassas, VA, USA). Cells of passage number 38 to 56 were seeded onto Transwell Clear® permeable filter inserts at a density of 100,000 cells/cm^2. Immediately on seeding, cells were grown in 500 µl apical and 1500 µl basolateral media (Eagle's minimum essential medium (PAA, Pasching, Austria) supplemented with 10% foetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin) at 37℃ in a 5% CO_2 incubator. For air interface culture 1 day after seeding the apical medium was removed and the basolateral medium was reduced to 500 µl. In the liquid condition culture the medium was changed every second day. Also the AIC cells were fed one over the other day with 500 µl medium basolaterally. The cells were cultivated until day 21 after seeding. In the LCC culture the tightness of the epithelial barrier was proofed by measuring the transepithelial electrical resistance.

7.2.4 Transport studies

The transport of salbutamol sulphate and budesonide across the cell monolayers was studied after air interface deposition (AID) of powders as well as after application of drug solution/suspension. We used hAEpC monolayers with TEER > 1,000 Ohm*cm^2 on days 6 to 9 post plating and Calu-3 cells in the age of 21 days. The integrity of the barrier was measured before and after the transport experiments. In cases of air interface deposition the complete cell culture medium was removed from the system, and only the basolateral compartment was refilled with 500 µl (Calu-3 cells) or 200 µl (hAEpC) prewarmed Krebs-Ringer solution. In case of MSLI deposition the filter inserts were placed after aerosol application back in the culture plates and the basolateral compartment was filled with 1,500 µl prewarmed KRB.

In all experiments after determined time points up to 4 hours samples of 120 µl were taken from the acceptor compartment and the sample volume was refilled with prewarmed KRB. At the end of the experiment the cells in the apical compartment were lysed with 200 µl DMSO (Sigma, Deisenhofen, Germany) and the apical compartment was unified with the basolateral compartment. A sample was taken to allow the calculation of the totally administered initial dose. The content of drug in the samples was quantified by HPLC. During the transport experiment the cell monolayers were agitated using an orbital shaker at constant stirring rate (100 rpm) at 37℃ under humidified conditions.

Air interface deposition was performed with the DP-4 insufflator syringe or with the MSLI on air interface cultivated cells. The transport of the drugs in solution (liquid interface deposition LID) across the monolayers was determined with submersed cultivated cell monolayers to which was added to the apical compartment a solution (30 µM for budesonide and 1,000 µM for salbutamol sulphate, respectively) of the powders in 200 µl KRB. Calculation of the apparent permeability coefficients was conducted in the same manner like described in chapter 6.2.3. In all experiments without a measurable amount of liquid in the donor compartment the transported amount in percent of the administered dose versus the time was plotted. Experiments were carried out with at least n = 6 using cells from different passages.

7.2.5 HPLC analytics

The contain of drug in the basolateral, apical and in the cellular compartment was determined on the one hand to get hints on intracellular storage of drug, and on the other hand for calculation of the complete administered dose.

The HPLC system consisted of a Dionex P680 HPLC pump and a Dionex UVD 340U detector operating at 277 nm (Dionex, Germering, Germany). Samples were injected using an ASI 100 automated sample injector. The analytical column used was a LiChrocart RP-18 column (125 x 4 mm i.d., particle size 5 µm, Merck, Darmstadt, Germany).

In case of salbutamol sulphate analytic the mobile phase was triethylamine - phosphate buffer pH 6.0 – methanol (90:10, v/v) and the column temperature was maintained at 40℃. A constant flow-rate of 1.0 ml/ min was employed throughout the analyses. Also the amounts of transported budesonide were analysed by HPLC. The same column and HPLC system like in the salbutamol sulphate analytic was used. A

more lipophilic mixture of 60% phosphate buffer pH 2.5 and 40% acetonitril was chosen as mobile phase. The flow rate was 1.7 ml/min and the temperature of the column oven was 40°C. The detection limit for the s albutamol sulphate analytic was determined with 25 ng/ml. The robustness of the method was checked in presence of proteins (bovine serum albumin and foetal calf serum) as well as in the presence of DMSO which is used to dissolve the cells after the transport experiments. In all cases an elution of the salbutamol sulphate was observed after 3.2 minutes run time. The linearity of the analytic was proofed for the range between 25 ng/ml and 250 µg/ml.
For budesonide the retention time was 3.7 minutes and the detection limit was 30 ng/ml and the linearity was proven in the range from 30 ng/ml to 400 µg/ml. DMSO and the cell culture medium did not influenced the analytic negatively.

7.3 Results

Liquid interface deposition was conducted by pipetting a solution in the fluid filled apical compartment of filter grown human alveolar epithelial cells and bronchial Calu-3 cell monolayers. The concentration of 30 µM for budesonide and 1000 µM for salbutamol sulphate were on the one hand below the saturation concentration and on the other hand high enough to quantify also the drug concentration in the basolateral compartment with the HPLC. No significant differences in the permeability across the two different cell models were measured. Budesonid was in both absorptive as well as secretive direction highly permeable. No preferred transport direction could be detected. The more hydrophilic salbutamol sulphate was transported with 10 fold reduced rate in comparison to budesonide. Only in the primary alveolar cells a slightly expressed favorized absorptive transport could be observed. For detailed transport rates see Table 2.

	Salbutamol sulphate	Budesonide
P_{app} A to B hAEpC	$0.67 \pm 0.34 * 10^{-6}$	$9.84 \pm 0.47 * 10^{-6}$
P_{app} B to A hAEpC	$0.37 \pm 0.23 * 10^{-6}$	$19.7 \pm 1.51 * 10^{-6}$
P_{app} A to B Calu-3	$0.28 \pm 0.16 * 10^{-6}$	$8.59 \pm 0.34 * 10^{-6}$
P_{app} B to A Calu-3	$0.24 \pm 0.09 * 10^{-6}$	$22.4 \pm 0.77 * 10^{-6}$

Table 2 Apparent permeability [cm/sec] of the two model drugs salbutamol sulphate and budesonide across monolayers of hAEpC and Calu-3 cells; data present mean ± standard deviation; n = 6.

In all air interface deposition experiments with micronized pure drurgs as well as with powder formulations only transport in resorptive direction was measured. Air interface deposition always resulted in significantly faster transport rate, compared with liquid interface deposition. The transport after air interface deposition was characterized by a pronounced initial burst effect. After 4 hours the transport of micronized budesonide after AID was 21.0 ± 9.0% compared to 5.9 ± 0.1% after LID (see figure 1). In both cases the complete amount of budesonide in the system was equal and with 30 µM below the saturation concentration. For the three dry formulation aerosols similar transport was found when the dry powder was dissolved. In all cases 4 hours after application between 26.4% and 29.5% was transported (see Figure 7-2).

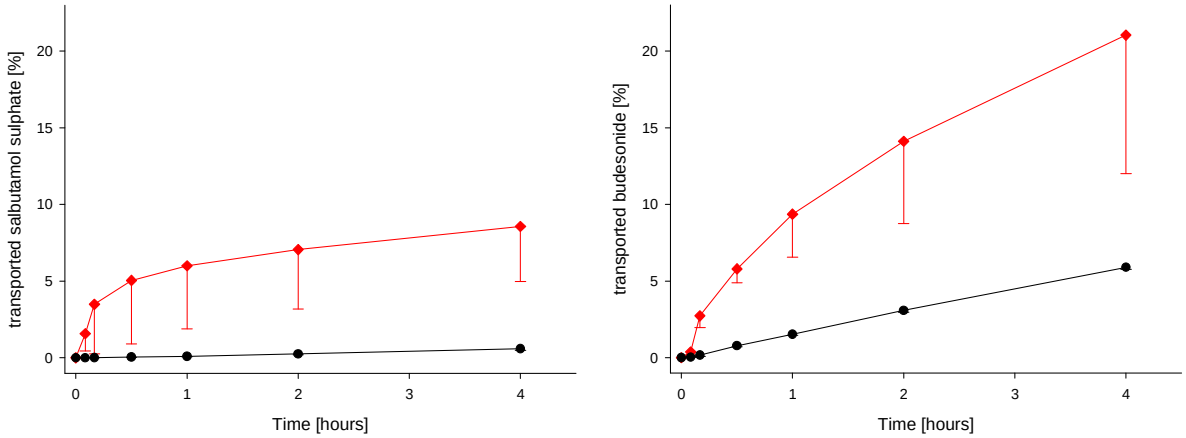

Figure 7-2 Transport of salbutamol sulphate and budesonide across Calu-3 cell monolayers. ♦ Transport after air interface deposition (AID); ● Transport after application as solution (data present mean ± standard deviation; n = 6)

In contrast, air interface deposition of the powder formulations revealed significant differences in the transport rates. After air interface application we measured after 4 hours 55.1% absorption for the powder taken from the Autoinhaler®. Easyhaler® and Cyclocaps® absorption was similar (47.0% and 46.9%, respectively) but significant slower then the Autoinhaler® formulation (see Figure 7-3 left).

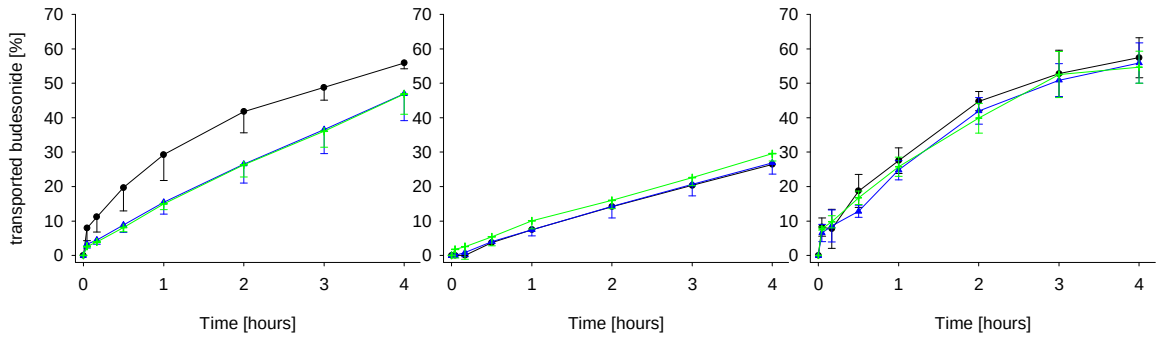

Figure 7-3 Transport of budesonide deposited (Δ Cyclocaps®; ● Autoinhaler®; ♦ Easyhaler®) as dry powder with the aid of DP-4 insufflator syringe (left side), or pipetted as suspension (middle), or applied with a Spinhaler device and the cell compatible MSLI (in all cases applied 30 µM on air interface cultivated hAEpC; data present mean ± standard deviation; n = 6)

Microscopic analysis of the particle sizes in the Autoinhaler® powder showed significant smaller particles with a diameter under 20 µm in comparison with diameters of approximately 100 µm in the Cyclocaps® and Easyhaler ® powders (see Figure 7-4).

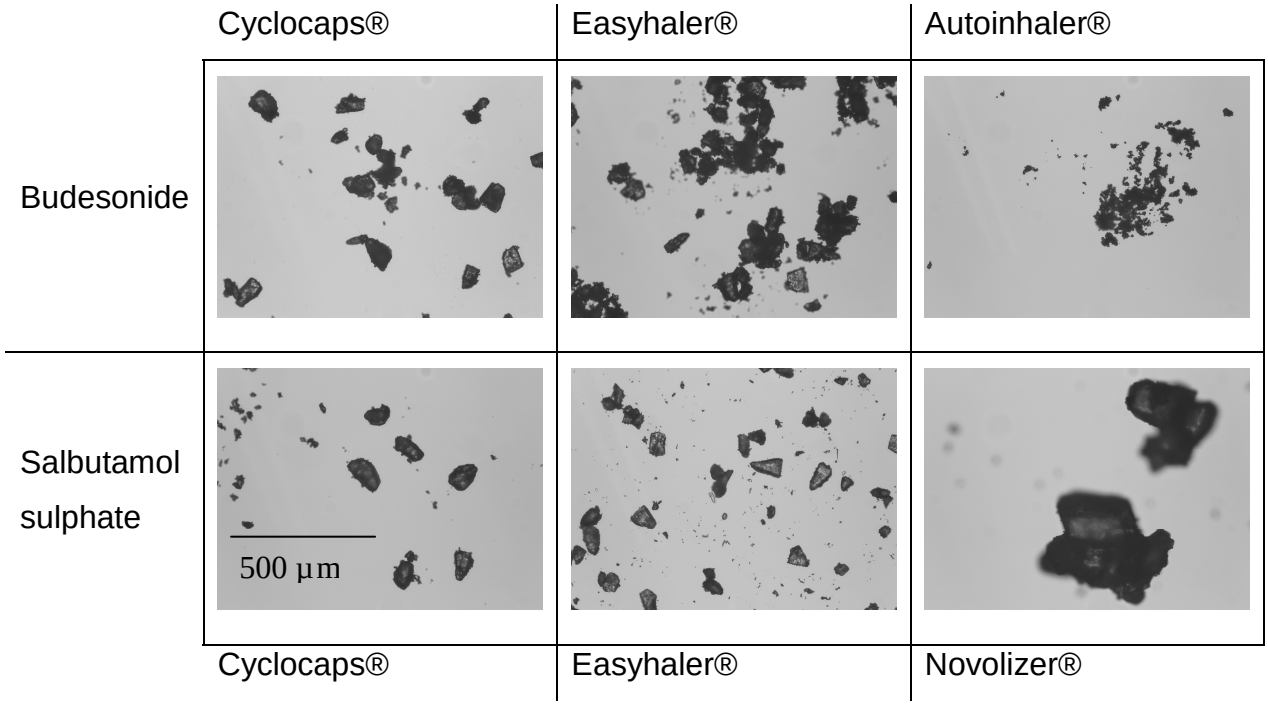

Figure 7-4 Light microscopy of the different dry powder aerosols. Scale bar presents 500 µm

Also the hydrophilic salbutamol sulphate was transported significantly faster after application as aerosol. As determined for both drugs in solution, salbutamol sulphate has an approximately 10 fold slower intrinsic permeability across cell monolayers than budesonide. Therefore all transported amounts of salbutamol were expected to be smaller in comparison to budesonide. 8.56 ± 3.58% of the air interface deposited salbutamol sulphate was transported after 4 hours. Simultaneously only 0.59 ± 0.09% of salbutamol sulphate was transported, when administered as solution (see Figure 7-2).

Likewise for the drug salbutamol sulphate, we tested three different aerosol products. Similar to the budesonide experiments no difference was detectable in the LID experiment. Only the AID resulted in different transport behaviour of the three aerosol powder formulations. Easyhaler® and Cyclocaps® powder showed the same particle size distribution in the microscopic picture and similar absorption rate (see Figure 7-5).

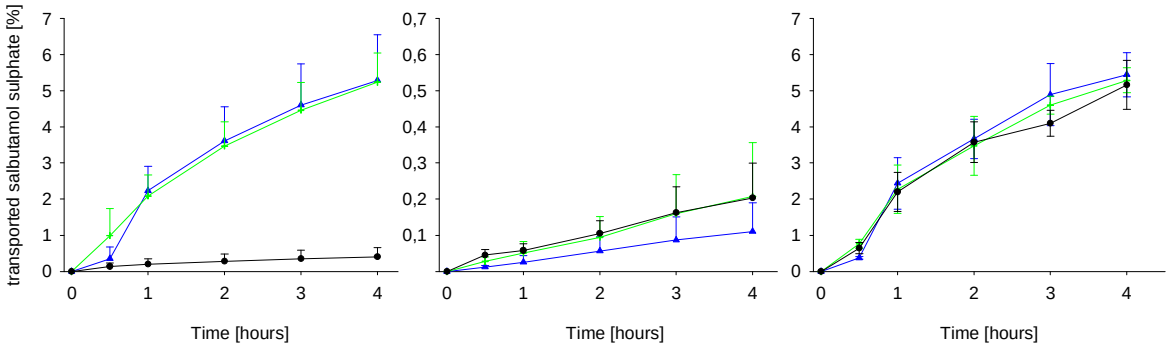

Figure 7-5 Transport of salbutamol sulphate deposited (Δ Cyclocaps®; • Ventilastin® Novolizer®; ♦ Easyhaler®) as dry powder with the insufflator syringe (left side), or pipetted as suspension (in the middle) or applied using a Spinhaler device and the MSLI (in all cases 1000 µM donor concentration and applicated on air interface cultivated hAEpC monolayers; data present mean ± standard deviation; n = 6)

Remarkable changes in the transport behaviour were detected only for the Novolizer® formulation. The transport of salbutamol sulphate from Novolizer® after AID was significant lower in comparison to the two other formulations.

