JOURNAL OF AEROSOL MEDICINE AND PULMONARY DRUG DELIVERY
Volume 30, Number 6, 2017
© Mary Ann Liebert, Inc.
Pp. 411–424
DOI: 10.1089/jamp.2016.1361

Biokinetics of Aerosolized Liposomal Ciclosporin A in Human Lung Cells *In Vitro* Using an Air-Liquid Cell Interface Exposure System

Otmar Schmid, PhD,[1,2] Corinne Jud, PhD,[3,*] Yuki Umehara,[3] Dominik Mueller,[4] Albert Bucholski,[4] Friedrich Gruber,[4] Oliver Denk, PhD,[4] Roman Egle, PhD,[4] Alke Petri-Fink, PhD,[3] and Barbara Rothen-Rutishauser, PhD[3]

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

Abstract

Background: Inhalation of aerosolized drugs is a promising route for noninvasive targeted drug delivery to the lung. Nanocarrier systems such as liposomes have been explored for inhalation therapy opening new avenues, including stabilization of nonsoluble drugs (e.g., Ciclosporin A [CsA]) and controlled release.
Methods: The biokinetic behavior of the immunosuppressive drug CsA encapsulated in liposomes (L-CsA) at the lung epithelial barrier was studied *in vitro*. Human lung epithelial cells (alveolar A549 and bronchial 16HBE14o- epithelial cells) were exposed to aerosolized L-CsA at the air–liquid interface (ALI) using a dose-controlled air-liquid interface cell exposure (ALICE) system and the temporal profile of the L-CsA dose in the apical, basal, and cell compartment was monitored up to 24 hours.
Results: Aerosolization of different volumes of L-CsA solution with the ALICE resulted in dose-controlled, spatially uniform, and reproducible L-CsA delivery. Cell viability at 24 hours postexposure was not impaired and immunofluorescence staining revealed the typical epithelial cell morphology in control as well as in L-CsA-exposed cells. The (pro-)inflammatory interleukin-8 levels were not elevated under any condition. The biokinetic analysis revealed that both cell types formed a tight, but imperfect, barrier for L-CsA resulting in initially high transbarrier L-CsA transport rates, which ceased after about 4 hours. Although substantial transbarrier L-CsA transport was observed for both cell types, respectively, a 150-fold higher L-CsA concentration was established in the apical and cell compared to the basal compartment. Most importantly, for pulmonary drug targeting, a high cellular L-CsA dose level (20%–25% of the delivered dose) was obtained rapidly (<1 hour) and maintained for at least 24 hours.
Conclusions: The ALICE system combined with lung epithelial cells cultured at the ALI offers a reliable and relevant *in vitro* platform technology to study the effects of inhalable substances such as L-CsA under biomimetic conditions.

Keywords: aerosolized liposomes, air–liquid exposures, biokinetics, ciclosporin A, lung cell cultures

Introduction

Encapsulation of drugs into liposomes has been used since the 1960s for the delivery of insoluble active substances, enhancement of tissue absorbance or protection against inadvertent enzymatic degradation, and immunological or chemical inactivation.[1] Currently, multifunctionalized nanodrug carriers (henceforth nanocarriers) are being studied intensively for combined therapeutic and diagnostic purposes (theranostics) with controlled release properties and cell-specific (e.g., cancer tissue) targeting.[2] Liposomes are artificially prepared biocompatible vesicles made of a phospholipid

[1]Comprehensive Pneumology Center (CPC), German Center for Lung Research (DZL), Munich, Germany.
[2]Institute of Lung Biology and Disease, Helmholtz Zentrum München—German Research Center for Environmental Health, Neuherberg, Germany.
[3]BioNanomaterials, Adolphe Merkle Institute, University of Fribourg, Fribourg, Switzerland.
[4]PARI Pharma GmbH, Starnberg, Germany.
*Present address: Agroscope, Competence Division Method Development and Analytics, Route de la Tioleyre 4, 1725 Posieux, Switzerland.

bilayer. Liposomes are the most clinically successful type of nanocarriers with more than 80 liposomal drug formulations on the market or in clinical trials[1] addressing indications ranging from cancer (>50% prevalence) over infectious diseases (e.g., fungal infection and hepatitis A) to vascular disease.

Currently, the lung is also considered a promising route for biomedical applications using the inhalation pathway to deliver such nanocarriers.[3] A few aerosolized liposomal formulations have been introduced for indications such as pain relief, cystic fibrosis, solid tumor, and bronchiolitis obliterans after lung transplantation. A 2009 call by the U.S. Food and Drug Administration (FDA) for the use of physiologically realistic preclinical drug testing methods[4] may have been, in part, due to the lack of easy-to-use, standardized *in vitro* test systems meeting these requirements for inhalable drugs.

One clinically relevant inhalable drug encapsulated into a nanocarrier (liposome) is ciclosporin A (CsA), which is an immunosuppressive drug widely used to reduce rejection of transplanted lungs.[5,6] The number of lung transplantations, which provide a therapeutic alternative for end-stage lung diseases, has increased significantly during recent years.[7] The only possibility to reduce the rate of acute and chronic rejection is to apply immunosuppressive drugs such as calcineurin inhibitors, antiproliferative agents, as well as corticosteroids.[8] The agents are usually given orally or by intravenous injections.

However, patients receiving these medications frequently suffer from adverse side effects. Hence, there is an urgent need to develop inhalation therapies for targeted drug delivery allowing for higher dosing at the site of disease (lung) and fewer systemic side effects than with conventional applications. Initial results with inhaled CsA or amphotericin-B are promising and Phase II clinical trials are ongoing. However, further research is needed to find an optimal drug preparation and aerosol delivery [for a review see Corcoran[9]]. The possible indication of aerosolized liposomal CsA (L-CsA) treatment is chronic lung allograft dysfunction or chronic rejection after lung transplantation, and target compartments would be the terminal and respiratory bronchioles as well as the periphery of the lung allograft, which all can be affected.[10]

For an animal model of lung transplantation, it has been shown that aerosolized pulmonary application of CsA formulated in propylene glycol did not induce any adverse effects even after 9 months of treatment. The drug was rapidly adsorbed and only low amounts remained in the lung tissue and blood after 24 hours.[11] A biokinetic study in lung transplant patients also using aerosolized CsA suspended in propylene glycol revealed a substantial delivery of CsA to the lungs. The translocation of CsA into the blood was high in the first hour, but then significantly dropped, falling below levels usually observed for oral applications.[12] A clinical study using aerosolized CsA in propylene glycol after lung transplantation did not lead to the desired improvement and raised questions regarding dosing and the suitability of the formulation.[13]

