European Journal of Pharmaceutics and Biopharmaceutics 72 (2009) 350–357

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

A novel cell compatible impingement system to study in vitro drug absorption from dry powder aerosol formulations

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ARTICLE INFO

Article history:
Received 29 April 2008
Accepted in revised form 27 July 2008
Available online 17 August 2008

Keywords:
Calu-3
Air interface deposition
Cell compatible impingement system
Multi stage liquid impinger
Transport experiment
Dry powder aerosol

ABSTRACT

A modified Astra type multistage liquid impinger (MSLI) with integrated bronchial cell monolayers was used to study deposition and subsequent drug absorption on in vitro models of the human airway epithelial barrier. Inverted cell culture of Calu-3 cells on the bottom side of cell culture filter inserts was integrated into a compendial MSLI. Upside down cultivation did not impair the barrier function, morphology and viability of Calu-3 cells. Size selective deposition with subsequent absorption was studied for three different commercially available dry powder formulations of salbutamol sulphate and budesonide. After deposition without size separation the absorption rates from the aerosol formulations differed but correlated with the size of the carrier lactose particles. However, after deposition in the MSLI, simulating relevant impaction and causing the separation of small drug crystals from the carrier lactose, the absorption rates of the three formulations were identical, confirming the bioequivalence of the three formulations.

1. Introduction

Progress in the development of modern inhalers and the upcoming aerosol formulation technology allow effective and safe treatment of local as well as systemic diseases by inhalation [1,2]. The inhalable insulin formulation Exubera® also induced euphoria for pulmonary drug delivery and demonstrated simultaneously the feasibility of successful large molecule administration by the inhalative route.

In vitro test systems are hardly substitutable in the development of new pharmaceutical aerosols. On the one hand, deposition in the different regions of the lung can be simulated with the aid of aerosol classification devices such as multistage liquid impinger (MSLI), next generation impactor or Andersen impactor. On the other hand, biological interactions of aerosols with the air–blood barrier such as absorption, toxic effects or clearance can be tested with cell culture experiments. But test systems combining deposition and absorption – as likewise critical steps of successful aerosol drug application – are still rare in pharmaceutical sciences. However, in environmental toxicology test systems in combination with cell culture systems [3,4] are more widely used to investigate the toxicology of dust, nanoparticles or air pollution. But the typical long-time and low-dose application in environmental toxicology is not comparable with the short-time bolus inhalation for therapeutic purposes. Also, the investigated particle–cell interactions in pharmaceutical sciences are quite different from environmental toxicology aims and objectives. Permeation and drug release are of same interest as biocompatibility and degradation of the formulation.

To measure drug absorption, tight cell monolayers generated from relevant cell types are needed. A549 cells, which fail to build up tight monolayers, or a single culture of macrophages allows statements regarding the cytotoxicity, but allows no quantification of drug permeation across the epithelial barrier. Also, submersed cell culture systems are of limited value for the question of the interaction between inhaled particles and cells lining the alveolar space. For the cells as well as for the particles, significant differences arise from the application of particles in the solid state on a more or less dry cellular surface instead of the application of a suspension or even solution of particles on submersed cells. The human lung in healthy patients is covered with very thin layers of pulmonary surface liquids. In the deep lung only few nanometers of surfactant [5] disassociate deposited particles from the apical cell membranes. Surfactant, a phospholipid mixture with tensid character, decreases the surface tension [6] and prevents the collapse of the alveoli. Even in the conducting airways the thickness of the high viscous mucus layers is in the range of few micrometers

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doi:10.1016/j.ejpb.2008.07.019

and therefore rather different from the situation in submersed cultivated filter inserts, where fluid layers of several millimeters are covering the cell monolayer.

Small particles with a diameter of 1 μm – leading to preferential deposition on the slightly wetted alveolar epithelium in the deep lung after inhalation – can interact after landing only with small volumes of the fluid phase. Dissolution, similar to that observed in submersed systems, can be limited by finite fluid volume. Slow erosion processes starting from the bottom by the lining fluid and on the whole surface by the humidified air space should lead to a slower degradation and erosion than the immersion of the particles in large fluid volumes. Also particle properties such as zeta potential or radicals on the surface can be influenced in a submersed system. In dependency of the particle size, it could be shown that ultrafine particles do not sediment in submersed cell culture systems, and in consequence reduced cellular uptake of ultrafine particles was reported [7].