In order to investigate the effects of aerodynamic properties of the generic aerosol powders we decided to perform additional experiments in the modified multistage liquid impinger. The differences in transport rates of the generic aerosols after application with the insufflator syringe disappeared by application of the powder with the aid of the multi stage liquid impinger. Air interface cultivated Calu-3 cells were placed under the nozzle of the fourth stage and the generic aerosols were aerosolized with a flow rate of 30 l/min. On the cell surface collected powder of all three generic aerosols showed same drug absorption characteristics. The faster absorption from the Autoinhaler® powder disappeared as well as the slower transport from the Novolizer® powder (see Figure 7-3 and Figure 7-5).

Mucus on Calu-3 cells, induced by air interface cultivation, did not influence the transport of salbutamol sulphate in a significant manner. The air interface culture initiates the production of mucus which covers the whole surface with a thin layer. The existence of mucus could be confirmed by alcian blue mucus staining (see Figure 5-10). There was no significant difference in the transport rate of salbutamol sulphate deposited at the air interface of these cultivated cell monolayers in comparison to a liquid interface cultivated cell monolayer where the apical

compartment was removed before the deposition of the aerosol on the cellular surface. Thus, the presence of mucus even slightly promoted the transport of salbutamol sulphate, possibly by accelerated dissolution of the aerosol particles (see Figure 7-6).

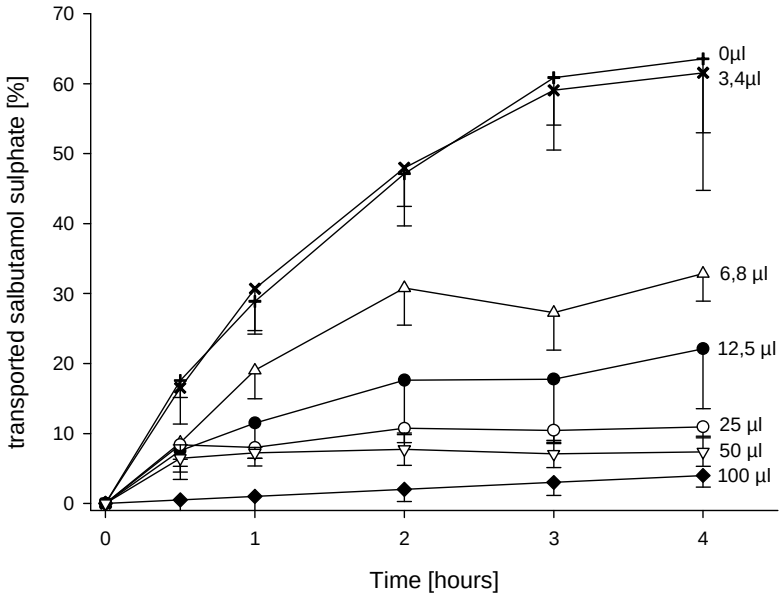

Figure 7-6 The fluid volume in the donor compartment as velocity limitation parameter in the transport of salbutamol sulphate (Easyhaler®) across hAEpC cell monolayers; + 3.4 µl; x 6.8 µl; Δ 12.5 µl; • 25 µl; ♦ 50 µl; o 100 µl (data present mean ± standard deviation; n = 4)

In a next set of experiments the influence of the total fluid volume in the donor compartment on the transport rate was investigated in more detailed. A significant decrease of the transport rate, corresponding with an increase of the fluid volume, was observed. Starting with only 3.4 µl buffer in the donor compartment of air interface hAEpC cell monolayer transport rate similar in “dry” cell monolayers was measured. By increasing of the buffer volume to 6.8 µl the transport rate decreased. Above 25 µl there was no further volume dependency of the transport rate detectable.

If the poorly water soluble budesonide (as budesonide Easyhaler® powder) was applicated as dry powder on the air interface of Calu-3 cells, and than overlaid with 25 or 200 µl of buffer, faster transport rates were detectable (see Figure 7-7).

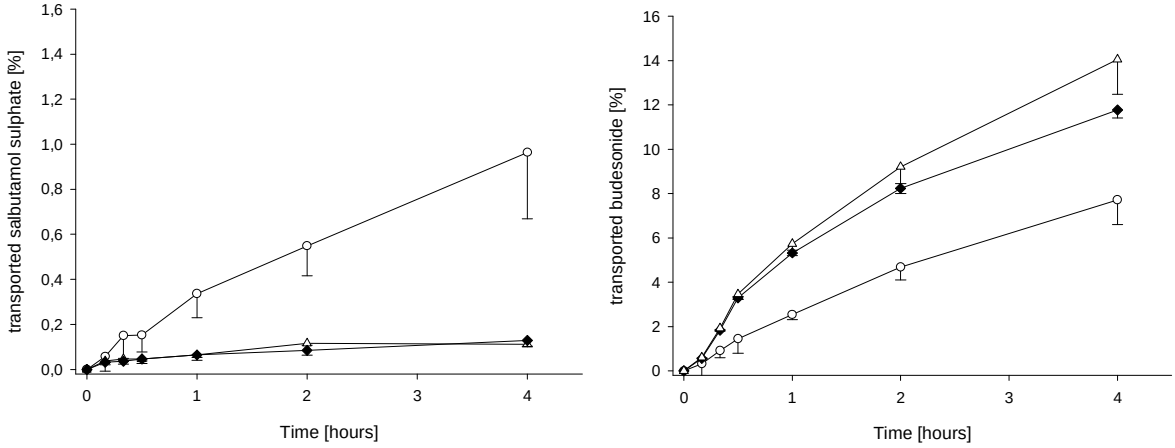

Figure 7-7 Wetability and solubility as rate limiting parameters in the transport of salbutamol sulphate (Easyhaler®) and budesonide (Easyhaler®) across hAEpC cell monolayers. Δ application as dry powder with subsequent adding of 25 µl buffer; O application as dry powder without any fluid adding; ♦ application as dry powder with subsequent adding of 200 µl buffer, (data present mean ± standard deviation; n = 4)

To investigate the influence of solubility on the transport rate the same experiment was conducted a second time in a slightly modified manner. The budesonide Easyhaler® powder was suspended/soluted in 25 µl and then applied on the cell monolayer. As consequence of the anticipated wetting and dissolution, faster transport rates were detectable. However, for the highly water soluble salbutamol sulphate it makes no difference whether the drug is administered in solid state on air interface cells and then covered with buffer or solvated at first in the buffer and then applied as solution.

7.4 Summary

Budesonide and salbutamol sulphate are two widely used aerosol drugs to treat pulmonary diseases. Permeability data for drug solutions across different cell culture systems are available. Our measured P_{app} values correspond very well with published data from different laboratories [35, 37, 102, 103]. The determined A to B and B to A ratio of the two drugs support in case of salbutamol sulphate and hAEpC the involvement of active transport processes in the absorption as was reported earlier by Ehrhardt et al [104].

To the best of our knowledge transport studies of pharmaceutical aerosols across cell cultures after air interface deposition have previously not been reported. An ultra fast absorption of amorphous pure drug aerosols after deep lung inhalation in canine was recently described by Rabinowitz et al. [105]. The two drugs alprazolam and prochlorperazine were detectable in the left ventricular blood after 20 seconds and 5 seconds earlier then after intravenous application. The enormous concentration gradient of the deposited drug served as an explanation for the rapid onset of absorption. Also in our *in vitro* experiments we measured very fast absorption with pronounced burst effect. Normally experiments to predict the uptake of aerosolized drugs across the human air-blood barrier are made in animal experiments or with submersed cell culture models of the pulmonary barrier. However, as our data demonstrate, working with cells submersed in a larger apical volume of donor fluid does not reflect the *in vivo* situation of the more or less dry resorption barriers in the lung. According to Bastacky et al. [106] and Scarpelli et al. [15] the thickness of the liquid layer which covers the epithelial surface in the deep lung was measured between 21 nm and 200 nm with differences dependent on the site of measurement. Particles with a diameter of 1 µm, which can land on the respiratory epithelium of the deep lung where no mucus is present, would be immersed only with 1/50 to 1/5 of their diameter in the liquid phase. Geiser et al. [19] postulated an interesting mechanism for the uptake of ultrafine insoluble particles after landing in the deep lung. Nonphagocytotic processes, interfacial and line tension effects and non membrane bound particles could be detected in macrophages and red blood cells. Without any doubt this mechanism is relevant for single small particles, but it is limited on insoluble particles. However, in case of pharmaceutical aerosols the situation is obviously different. Prior to absorption of a compound in molecularly dispersed state, the preceding drug release governed by wetting, spreading and

dissolution must be started. A transport of complete, non dissolved particles is unlikely, mainly for particle sizes of aerosolized drugs larger than the nanoscale. By analysis of the cell lysat in our experiments, neither in case of budesonide nor in the case of salbutamol sulphate formulations significant amounts of the drug was found internalized in cells. The absorption of drugs from such aerosolized solid state drug particles across the air-blood barrier can be assumed to occur in two steps. First the drug must dissolve in the relatively small volume of the apical lining fluid, resulting in a highly concentrated drug solution with a steep concentration gradient. In a second step, absorption occurs by transport across the cellular epithelial barrier. Provided that drug absorption occurs relatively fast, the rate of the overall process is governed by the rate of particle dissolution/drug release. The onset and rate of the transport is determined by the rate of particle dissolution. After dissolution of the drug the following transport process can be described by Fick's first law:

$$J = -D\,(dc/dx)$$

where J is the flux across a membrane which is the product from the diffusion constant D and the concentration gradient (dc/dx). According the equation the amount of the aqueous phase influences the concentration gradient. We could show that the transport rate of non solubility limited salbutamol sulphate - released from lactose carrier particles - decreased with rising amounts of transport buffer in the donor compartment. By stepwise increasing the liquid phase the point can be estimated where the volume of the aqueous phase has no longer any influence on the transport rate. In case of hAEpC cells and salbutamol sulphate we measured above 12.5 µl no influence of the volume of the liquid phase on the transport rate. A volume of 12.5 µl corresponds with a fluid height of 110 µm. The used Easyhaler® contains lactose carrier particles with a diameter of approximately 100 µm. That implies the total submersion of the particle in the next higher fluid volumes of 25 µl or even more. The lactose carrier dissolves very fast and the resulting solution contains the total amount of free drug.

No significant differences between cells with mucus layer and cells without mucus could be detected. Mucus seems not to influence the transport velocity in the chosen experimental setup; the mucus hydrogel seems not to represent a significant additional biological barrier, at least not for budesonide and salbutamol.

A change of epithelial barrier function could be not detected after aerosol application on the cell monolayer with the aid of the DP-4 insufflator. Also Fiegel et al.[107], observed no damage after particles bombardment on mucus covered air interface cultivated cells. However, leakage of the barrier after particle impaction on washed, mucus-free cells support the hypothesis that mucus protects the cells by absorbing of the impaction forces.

In the submersed setup no differences between the model formulations of salbutamol sulphate and budesonide were detected. The P_{app} values did not differ from the P_{app} value of the blank drug. Only in the air interface deposition setup differences in the transport rate could be measured.

The slower transport of salbutamol sulphate deposited as Ventilastin® Novolizer® ($0.41 \pm 0.26\%$ after 4 hours for powder from the Ventilastin® Novolizer® compared to $5.28 \pm 1.26\%$ and $5.23 \pm 0.80\%$ for the Cyclocaps and Easyhaler powders) can be explained with the larger size of the carrier lactose, and therefore slower dissolution in the limited apical fluid volume. The effect of the carrier lactose disappeared by application of the powder with the aid of the MSLI. The particle degradation by impaction forces in the mouthpiece and stages of the MSLI separates the salbutamol sulphate crystals from the carrier lactose [108]. The resulting small salbutamol sulphate particles can be solvated very fast in the small amounts of fluid on the cell surface. In consequence no differences regarding the drug transport rate after application of the salbutamol sulphate formulations in the MSLI could be observed.

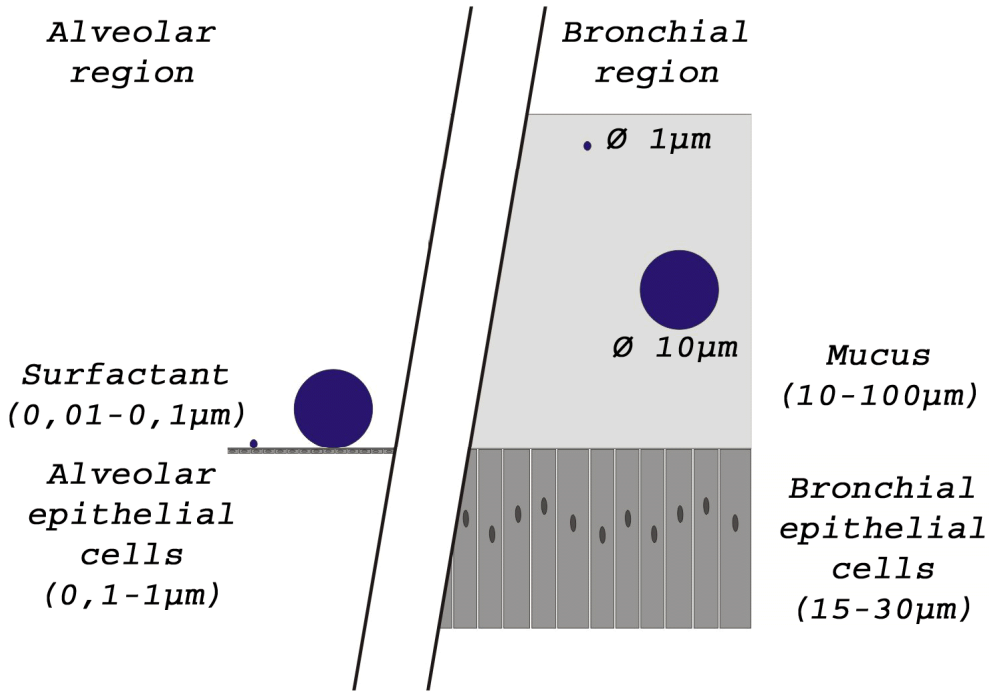

Figure 7-8 Different liquid layer thickness decides about wetting or submersion of deposited particles.

Also the budesonide formulations showed size dependent effects on transport rate. The pulverized Autoinhaler® tablet showed the fastest transport rate which can be explained by the smallest particle size. Cyclocaps® and Easyhaler® formulations led for both drugs to similar, but slower transport behaviour. Also in the size of the powder we found no significant differences. However, it must be pointed out that this phenomenon disappeared after size separating deposition in the MSLI.

By using such a model that also address aerodynamic properties of aerosol powders by mimicking the *in vivo* deposition mechanisms impaction, diffusion, and sedimentation, similar transport rates for all model formulations could be shown. Especially the impaction force which separates the micronized drug crystals from the big lactose carriers seems to be a critical factor in air interface deposition of powder aerosols. The prepared powder from the Autoinhaler® by manually grinding was not comparable with the powder generated from the device in case of regular use. The abrading of the ring tablet results to larger particles then the pulverisation of the tablet in a mill and subsequent sieving. However not only for drug delivery studies but

also for cytotoxicity investigations the air interface deposition offers new possibilities. It was demonstrated by Limbach et al. [109] that very small particles (Ceria nanoparticles with a diameter of 50 nm) underlie not the sedimentation forces in submersed cell culture systems. In consequence small particle uptake could not be measured. These results do not correlate with *in vivo* data where especially very small particles show extremely high permeation rates in cells and tissues [19].

In summary the air interface deposition on dry epithelial surface appears to mimic better the *in vivo* situation on the human air-blood barrier than the typically used submersed cell culture models. The fluid volume controls at first the drug release rate from particles. Thereafter, the fluid volume influences the concentration gradient, which is according to Fick´s first law the motor of passive transport processes. The deposition by the insufflator syringe simulates sufficiently realistic the dissolution and absorption processes occurring after deposition on a wet mucosal surface, but not aerosol deposition itself. The limitations of the air interface deposition with a relatively simple insufflator syringe, however, become apparent in cases of drug formulations where aerodynamic properties of the aerosolized powder particles get critical. Nevertheless the application by insufflator syringe offers the possibility to study the effects of particles and powder formulations on pulmonary drug absorption and epithelial barrier functions by direct deposition on such filter-grown epithelial cells.