Due to the low solubility and permeability of CsA, the encapsulation of drugs in nanometer-sized liposomes is counted among the most advanced methods for targeted drug delivery [for reviews see Klyashchitsky and Owen[14] and Liu et al.[15]]. The aerosolized delivery of L-CsA in animals showed effective and selective adsorption into the lungs.[16] In addition to animal experiments, aerosolized L-CsA has already been applied in lung transplant patients, where it was well tolerated and selectively deposited in the lung.[5,6] However, association/internalization of L-CsA with/into the cells, release of the CsA in lung cells, and its molecular mechanisms are poorly understood, in part, due to a lack of suitable *in vitro* platform technologies.

Typically, preclinical testing of inhalable (liposomal) drugs is performed under submerged cell culture conditions, where the liposomal drug (or any other nanocarrier-based drug) is pipetted into the cell culture medium, which completely covers the pulmonary cell culture model.[17] This does not resemble the physiological situation of inhaled drugs in the lung, where the drug is deposited on the epithelial cells that form the air–liquid tissue barrier of the lung.[18] Upon deposition of the micron-sized aerosol droplets (or dry powder particles) containing nanosized L-CsA onto the thin layer of protective lining fluid [<70 nm thick in the alveolar region[17]], the initially high L-CsA concentration in the lining fluid will result in steep concentration gradients, which are rapidly depleted with the onset of transbarrier transport of L-CsA.

Consequently, the observed specific drug release profile for inhaled drugs is characterized by a steep initial ''burst'' and a subsequent slow absorption phase. Moreover, it is well known that submerged culturing of pulmonary cells does not allow realistic cell polarization, cell differentiation, and mucus/surfactant lining of the epithelial cells.[18] Finally, for nanocarriers suspended in cell culture media, it is difficult to determine the dose delivered to the cells,[19,20] while in air–liquid interface (ALI) settings, the aerosol is directly deposited on the cell allowing for accurate control of the cell-delivered dose.[21] Thus, the development of biomimetic *in vitro* systems allowing aerosolized, dose-controlled delivery of nanocarrier-based drugs to pulmonary barrier models cultured at the ALI is essential for efficient and reliable efficacy testing of inhalable nanocarrier-based drugs.[18]

In this work, a recently introduced air–liquid interface cell exposure (ALICE) system was used[22] to establish controlled and reproducible nebulization of reconstituted L-CsA over lung epithelial cell cultures at the ALI, mimicking a realistic inhalation of L-CsA. Two different epithelial cell lines were compared: (1) A549 cells originating from human lung carcinoma,[23] which belong to the best characterized and most widely used *in vitro* models mimicking alveolar epithelial type II cells,[24] and (2) immortalized 16HBE14o- cells, an SV-40 large T antigen transformed bronchial epithelial cell line, representing healthy human airway epithelial cells[25,26] [for a review, see Rothen-Rutishauser et al.[27]]. The cells were cultured at the ALI, a more physiological setting than the conventional submerged culture conditions, allowing direct exposure of the cells to aerosolized drugs and mimicking the *in vivo* conditions during inhalation therapy.

As described above, this not only avoids unrealistic interactions of L-CsA with cell culture medium but also allows determination of the exact dose that has been deposited on the cell wall (cell-delivered dose). The epithelial cell morphology, viability, barrier tightness, and (pro-)inflammatory responses were measured upon L-CsA exposure and the biokinetic behavior of the L-CsA formulations at the epithelial barrier was determined after various time points up to 24 hours after exposure.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

Material and Methods

Cell cultures

A549 epithelial cells. A549 cells (source: American Type Culture Collection) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (with 25 mM HEPES, without L-glutamine; Gibco, Life Technologies Europe B.V., Zug, Switzerland), supplemented with 1% penicillin G/streptomycin sulfate (P/S; 10,000 units/mL or 10,000 μg/mL, Gibco), 1% L-glutamine (L-Glut; Life Technologies Europe B.V.), and 10% fetal bovine serum (FBS Gold; PAA Laboratories, Chemie Brunschwig AG, Basel, Switzerland), subsequently referred to as the ''RPMI complete medium.''

For exposure experiments, cells were seeded in BD Falcon™ cell culture inserts (high pore density polyethylene terephthalate (PET) membranes with a growth area of 4.2 cm^2 and pores with 3.0 μm diameter; Becton Dickinson AG, Allschwil, Switzerland) placed in BD Falcon six-well tissue culture plates (Becton Dickinson AG) at a density of 0.5×10^6 cells/mL per insert with a total volume of the suspension of 2 mL. Cells were grown to confluence for 7 days under submerged conditions (2 mL RPMI complete medium in the upper and 3 mL in the lower transwell chamber) and then transferred from submerged to ALI conditions. The cell culture medium from the upper (apical) transwell chamber was removed and the cell culture medium in the lower (basal) transwell chamber was replaced by 1.2 mL of fresh medium. After an additional 24 hours in the incubator at the ALI, the cultures were used for exposure experiments.

16HBE14o- epithelial cells. The human bronchial epithelial cell line 16HBE14o- (a generous gift from Dr. D. Gruenert, Cardiovascular Research Institute, University of California, San Francisco, CA) was used as previously described.[28] Briefly, 16HBE14o- cells were maintained in Minimum Essential Medium (MEM) 1×, with Earle's Salts, 25 mM and HEPES, without L-glutamine (Gibco BRL Life Technologies Invitrogen AG, Basel, Switzerland); supplemented with 1% L-glutamine (LabForce AG, Nunningen, Switzerland), 1% penicillin/streptomycin (Gibco BRL), and 10% fetal calf serum (PAA Laboratories, Lucerna-Chem AG, Lucerne, Switzerland).

The inserts were treated with fibronectin coating solution containing 0.1 mg/mL bovine serum albumin (BSA; Sigma, Fluka Chemie GmbH, Buchs, Switzerland), 0.03 mg/mL Vitrogen 100 (Bovine Collagen Type I, FXP-019; Cohesion Technologies), and 0.01 mg/mL Human Fibronectin (40008; BD Laboratories) in LHC Basal Medium (Biosource Int'/Biofluids, 118–500; Lucerna Chemie AG) for either 3 hours at 37°C or overnight at room temperature. The culture conditions for 16HBE14o- cells were the same as described above for A549 cells.