Therefore, the best possible deposition/permeation system for pharmaceutical investigations of particle–cell interaction after inhalation will probably consist of an air exposed tight cell monolayer and a deposition system providing the imitation of bolus inhalation. The European Pharmacopoeia describes different aerosol classification systems. Especially, the Astra type multistage liquid impinger (MSLI) seems to reflect the in vivo deposition pattern sufficiently precisely, and is one of the most used impingement system in pharmaceutical research [8]. Impaction-, gravimetric- and sedimentation forces represent very realistically the in vivo deposition mechanisms. A thin liquid layer on the stages simulates the surfactant layer in the lung and prevents the rebound of particles after impaction. The common acceptance as well as simple construction and handling recommends the MSLI as a good development basis for cell compatible pharmaceutical impingement systems.

However, not only the impingement system, but also the inserted cell culture is a critical factor for the successful simulation of the deposition of pharmaceutically relevant aerosols on the lung surface. Essentially, the cells have to build up a tight and rigid transport barrier. Hence, A549 cells, which express even under optimized culture conditions [9] only electrical epithelial resistance of less than 200 Ohm cm^2, do not present a realistic absorption model of the tight in vivo air–blood barrier.

In addition to the tightness of the cell monolayer the requirement of an air interface culture (AIC) exists. Different pulmonary immortalized cells cultivated on air interface express mucus and other bronchial typical features such as cilia [10]. Particularly, monolayers of Calu-3 cells have been proven of their suitability as a model of the human airway epithelial barrier [11,12].

An integration of cell monolayers in an impinger is described in several publications. First of all, the works from Cooney [13] and Fiegel [14] have to be cited, but in both cases the cell monolayers