This may be more relevant for future studying pulmonary controlled release formulations, where the amount of available apical liquid is likely to affect both drug releases as well as permeability modulating effects (e.g. permeability enhancement) as well as for toxicological studies on engineered or environmentally born nanoparticles.

Air interface deposition yields higher transport rates and better reflects the situation *in vivo* than liquid interface deposition. Application by insufflator may suffice if aerodynamic properties of the aerosol particles are not critical. To address also aerodynamic properties more sophisticated setups like the cell compatible multi stage liquid impinger can be used. A use in drug delivery as well as in toxicological investigations seems possible and advantageous. Under such conditions, pulmonary cell culture models offer a way to simulate the most important peculiarity of aerosol drug delivery: absorption of a relatively high dose within a relatively short time after deposition on slightly wetted epithelial surface.

8 Development of a cell compatible aerosol deposition system to investigate aerosol particle – alveolar cell interactions

Abstract:

The following chapter gives an overview about instrumented approaches to investigate the interactions of pulmonary administered formulations with in vitro cultivated epithelial cells. Different cell compatible aerosol application devices, which allow simultaneously deposition and drug absorption quantification, are described. Differences between long time but low dose aerosol deposition in environmental toxicology and short time bolus inhalation of pharmaceutical aerosols are elucidated.

Furthermore, a modified Astra type multi stage liquid impinger (MSLI) with integrated bronchial cell monolayers was used to mimic pharmaceutical aerosol deposition on air interface cultivated Calu-3 cells. The practicability of a size selective deposition experiment with subsequent absorption study was proven for three different salbutamol sulphate and budesonide formulations. In case of application without size separation the absorption rates of the model aerosols differed but correlated with the size of the carrier lactose particles. However, after deposition in the MSLI, which is simulating also in vivo relevant impaction and in consequence the separation of the drug crystals from the carrier lactose, the absorption rates of the formulations have been equivalent.

Parts of this chapter are submitted for publication in:

Bur M, Rothen-Rutishauser B, Lehr CM; A novel cell compatible impingement system to study drug absorption after pharmaceutical aerosol inhalation; Journal of Aerosol Medicine submitted

8.1 Introduction

The following chapter gives in the first part an overview about the existing instrumented approaches to investigate the interactions of pulmonary administered formulations with epithelial cell cultures *in vitro* and describes in the second part a new setup developed in our laboratory. Different experimental approaches to simultaneously assess deposition and subsequent absorption of pharmaceutical aerosol formulations, typically by adapting some existing impactor/impinger devices to accommodate pulmonary epithelial cell culture systems are described. Differences between long time but low dose aerosol deposition in environmental toxicology and short time bolus inhalation of pharmaceutical aerosols are elucidated.

The inhalation route is of general interest for the application of drugs in order to treat systemic and local diseases [96, 97, 110]. Recent advances in the development of inhalation devices and particle technology are allowing to deliver small molecules as well as proteins and peptides with sufficient efficacy to the lung [30, 111]. However, this option is limited mainly due to missing data regarding safety and uptake of drugs and excipients after inhalation. Proven safety and high bioavailability of a drug or excipient after oral administration does not warrant safety and efficacy after inhalation. The lack of safety data is generally regarded as the reason that at the moment only few drugs and excipients are approved for pulmonary application. Especially missing *in vitro* test systems decelerate development of modern inhalable medicines. One cause for this drawback is the complexity of inhalation [112] and pulmonary deposition, which is hardly to simulate *in vitro*.

Also application of drug to the *in vitro* model accounts for conduction in a physiologically relevant manner. Adding a drug solution to the apical compartment of a cell culture, or in a filled Ussing chamber does not mimic the *in vivo* situation, where an aerosol deposits on a moistened surface. The deposition directly on the cells leads to enormous differences compared to LID. There are enormous differences for both, for the aerosol as well as for the cells. Regarding the fact that predominantly particles with diameters of 1 - 5 µm are deposited in the alveolar region, where a 7 - 70 nm deep fluid layer covers the cells, the particle will be, subsequent to deposition, only with its “toes” in the water. Erosion from the minor liquid layer on the underneath and degradation starting on the complete surface by

the humidified atmosphere is observed *in vivo*. However, surface properties like zeta potential and roughness, etc. are conserved. These parameters are probably not important to drug release and toxicity [113] but will be lost after dispersion/dissolution of the aerosol powder in transport buffer. Therefore, particles excessively modified or even solvated by the dispersion fluid may not be relevant for *in vivo* situation and such approaches should be avoided in *in vitro* experiments. Also for droplet aerosols, one will observe a much higher concentration gradient after application of aerosol droplets compared to a diluted drug solution. Consequently, droplet aerosols should not be diluted in *in vitro* experiments by using LID models.

8.2 The optimal cell compatible aerosol deposition system

As addressed above, application of drugs in a physiologically relevant manner can solely be conducted as aerosol deposition on air interface cell culture inserts. Regarding the techniques to deposit aerosols on cell surfaces, pharmaceutical science may profit from the experience in environmental toxicology. However, whilst toxicological studies on aerosols are typically focusing on the exposure of xenobiotic compounds at a certain concentration over a given period of time, pharmaceutically relevant aerosols are to be administered usually as a metered, single dose.

8.2.1 Aerosol classification systems

8.2.1.1 The MSLI

The pharmacopoeias describe different aerosol classification devices for metered bolus inhalation. Especially, the multi stage liquid impinger (MSLI) shows good correlation to the *in vivo* deposition and is the most applied impingement system in pharmaceutical research. Due to the high air flow rate in the device (30 l/min), only impaction and sedimentation as deposition mechanism are simulated sufficiently in the MSLI; however, diffusion, the major deposition process in the deep lung *in vivo*, is not reflected correctly. A thin liquid layer on the stages mimics he surfactant layer in the lung and avoids the rebound of particles after impaction. The common acceptance as well as the simple construction and handling make the MSLI a good development basis for a cell compatible pharmaceutical impingement system

8.2.1.2 Deposition systems used in environmental toxicology

The recently published aerosol deposition system CULTEX® [114] allows continuous exposure of lung cell monolayers to complex atmospheres. The device was developed as a tool for the assessment of environmental lung toxicology. CULTEX® enables treatment of epithelial cells, cultivated on permeable filter inserts with aerosols and subsequent to impingement *in vitro* assays and permeability measurement [115]. By controlling pO_2, pCO_2, and humidity, cells and lung slices stay viable for at least 48 h. The apparatus setup is shown in Figure 8-1. As it can be seen there pulmonary cell monolayers are placed on membranes inside a deposition chamber. The CULTEX® system is entirely made of a glass facilitating the housing of three vessels with cell culture inserts, whereas the temperature of these vessels is controllable. Nutrient medium is directed to the cell culture insert vessels via a tube system. The device allows sampling of medium for biological analysis during the experiment, e.g. for measurement of transported drug amount. The test aerosol is drawn into the deposition chamber via a tube system using negative pressure. The analysis of several aerosol compounds including particle concentrations can be performed online parallel to the cell exposure.

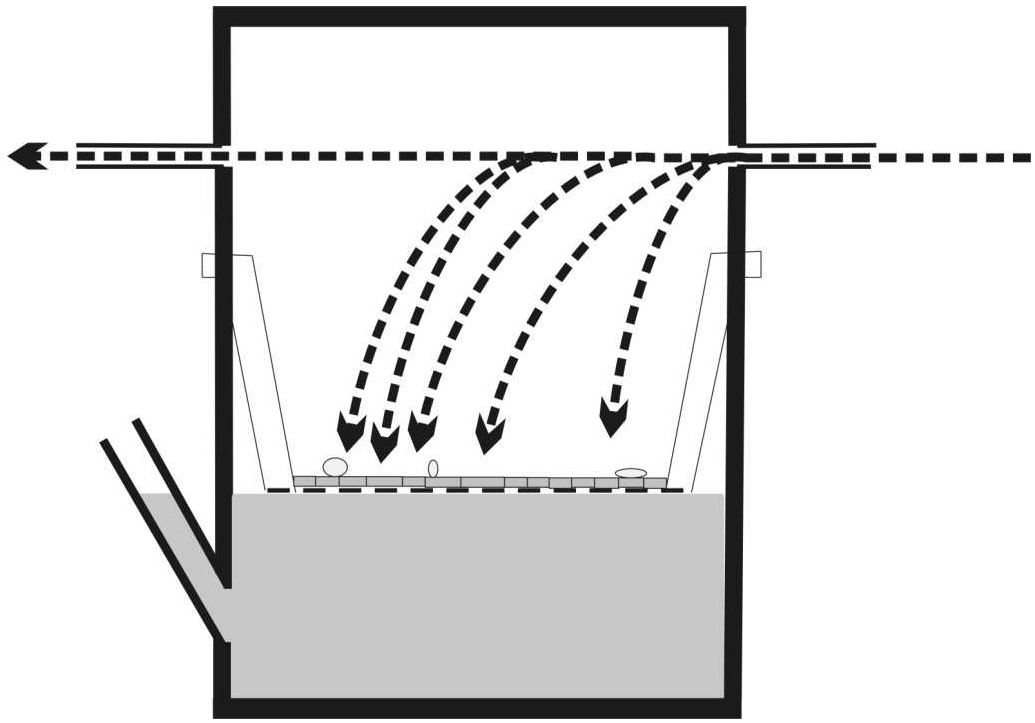

Figure 8-1 Principle of the Cultex® setup, mainly impaction and sedimentation processes are simulated.

Aufderheide et al. [115] performed experiments with human lung cells directly exposed to a diesel exhaust line. In contrast to any other exposure concepts for complex mixtures, this experimental setup facilitates a direct and reproducible contact between the cell monolayer and the test atmosphere. This could be achieved by following improvements: i) strict separation of the medium and gas supply, ii) the application of cell culture membranes with small pore size thus preventing accidental donor aerosol contact during the exposure, iii) transport of the test atmosphere directly to the apical side of the cells.

Results clearly indicate effects on the cells of native diesel exhaust from different engine operating conditions already after 1 h of exposure. However, a 1 hour exposure to a pharmaceutical aerosol is not clinically relevant, and thus, for pharmaceutical realistic exposure, high deposition within one breath, i.e. five seconds, would be necessary and of vital importance.

A another setup developed for environmental toxicology questions is described by Tippe et al. [116]. A commercially available perfusion unit (MINUCELL, Bad Abbach, Germany) is adapted to study biological effects of fine and ultrafine particles on cells [117]. The radially symmetric stagnation point flow arrangement deposits particles uniformly and quantifiably onto a cell layer. Due to the low flow velocity over the membrane (approximately 5 ml/min), mechanisms of particle deposition are convective transport and diffusion and only less impaction. For cell exposure, Anodisc membranes (Whatman, Maidstone, UK) with 47 mm diameter and a pore size of 0.2 µm were used. These membranes remain completely plane after humidification, which is important for homogeneous nutrient medium transport from the basolateral compartment and for homogenous particle deposition during exposure. Confluent A549 epithelial cells were integrated in the chamber system. Ultrafine carbonaceous model particles with a count median mobility diameter of about 95 ± 5 nm were delivered to the exposure system. After six hours, 87 ± 23 ng/cm^2 of particles were deposited homogenously on the cell surface (deposition efficiency of 2%). Compared with therapeutically single doses of pulmonary administered drugs such as budesonide or salbutamol (100 µg or more), this deposition efficacy is significantly too low. Nevertheless, the aerosol deposition

system of Tippe et al. could be used after minor modifications in order to increase the deposition efficacy as an aerosol deposition model for pharmaceutical questions.

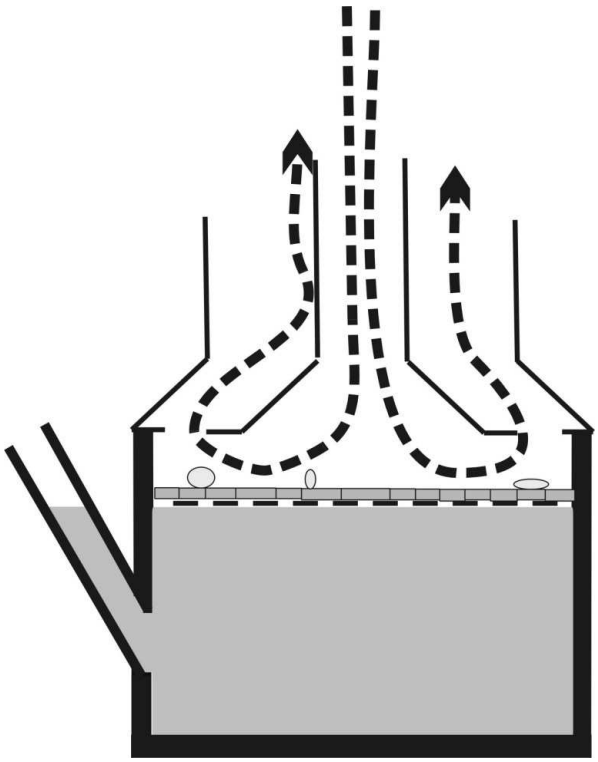

Figure 8-2 Setup from Tippe et al., low flow rate but simulation of deposition by diffusion.

Schreier et al. [118] used the MSLI to generate a simulation aerosol system for studying gene delivery. Hereby, the device can be used to study the characteristics of aerosol formulation, stability, delivery efficacy and expression efficacy of delivered gene products. The impinger consists of a PARI® nebulizer, a controller for temperature and humidity and an Andersen cascade impactor, in which stages are seeded with pulmonary cells (2-CFSME0-, derived from submucosal tracheobronchial glands of a CF patient). Cell viability retains over 95% subsequent to deposition experiments. A fluorescent dye was used to visualize aerosol distribution and to monitor cellular uptake. Additionally, the majority of a gene product was delivered to stages 1 through 5 which are corresponding to the *in vivo* area from the pharynx to the terminal bronchi excluding the alveolar space. Then, a corresponding, although very low, transfection of cells was found with the majority of transfected cells on stages 4 and 5. This experiment was the first application of a cell compatible cascade impinger and inspired further experiments with viable impactors.

A crucial question for pulmonary drug delivery is the effect of aerosol impaction on cell viability. In difference to slow air streams applied in environmental toxicology setup (only a few ml per minute), pharmaceutical setup are working with high air velocity between 30 and 60 l/min. For such high air velocity even small particles have high impaction forces and hence might induce toxic effects. In a study from Fiegel et al., the integrity of Calu-3 cell monolayers impinged with polymeric large porous particles was investigated by means of measurement of the transepithelial electrical resistance and transport of the paracellular transport marker fluorescein-sodium. Filter inserts containing cell monolayers were placed directly under the second stage nozzle of an Astra-type liquid impinger (Erweka, Heusenstamm, Germany) as shown in Figure 8-3. Microparticles were aerosolized onto the monolayers for 30 s at 30 l/min via a Spinhaler device. The filter insert was then returned to a cell culture plate and placed back in an incubator. Light microscopy images of the monolayers confirmed that particles were not aggregated when deposited on the monolayers and those particles were dispersed homogeneously across the entire cell monolayer surface. Also scanning electron microscopy (SEM) images of both AIC and LCC grown monolayers impinged with microparticles, revealed no apparent damage of the monolayers. Although there was no detectable effect on the transepithelial electrical resistance, monolayers grown under LCC conditions showed an increased flux of the paracellular marker fluorescein-sodium, when compared to the AIC monolayers. Mucus staining of the cell monolayers exhibited positive staining only for Calu-3 cells grown under AIC conditions. This mucus on monolayers may protect cells against microparticle impingement by means of a protective coating, thus cushioning the landing of the particles. In contrary, mucus produced by cells grown under LCC conditions likely dissolves into the apical fluid as it is produced and is removed by aspiration of the supernatant prior to particle impinging. Therefore, microparticles landing directly on the surface of cells grown under LCC conditions may cause damage to the barrier properties.