Preparation and reconstitution of L-CsA

To prepare liposomal CsA, a buffered aqueous suspension of CsA and soy phospholipids was high-pressure homogenized followed by sterile filtration (0.2 μm) of the resulting colloidal formulation. To preserve the liposomal structure, the formulation was lyophilized in small aliquots using a sugar as lyoprotectant.

The lyophilized L-CsA was reconstituted by addition of 1.2 mL of a 0.25% NaCl solution using a syringe. After 5 minutes of slow swirling, a final concentration of 4 mg/mL (0.4% CsA) was reached. The reconstituted L-CsA suspension (stock suspension) was used on the same day.

Photon correlation spectroscopy measurements result in a typical size (measured as the z-average) of 40–100 nm for the liposomal formulation. The size range is also backed by Cryo Transmission Electron Microscopy (Cryo-TEM) showing mostly unilamellar vesicles (Fig. 1).

ALICE system and L-CsA exposure

Nebulization of L-CsA and dose-controlled aerosol deposition onto the cells was performed using the ALICE as described by Lenz et al.[22] Briefly, the ALICE consists of a nebulizer (investigational eFlow vibrating membrane nebulizer; PARI Pharma GmbH, Munich, Germany), an incubation chamber, an exposure chamber (which is connected to

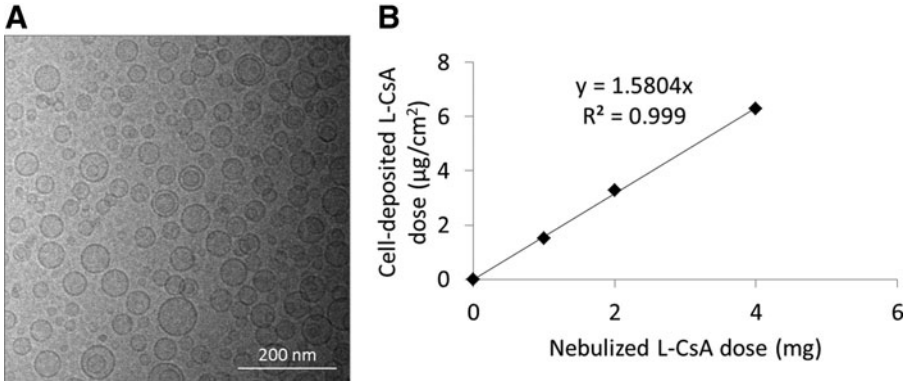

FIG. 1. Morphology and dosimetry of the L-CsA. **(A)** Cryo-TEM images show mostly unilamellar vesicles (liposomes) with a size range of 40–100 nm. **(B)** HPLC-MS/MS analytics of CsA show that there is a strong linear correlation ($R^2 = 0.999$) between the invested (nebulized) and cell-deposited L-CsA dose obtained with the ALICE system. Hence, the cell-delivered L-CsA dose can be controlled by varying the nebulized L-CsA dose. ALICE, air–liquid interface cell exposure; Cryo-TEM, Cryo Transmission Electron Microscopy; L-CsA, liposomal ciclosporin A.

a flow system providing humidity and temperature conditions suitable for exposure of up to 14 transwell inserts for cell cultivation), as well as a quartz crystal microbalance (QCM 200/25, 5 MHz; Stanford Research Systems, GMP SA, Renens, Switzerland) for real-time determination of the deposited material.

The QCM was not used in this study, since it is not able to distinguish between the mass of CsA (active agent) and liposome material (passive carrier). For exposures with variable doses, different amounts of the stock suspension (0.1, 0.25, or 0.5 mL) were nebulized within less than a minute and transported as aerosol into the exposure chamber (flow rate: 5 L/min), where it gently deposits onto cells cultured at ALI over 20 minutes. The chosen flow rate is optimal for the aerosol cloud to be sufficiently mixed and diverted to all sides of the exposure chamber, thus resulting in spatially uniform droplet deposition onto the cells cultured in transwell inserts.

As mentioned above, cells were cultured for 7 days under submerged culture conditions and then acclimatized to ALI conditions 24 hours before aerosol exposure. Two hours before the experiment, the medium was removed and 1.2 mL of fresh medium was added to the lower chamber. Control cultures (sham) were exposed to 0.25% NaCl solution (0.5 mL volume) and experimental cultures to 0.1, 0.25, or 0.5 mL of the reconstituted L-CsA suspension (i.e., 0.4, 1, and 2 mg L-CsA, respectively). The cells were then further analyzed after a 24-hour postincubation period in the incubator.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

Determination of L-CsA biokinetics

After exposure and postincubation, an aliquot of the medium (basal compartment) was collected; the cells (apical compartment) were washed thrice with 0.5 mL of an isotonic 0.9% NaCl solution to determine the CsA concentration that had adhered to the apical side of the cells. Afterward, the membranes of the inserts (carrying the cells) were cut out and placed in Eppendorf tubes, covered with 1.5 mL of sterile water, frozen in liquid nitrogen, and then thawed in a 37°C water bath; the procedure was repeated thrice to achieve complete lysis of the cells. The Eppendorf tubes were stored at −20°C until analysis. The complete medium from the well in the basal compartment was collected and stored at −20°C.

Samples were measured after appropriate sample preparation (dilution and centrifugation) by HPLC-MS/MS. The mass transfer measured was 1220–1203 Da. The method was calibrated in a concentration range from 0.025 to 2.00 μg/mL.

Lactate dehydrogenase assay

To determine cytotoxicity, supernatant was sampled and stored at 4°C for the lactate dehydrogenase (LDH) assay. To obtain a 100% reference value (positive control) representing the maximum amount of LDH that could have been released by the cells, Triton X-100 detergent (0.2% in medium) was used for cell lysis. The supernatant of cells exposed to only 0.25% NaCl was used as negative control. The LDH assay was performed with the Cytotoxicity Detection Kit (Roche Applied Science) according to the supplier's manual. Samples were diluted 1:10. LDH was quantified photometrically by measuring at 490 nm, with 630 nm as reference wavelength. Each sample was assessed in triplicate. The values were normalized to the positive control at respective postexposure times.