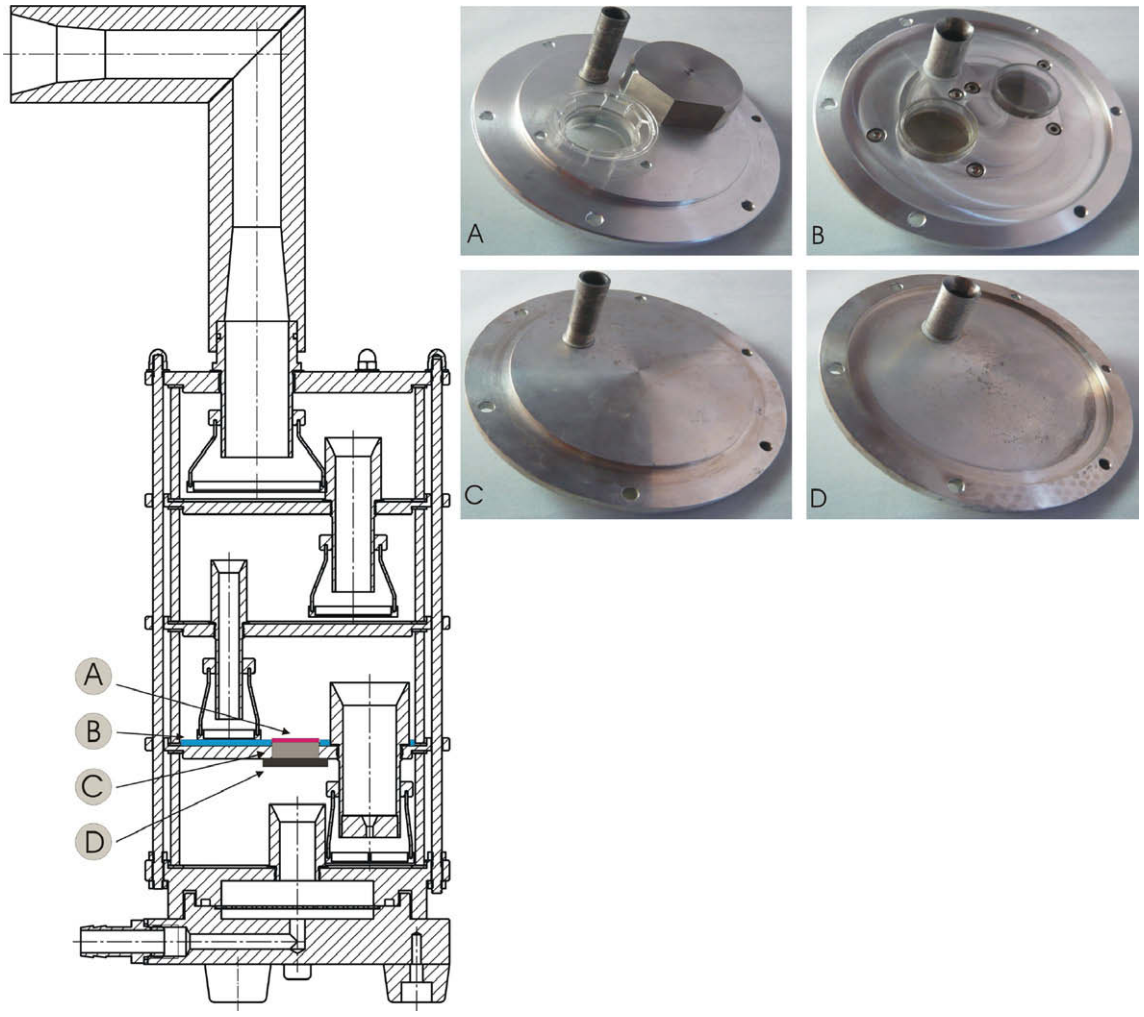

Fig. 1. Cross-section of the modified multistage liquid impinger (A: cell monolayer B: fluid layer on the stage C: filter insert D: stainless steel capsulae) and the picture of a modified second stage with inserted Transwell® filter in comparison to a non-modified stage.

were simply placed under the nozzles of the impinger stages. In consequence, the bowel-like form of the filter inserts caused turbulences in the whole stage and especially in the filter inserts a low deposition efficacy was observed. To increase the deposition efficacy and to decrease the cell stress, we decided to develop a cell compatible aerosol deposition model, which integrates cell monolayers without any turbulences in the bottom of the stage.

A simple but effective method offers the cultivation of the cells on the underside of filter inserts (Transwell®) and the insertion of the inverted filters from the under stage through holes in the surface of the stage of the MSLI (see Fig. 1).

In the following study, we investigated first the influence of the impinger modification on the deposition of aerosols on the inserted cell cultures. In addition, unchanged morphology and transport barrier function of the inverted Calu-3 cell culture were demonstrated. The suitability of the modified impingement system for pharmaceutical aerosol deposition and subsequent absorption measurement was confirmed by deposition and transport experiments with different commercially available dry powder aerosol formulations.

2. Methods and materials

2.1. Cell culture

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 of the cell culture plates – holes with a diameter of 25 mm instead of 22 mm – was necessary to allow the inversion of the filter inserts (see Fig. 2) 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.

Calu-3 cells (passage number 41–46) were seeded onto the bottom side of the 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. The seeding in the inverted style was necessary to allow the adherence of the cells on the cell culture insert membrane. Immediately after seeding, cells were grown in 500 μl apical and 2000 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 °C in a 5% CO_2 incubator. The inserts were turned around two days after seeding and after adhesion of the cells (liquid interface culture LIC). In the case of air interface culture, the bottom side-seeded filter inserts were not inverted, and cells cultivated under air interface culture (AIC) conditions were fed for 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.

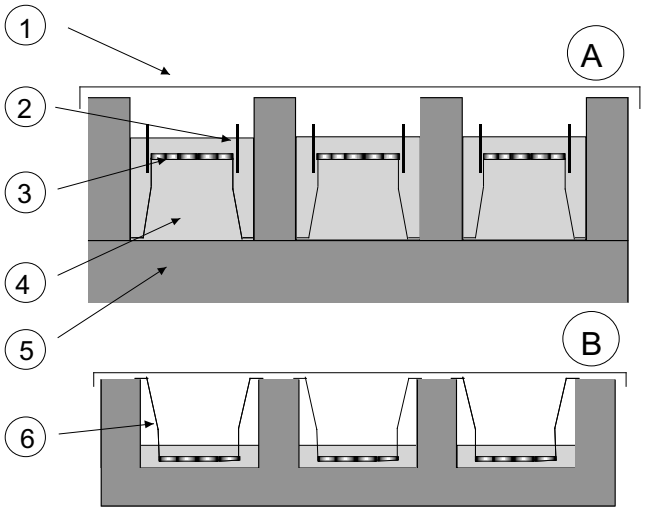

Fig. 2. The principle of the inverted cell culture.1: lid 2: cartridge 3: cell monlayer 4: cell culture medium 5: stainless steel culture plate 6: filter insert A: inverted style; B: normal style.

2.2. Bioelectric measurements

The development of a tight cell monolayer during culture was controlled by measuring the transepithelial electrical resistance (TEER) every other day. To assess the integrity of the cell layer during the transport experiment, the TEER across the monolayers was measured before and after each transport experiment. The long-time effect of inverted culture condition was analyzed by monitoring TEER for 1–14 days after seeding. Experiments were carried out with n = 4–22 filter inserts. TEER was measured with an EVOM device (WPI, Berlin, Germany) equipped with chopstick electrodes. In the case of air interface culture the apical compartment was filled with prewarmed medium before the measurement, equilibrated for 1 h and thereafter removed again.