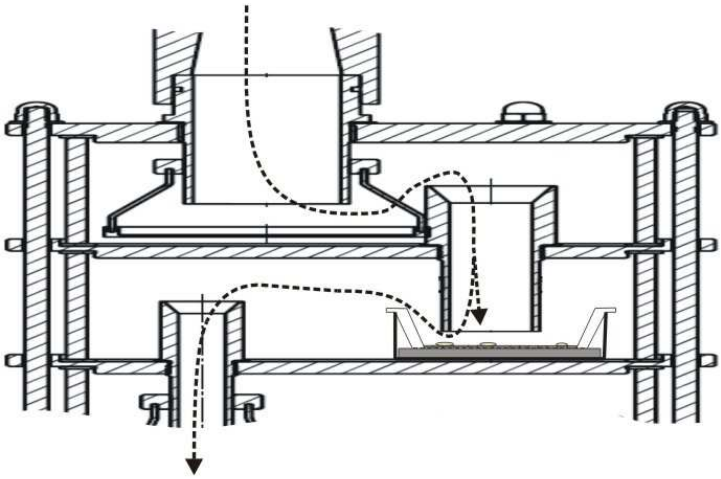

Figure 8-3 MSLI with inserted cell culture, impaction forces are the critical factor.

A further “proof of principle” was conducted by Cooney et al. [119] who demonstrated the feasibility of the Andersen six-stage viable particle sampler as a cell compatible deposition device. Permeability coefficients of FITC - dextranes after impaction as aerosols on Calu-3 cells could be calculated. Deposition did not negatively affect cell monolayers integrity.

In both described setups, deposition on the inserts was approximately 50% or less of the amount usually anticipated on an equal sized area without cell culture inserts. This difference may be explained by the changed distance between orifice plate and impaction surface in the cell culture insert. The collection efficacy of an impactor decreases with an increase in the ratio of the orifice plate to impaction plate distance over jet width. Observations indicate that, though the inserts are not at an optimal distance from the orifice plate and are not exactly at the same position compared to the original collection stage, the cell culture inserts do function as an impaction surface for particles in a relevant size range. The previously described approaches may be seen as disadvantageous because of the low deposition rates. In order to circumvent this issue, an improved cell compatible MSLI with cell culture monolayers inserts was designed. In this approach, the cell culture inserts do not interfere with the air stream in the stage and as a consequence deposition efficacy and deposition pattern is not altered compared to non modified MSLI. But not only the deposition system, but also the inserted cell culture decides about the value of the model.

8.2.2 Cell culture used in aerosol deposition systems

Depending on particle characteristics and breathing pattern, particulate aerosols are impinging in various regions of the lung. In the bronchial as well as in the alveolar region, the particles are sedimentating on an epithelium, in both cases the main barrier for pulmonary drug absorption. Already in the 1980's, attempts have been made to simulate the respiratory epithelia using isolated organs or organ slices [120]. However, these approaches have been limited by functional breakdown of the tissue [32], missing reproducibility and high costs (see chapter 5.3.1) [121]. Finally, progress in cell culture overcame these issues and led to standardized and validated models of the air-blood barrier. For instance, the immortalized bronchial cell lines Calu-3 [35] and 16HBE14o- [31] develop when grown on semi-permeable filter inserts tight and polarized monolayers suitable for transport studies. For the alveolar region, up to now, no immortalized cell line with sufficient barrier properties is available; however, primary cell cultures isolated from different species [51], including from human origin, are reported in literature [48, 49, 101, 122].

Inspired by the successful employment of the intestinal cell line Caco-2 as model to predict oral absorption, pulmonary cell culture based models were used in the same manner. However, the cultivation of pulmonary cells under a deep fluid layer (liquid culture conditions, LCC), which may be acceptable for an *in vitro* model of the intestinal barrier, is not an adequate method for the air-blood barrier [45]. Especially, for the Calu-3, 16HBE14o-, and the primary alveolar models, the possibility of air interface culture was confirmed [39]. Also co-culture approaches with endothelial cells [123] [124], dendritic cells [42], or macrophages [125] were conducted in order to increase the complexity and the explanatory power of these models. In conclusion, sufficiently realistic barrier models for measuring safety and uptake/transport of drugs in viable impinger are available [100, 126, 127]. For our purpose we have chosen AIC cultivated Calu-3 cells.

8.3 Materials and Methods

8.3.1 Inverted cell culture of Calu-3 cells

The human adenocarcinoma cell line Calu-3 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Fluorescein-sodium (flu-Na), tissue culture media, and all other reagents were obtained from Sigma (Deisenhofen, Germany). Transwell Clear® inserts (12.0 mm inner diameter, pore size 0.4 µm) were purchased from Corning Costar (Bodenheim, Germany). The modified stainless steel cell culture plate for the inverted cell culture was custom-made by Erweka (Heussenstamm, Germany). The modification – holes with bigger diameter - was necessary to allow the inversion of the filter inserts (see Figure 8-4) during seeding and culture. Also the special tubes, which are slipped over the lower part of the inverted filter, to generate a new separated apical compartment during seeding, enforced a higher construction.

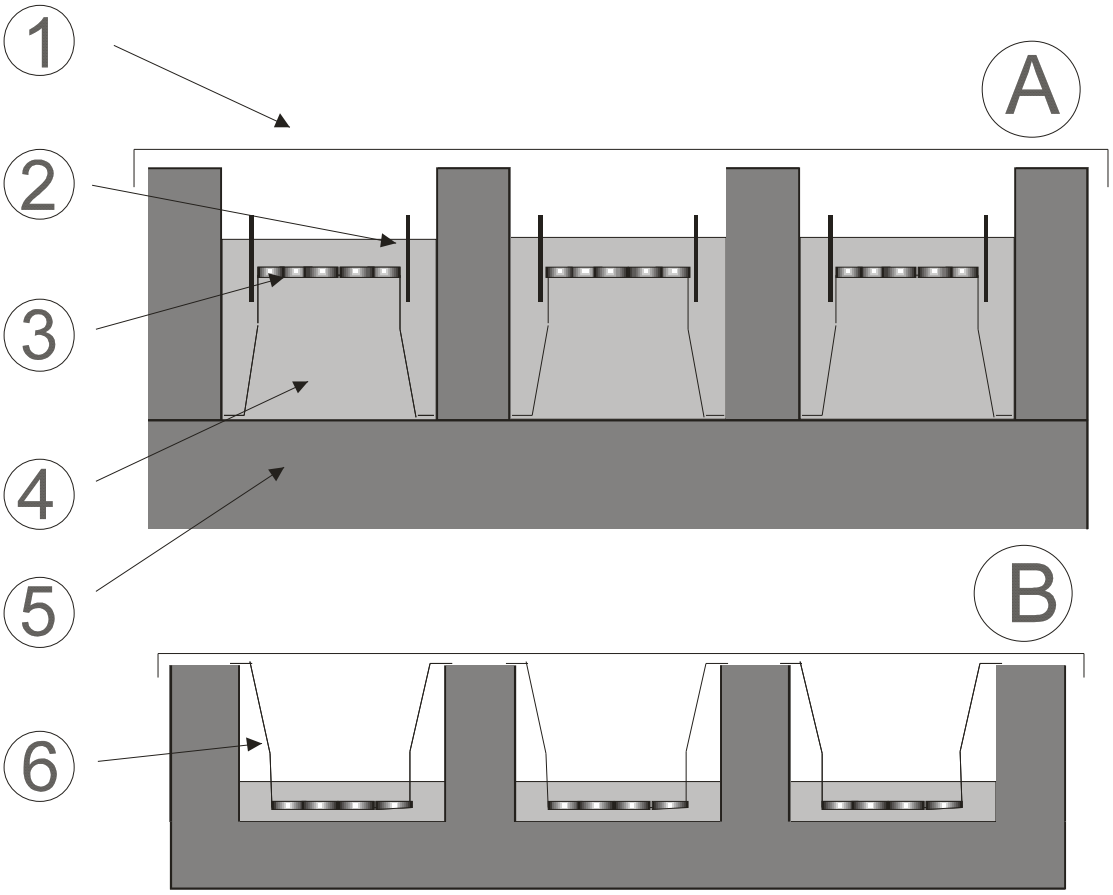

Figure 8-4 The principle of the inverted cell culture. 1: lid 2: cartridge 3: cell monlayer 4: cell culture medium 5: stainless steel culture plate 6: filter insert; A: inverted style; B: normal style

Calu-3 cells (passage number 41 to 46) were seeded onto the bottom site of inverted Transwell Clear® permeable filter inserts at a density of 10^5 cells/cm^2. Before seeding, special tubes were sheathed over the inverted cell culture insert to generate a liquid-tight new apical compartment in which the seeding can take place. Immediately after seeding, cells were grown in 500 µl apical and 2,000 µl basolateral media (Eagle's minimum essential medium supplemented with 10% foetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin) at 37℃ in a 5% CO_2 incubator. The inserts could be reversed two days after seeding and adhesion of the cells. In case of air interface culture the bottom side seeded filter inserts were not upturned, but cells cultivated under AIC conditions were fed all two days with 1000 µl fresh media basolaterally only. Simultaneously Calu-3 cells were seeded under identical conditions on the top side of non inverted filter inserts.

8.3.2 The modified MSLI

The MSLI consists of a mouthpiece, 4 impaction stages, each containing 10 ml KRB buffer during operation, and a final filter stage. The liquid in the impinger can be expected to reduce particle bounce and re-entrainment. Specified as apparatus A in the European Pharmacopoeia, the multi stage liquid impinger originally designed by Astra Draco, Lund, Sweden is a versatile cascade device that is used for testing both MDIs and DPIs for the determination of particle size distribution. Such a commercial available MSLI was modified by Erweka (Heussenstamm, Germany) with the objective of integration of cell monolayers in the bottom of the stages. Therefore, two holes per stage were drilled into the bottom of the stages 2 and 3, housing the inverted cell culture inserts. Consequently, cultivation of pulmonary cells on the underneath of a cell culture insert was necessary. The inner diameter of the drilled wholes is exactly the external diameter of commercially available Transwell® filter inserts. Since the cell culture insert extends into the lower stage, and thus might affect aerodynamic properties in this particular stage, the filter was encapsulated with stainless steel.

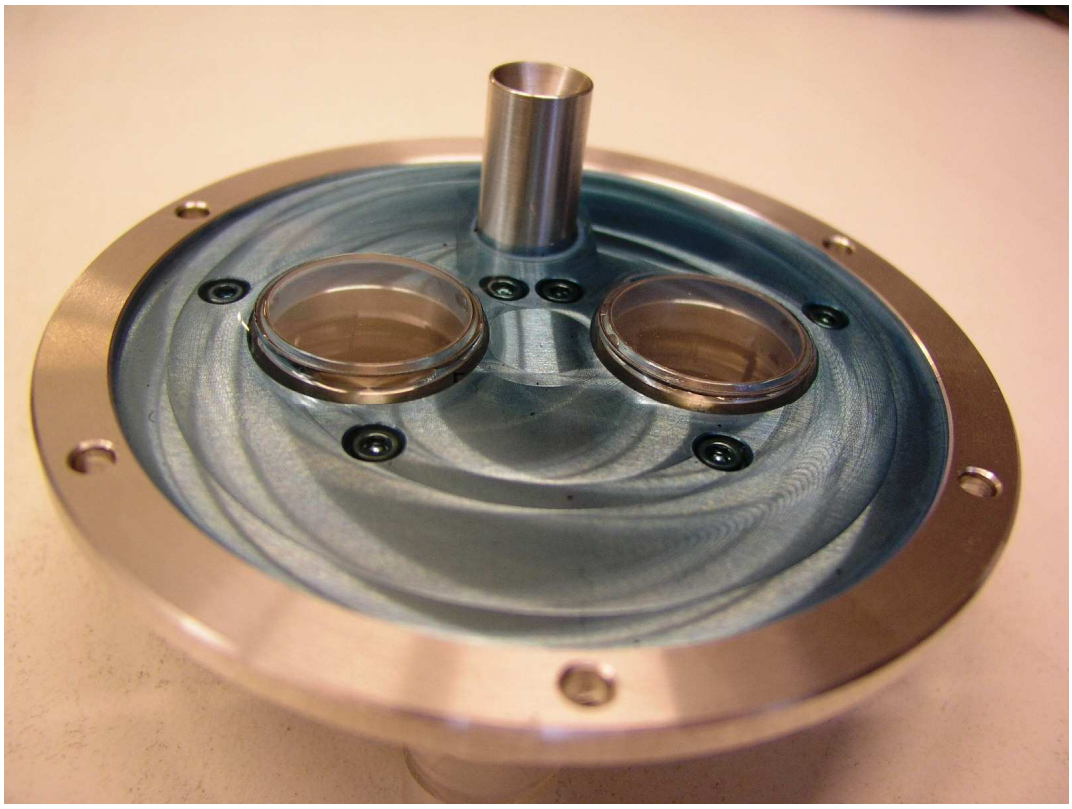

Figure 8-5 The third stage of a MSLI with inserted Transwell® filter inserts.

After modification a cell crown filter can be inserted in the stage without any aerodynamic changes in the upper stage (see Figure 8-6).

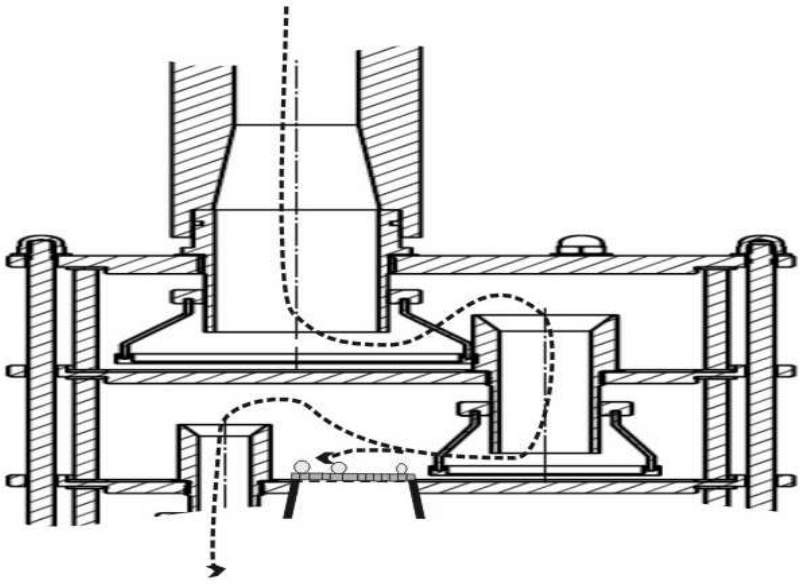

Figure 8-6 Cross-section of a MSLI stage with inserted cell culture.

The cell monolayer is perfectly integrated in the stage lining fluid (10ml) and the cell surface is flush with the fluid layer in the stage. The MSLI was operated with an Erweka Vacuum Pump (H.D.-Pump) and a pump rate of 30 l/min. The air flow was controlled by a digital flow meter (Model M1A, Copley, Therwil, Switzerland). The time of inhalation was managed by a testing unit for dry powder inhalations (Erweka, Model FG1) which allows adjusting the duration of inhalation and amount of air.

8.3.3 Electron microscopy

For SEM analysis, cells were fixed with 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer (pH 7.4), dehydrated in a graded ethanol series and placed finally in absolute ethanol. Following critical point drying with carbon dioxide, the specimens were mounted on stubs and sputtered with gold to a layer thickness of 10nm. Scanning electron micrographs were recorded on a Philips XL30 SEM (FEI Co. Philips Electron Optics, Zurich, Switzerland) at 10kV.

For TEM analysis cells were fixed with 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. The cells were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, and with 0.5% uranyl acetate in 0.05 M maleate buffer. Cells were then dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections were cut and transferred on 200 - mesh uncoated copper grids, stained with uranyl acetate, counter-stained with lead citrate and observed with a Philips 300 TEM at 60 kV (FEI Company Philips Electron Optics, Zuerich, Switzerland).