Dextran blue assay

Blue dextran 2000 (GE Healthcare) was used to assess the membrane integrity and tight junction formation [as described by Horvath et al.[29]] of the cells exposed to L-CsA and 0.25% NaCl. The cell culture medium was removed and the cells were washed once with 1 × phosphate-buffered saline (PBS; Gibco, Invitrogen). Then, 0.5 mL of supplemented phenol red-free medium was added to the upper (apical), and 1 mL to the lower (basal), chamber. To the upper chamber, 0.5 mL of 1% blue dextran 2000 in PBS was added and the cells were incubated for 2 hours at 37°C and under 5% CO_2. The content of the lower chamber was collected and the optical densities (proportional to dextran blue concentration) were determined photometrically (600 nm). Supplemented phenol red-free medium was used as a blank for photometric analysis. As a reference value, insert-only controls (with no cells) were used.

Laser scanning microscopy

After the postincubation period, the cells were fixed for 15 minutes in 3% paraformaldehyde (PFA) in PBS and treated with 0.1 M glycine in PBS for another 15 minutes, while stored at 4°C. To permeabilize the cell membrane, cells were treated with 0.2% Triton X-100 in PBS for 15 minutes. Phalloidin rhodamine (R-415; Molecular Probes, Life Technologies Europe B.V.) was used to stain the F-actin cytoskeleton at a 1:50 dilution, while the nucleus was stained with DAPI ([1 μg/mL] in PBS) (Sigma-Aldrich), respectively. The dyes were then incubated for 60 minutes at room temperature. For optical/microscopic analysis, samples were embedded in Glycergel (DAKO Schweiz AG, Baar, Switzerland). Visualization of the samples was achieved by using an inverted laser scanning confocal microscope (LSM 710, Axio Observer.Z1; Carl Zeiss). Image processing was performed using the restoration software IMARIS (Bitplane AG, Zurich, Switzerland).

Interleukin-8 quantification

Protein release of the (pro-)inflammatory mediator interleukin-8 (IL-8) was quantified by a commercially available DuoSet ELISA Development Kit (R&D Systems) according to the manufacturer's protocol. Tumor necrosis factor alpha (TNF-α) was used as a positive control at a concentration of 15 ng/mL.

Statistics

To investigate the significance ($p < 0.05$) of the blue dextran, LDH, and cytokine measurements, the GraphPad Prism software for Windows (Version 6; GraphPad Prism, Inc., La Jolla, CA) was used. With one-way analysis of variance (ANOVA), pairwise multiple comparison procedure (Student-Newman-Keuls) was tested. Unless stated otherwise, results are presented as mean ($n = 3$) ± standard error of the mean (SEM).

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

Results

Determination of the delivered L-CsA dose

A six-well plate with 3 mL completed cell culture medium in each well (surface area of 9.6 cm^2 per well) was placed into the ALICE chamber and different volumes of reconstituted L-CsA suspension, that is, 0.25, 0.5, and 1 mL (corresponding to a total mass of 1, 2, and 4 mg CsA, respectively), were filled in the nebulizer and nebulized. A control exposure with 1 mL 0.25% NaCl was performed (blank value for CsA analysis). The L-CsA concentration in 3 mL of cell culture media in each well was measured by HPLC-MS/MS and converted into the well-deposited L-CsA dose per surface area of the well ($\mu g/cm^2$). This revealed a linear relationship ($R^2 = 0.999$) between the nebulized mass of L-CsA and the well-/cell-deposited dose (Fig. 1). The mean CsA concentration per well was determined to be 4.8 (standard deviation [SD] 0.1), 10.5 (SD 0.2), and 20.1 (SD 0.8) μg/mL, which is equal to a dose of 1.5, 3.3, and 6.3 $\mu g/cm^2$, respectively (Table 1). No L-CsA was detected in the wells where only NaCl had been nebulized.

For comparison with previous performance characterization of the ALICE system (with different substances), it is instructive to calculate the fraction of L-CsA deposited at the bottom plate of the ALICE chamber (where the wells/inserts are located), henceforth referred to as deposition factor. Considering that the bottom plate has an area of 400 cm^2,[22] the total L-CsA dose reaching the bottom of the exposure chamber was 0.6, 1.3, and 2.5 mg for nebulized L-CsA masses of 1, 2, and 4 mg, respectively. Comparing these doses with the nebulized amount of L-CsA yielded a deposition factor of 0.600, 0.656, and 0.628, which is constant within experimental uncertainty, that is, 0.628 ± 0.028 (SD) % of the invested dose is deposited at the bottom of the exposure chamber (Table 1).

Response of A549 cells to L-CsA

A549 cells were grown on membrane inserts in two chamber systems and grown to confluence under suspension. After 7 days, the cells were kept at the ALI for 1 day and then exposed either to NaCl or L-CsA. The cells were further analyzed after a 24-hour postincubation period in the incubator. Control cultures were always exposed to 0.25% NaCl (substrate of L-CsA suspension) and experimental cultures to 0.63, 1.5 and 3.3 $\mu g/cm^2$, that is, 0.1, 0.25, and 0.5 mL of the reconstituted L-CsA suspension was nebulized, respectively. For the entire dose range, no cell response, that is, cytotoxicity, disruption of epithelial barrier tightness, or induction of a (pro-)inflammatory response (Fig. 2A–C), was observed, nor were changes in the cell morphology (cytoskeleton and the cell nuclei) (Fig. 2D).

The same experiments were performed with the bronchial cell line 16HBE14o- and similar results were found for all endpoints measured (data not shown).

Biokinetic behavior of L-CsA over 24 hours

Since the highest L-CsA dose for response testing (3.3 $\mu g/cm^2$; in this study: 10–16 μg per insert) did not induce any adverse effects, we have decided to continue with this dose for the biokinetic studies. At 0, 0.5, 2, 4, and 24 hours after aerosol exposure in the ALICE (nebulization of 0.5 mL of the L-CsA stock suspension) of A549 or 16HBE14o- cells cultured at ALI, the CsA concentration was determined for three different compartments (see also scheme in Fig. 3): (1) the air-exposed (apical) cell side was washed with 0.5 mL isotonic 0.9% NaCl solution to measure the L-CsA that is attached to the apical cell surface, (2) the insert with the attached cells to determine the intracellular L-CsA content, and (3) the medium in the lower (basal) compartment to determine the translocated L-CsA.