2.3. Fluorescein–sodium transport across monolayers in submersed culture

The transport of fluorescein–sodium (flu–Na) across the cell monolayers was performed to determine the differences in the barrier function in consequence of the culture conditions. Experiments were carried out with n = 6, using cells in the age of 10–15 days from five different passages. 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 and pH 7.4) was used as a transport buffer. Flu-Na solution (50 μM in KRB) was added to the apical (500 μl) or basolateral (1500 μl in the normal culture and 2000 μl in the inverted culture) compartment of each well. The cell monolayers were agitated using an orbital shaker at a constant stirring rate (100 rpm) at 37 °C 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 by

$$P_{app} = (dQ/dt)/(A * C_0)$$

where dQ/dt (mol/s) is the transport rate and indicative of the increase in the concentration of the drug in the receiver chamber per time interval. A (cm^2) is the surface area of the cell culture support, and C_0 (mol/ml) is the initial drug concentration in the donor compartment.

2.4. Modification on the MSLI

The MSLI consists of a mouthpiece, 4 impaction stages, each containing 10 ml KRB during operation, and a final filter stage. The liquid in the impinger can be expected to reduce particle bounce and re-entrainment. A commercially available MSLI was modified by Erweka (Heussenstamm, Germany) with the objective of integration of filter grown cell monolayers in the bottom of the stages. Two holes were drilled in the bottom of the stages 2 and 3. The inner diameter of the holes was exactly the same as the external diameter of the commercially available Transwell® (model 3460) filter inserts. After adoption of the filter inserts in the holes, the underside of the filter insert is in the upper stage and some parts of the filter insert protrude in the lower stage. To minimize turbulences in the air streams, the parts protruding in the lower stage were encapsulated (see Fig. 1A). The filter membrane of the adopted transwell insert is perfectly integrated in the stage lining fluid (10 ml), and the cells grown on the top of the membrane are flushed with the fluid layer in the stage (see Fig. 1B). To avoid fluid spills over the cell monolayer, the MSLI was operated with an Erweka Vacuum Pump (H.D.-Pump) at a pump rate of 30 l/min on a shock-absorbing desk. 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 the amount of air.

2.5. Determination of the deposition pattern after modification

Flu-Na solution (13 μM, prepared with KRB) 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 the excitation and emission wavelengths of 485 and 530 nm, respectively.

2.6. Budesonide and salbutamol sulphate transport after deposition on AIC Calu-3 monolayers in the modified impinger

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). All the used powder formulations consist of large carrier lactose particles and adherent micronized drug particles. 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 after powder application, the filters were placed upside down in the modified stainless steel plate and incubated at 37 °C. The basolateral compartment was filled with KRB. Samples were taken after determined time points from the receptor compartment, and the amount of transported drug was quantified by HPLC.

Furthermore, the same experiment was carried out a second time with normally cultivated Calu-3 cells by powder application with a spatula on the air exposed cellular surface, and a third time after dissolving of the formulations in buffer and pipetting the resulting solution to the liquid-filled apical cell compartment. A donor concentration of 1000 μM for salbutamol sulphate and 30 μM for the higher permeable budesonide was planned. In all experiments, the actual deposited drug amount was determined after the experiment by solving the cells with Dimethylsulfoxide, unifying apical and basolateral compartments and measuring the total drug concentration.

2.7. Particle size determination

The size of the particles in the generic salbutamol and budesonide formulations was determined with the aid of laser diffractometry (Sympatec, dry disperser RODOS/M equipped with vibratory feeder VIBRI, Clausthal-Zellerfeld, Germany).

2.8. HPLC analytics

The deposited and transported amounts of salbutamol sulphate were analysed by reversed phase HPLC on a Dionex System comprised of a P690 pump, ASI100 automated sampler and UVD 340 U UV/VIS detector (Dionex, Idstein, Germany). A Lichrospher® C_8 column (125 mm × 4.0 mm i.d., Merck, Darmstadt, Germany) was used, and the samples were analysed at 276 nm using a triethylamine-phosphate buffer (pH 6.0): methanol (10:90, v/v) eluent at a flow rate of 1.0 ml/min and column temperature of 40 °C.

A similar HPLC method was utilised for the budesonide quantification. The mobile phase consisted of phosphate buffer, pH 2.5, and acetonitrile (60:40, v/v). The flow rate was chosen as 1.7 ml/min and the column temperature was maintained at 40 °C.