8.3.4 Measurement of Interleukin-8

Cytokine concentrations were quantified using commercial available sandwich ELISA kits (PromoCell, Heidelberg, Germany). In brief, quantification of IL-8 took place using mouse monoclonal anti-human IL-8 antibody diluted to 4.0 µg/ml in phosphate buffered saline PBS. Recombinant human IL-8, serially diluted from 5,000 pg/ml was utilised as standard. Cell culture medium samples were diluted 1:20 with 0.1% bovine serum albumin, 0.05% Tween 20 in PBS, immediately before use. Secondary detection antibody was rabbit anti-human IL-8 antibody. Incubation of samples and standards, and then of the secondary antibody, took place on a plate agitator for 1 h

at 37℃. Between each stage, all wells were aspirated, washed forcefully five times with wash buffer (0.05% Tween 20 in PBS), and blotted dry. Measurement of absorbance took place using wavelengths of 450 and 550 nm. All samples were analyzed in duplicate. Cytokine concentrations are expressed per volume of cell culture medium (ng/ml).

8.3.5 Salbutamol and budesonide as hydrophilic and lipophilic model drugs

Budesonide and salbutamol powders taken from Cyclocaps® (Jenapharm, Jena, Germany) Easyhaler® (HEXAL, Holzkirchen, Germany), Ventilastin® Novolizer® (Viatris, Bad Homburg, Germany), and Autoinhaler® (CT Pharma, Berlin, Germany) were aerosolized onto Calu-3 monolayers using a Spinhaler device (Fisons, Bedford, MA, USA). The powders were transferred in capsules to allow the aerosolisation in the Spinhaler device for 30 s with a flow rate of 30 L/min. Upside down cultivated Calu-3 cells were inserted in the third stage of the modified MSLI and the impinger was sealed. The stages were flooded with 10 ml warmed KRB and 10 mg of the powder were aerosolized. After powder application the filters were placed upside down in the modified stainless steel plate and placed back into an incubator at 37℃. The basolateral compartment was filled with KRB. Samples were taken after determined time points from the receptor compartment.

Furthermore the same experiment was carried out a second time by powder application with a spatula on the dry cellular surface and a third time after solution of the formulations in buffer and application of the resulting solution in the apical cell compartment.

The deposited and transported amount of salbutamol sulphate was analysed by reversed phase HPLC (see chapter 7.2.5).

8.4 Results

8.4.1 The inverted cell culture

8.4.1.1 Cell morphology

A first comparison of the morphology of normal and inverted Calu-3 cell culture was performed by electron microscopy pictures. As well in transmission as in scanning mode no significant differences in the dimensions and in the structure are detectable. In the first days of culture the inverted cells showed a higher attitude in cross-section. Also in the scanning picture a stronger profiled surface was observable. But during the culture until day 10 the cells get more and more flattened and on day 17 no optical difference between the two cell cultures was visible (see Figure 8-7). A belt of tight-junctions circumrounding the cell bodies and ciliary structures were detectable independent of the culture style.

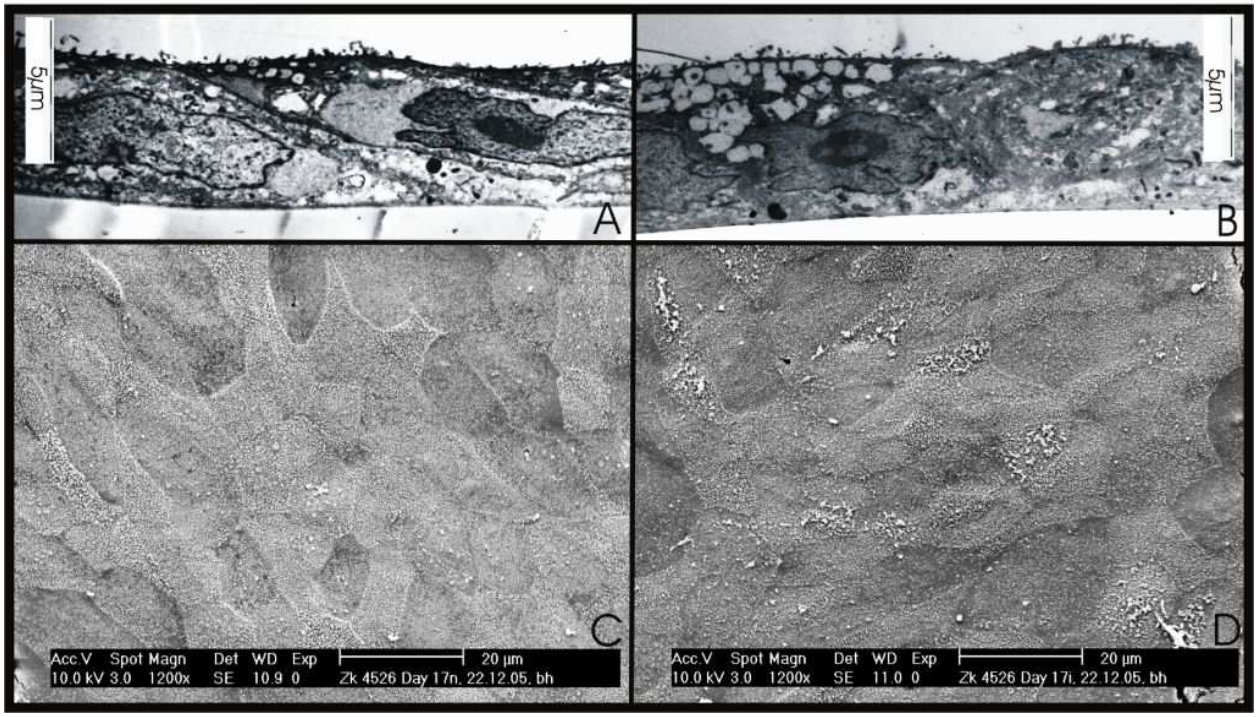

Figure 8-7 SEM and TEM pictures of Calu 3 cells in the age of 17 days (B and D inverted culture, A and C normal culture).

8.4.1.2 Barrier properties

The inverted cell culture developed tight-junctions slightly slower than under normal conditions, but reached the same value after 10 days in culture.

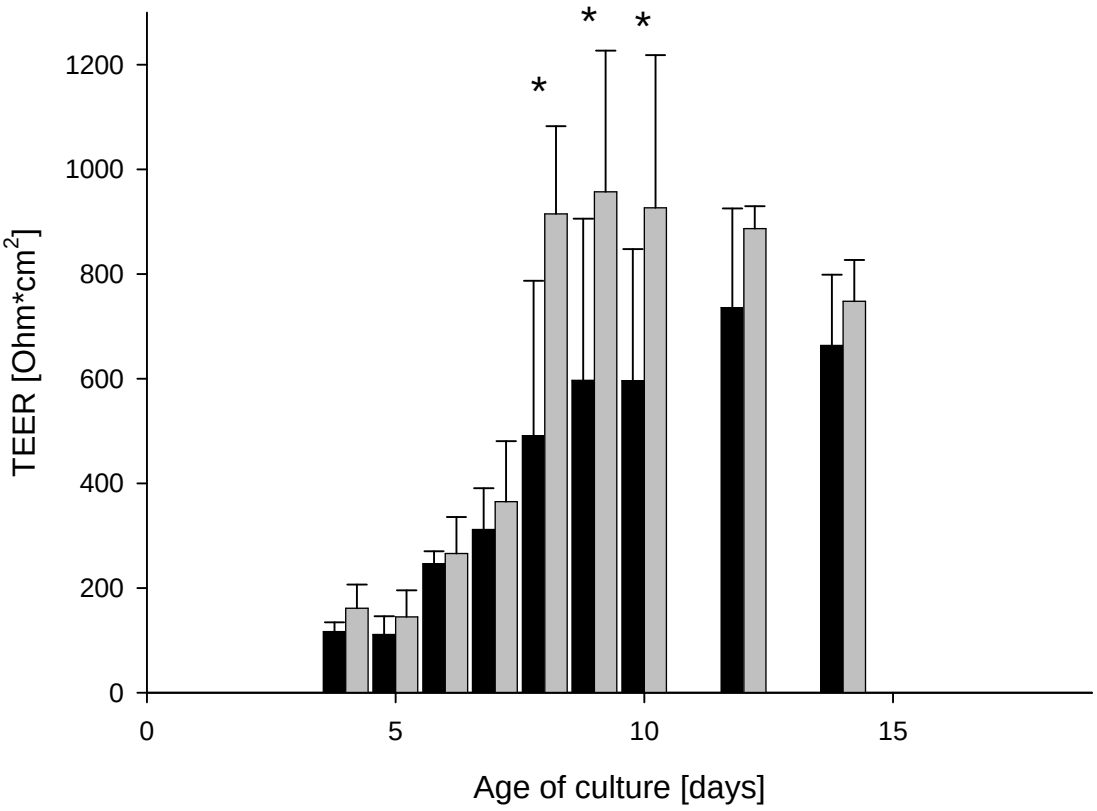

Figure 8-8: TEER development of LCC Calu-3 cells in dependency of the culture style; black bars represent inverted style, grey bars normal style (Mean ± std. dev. n= 6-18; P < 0,05).

The TEER of the normal culture peaked on day 9 (TEER 957.2 ± 269.4 Ohm*cm^2; n=18) in contrast to the lower maximum in the inverted cell culture on day 12 after seeding (TEER 735.83 ± 269.4 Ohm*cm^2; n=6). Statistical significant differences in the TEER values between normal and inverted culture was found only for the time period between day 8 and 10 of culture (see Figure 8-8). In consequence all transport experiments were carried out after day 10 or more in culture.

The transport of fluorescein-sodium (flu-Na) across the cell monolayers was performed to determine differences in the barrier function in consequence of the culture conditions. Experiments were carried out with n = 6, using cells from five different passages in the age of 10-15 days. Krebs Ringer Buffer (KRB; pH 7.4) was used as transport buffer. Flu-Na solution (50 µM in KRB buffer) was added to the apical (500µl) or basolateral (1,500µl) compartment of each well. The cell monolayers were agitated using an orbital shaker at constant stirring rate (100 rpm) at 37℃ under humidified conditions. The initial concentration of flu-Na in the donor fluid was assayed by taking a 20 µl sample. 200 µl samples were taken at predetermined time points up to 360 min from the receptor compartment and replaced with an equal amount of fresh warmed buffer. The fluorescence of flu-Na was measured in 96-well plates using a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively. Samples were diluted with KRB where appropriate. Apparent permeability coefficients of the cell monolayers, P_{app}, were calculated according to the equation in chapter 6.2.3.

Flu-Na was transported in normal and inverted cell culture with the same absorption rate. No asymmetry between absorption and secretion was found. Apparent permeability coefficients were calculated for the absorptive as well as for the secretive direction. In normal culture $1.74 \pm 0.113 * 10^{-6}$ cm/sec for the absorption and $1.51 \pm 0.112 * 10^{-6}$ cm/sec for the secretive direction was calculated. The upturned cell culture resulted in similar rates $1.75 \pm 0.165 * 10^{-6}$ cm/sec for the absorption and $1.47 \pm 0.179 * 10^{-6}$ cm/sec for the secretion (see Figure 8-9). The integrity of the cellular barrier was confirmed before and after each transport experiment by measuring the TEER value.

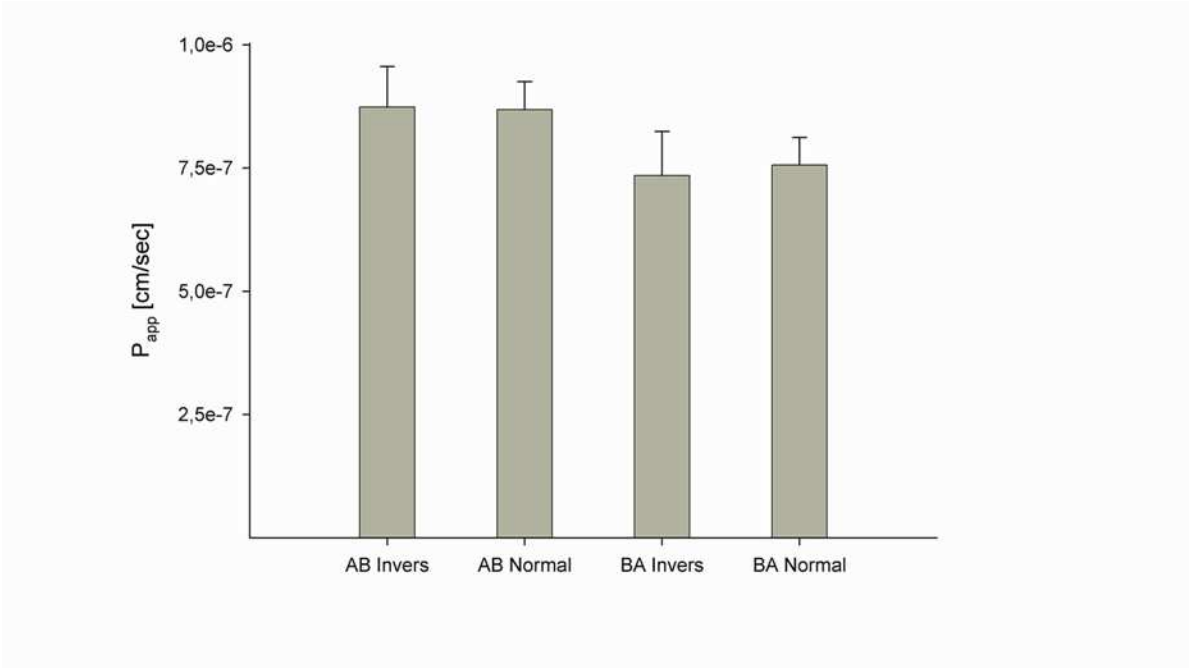

Figure 8-9 Permeability of flu-Na across normal and inverted cultivated Calu-3 cell monolayers (Mean ± std. dev. n=8; P < 0,05).

Also in the interleukin 8 levels in normal and inverted cultured Calu-3 cells we found no statistically significant difference. The inverted cell culture leaded to slightly non significant increased interleukin 8 levels (4.32 ± 0.67 ng/ml; n=6) in comparison to the normal culture (4.01 ± 0.43 ng/ml; n=6).

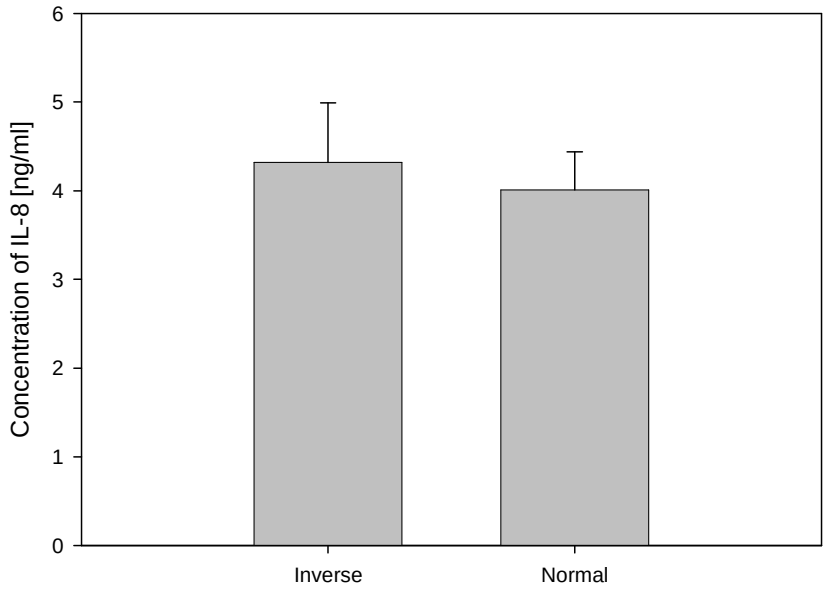

Figure 8-10 IL-8 levels in normal and inverted Calu-3 cell culture supernatant on day 12. (Mean ± std. dev. n=6)

8.4.2 Short characterisation of the modified impinger

8.4.2.1 Deposition pattern before and after modification

Flu-Na solution (13 µM, prepared with KRB buffer) was aerosolized by a PARI® nebulizer (Pari GmbH, Starnberg, Germany) in a sealed MSLI before and after modification. The deposited amounts in the mouthpiece, the stages, and the terminal filter were measured by quantification of the fluorescence (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively. Modifications solely in stage 2 or in stage 3 resulted in significant increased deposition on the modified stage itself. But in case of simultaneously modification of stage 2 and stage 3 we found no significant changed deposition patterns in comparison to the non modified MSLI.