Independent of cell type, the biokinetic analysis revealed a relatively high and constant intracellular L-CsA dose over the entire 24-hour period and substantial apical-to-basal translocation of L-CsA with a burst-like profile (Fig. 3). The burst-like transbarrier L-CsA transport rate was initially high (steep slope in apical and basal dose profile) at 0.22% and 0.36% per min/cm^2 [or ca. 50 ng/(min·cm^2)] for A549 and 16HBE14o- cells, respectively (Table 2), followed by a sharp decline (ca. 40-fold reduced rates for the 2–4 hour time interval) and ceased almost completely after 4 hours. Consistent with the observed rates, there was a significantly higher cumulative transport across 16HBE14o- cells ($65.2\% \pm 3.0\%$ of the delivered dose at 24 hours) compared to A549 cells ($38.9\% \pm 1.3\%$ at 24 hours).

On the other hand, the L-CsA dose residing in the cell compartment (i.e., L-CsA is either inside the cells or tightly

Table 1. Relationship Between Nebulized (Invested) and Corresponding Cell-Deposited Liposomal Ciclosporin A Dose

Amount of nebulized (invested) L-CsA	*L-CsA concentration in wells ($\mu g/mL$)*	*L-CsA dose per surface area*[a] *($\mu g/cm^2$)*	*L-CsA mass on bottom of ALICE chamber*[b] *(mg)*	*Deposition factor*[c]
One milligram (in 0.25 mL stock suspension)	4.8 (SD 0.1)	1.5	0.60	0.600
Two milligrams (in 0.5 mL)	10.5 (SD 0.2)	3.3	1.3	0.656
Four milligrams (in 1 mL)	20.1 (SD 0.8)	6.3	2.5	0.628

[a]L-CsA dose per surface area = L-CsA concentration × 3 mL (amount of medium per well)/9.6 cm^2 (area of well).
[b]L-CsA mass on bottom of ALICE chamber = L-CsA dose per surface area × 400 cm^2 (bottom area of ALICE chamber).
[c]Deposition factor = L-CsA mass on bottom of ALICE chamber/invested L-CsA mass (L-CsA mass filled into nebulizer). The deposition factor represents the fraction of the invested dose, which is deposited at the bottom of the ALICE chamber. Mean ± SD = 0.626 ± 0.028.

ALICE, air–liquid interface cell exposure; L-CsA, liposomal ciclosporin A; SD, standard deviation.

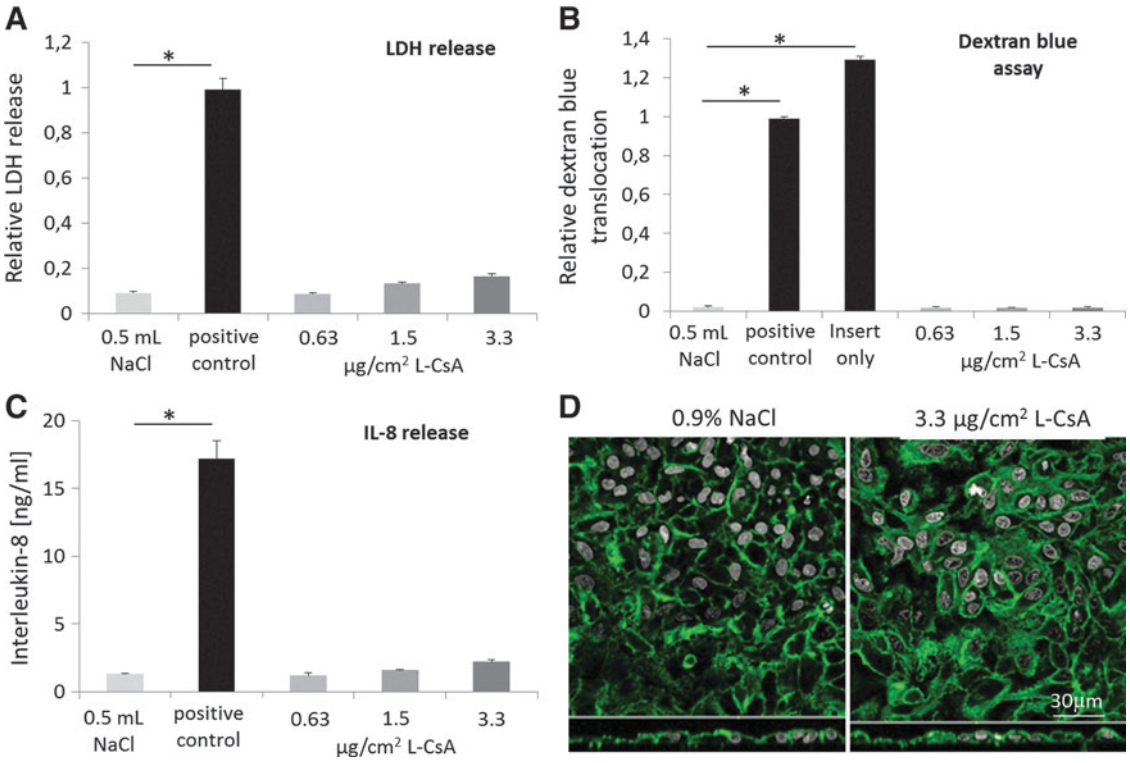

FIG. 2. Determination of viability (cytotoxicity), barrier integrity, proinflammatory response, as well as morphology of A549 cells 24 hours after exposure to L-CsA. For all cases, 0.5 mL of nebulized 0.9% NaCl served as negative control. **(A)** L-CsA did not induce any cytotoxic response as indicated by the low LDH values. Results are normalized to the positive control (cells lysed with Triton-X 100; $n = 3$). **(B)** As shown by relative light absorbance (at 600 nm) in the (basal) cell culture medium, L-CsA exposure did not affect the apical-to-basal translocation of dextran blue (positive controls: cell cultures treated with EDTA or inserts only), that is, the tightness of the cell barrier remained unaffected by L-CsA. **(C)** Low levels of IL-8 in the medium reveals that L-CsA did not induce a (pro-)inflammatory response. (Positive control: cells were treated with TNF-α; $n = 3$). **(D)** The cells were fixed and stained for F-Actin (green) and cell nuclei (white), and analyzed by laser scanning microscopy. The upper pictures represent xy-sections, the lower xz-sections. Cells formed confluent monolayers and no differences in cell morphology were observed between L-CsA- and NaCl- treated cells. IL-8, interleukin-8; LDH, lactate dehydrogenase; TNF-α, tumor necrosis factor alpha. *Indicates statistical significance compared to negative control ($p < 0.05$).