Chromatograms were analysed by estimating the area under the peak in the curve by employing a computerised data integration program (Chromeleon 6.50, Dionex). Samples were diluted with KRB, where appropriate. Acetonitrile (HPLC grade), monobasic potassium phosphate (HPLC grade), hydrochloric acid and all other chemicals were purchased from Sigma Aldrich (Steinheim, Germany).

2.9. Specimen Preparation for SEM (Scanning 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 10 nm. Scanning electron micrographs were recorded on a PhilipsXL30 SEM (FEI Co. Philips Electron Optics, Zuerich, Switzerland) at 10 kV.

2.10. Transmission electron microscopy

For TEM analysis cells were fixed with 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. The cells were post-fixed 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).

2.11. Measurement of Interleukin-8

Cytokine concentrations were quantified using commercially available sandwich ELISA kits (PromoCell, Heidelberg, Germany). In brief, quantification of IL-8 was done 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 5000 pg/ml, was utilised as a 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 the

samples and standards, and then of the secondary antibody, was done on a plate agitator for 1 h at 37 °C. 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 was done 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).

3. Results

The first comparison of the morphology of normal and inverted Calu-3 cell cultures was performed by electron microscopy. In transmission mode as well in scanning mode no significant differences in the dimensions and in the structure are detectable. During the first few 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 on day 10, the cells got more and more flattened, and on day 17 no essential optical difference between the two cell cultures was visible (see Fig. 3). A belt of tight-junctions surrounding the cell bodies and ciliary structures were detectable in the scanning electron microscopic images independent of the culture style.

Nevertheless, the inverted cell culture method led to a weaker electrical resistance, which was measured with the aid of an EVOM. Furthermore, the inverted cell culture developed time delayed increase of transepithelial resistance. The TEER of the normal culture peaked on day 9 (TEER 957.2 ± 239.4 Ohm cm^2; $n = 18$), in contrast to the lower maximum in the inverted cell culture on day 12 after seeding (TEER 735.83 ± 299.7 Ohm cm^2; $n = 6$). However, statistically significant differences in the TEER values between normal and inverted cultures were not found (see Fig. 4).

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

Also in the interleukin 8 levels – as a marker for cellular stress – in normal and inverted cultured Calu-3 cells, we found no statistically significant difference. The inverted cell culture led 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; $n = 6$).

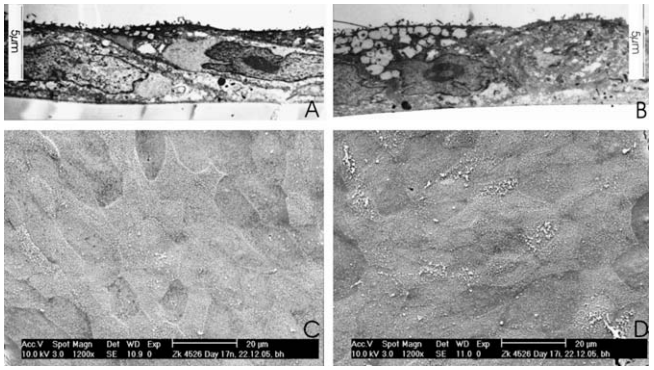

Fig. 3. Electron microscopic pictures of Calu-3 cells in the age of 17 days. Pictures A (normal style) and B (inverted style) show cross-section. Whereas pictures C (normal style) and D (inverted style) give an impression about the surface properties of the tight Calu-3 cell monolayer; A and D are transmission electron micrographs, C and D are scanning electron micrographs.

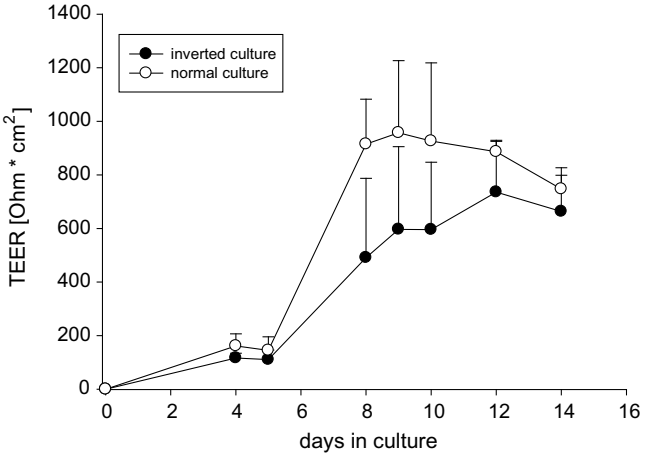

Fig. 4. TEER development in dependency of the culture style, black dots = inverted style and white dots = normal style (data represent mean ± SD; $n = 6$–22; no significant differences found by statistical analysis).