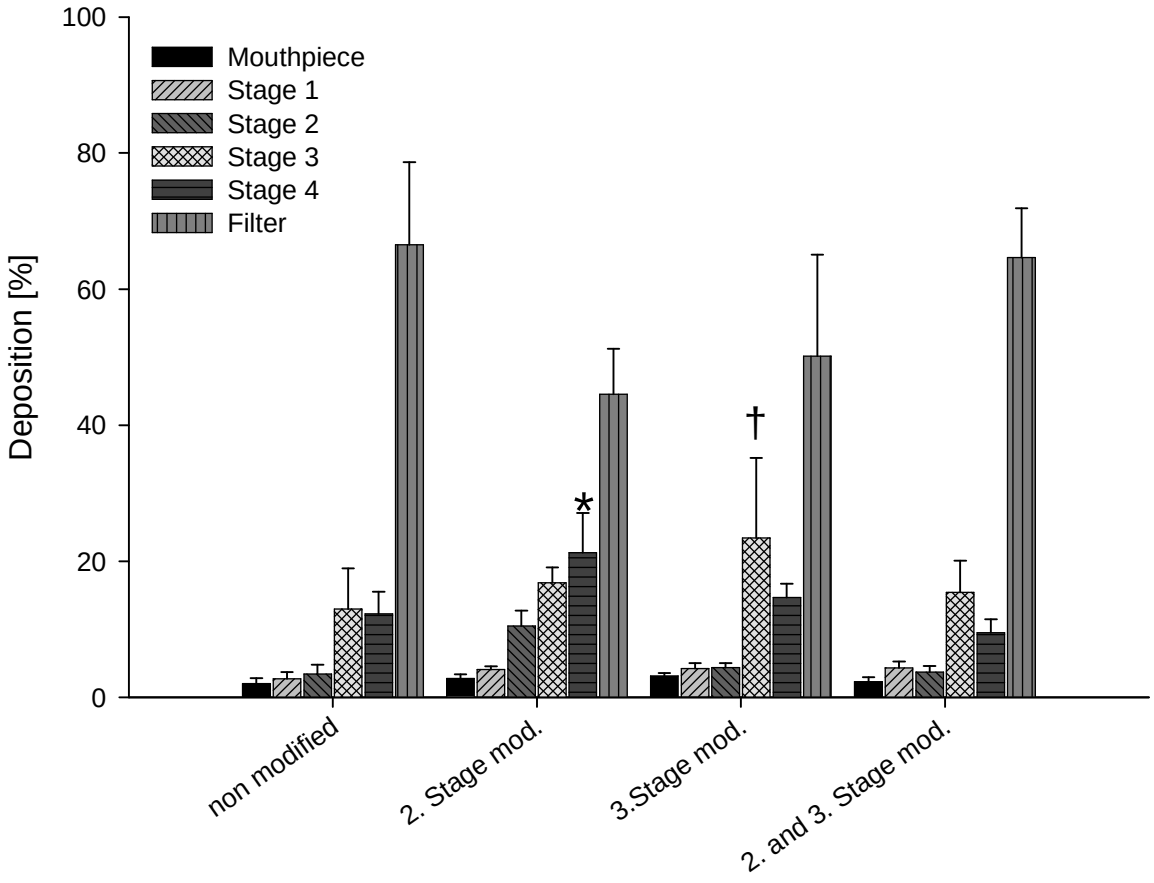

Figure 8-11 Deposition of flu-Na droplet aerosol in normal and modified MSLI. †,* significant differences from the non modified MSLI, deposited amount in the modified stages compared to the stages of the non modified system (Mean ± std. dev. n = 8-12. P < 0,05)

In consequence, deposition of flu-Na aerosol on Calu-3 cells was accomplished in the second and third stage modified MSLI.

8.4.2.2 Deposition of droplet and powder aerosols in the viable MSLI

Filter inserts containing cell monolayers on the bottom side were mounted in the second and third stage respectively of our modified impinger and the impinger was sealed. Flu-Na solution (13 µM) was aerosolized onto the monolayers for 30 s at 30 litre/min from a PARI® nebulizer. Then the filter insert was returned to the 12-well plate and placed back into an incubator at 37°C. Samples from the receiver compartment were taken and analyzed for flu-Na using a fluorescence plate reader. Samples were diluted with KRB where appropriate. To obtain data on the reproducibility of the aerosol application and to measure the apical deposited amount, slightly wetted Transwell Clear® filter inserts containing no cells (n = 6) were placed in the impinger and treated as described above. In the inverted culture with a liquid donor compartment a P_{app} of $1.75 \pm 0.165 * 10^{-6}$ cm/sec (n=6) was measured after LID in contrast to a P_{app} of $5.81 \pm 2.271 * 10^{-6}$ cm/sec (n=6) in a system with a dry donor compartment and MSLI mediated air interface deposition.

The two drugs salbutamol sulphate and budesonide were chosen as model for highly water soluble and poorly water soluble drugs. The absorption rate of the bioequivalent generics were tested in three different setups. At first solutions prepared from the drug powders were applicated in a submersed cell culture. In a second experiment the drug powder was added on the dry cell surface with a spatula. In a third experiment we aerosolized the generics with the MSLI on inserted air interface cultivated Calu-3 cells. In liquid interface deposition conditions all three generics showed the same low absorption rate. However, after air interface deposition the transported drug amounts were after 4 hours approximately 30 times higher for salbutamol sulphate and 3 times higher for budesonide compared to the liquid interface deposition experiment. Application of the powder with the spatula resulted in a significant slower salbutamol sulphate transport from Novolizer® Ventilastin® (see Table 3) and a significantly faster absorption rate of budesonide from the Autoinhaler® powder.

		LID	AID / spatula	AID / MSLI
Salbutamol sulphate	Easyhaler®	0.4099	12.6017	12.8107
	Cyclocaps®	0.2230	12.7093	13.2954
	Ventilastin®	0.4356	1.0472	12.2323
Budesonide	Easyhaler®	55.8130	103.1139	147.2448
	Cyclocaps®	63.9254	101.6106	148.9616
	Autoinhaler®	54.8950	151.8714	157.2196

Table 3 Area under the curve of the powder formulations. Data represent mean of 4 independent experiments.

All these differences disappeared after powder impingement with the MSLI in a third experiment and an equivalent high absorption rate for all generics could be detected.

8.5 Discussion

The cultivation of cells on the bottom side of filter inserts is described in the literature, but in all cases for experiments regarding the uptake of particles in coculture models consisting of two or three different cell types, or to investigate the communicative network of cells with different origin and function like epithelial, endothelial, and dendritic cells [123, 124]. Tightness and robustness of these cell monolayers are not strictly necessary for such purposes. However, for drug transport experiments the barrier tightness is a critical factor. Per definition an epithelium is called tight, if it builds up electrical resistance higher than 500 Ohm*cm^2 [128]. Our measured Calu-3 TEER values are in good correlation to other published TEER data [100, 103, 104]. Also the development and peaking of the TEER value over time fit well with data from literature. The morphological differences disappeared after a few days in culture and on day 17 no significant differences in the thickness of the cell bodies were found. Also the cell surface was under both culture conditions in the same manner structured, especially the ciliary like structures and vesicles filled with mucus could be observed in both cultures. In cross sections tight-junctions could be detected. The TEER development peaked time delayed in the inverted cell culture, which is also caused by the morphological differences until day 10. Because of the upturning of the

cells 2 days after seeding gravimetric forces could be the source for the higher height and time delayed barrier development of the inverted cell culture.

To check the suitability as drug transport model the absorption of flu-Na was measured. This fluorescent lipophilic model substance is transported mainly by passive transport through tight junctions and intercellular spaces. Only if functional tight-junctions are expressed low transport rates will be measured. The resulting calculated P_{app} values confirmed the electrical measured tightness of the barrier.

In consequence also the inverted cell culture of Calu-3 can be looked upon as a suitable model of the human air-blood barrier for drug uptake studies.

The MSLI is a well established aerosol classification device in pharmaceutical sciences. The necessary modifications to integrate the cells in the stages resulted in slightly but non significant changed deposition patterns in case of simultaneously modification of stage 2 and stage 3. The deposition pattern was controlled by collecting the polydispers droplets from an aerosolized fluorescent solution. Further investigations of the deposition pattern were not carried out due to effective and short time deposition of drug aerosols on dry cell monolayers were the main objectives of the MSLI modification. While measuring the particle size distribution of aerosols was not the scope of the modification, effective deposition of aerosol particles in a physiological manner on a cell surface was possible.

Not only the deposition on the cell surface should be designed as realistic as possible, but also the cellular surface by itself. *In vivo* the fluid layer seems to play a major role in the complex network of particle uptake and cleaning. After landing soluble drug particles will be dissolved in the more or less big lung surface fluid layers. The dissolution rate will be controlled by the volume of fluid on the place of particle deposition. Especially the particle size influences also the dissolution rate. Particles with diameter larger than the thickness of the fluid layer will be only with parts of their surface in the fluid and consequently slower dissolved. In contrast small particles can be submersed totally. Regarding the solubility and the wetting properties of the particles complex dissolution processes are resulting. After dissolution passive or active transport processes of the substance across the cell monolayer occurs. The permeation step is controlled by the cell monolayer itself, and is dependent of the tightness and expression of uptake transporters or efflux systems. In consequence, in transport experiments conducted in air interface cultured Calu-3 cell system two serial connected processes – dissolution and

transport - can be observed. By working with drug solutions in liquid interface deposition experiments the dissolution step is missing.

A second difference between adding solution or dry powder aerosol in the donor compartment is given by different contact area. If solutions are added to the donor compartment a maximal contact area between substance and cell surface will be caused. By application of single drug particles, only parts of the cell surface or the lining fluid layer will be in contact with the applied substance.

The transport of drugs across cellular barriers can be described with Fick`s first law. The concentration gradient is one of the major forces which regulates the absorption rate. After landing of single drug crystal, local high drug concentrations will enforce drug absorption rate *in vivo*. This so called ultrafast absorption of drugs was described by several authors [105, 129] and explained with big resorption surface coupled with steep concentration gradients. Also our results can be interpreted with the enormous local concentration gradients. We measured in all cases of aerosol application higher absorption rates compared with the typically used submersed experiments.

The area under the curve was chosen as parameter to judge equivalence of the generic formulations of salbutamol and budesonide. Very similar absorption rates resulted after liquid interface deposition. By dissolving of the powders, which consist of large carrier lactose particles and small adherent drug crystals, differences in particle size disappeared and the drug solutions showed the same absorption rates. The air interface application with the spatula does not include size separation and fractionation of the powder in drug crystals and carrier particles. In dependency of the lactose carrier size we observed different absorption rates. Easyhaler® and Cyclocaps® formulations contained carrier lactose particles in the same range of diameter. But Ventilastin® consisted of significant larger carrier particles which are after deposition on the slightly wetted cellular surface only with parts of their surface in the liquid layer. Slower dissolution of the lactose and therefore also slower absorption were the consequence. In case of the budesonide containing Autoinhaler® powder the carrier lactose particle size was significantly smaller and particles deposited on the surface liquid can be completely submersed. Fast dissolution and absorption rates can be measured.

The deposition in the MSLI simulates the *in vivo* inhalation where a separation of the adherent drug crystals from the carrier lactose occurs by impaction forces. Large

lactose carrier particles will be deposited in the pharynx region, and only the drug crystals with suitable aerodynamic properties will reach the deep lung. Also in our case the different generics were size separated and only drug crystals, but no carrier lactose particles were deposited on the cell monolayer in the third stage. Due to all drug crystals in the generics have similar diameters comparable absorption rates could be measured. Mainly the size of the particles seems to influence the absorption rate after air interface deposition of soluble drug particles.

Application with a spatula is not able to simulate the complex inhalation processes *in vivo*.

The successful separation of the micronized drug from the different sized carrier lactose particles could be confirmed with the equal transport rate of the three generics after application in the cell compatible MSLI.

8.6 Conclusion

Based on a commercial available aerosol classification system a first prototype of a cell compatible pharmaceutical aerosol deposition model was developed. This model together with on the bottom side air interface cultivated Calu-3 cells allows the prediction of drug absorption rates after application in the human lung in a physiological relevant manner.

First experiments with the permeability marker flu-Na suggest successful application of aerosols on cell monolayers and thereafter the possibility to measure substance transport across epithelial barriers.

The importance of a realistic deposition simulation could be shown for three commercial available bioequivalent aerosols. Only after deposition in the cell compatible impingement system, where a separation of micronized drug crystals from the carrier lactose takes place, the bioequivalence of the three generics could be confirmed.

9 Perspectives

In order to characterize pharmaceutical aerosols *in vitro*, especially in regard to the cellular reaction on the deposition, various experimental setup are reported in literature. Devices having their roots in environmental toxicology, pose promising starting points for further development of *in vitro* devices, which should allow application of high metered single dose aerosols. Further attempts have been made using compendial MSLI systems integrating pulmonary cell culture monolayers. Here, the conditions are more related to pharmaceutically relevant applications. However, the high deposition rate using these systems can only be achieved by applying high flow rates, solely allowing impaction and sedimentation as deposition mechanism and excluding diffusion. Thus, these devices are mainly suitable for modelling deposition in upper airways. Hereby, the most effective experimental approach is based upon the principle of the air/liquid exposure technique in the MSLI during deposition. However, by using liquid/liquid exposure technique, for instance with Ussing chamber, the aerosol is chemically and physically altered prior to the contact with the cell monolayer and findings concerning aerosol cell interaction may be limited.

A perfect *in vitro* model characterizing aerosol formulations would incorporate cell types from various regions of the lung (tracheal, bronchial and alveolar) and would facilitate simulation of deposition mechanisms by impaction, sedimentation, and diffusion of a high metered single bolus inhalation. Furthermore, the application of targeted cells from human lung epithelium may reduce in the future the need for animal studies and may offer extrapolation of *in vitro* test results of aerosol formulation to *in vivo* situations.

10 Zusammenfassung

Die Behandlung sowohl lokaler als auch systemischer Erkrankungen mit Inhalanda erfreut sich immer größerer Beliebtheit. Neben Weiterentwicklungen auf dem Gebiet der Asthmatika und Kortikoide als lokal wirksame Arzneimittel wurde mit Exubera® zum ersten Mal die systemische Applikation eines hochmolekularen Peptides über die Lunge verwirklicht. Obwohl sich weitere innovative Formulierungen zur Behandlung der verschiedensten Erkrankungen in der Pipeline befinden, ist es zum Beispiel immer noch nicht möglich retardierte Arzneiformen für die pulmonale Applikation herzustellen. Vor allem der Mangel an Daten bezüglich Arznei- und Hilfsstoffverträglichkeit nach Inhalation und Arzneistoffpermeation nach Deposition erweist sich als Hemmschuh der Entwicklung moderner Inhalanda. Auch das Fehlen geeigneter Zellkulturmodelle, welche wesentlichen Anteil an der Entwicklung moderner oraler Arzneiformen haben, erschwert die Entwicklung sicherer und effektiver pulmonaler Arzneiformen. Im Rahmen dieser Dissertation wird der Einsatz humaner pulmonaler Zellkulturen zur Vorhersage der Arzneistoffabsorption *in vivo* erläutert.

Im ersten Teil der Dissertation wurde die Permeation von Peptiden mit verschiedenem Molekulargewicht durch Monolayer humaner alveolarer Epithelzellen untersucht. Für diese Experimente wurden Lösungen der Peptide hergestellt, und diese in die flüssigkeitsgefüllten Donorkompartimente von humanen alveolaren Epithelzellkulturen gegeben. Auch wenn das Arbeiten mit Lösungen / Suspensionen und submersen Zellkulturen weit verbreitet ist, und auch für die Untersuchung intestinaler Absorptionsvorgänge die *in vivo* Situation ausreichend genau wiedergibt, stellt diese Methode keine realistische Applikationsart für Aerosole dar. Die menschliche Luft-Blut Schranke ist beim gesunden Patienten mit einem ausgesprochen dünnen Flüssigkeitsfilm bedeckt, der nur den hundertsten Teil der Dicke üblicher Flüssigkeitsschichten in submersen Zellkulturen ausmacht. Eine realitätsnahe Kultivierung an der Luft-Grenzschicht ist zumindest für verschiedene pulmonale Zellkulturen möglich. Vor allem Calu-3 Zellen als Modelle des Bronchialepithels, und primäre humane alveolare Epithelzellen als Modell der alveolaren Bereiche der Lunge, lassen sich ohne flüssigkeitsgefülltes apikales

Kompartiment kultivieren. Als Folge der Kultur an der Luft-Grenzschicht produzieren die Zellen verstärkt Mucus oder Surfactant ähnliche Substanzen.