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

attached to the cells) was constant with 18.7% ± 0.7% (A549) and 26.1% ± 2.3% (16HBE16o-) of the cell-delivered L-CsA dose for almost the entire investigated time period (between 0.5 and 24 hours) independent of cell type. Expressed in absolute dose, the relative difference between intracellular L-CsA dose is even lower (2.8 and 3.4 μg for A549 and 16HBEo-, respectively). This is an essential finding for pulmonary drug targeting, since it suggests that rapid (<1 hour) and constant dosing of CsA in the lung tissue for up to at least 24 hours is possible with aerosolized L-CsA.

It is important to note that the initial apical dose at 0 hour is significantly lower than 100% (52% and 56% for A549 and 16HBE14o- cells, respectively), because there is already substantial L-CsA translocation during the 20 minutes of aerosol exposure time in the ALICE system. This is consistent with previously performed aerosol deposition measurements in the ALICE,[22] which show that aerosolized L-CsA starts to be deposited on the cells within the first minute of exposure. Thus, the first time point presented in this study (0 hour) reflects the situation ca. 20 minutes after the first contact between L-CsA and cells (Fig. 3).

Asymptotic biodistribution of L-CsA

As the translocation of L-CsA has virtually ceased after 4 hours, we consider the L-CsA biodistribution at 24 hours as the asymptotic biodistribution. The measured asymptotic L-CsA doses in each of the compartments were a few micrograms (between 2.2 and 8.5 μg) for both cell types (A549 and 16HBE14o-) (Table 3). If the cells would not fulfill their barrier function, one would expect that the L-CsA concentrations would be similar in each of the compartments. For conversion of measured L-CsA mass into concentration, the volume for each of the compartments has to be known. While this is trivial for the basal compartment (1.2 mL medium), certain assumptions have to be made for the apical and cell compartments. The apical volume can be estimated as the aerosol volume deposited onto the cells, which is 3.3 μL as readily derived from the surface area of the insert (4.2 cm^2), the nebulized liquid volume (0.5 mL), as well as the deposition factor (0.626) and the area (bottom plate, 400 cm^2) of the ALICE system (see Table 1, footnote 1).

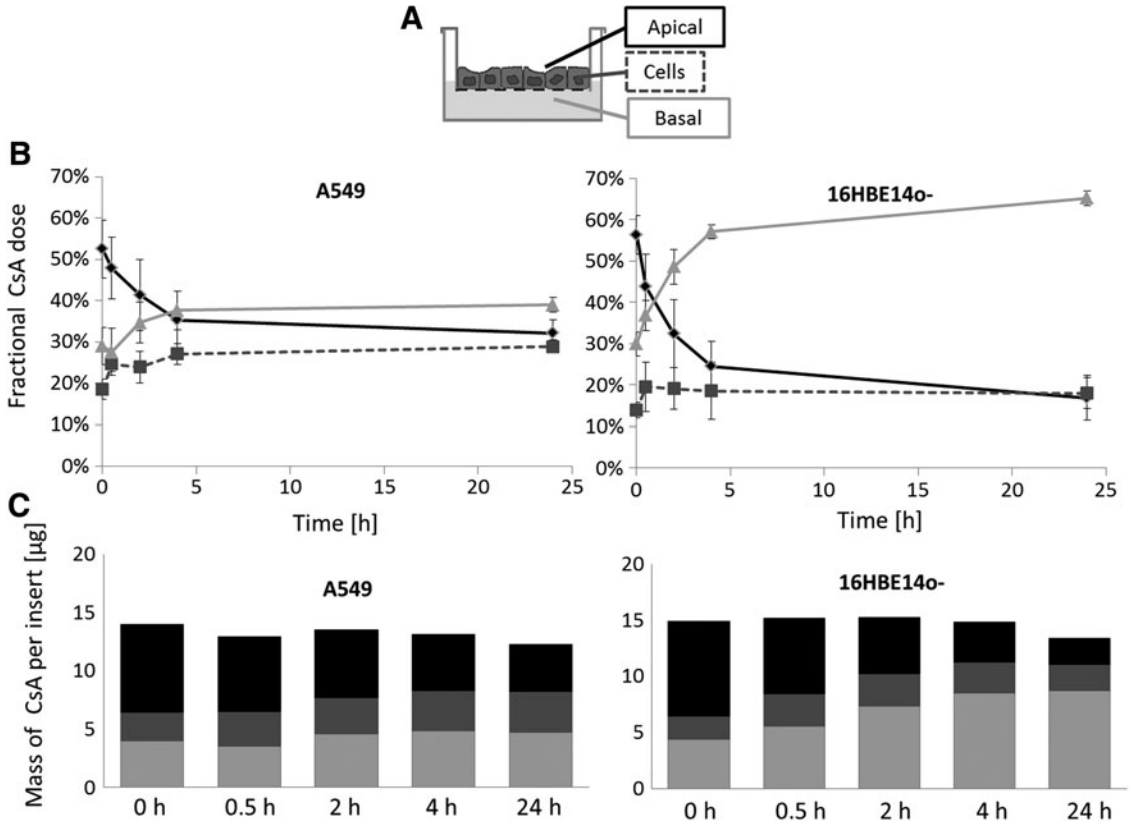

FIG. 3. Similar biokinetic behavior of L-CsA in A549 and 16HBE14o- cells. **(A)** Schematic indicating the compartment-specific color coding for the other panels. **(B)** The biokinetic measurements show a constant fractional CsA dose in the epithelial tissue for both A549 and 16HBE14o- cells starting from 0.5 hours after the exposure, which is conducive for targeted drug delivery by inhalation therapy. Moreover, an initially high, but rapidly decreasing translocation rate of L-CsA (slope of curves) from the apical to the basal compartment was observed with a higher asymptotic basal dose at 24 hours for 16HBE14o- (65%) compared to A549 cells (39%). **(C)** Same data as given in **(B),** but presented in terms of absolute L-CsA dose (mass) per insert ($n = 3$). Such burst-like transport kinetics are typical for inhalation therapy.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

It is noteworthy that the 3.3 μL of aerosol deposited per insert (4.2 cm^2) corresponds to an 8 μm thick liquid layer on the cells. For a confluent cell layer, the volume of the cell compartment is the product of the cell-covered area (4.2 cm^2 per insert) and the thickness of the cell layer. Assuming an average cell thickness of 10 μm [estimated from Fig. 2D; consistent with Lenz et al.[30] and Bachler et al. [31]], the volume of the cell compartment is 4.2 μL. With these considerations, it is evident that the concentrations in the apical and cell compartments are of the same order of magnitude (560–1280 μg/mL), while a 100- to 200-fold (approximately 150-fold) lower L-CsA concentration (4.3–7.1 μg/mL) is found in the basal compartment (Table 3).