Flu-Na droplets generated by a PARI® nebulizer were used to check the deposition pattern after modification. The proportional deposited amounts in mouthpiece, stages and filters were compared. Modifications solely in stage 2 or in stage 3 resulted in a significantly increased deposition on the modified stage itself. But in the case of simultaneous modification of stage 2 and stage 3, we found no significant changed deposition patterns in comparison to the non-modified MSLI (see Fig. 5).

In consequence, deposition of the dry powder aerosols on Calu-3 cells was accomplished in a MSLI modified on both stages.

The two drugs, salbutamol sulphate and budesonide, were chosen as the model drugs. Salbutamol sulphate represents a drug of the biopharmaceutics classification system (BCS) class III characterized by a low solubility and a high permeability. The highly permeable but lowly soluble budesonide is classified in class II of the BCS.

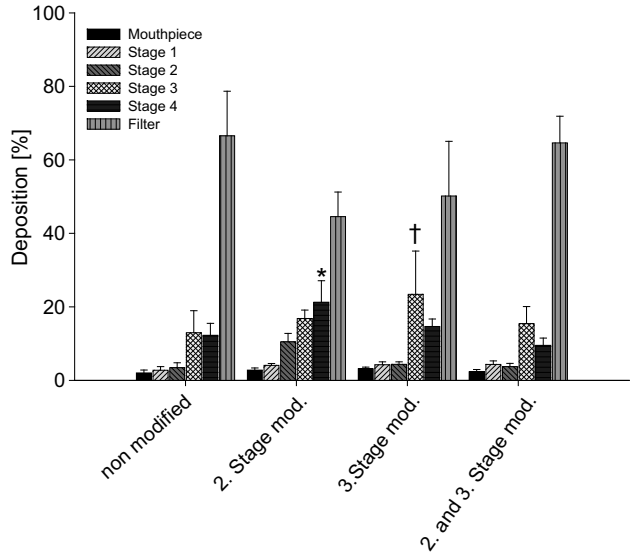

Fig. 5. Deposition of flu-Na droplet aerosols in normal MSLI and modified MSLI. †,*Significant differences from the non-modified MSLI (data present mean ± standard deviation; $n = 8$).

The absorption rate of the model aerosols was tested in three different setups. In the first experiment, solutions prepared from the drug powders were applied to a submersed cell culture. In the second experiment, a weighted amount of the drug powder was added on the air exposed cell surface with a spatula. In the third experiment, the dry powder formulations were aerosolized in the modified MSLI and deposited on air exposed cell cultures. As expected, the solutions of all the three formulations showed the same low absorption rate (see Figs. 6.1. and 6.2.). However, after air interface deposition with a spatula the transported drug amounts were approximately 30 times higher for salbutamol sulphate and 3 times higher for budesonide after 4 h compared to the liquid interface deposition experiment. But at the same time, application of the powder with the spatula resulted in a significantly slower salbutamol sulphate transport from Novolizer®, and a significantly faster absorption rate of budesonide from the Autoinhaler® powder. These differences disappeared after powder impingement with the MSLI, and equivalent high absorption rates for all the formulations could be detected.

4. Discussion

The cultivation of cells on the bottom side of the filter inserts has already been 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 a different origin and function such as epithelial, endothelial and dendritic cells [15,16]. 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 an electrical resistance higher than 500 Ohm cm^2 [17]. Our measured Calu-3 TEER values are in good correlation to other published TEER data [18–20]. Also the development and peaking of the TEER value over time fit well with data from the 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 between normal and inverted cultured cells were found. Also the cell surface was structured in the same manner under both the culture conditions, especially the ciliary like structures and vesicles filled with mucus could be observed in both the cultures. In cross-sections, tight-junctions could be detected. The TEER increased time delayed in the inverted cell culture, which is also caused by the morphological differences on day 10. Because of the upturning of the cells 2 days after seeding, gravimetric forces combined with the cellular stress could be the source for the increased height and time delayed barrier development of the inverted cell culture.

Since the barrier function is essential for the use as transport model, the absorption of flu–Na across the cell monolayer was measured. This fluorescent hydrophilic model substance is transported mainly by passive transport through tight-junctions and intercellular spaces. Low transport rates will be measured only if functional tight-junctions are expressed. The resulting calculated P_{app} values confirmed the electrically measured tightness of the barrier.