Im zweiten Teil der Arbeit wurde mittels solcher an der Luft-Grenzschicht kultivierter zellulärer Modelle untersucht, inwiefern die Applikation als Lösung oder in Form eines trockenen Pulveraerosols den Transport von Arzneistoffen beeinflusst. Nach Deposition trockener Aerosolformulierungen auf an der Luft-Grenzschicht kultivierte Zellen konnten signifikant schnellere Resorptionsvorgänge gemessen werden. Die Applikation erfolgte mittels einer einfachen Insufflator Spritze, welche normaler weise zur intratrachealen Applikation von Aerosolen bei Versuchstieren benutzt wird.

Obwohl diese Art der Deposition an sich schon eine sinnvolle Verbesserung von Transportexperimente an Modellen der Luft-Blutschranke darstellt, berücksichtigt die Insufflator Spritze nicht alle Aerosol Charakteristika. Vor allem im Falle von Aerosolen mit Laktose Partikeln als Wirkstoffträger ist die Insufflator Spritze nicht in der Lage die Separation der mikronisierten Arzneistoffkristalle von den wesentlich größeren Laktose Trägern zu bewerkstelligen.

Um auch diese Prozesse wirklichkeitsnah zu simulieren wurde im dritten Abschnitt der Arbeit ein zellkompatibler Kaskaden-Impaktor entwickelt. In diesem war es möglich sowohl eine realistische Auftrennung der Aerosole nach der Partikelgröße als auch die Deposition der einzelnen Partikelfraktionen auf Luft-Grenzschicht kultivierte Zellmonolayer nach zu ahmen. Um eine störungsfreie Integration von dichten Zellmonolayer in den Kaskaden-Impaktor zur ermöglichen wurden in den einzelnen Impaktor Etagen Bohrungen in den Platten angebracht, deren Durchmesser exakt dem Außendurchmesser von Zellkultureinsätzen entsprachen. In diese Bohrungen wurden dann umgedrehte und auf der Unterseite der Membran mit Calu-3 Zellen bewachsene Zellkultureinsätze eingesetzt. Nach einer Charakterisierung der invers kultivierten Zellen hinsichtlich Differenzierung und Barriereeigenschaften konnten mittels dieses Modells sehr realitätsnah verschiedene Pulver Aerosol Formulierungen auf pulmonalen Zellen abgeschieden werden.

Es wurden mit den entwickelten Modellen zum ersten Mal pharmazeutische Aerosole aus als Arzneimittel zugelassenen Trockenpulver-Inhalatoren realitätsnah auf Zellkultur basierte Modelle der menschlichen Luft-Blutschranke appliziert.

11 References

[1] Gehr P (1984) Respiratory tract structure and function. J Toxicol Environ Health 13: 235-49

[2] Weibel ER (1963) Morphometry of the Human Lung. Springer Verlag/Academic Press, Heidelberg, New York,

[3] Nakajima M, Kawanami O, Jin E, Ghazizadeh M, Honda M, Asano G, Horiba K, Ferrans VJ (1998) Immunohistochemical and ultrastructural studies of basal cells, Clara cells and bronchiolar cuboidal cells in normal human airways. Pathol Int 48: 944-53

[4] Fehrenbach H (2001) Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2: 33-46

[5] Tsukita S, Furuse M (2000) The structure and function of claudins, cell adhesion molecules at tight junctions. Ann N Y Acad Sci 915: 129-35

[6] Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 123: 1777-88

[7] Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S (1998) Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 141: 1539-50

[8] Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 142: 117-27

[9] Wong V, Gumbiner BM (1997) A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol 136: 399-409

[10] D'Atri F, Citi S (2002) Molecular complexity of vertebrate tight junctions (Review). Mol Membr Biol 19: 103-12

[11] Bojarski C, Weiske J, Schoneberg T, Schroder W, Mankertz J, Schulzke JD, Florian P, Fromm M, Tauber R, Huber O (2004) The specific fates of tight junction proteins in apoptotic epithelial cells. J Cell Sci 117: 2097-107

[12] Simionescu M, Simionescu N (1991) Endothelial transport of macromolecules: transcytosis and endocytosis. A look from cell biology. Cell Biol Rev 25: 1-78

[13] Schnitzer JE (2001) Caveolae: from basic trafficking mechanisms to targeting transcytosis for tissue-specific drug and gene delivery in vivo. Adv Drug Deliv Rev 49: 265-80

[14] Eljamal M, Nagarajan S, Patton JS (1996) In situ and in vivo methods for pulmonary delivery. Pharm Biotechnol 8: 361-74

[15] Scarpelli EM (2003) Physiology of the alveolar surface network. Comp Biochem Physiol A Mol Integr Physiol 135: 39-104

[16] Batenburg JJ (1992) Surfactant phospholipids: synthesis and storage. Am J Physiol 262: L367-85

[17] Hamm H, Fabel H, Bartsch W (1992) The surfactant system of the adult lung: physiology and

clinical perspectives. Clin Investig 70: 637-57

[18] Anand Balakrishnan BDR, Gordon L. Amidon, James E. Polli, (2004) Surfactant-mediated dissolution: Contributions of solubility enhancement and relatively low micelle diffusivity. Journal of Pharmaceutical Sciences 93: 2064-2075

[19] Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J, Gehr P (2005) Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environ Health Perspect 113: 1555-60

[20] Seiler MP, Luner P, Moninger TO, Karp PH, Keshavjee S, Zabner J (2002) Thixotropic solutions enhance viral-mediated gene transfer to airway epithelia. Am J Respir Cell Mol Biol 27: 133-40

[21] Widdicombe J (1997) Airway and alveolar permeability and surface liquid thickness: theory. J Appl Physiol 82: 3-12

[22] Samet JM, Cheng PW (1994) The role of airway mucus in pulmonary toxicology. Environ Health Perspect 102 Suppl 2: 89-103

[23] Ding X, Kaminsky LS (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. Annu Rev Pharmacol Toxicol 43: 149-73

[24] Hukkanen J, Pelkonen O, Hakkola J, Raunio H (2002) Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. Crit Rev Toxicol 32: 391-411

[25] Evans JP, Tudball N, Dickinson PA, Farr SJ, Kellaway IW (1998) Transport of a series of D-phenylalanine-glycine hexapeptides across rat alveolar epithelia in vitro. J Drug Target 6: 251-9

[26] Forbes B, Wilson CG, Gumbleton M (1999) Temporal dependence of ectopeptidase expression in alveolar epithelial cell culture: implications for study of peptide absorption. Int J Pharm 180: 225-34

[27] Lehr C-M (2004) Deutsche Apotheker Zeitung

[28] Hastings RH, Folkesson HG, Matthay MA (2004) Mechanisms of alveolar protein clearance in the intact lung. Am J Physiol Lung Cell Mol Physiol 286: L679-89

[29] Hamilton KO, Yazdanian MA, Audus KL (2002) Contribution of efflux pump activity to the delivery of pulmonary therapeutics. Curr Drug Metab 3: 1-12

[30] Byron PR, Patton JS (1994) Drug delivery via the respiratory tract. J Aerosol Med 7: 49-75

[31] Manford F, Tronde A, Jeppsson AB, Patel N, Johansson F, Forbes B (2005) Drug permeability in 16HBE14o- airway cell layers correlates with absorption from the isolated perfused rat lung. Eur J Pharm Sci 26: 414-20

[32] Saldias FJ, Comellas A, Guerrero C, Ridge KM, Rutschman DH, Sznajder JI (1998) Time course of active and passive liquid and solute movement in the isolated perfused rat lung model. J Appl Physiol 85: 1572-7

[33] Daniele N, Halse R, Grinyo E, Yeaman SJ, Shepherd PR (2002) Conditionally immortalized cell lines as model systems for high-throughput biology in drug discovery. Biochem Soc Trans 30: 800-2

[34] Wan H, Winton HL, Soeller C, Stewart GA, Thompson PJ, Gruenert DC, Cannell MB, Garrod DR, Robinson C (2000) Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o. Eur Respir J 15: 1058-68

[35] Mathia NR, Timoszyk J, Stetsko PI, Megill JR, Smith RL, Wall DA (2002) Permeability characteristics of calu-3 human bronchial epithelial cells: in vitro-in vivo correlation to predict lung absorption in rats. J Drug Target 10: 31-40

[36] Pezron I, Mitra R, Pal D, Mitra AK (2002) Insulin aggregation and asymmetric transport across human bronchial epithelial cell monolayers (Calu-3). J Pharm Sci 91: 1135-46

[37] Borchard G, Cassara ML, Roemele PE, Florea BI, Junginger HE (2002) Transport and local metabolism of budesonide and fluticasone propionate in a human bronchial epithelial cell line (Calu-3). J Pharm Sci 91: 1561-7

[38] Hamilton KO, Yazdanian MA, Audus KL (2001) Modulation of P-glycoprotein activity in Calu-3 cells using steroids and beta-ligands. Int J Pharm 228: 171-9

[39] Ehrhardt C, Kneuer C, Fiegel J, Hanes J, Schaefer UF, Kim KJ, Lehr CM (2002) Influence of apical fluid volume on the development of functional intercellular junctions in the human epithelial cell line 16HBE14o-: implications for the use of this cell line as an in vitro model for bronchial drug absorption studies. Cell Tissue Res 308: 391-400

[40] Ehrhardt C, Kneuer C, Laue M, Schaefer UF, Kim KJ, Lehr CM (2003) 16HBE14o- human bronchial epithelial cell layers express P-glycoprotein, lung resistance-related protein, and caveolin-1. Pharm Res 20: 545-51

[41] Ehrhardt C, Collnot EM, Baldes C, Becker U, Laue M, Kim KJ, Lehr CM (2005) Towards an in vitro model of cystic fibrosis small airway epithelium: characterisation of the human bronchial epithelial cell line CFBE41o. Cell Tissue Res 1-11

[42] Rothen-Rutishauser BM, Kiama SG, Gehr P (2005) A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol 32: 281-9

[43] Kim KJ, Matsukawa Y, Yamahara H, Kalra VK, Lee VH, Crandall ED (2003) Absorption of intact albumin across rat alveolar epithelial cell monolayers. Am J Physiol Lung Cell Mol Physiol 284: L458-65

[44] Wadell C, Bjork E, Camber O (1999) Nasal drug delivery--evaluation of an in vitro model using porcine nasal mucosa. Eur J Pharm Sci 7: 197-206

[45] Steimer A, Haltner E, Lehr CM (2005) Cell culture models of the respiratory tract relevant to pulmonary drug delivery. J Aerosol Med 18: 137-82

[46] Uhal BD (1997) Cell cycle kinetics in the alveolar epithelium. Am J Physiol 272: L1031-45

[47] Alcorn JL, Smith ME, Smith JF, Margraf LR, Mendelson CR (1997) Primary cell culture of human type II pneumonocytes: maintenance of a differentiated phenotype and transfection with recombinant adenoviruses. Am J Respir Cell Mol Biol 17: 672-82

[48] Elbert KJ, Schafer UF, Schafers HJ, Kim KJ, Lee VH, Lehr CM (1999) Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies. Pharm Res 16: 601-8

[49] Fuchs S, Hollins AJ, Laue M, Schaefer UF, Roemer K, Gumbleton M, Lehr CM (2003)

Differentiation of human alveolar epithelial cells in primary culture: morphological characterization and synthesis of caveolin-1 and surfactant protein-C. Cell Tissue Res 311: 31-45

[50] Gumbleton M, Abulrob AG, Campbell L (2000) Caveolae: an alternative membrane transport compartment. Pharm Res 17: 1035-48

[51] Kim KJ, Malik AB (2003) Protein transport across the lung epithelial barrier. Am J Physiol Lung Cell Mol Physiol 284: L247-59

[52] Matsukawa Y, Yamahara H, Yamashita F, Lee VH, Crandall ED, Kim KJ (2000) Rates of protein transport across rat alveolar epithelial cell monolayers. J Drug Target 7: 335-42

[53] Kanse SM, Kreymann B, Ghatei MA, Bloom SR (1988) Identification and characterization of glucagon-like peptide-1 7-36 amide-binding sites in the rat brain and lung. FEBS Lett 241: 209-12

[54] Richter G, Goke R, Goke B, Arnold R (1990) Characterization of receptors for glucagon-like peptide-1(7-36)amide on rat lung membranes. FEBS Lett 267: 78-80

[55] Wei Y, Mojsov S (1995) Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences. FEBS Lett 358: 219-24

[56] Hassan M, Eskilsson A, Nilsson C, Jonsson C, Jacobsson H, Refai E, Larsson S, Efendic S (1999) In vivo dynamic distribution of 131I-glucagon-like peptide-1 (7-36) amide in the rat studied by gamma camera. Nucl Med Biol 26: 413-20

[57] Vara E, Arias-Diaz J, Garcia C, Balibrea JL, Blazquez E (2001) Glucagon-like peptide-1(7-36) amide stimulates surfactant secretion in human type II pneumocytes. Am J Respir Crit Care Med 163: 840-6

[58] Benito E, Blazquez E, Bosch MA (1998) Glucagon-like peptide-1-(7-36)amide increases pulmonary surfactant secretion through a cyclic adenosine 3',5'-monophosphate-dependent protein kinase mechanism in rat type II pneumocytes. Endocrinology 139: 2363-8

[59] Adams AE, Pines M, Nakamoto C, Behar V, Yang QM, Bessalle R, Chorev M, Rosenblatt M, Levine MA, Suva LJ (1995) Probing the bimolecular interactions of parathyroid hormone and the human parathyroid hormone/parathyroid hormone-related protein receptor. 2. Cloning, characterization, and photoaffinity labeling of the recombinant human receptor. Biochemistry 34: 10553-9

[60] Urena P, Kong XF, Abou-Samra AB, Juppner H, Kronenberg HM, Potts JT, Jr., Segre GV (1993) Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 133: 617-23

[61] Usdin TB, Bonner TI, Harta G, Mezey E (1996) Distribution of parathyroid hormone-2 receptor messenger ribonucleic acid in rat. Endocrinology 137: 4285-97

[62] Hastings RH, Berg JT, Summers-Torres D, Burton DW, Deftos LJ (2000) Parathyroid hormone-related protein reduces alveolar epithelial cell proliferation during lung injury in rats. Am J Physiol Lung Cell Mol Physiol 279: L194-200

[63] Sasaki Y, Morimoto T, Saito H, Suzuki M, Ichizuka K, Yanaihara T (2000) The role of parathyroid hormone-related protein in intra-tracheal fluid. Endocr J 47: 169-75

[64] Codrons V, Vanderbist F, Verbeeck RK, Arras M, Lison D, Preat V, Vanbever R (2003) Systemic delivery of parathyroid hormone (1-34) using inhalation dry powders in rats. J Pharm Sci 92: 938-50

[65] Codrons V, Vanderbist F, Ucakar B, Preat V, Vanbever R (2004) Impact of formulation and methods of pulmonary delivery on absorption of parathyroid hormone (1-34) from rat lungs. J Pharm Sci 93: 1241-52

[66] Ahsan F, Arnold JJ, Yang T, Meezan E, Schwiebert EM, Pillion DJ (2003) Effects of the permeability enhancers, tetradecylmaltoside and dimethyl-beta-cyclodextrin, on insulin movement across human bronchial epithelial cells (16HBE14o-). Eur J Pharm Sci 20: 27-34

[67] Morishige WK, Uetake CA, Greenwood FC, Akaka J (1977) Pulmonary insulin responsivitiy: in vivo effects of insulin on the diabetic rat lung and specific insulin binding to lung receptors in normal rats. Endocrinology 100: 1710-22

[68] Shapiro DL, Livingston JN, Maniscalco WM, Finkelstein JN (1986) Insulin receptors and insulin effects on type II alveolar epithelial cells. Biochim Biophys Acta 885: 216-20

[69] Iozzo P, Osman S, Glaser M, Knickmeier M, Ferrannini E, Pike VW, Camici PG, Law MP (2002) In vivo imaging of insulin receptors by PET: preclinical evaluation of iodine-125 and iodine-124 labelled human insulin. Nucl Med Biol 29: 73-82