Taking into account the uncertainties in thickness of the cell layer and apical volume (cell-secreted liquid and/or (partial) resorption of aerosol liquid by the cells), we consider concentration differences of a factor of <2 (as observed in this study) to still be within experimental uncertainties. Thus, there is no significant difference between the L-CsA concentrations in the apical and cell compartment and there are no significant differences between A549 and 16HBE14o- cells. Thus, apically delivered L-CsA is readily available to

Table 2. Compartment-Specific, Initial Liposomal Ciclosporin A Dose Rate

	Initial relative L-CsA dose rate [%/(min·cm²)]		*Initial absolute L-CsA dose rate [ng/(min·cm²)]*	
Compartment	*A549*	*16HBE14o-*	*A549*	*16HBE14o-*
Apical	−0.57 (SD 0.11)	−0.52 (SD 0.06)	−66.6 (SD 0.3)	−74.5 (SD 10.0)
Cell	0.35 (SD 0.04)	0.17 (SD 0.05)	29.2 (SD 3.1)	24.1 (SD 4.5)
Basal	0.22 (SD 0.08)	0.36 (SD 0.03)	47.3 (SD 5.7)	52.6 (SD 1.6)

The initial rate at which L-CsA is transported into (positive sign) or out of (negative sign) a given compartment. Relative and absolute values are calculated based on the first dose measurement (0 hour) assuming that 100% of the insert-delivered dose was deposited onto the cells (apical) almost immediately after the nebulizer was started (i.e., 20 minutes before 0 hour). Both cell types display identical (within experimental uncertainty) absolute L-CsA transport rates, suggesting that L-CsA is transported passively across the cell barrier.

Table 3. Compartment-Specific, Asymptotic Dose and Concentration of Liposomal Ciclosporin A

	L-CsA dose (μg)[a]			*L-CsA concentration (μg/mL)*	
Compartment	*A549*	*16HBE14o-*	*Liquid volume (μL)*	*A549*	*16HBE14o-*
Apical	4.2	2.2	3.3[a]	1280	680
Cell	3.8	2.4	4.2[b]	910	560
Basal	5.1	8.5	1200[c]	4.3	7.1

The asymptotic L-CsA concentration was calculated from the ratio of the asymptotic (24 hours) L-CsA dose and the (liquid) volume of each compartment.

[a]The volume of L-CsA suspension deposited in each insert is $3.3\,\mu L = 4.2/400 \times 0.626 \times 500\,\mu L$, where we used the following values: nebulized volume (500 μL, 4 mg L-CsA/mL), surface area of the insert (4.2 cm^2), surface area of the ALICE system (400 cm^2), and deposition fraction (0.626; see Table 1). Limitation: The apical volume may be different due to cell-secreted liquid and/or resorption of aerosol-deposited liquid by the cell layer.

[b]Volume of cell compartment (4.2 μL) is estimated from the cell-covered surface area of the insert (4.2 cm^2) and the (estimated) thickness of the cell layer (ca. 10 μm) as estimated from Figure 2D.

[c]Volume of basal medium was 1.2 mL.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

the epithelial cells, while both cell types represent a relatively tight barrier for L-CsA toward systemic translocation as evidenced by the more than 150-fold lower L-CsA concentration in the basal compartment (medium).

Comparison of different CsA formulations

As an additional application of the ALICE method, we assessed the effect of different L-CsA production processes on the biokinetics of L-CsA. For this alternate formulation, L-CsA liposomes were processed by 10-fold extrusion through a 100 nm membrane (instead of high-pressure homogenization), which resulted in liposomes that were larger (Z-average ~93 nm) and had a more narrow size distribution (polydispersity index ~0.1) compared to the liposomes derived from high-pressure homogenization. The formulation was used directly as liquid formulation without the lyophilization step for preservation.

For this comparative study, A549 cells were used and a volume of 0.5 mL was nebulized (3.3 $\mu g/cm^2$). Sample collection was performed as described above. The biokinetic behavior of the extruded L-CsA batch (formulation 2 in Fig. 4) showed a similar pattern to that of the first pressure-homogenized L-CsA formulation (Fig. 4) with a rapidly saturated and constant intracellular CsA concentration of about 25% (23.7% ± 3.1% and 28.9% ± 1.5% for formulations 2 and 1, respectively) of the cell-delivered dose. After longer incubation times, that is, 4 and 24 hours, there is a slightly (but significantly) higher translocation across the epithelial barrier for the extrusion formulation (52.9% ± 2.7% compared to 39.0% ± 1.3%).