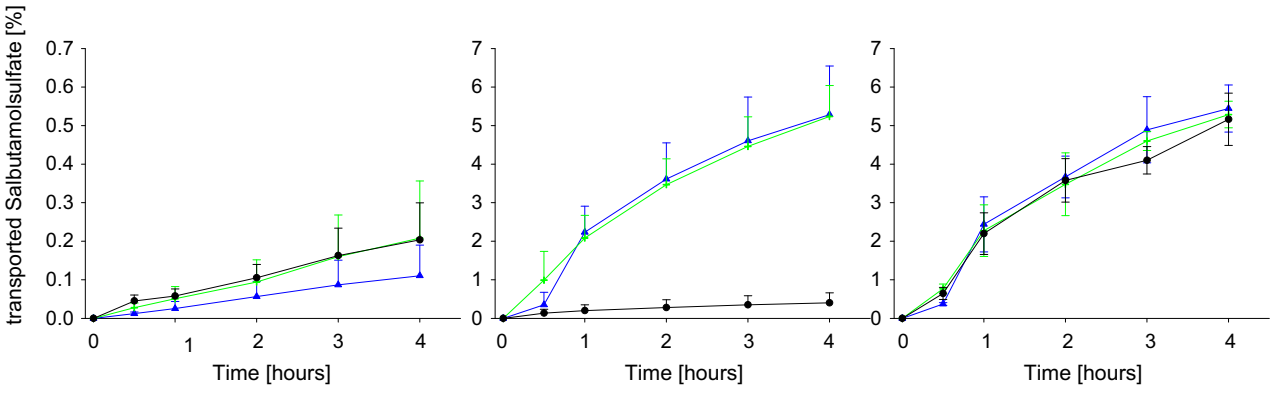

Fig. 6.1. Transport of deposited salbutamol sulphate (Δ Cyclocaps®; • Ventilastin® Novolizer®; ♦ Easyhaler®): pipetted as suspension (left side), as dry powder applied with a spatula (in the middle) or applied using a Spinhaler device and the MSLI (on the right side) (in all cases a donor concentration of 1000 μM was applied on air interface cultivated Calu-3 monolayers; data present mean ± standard deviation; $n = 6$).

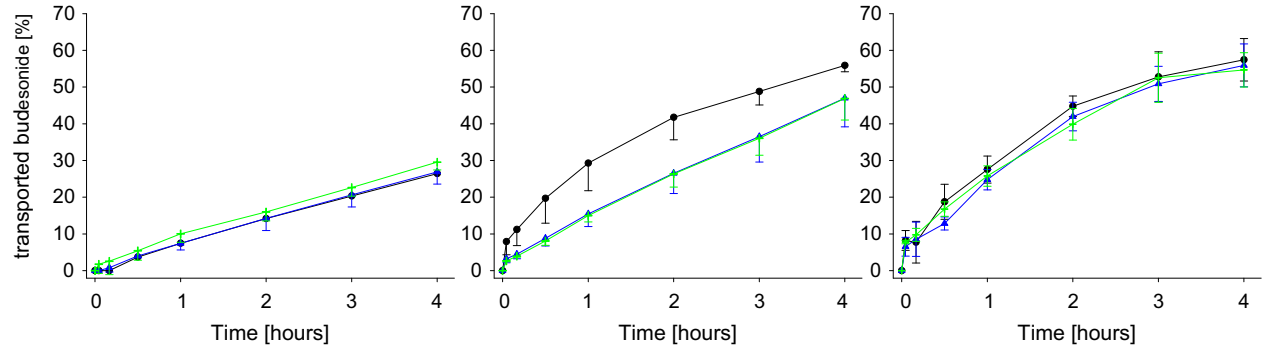

Fig. 6.2. Transport of deposited budesonide (Δ Cyclocaps®; • Autoinhaler®; ♦ Easyhaler®): pipetted as suspension (left side), as dry powder with the aid of a spatula (in the middle) or applied with a Spinhaler device and the cell compatible MSLI (on the right side) (in all cases a donor concentration of 30 μM was applied on air interface cultivated Calu-3 monolayers; data present mean ± standard deviation; $n = 6$).

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

The MSLI is a well-established aerosol classification device in pharmaceutical sciences. Specified as apparatus A in the European Pharmacopoeia, the MSLI 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. The necessary modifications to integrate the cells in the stages resulted in slightly but non-significant changed deposition patterns in the case of simultaneous modification of second and third stages. The deposition pattern was controlled by collecting the polydispersed droplets from an aerosolized fluorescent solution. Further investigations of the deposition pattern were not carried out, as effective and short-time deposition of drug aerosols on air exposed cell monolayers was the main objective of the MSLI modification, and not aerosol size characterisation.