[70] Amit T, Barkey RJ, Guy J, Youdim MB (1987) Specific binding sites for prolactin and growth hormone in the adult rabbit lung. Mol Cell Endocrinol 49: 17-24

[71] Walker JL, Moats-Staats BM, Stiles AD, Underwood LE (1992) Tissue-specific developmental regulation of the messenger ribonucleic acids encoding the growth hormone receptor and the growth hormone binding protein in rat fetal and postnatal tissues. Pediatr Res 31: 335-9

[72] Allen JT, Bloor CA, Kedia RK, Knight RA, Spiteri MA (2000) Expression of growth hormone-releasing factor, growth hormone, insulin-like growth factor-1 and its binding proteins in human lung. Neuropeptides 34: 98-107

[73] Hill DJ, Riley SC, Bassett NS, Waters MJ (1992) Localization of the growth hormone receptor, identified by immunocytochemistry, in second trimester human fetal tissues and in placenta throughout gestation. J Clin Endocrinol Metab 75: 646-50

[74] Menon RK, Shaufl A, Yu JH, Stephan DA, Friday RP (2001) Identification and characterization of a novel transcript of the murine growth hormone receptor gene exhibiting development- and tissue-specific expression. Mol Cell Endocrinol 172: 135-46

[75] Bogazzi F, Ultimieri F, Raggi F, Russo D, Vanacore R, Guida C, Brogioni S, Cosci C, Gasperi M, Bartalena L, Martino E (2004) Growth hormone inhibits apoptosis in human colonic cancer cell lines: antagonistic effects of peroxisome proliferator activated receptor-gamma ligands. Endocrinology 145: 3353-62

[76] Wu SJ, Robinson JR (1999) Transcellular and lipophilic complex-enhanced intestinal absorption of human growth hormone. Pharm Res 16: 1266-72

[77] Patton JS, McCabe JG, Hansen SE, Daugherty AL (1989) Absorption of human growth hormone from the rat lung. Biotechnol Ther 1: 213-28

[78] Folkesson HG, Hedin L, Westrom BR (1992) Lung to blood passage of human growth hormone after intratracheal instillation: stimulation of growth in hypophysectomized rats. J

Endocrinol 134: 197-203

[79] Colthorpe P, Farr SJ, Smith IJ, Wyatt D, Taylor G (1995) The influence of regional deposition on the pharmacokinetics of pulmonary-delivered human growth hormone in rabbits. Pharm Res 12: 356-9

[80] Bosquillon C, Preat V, Vanbever R (2004) Pulmonary delivery of growth hormone using dry powders and visualization of its local fate in rats. J Control Release 96: 233-44

[81] Caillard I, Tome D (1995) Transport of beta-lactoglobulin and alpha-lactalbumin in enterocyte-like Caco-2 cells. Reprod Nutr Dev 35: 179-88

[82] John TA, Vogel SM, Tiruppathi C, Malik AB, Minshall RD (2003) Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer. Am J Physiol Lung Cell Mol Physiol 284: L187-96

[83] John TA, Vogel SM, Minshall RD, Ridge K, Tiruppathi C, Malik AB (2001) Evidence for the role of alveolar epithelial gp60 in active transalveolar albumin transport in the rat lung. J Physiol 533: 547-59

[84] Ghinea N, Fixman A, Alexandru D, Popov D, Hasu M, Ghitescu L, Eskenasy M, Simionescu M, Simionescu N (1988) Identification of albumin-binding proteins in capillary endothelial cells. J Cell Biol 107: 231-9

[85] Shah D, Shen WC (1994) The establishment of polarity and enhanced transcytosis of transferrin receptors in enterocyte-like Caco-2 cells. J Drug Target 2: 93-9

[86] Franklin WA, Folkvord JM, Varella-Garcia M, Kennedy T, Proudfoot S, Cook R, Dempsey EC, Helm K, Bunn PA, Miller YE (1996) Expansion of bronchial epithelial cell populations by in vitro culture of explants from dysplastic and histologically normal sites. Am J Respir Cell Mol Biol 15: 297-304

[87] Widera A, Beloussow K, Kim KJ, Crandall ED, Shen WC (2003) Phenotype-dependent synthesis of transferrin receptor in rat alveolar epithelial cell monolayers. Cell Tissue Res 312: 313-8

[88] Foster KA, Avery ML, Yazdanian M, Audus KL (2000) Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. Int J Pharm 208: 1-11

[89] Widera A, Bai Y, Shen WC (2004) The transepithelial transport of a G-CSF-transferrin conjugate in Caco-2 cells and its myelopoietic effect in BDF1 mice. Pharm Res 21: 278-84

[90] Widera A, Kim KJ, Crandall ED, Shen WC (2003) Transcytosis of GCSF-transferrin across rat alveolar epithelial cell monolayers. Pharm Res 20: 1231-8

[91] Hastings RH, Wright JR, Albertine KH, Ciriales R, Matthay MA (1994) Effect of endocytosis inhibitors on alveolar clearance of albumin, immunoglobulin G, and SP-A in rabbits. Am J Physiol 266: L544-52

[92] Bernaudin JF, Bellon B, Pinchon MC, Kuhn J, Druet P, Bignon J (1982) Permeability of the blood-air barrier to antiperoxidase antibodies and their fragments in the normal rat lung. Am Rev Respir Dis 125: 734-9

[93] Shah U, Dickinson BL, Blumberg RS, Simister NE, Lencer WI, Walker WA (2003) Distribution of the IgG Fc receptor, FcRn, in the human fetal intestine. Pediatr Res 53: 295-301

[94] Spiekermann GM, Finn PW, Ward ES, Dumont J, Dickinson BL, Blumberg RS, Lencer WI

(2002) Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung. J Exp Med 196: 303-10

[95] Kim KJ, Fandy TE, Lee VH, Ann DK, Borok Z, Crandall ED (2004) Net absorption of IgG via FcRn-mediated transcytosis across rat alveolar epithelial cell monolayers. Am J Physiol Lung Cell Mol Physiol 287: L616-22

[96] Gonda I (2006) Systemic delivery of drugs to humans via inhalation. J Aerosol Med 19: 47-53

[97] Patton JS, Fishburn CS, Weers JG (2004) The lungs as a portal of entry for systemic drug delivery. Proc Am Thorac Soc 1: 338-44

[98] Wustneck R, Perez-Gil J, Wustneck N, Cruz A, Fainerman VB, Pison U (2005) Interfacial properties of pulmonary surfactant layers. Adv Colloid Interface Sci 117: 33-58

[99] Geiser M, Im Hof V, Siegenthaler W, Grunder R, Gehr P (1997) Ultrastructure of the aqueous lining layer in hamster airways: is there a two-phase system? Microsc Res Tech 36: 428-37

[100] Forbes B, Ehrhardt C (2005) Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm 60: 193-205

[101] Ehrhardt C, Kim KJ, Lehr CM (2005) Isolation and culture of human alveolar epithelial cells. Methods Mol Med 107: 207-16

[102] Lin H, Yoo JW, Roh HJ, Lee MK, Chung SJ, Shim CK, Kim DD (2005) Transport of anti-allergic drugs across the passage cultured human nasal epithelial cell monolayer. Eur J Pharm Sci 26: 203-10

[103] Ehrhardt C (2003) Characterisation of epithelial cell culture models of the lung for in vitro studies of pulmonary drug delivery; Thesis, Saarbruecken

[104] Ehrhardt C, Kneuer C, Bies C, Lehr CM, Kim KJ, Bakowsky U (2005) Salbutamol is actively absorbed across human bronchial epithelial cell layers. Pulm Pharmacol Ther 18: 165-70

[105] Rabinowitz JD, Lloyd PM, Munzar P, Myers DJ, Cross S, Damani R, Quintana R, Spyker DA, Soni P, Cassella JV (2006) Ultra-fast absorption of amorphous pure drug aerosols via deep lung inhalation. J Pharm Sci 95: 2438-51

[106] Bastacky J, Lee CY, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA (1995) Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. J Appl Physiol 79: 1615-28

[107] Fiegel J, Ehrhardt C, Schaefer UF, Lehr CM, Hanes J (2003) Large porous particle impingement on lung epithelial cell monolayers--toward improved particle characterization in the lung. Pharm Res 20: 788-96

[108] Weda M, Zanen P, de Boer AH, Barends DM, Frijlink HW (2004) An investigation into the predictive value of cascade impactor results for side effects of inhaled salbutamol. Int J Pharm 287: 79-87

[109] Limbach LK, Li Y, Grass RN, Brunner TJ, Hintermann MA, Muller M, Gunther D, Stark WJ (2005) Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. Environ Sci Technol 39: 9370-6

[110] Laube BL (2005) The expanding role of aerosols in systemic drug delivery, gene therapy, and vaccination. Respir Care 50: 1161-76

[111] Patton JS, Bukar J, Nagarajan S (1999) Inhaled insulin. Adv Drug Deliv Rev 35: 235-247

[112] Luo XY, Hinton JS, Liew TT, Tan KK (2004) LES modelling of flow in a simple airway model. Med Eng Phys 26: 403-13

[113] Albrecht C, Knaapen AM, Becker A, Hohr D, Haberzettl P, van Schooten FJ, Borm PJ, Schins RP (2005) The crucial role of particle surface reactivity in respirable quartz-induced reactive oxygen/nitrogen species formation and APE/Ref-1 induction in rat lung. Respir Res 6: 129

[114] Aufderheide M, Mohr U (1999) CULTEX--a new system and technique for the cultivation and exposure of cells at the air/liquid interface. Exp Toxicol Pathol 51: 489-90

[115] Aufderheide M, Mohr U (2000) CULTEX--an alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface. Exp Toxicol Pathol 52: 265-70

[116] Tippe AH, U. Roth, C. (2002) Deposition of fine and ultrafine aerosol particles during exposure at the air/cell interface. Journal of Aerosol Science 207-218

[117] Bitterle E, Karg E, Schroeppel A, Kreyling WG, Tippe A, Ferron GA, Schmid O, Heyder J, Maier KL, Hofer T (2006) Dose-controlled exposure of A549 epithelial cells at the air-liquid interface to airborne ultrafine carbonaceous particles. Chemosphere

[118] Schreier H, Gagne L, Conary JT, Laurian G (1998) Simulated lung transfection by nebulization of liposome cDNA complexes using a cascade impactor seeded with 2-CFSME0-cells. J Aerosol Med 11: 1-13

[119] Cooney D, Kazantseva M, Hickey AJ (2004) Development of a size-dependent aerosol deposition model utilising human airway epithelial cells for evaluating aerosol drug delivery. Altern Lab Anim 32: 581-90

[120] Niemeier RW (1984) The isolated perfused lung. Environ Health Perspect 56: 35-41

[121] Tronde A, Norden B, Jeppsson AB, Brunmark P, Nilsson E, Lennernas H, Bengtsson UH (2003) Drug absorption from the isolated perfused rat lung--correlations with drug physicochemical properties and epithelial permeability. J Drug Target 11: 61-74

[122] Lehr CM (2001) [In vitro models of intestinal and alveolar epithelium cultures in pharmaceutical research]. Altex 18: 59-63

[123] Gueven N, Glatthaar B, Manke HG, Haemmerle H (1996) Co-cultivation of rat pneumocytes and bovine endothelial cells on a liquid-air interface. Eur Respir J 9: 968-75

[124] Hermanns MI, Unger RE, Kehe K, Peters K, Kirkpatrick CJ (2004) Lung epithelial cell lines in coculture with human pulmonary microvascular endothelial cells: development of an alveolo-capillary barrier in vitro. Lab Invest 84: 736-52

[125] Wottrich R, Diabate S, Krug HF (2004) Biological effects of ultrafine model particles in human macrophages and epithelial cells in mono- and co-culture. Int J Hyg Environ Health 207: 353-61

[126] Ehrhardt C, Fiegel J, Fuchs S, Abu-Dahab R, Schaefer UF, Hanes J, Lehr CM (2002) Drug absorption by the respiratory mucosa: cell culture models and particulate drug carriers. J Aerosol Med 15: 131-9

[127] Florea BI, Cassara ML, Junginger HE, Borchard G (2003) Drug transport and metabolism characteristics of the human airway epithelial cell line Calu-3. J Control Release 87: 131-8

[128] Nancy K. Wills LR, Simon A. Lewis (1996) Epithelial Transport. Chapman & Hall,

[129] Farr SJ, McElduff A, Mather LE, Okikawa J, Ward ME, Gonda I, Licko V, Rubsamen RM (2000) Pulmonary insulin administration using the AERx system: physiological and physicochemical factors influencing insulin effectiveness in healthy fasting subjects. Diabetes Technol Ther 2: 185-97

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13 List of publications

Scientific publications

Bur M, Huwer H, Lehr CM, Hagen N, Guldbrandt M, Kim KJ, Ehrhardt C; Assessment of transport rates of proteins and peptides across primary human alveolar epithelial cell monolayers; European Journal of Pharmaceutical Sciences. 2006 Jun; 28(3): 196-203.

Bur M, Huwer H, Muys L, Lehr CM; Effect of air versus liquid interface deposition on transport of salbutamol sulphate and budesonide across pulmonary cell monolayers; Pharmaceutical Research submitted

Bur M, Rothen-Rutishauser B, Lehr CM; A novel cell compatible impingement system to study drug absorption after pharmaceutical aerosol inhalation; Journal of Aerosol Medicine submitted

Bur M, Henning A, Lehr C-M, Alveolar epithelial cell culture – a useful tool in aerosol drug delivery research, Respiratory Drug delivery X; Biological, Pharmaceutical, Clinical, and Regulatory Issues Relating to Optimized drug Delivery by Aerosol; Boca Raton Resort and Club, Florida, April 23-27, 2006.

Bur M, Anabousi S und Lehr CM; Transport von Arzneistoffmolekülen über die zelluläre Luft-Blut-Schranke, in G. Scheuch (ed.) "Aerosole in der Inhalationstherapie IX", dustriTBmed, München, (2005), pp.38-51.

Bur M, Bock U, Haltner-Ukomadu E and Lehr CM; In vitro Models for Pulmonary Drug Absorption", in K. Bechtold-Peters; H. Luessen (ed.) "Pulmonary Drug Delivery", Editio Cantor Verlag, Aulendorf, (2007), pp.58-81.

Oral presentations

Bur M, Ehrhardt C, Kim K.J, Lehr C.M, Bioelectric properties of primary cultured tight monolayers of human alveolar epithelial cells, DPhG Jahrestagung und Joint-Meeting 2004, October 7-9, 2004

Bur M, Lehr CM; Between deposition and absorption: water solubility, particle size, and apical liquid volume as critical factors to epithelial transport of aerosol drugs International Society for Aerosols in Medicine 2007, Tours, June 16 - 20, 2007

Poster/Abstracts

Bur M, Ehrhardt C, Kim K.J, Lehr C.M, Bioelectric properties of primary cultured tight monolayers of human alveolar epithelial cells, DPhG Jahrestagung und Joint-Meeting 2004, October 7-9, 2004

Bur M, Lehr CM, IL-8 release from bronchial epithelial cell monolayers (Calu-3) under impingement by pharmaceutical excipients, IX. Deutsches Aerosol Therapie Seminar, Nürnberg, Germany, November 12 - 13; 2004, 2004, awarded with a poster award for outstanding scientific effort

Ehrhardt C, Bur M, Schaefer UF, Lehr CM and Kim KJ; Bioelectric properties of primary cultured tight monolayers of human alveolar epithelial cells, FASEB J., (2004), 18(4):A723.

Bur M, Schaefer UF, Lehr CM, Cell culture models of the air-blood barrier for the development of new aerosol medicines, 5th World Congress on Alternatives & Animal Use in the Life Sciences, August 21-25, Berlin 2005

Bur M, Lehr CM, Cell culture compatible aerosol impinger to study drug transport across pulmonary epithelia in vitro; 33rd Annual Meeting & Exposition of the Controlled Release Society, Vienna, July 22 – 26, 2006

Bur M, Lehr CM; Between deposition and absorption: water solubility, particle size, and apical liquid volume as critical factors to epithelial transport of aerosol drugs International Society for Aerosols in Medicine 2007, Tours, June 16 - 20, 2007

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