Not only the deposition on the cell surface, but also the cellular surface should be simulated as realistically as possible. In vivo, the fluid layer seems to play a major role in the complex network of particle uptake and elimination. After landing, soluble drug particles will dissolve rather quickly in the more or less infinite lung surface fluid. The dissolution rate will be controlled not only by the volume of fluid at the place of particle deposition, but also by the composition of the lung surface lining fluid at the place of deposition. Davies et al. [21] showed that the dissolution rate of three glucocorticoids in the presence of the surfactant ingredient L–α phosphatidylcholine increased significantly in comparison to the dissolution rate in the absence of tensidic active surfactant substances. In our case, the fluid layer on the top of the Calu-3 cells consists of mucus-like structures secreted by the cells themselves.

In this work, the influence of the particle size on the dissolution rate is in the main focus. Particles with diameter larger than the thickness of the fluid layer will be only with parts of their surface in the fluid, and consequently, will dissolved slowly. In contrast, small particles can be submersed totally. Regarding the solubility and the wetting properties of the particles, complex dissolution processes resulted. After dissolution, passive or active transport processes of the substance across the cell monolayer occur. The permeation step is controlled by the cell monolayer itself and is dependent of the tightness, intrinsic permeability of the drug and expression of uptake transporters or efflux systems. In consequence, in transport experiments conducted in air interface cultured Calu-3 cell system, two serially connected processes – dissolution and permeation – can be observed. By working with drug solutions in liquid interface deposition experiments, the dissolution step is neglected.

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 crystals the resulting local high drug concentration controls the absorption rate. The so-called ultrafast absorption of drugs has been described by several authors [22,23] and explained by a large absorption surface in combination with steep concentration gradients. Also our results can be interpreted with the enormous local concentration gradients. In all cases of aerosol application, we measured higher absorption rates compared with the typically used submersed experiments.

The area under the curve was chosen as a parameter to judge the absorption rate of the model formulations of salbutamol and budesonide. Very similar absorption rates resulted after liquid interface deposition (see Table 1). 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 the 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 (see Table 1). But Ventilastin® consisted of fourfold 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 the case of the budesonide containing Autoinhaler® powder, the carrier lactose particle size was threefold smaller and particles deposited on the surface liquid can be completely submersed. Fast dissolution and absorption rates could be measured.

Table 1
Average diameter of the powder particles and area under the curve [ng/ml h] of the different salbutamol and budesonide formulations in dependency of deposition mode (the data were calculated from 6 independent experiments, and expressed as mean ± standard deviation)

		Particle Diameter X_{50} (μm)	LID AUC (ng/ml h)	AID/spatula AUC (ng/ml h)	AID/MSLI AUC (ng/ml h)
Salbutamol sulphate	Easyhaler®	49.56	0.40 ± 0.15	12.63 ± 1.78	12.87 ± 1.02
	Cyclocaps®	55.64	0.23 ± 0.19	12.74 ± 0.97	13.35 ± 0.87
	Ventilastin®	188.99	0.43 ± 0.25	1.051 ± 0.11	12.24 ± 1.07
Budesonide	Easyhaler®	58.33	55.81 ± 2.84	103.10 ± 5.16	147.27 ± 4.87
	Cyclocaps®	45.15	63.92 ± 4.97	101.65 ± 2.47	149.04 ± 5.47
	Autoinhaler®	18.00	54.90 ± 3.73	151.98 ± 8.45	157.21 ± 6.19

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 inserted cell monolayer. The fact that all drug crystals in the formulation have similar respirable diameters (between 1 and 5 μm) resulted in comparable absorption rates. Mainly, the size of the particles seems to influence the absorption rate after air interface deposition of the soluble drug particles.

While air interface deposition appears to be more relevant for testing dry powder aerosols than dissolving under liquid interface conditions, relevant results from such in vitro tests can only be expected, if one succeeds in separating the micronized drug from the different sized carrier lactose particles.

5. Conclusion

By adapting a compendial multistage liquid impinger, a prototype of a cell compatible pharmaceutical aerosol deposition model was developed. This model together with integrated lung epithelial cell monolayers allows to study transepithelial drug absorption from dry powder aerosol formulations.

The importance of the deposition step could be demonstrated for three generic formulations of the drugs budesonide and salbutamol sulphate. Only after deposition in the cell compatible impingement system, where a separation of micronized drug crystals from the carrier lactose takes place, similar absorption rates of the formulations could be recorded, confirming the bioequivalence of the pharmaceutical products.

Acknowledgments

We are grateful to A. Luginbuehl and B. Haenni for their excellent technical assistance. The authors also wish to thank G. Hierl

(ERWEKA, Heussenstamm, Germany) for the modification of the MSLI.

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