Discussion

The use of the inhalation pathway for medical treatments has a long history—asthma was already being treated by inhalation in the 19th century. Later, in the 20th and 21st centuries, research into inhalation treatments and the development of inhalation devices exploded and there is now a wide range of device types and numerous medical applications on the market [for a review see Patton and Byron[32]]. Currently, pulmonary drug targeting by aerosol inhalation is the preferred delivery route for the treatment of various lung diseases such as asthma treatment with corticosteroids,[33,34] antibiotic treatment of pseudomonas in cystic fibrosis patients,[35,36] or chemotherapy in lung cancer patients.[37]

With the development of engineered nanocarriers for drug delivery, medical imaging, and diagnostic purposes, the inhalation pathway is becoming increasingly important. Due

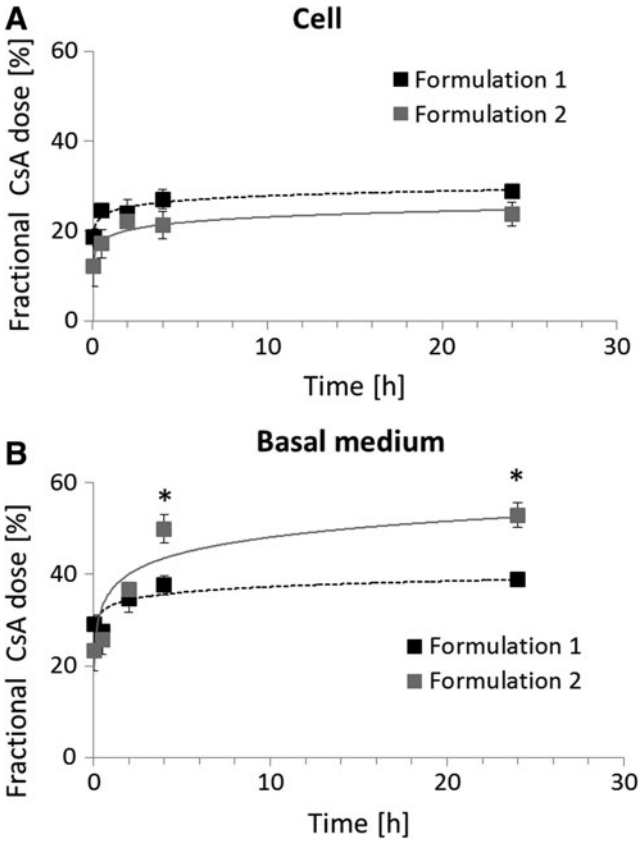

FIG. 4. Comparison of the biokinetics of two different formulations of L-CsA for A549 cells. The data of formulation 1 are already presented in Figure 3. **(A)** There is no statistically significant difference in the relative dose found in the cells for any given time point. **(B)** This is also the case for the pharmacokinetic profile in the basal medium with exception of a slight, but statistically significant dose enhancement for formulation 2 at 4 and 24 hours after the exposure. As the tissue dose profile is the therapeutically most relevant parameter, this indicates bioequivalence of the two formulations.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

to their small size [especially smaller than 20 nm in diameter;[38]] and the large surface area of the lung (140 m^2), nanocarriers reach the lung parenchyma[32] and rapidly cross the pulmonary air-blood barrier reaching secondary target organs through the systemic circulation without the problem of the first-pass effect.[39] This is exemplified by liposomes, which are artificial vesicles consisting of a phospholipid bilayer that have been promoted for many years as future drug delivery vehicles.[1] The contributions of numerous researchers over five decades have led to significant advances in the field, and liposomes are the first nanocarriers that have found success in the marketplace.[40]

L-CsA suspensions containing liposomes with a diameter of 50 nm have been used for clinical inhalation studies with a customized electronic nebulizer (eFlow; PARI Pharma GmbH, Starnberg, Germany), which produces droplets of appropriate size for peripheral lung deposition. With two inhalations per day, a therapeutically effective dose was reached in the peripheral lungs.[41] Inhaled L-CsA may be a promising new approach to optimize immunosuppression and thus improve long-term lung transplantation outcomes not only in animal experiments[42] but also in patients with lung transplants.[5]

Although many clinical trials are underway, a fundamental understanding of how L-CsA aerosols interact with lung cells is still unavailable. This may, in part, be due to the fact that *in vitro* biokinetic studies with liposomes (and other nanocarriers) have almost exclusively been performed with standard submerged cell culture systems[43–45] using Fick's law to calculate the apparent permeability coefficient (P_{app}). For *in vivo* conditions during inhalation therapy, a small volume of highly concentrated drug is applied directly to the epithelial barrier resulting in large, rapidly changing concentration gradients due to the fast depletion of liposomes in the donor compartment. Since Fick's law cannot be applied under these conditions,[17] the typical initial burst of liposome transport directly after aerosol application cannot be adequately captured with submerged cell culture systems.

Moreover, the absence of a lung lining fluid is another severe deficiency of submerged cell cultures, since it has been shown that the protein corona formed upon interaction with the lung lining fluid, the first bodily fluid encountered by lung-deposited particles, may affect the biokinetics of lung-deposited particles.[46] Finally, direct aerosol deposition onto ALI cell cultures allows accurate determination of the cell-delivered dose either by placing a filter substrate for aerosol collection at the site of the cells or by using a real-time dosimetry device such as a QCM.[22,47] However, for submerged exposures, the cell-delivered dose may be obscured by ''floating'' of the nanocarriers due to buoyancy effects depending on the effective density of these carriers.[48,49]

To the best of our knowledge, the study presented here is the first *in vitro* study on reporting the translocation biokinetics of aerosolized nanocarriers delivered to air-liquid pulmonary cells. While there have been numerous studies with soluble drugs [e.g., Lenz et al.[30]], only Schreier et al. deposited aerosolized nanocarriers (liposomes) directly onto human lung cells (2-CFSME0), to investigate the potential of complementary DNA (cDNA)-loaded liposomes for gene transfection.[50] Their aerosol-cell exposure system was limited as it was not adapted to the transwell insert cell culture technology, it required handling of each of the different aerosol impactor (delivery) stages separately, and the cell-delivered cDNA dose for each of the impactor stage depended on the aerosol size distribution. The ALICE system used in our work was designed for ease of use, while also providing dose-controlled, rapid, and efficient delivery of aerosolized liquids to air–liquid cell cultures, allowing us to closely mimic drug delivery through the inhalation route.[22]

We have demonstrated in earlier studies that the ALICE is an efficient nebulization system for spherical nanoparticles such as zinc,[22] gold,[51] or silver,[52] and the system is also applicable for the aerosolized delivery of fiber-shaped cellulose nanocrystals[47] as well as carbon nanotubes.[53] Recently, a refined version of the ALICE system, the ALICE-CLOUD system (commercially available as VITROCELL-CLOUD; Vitrocell Systems, Waldkirch, Germany), has successfully been used to investigate the efficacy of a molecular drug formulation (Bortezomib) on A549 cells,[30] but it has not yet been used for therapeutically active nanocarriers.

In this work the ALICE is used for dose-controlled aerosolized delivery of L-CsA to two widely accepted *in vitro* models of the human epithelial tissue barrier, that is, A549 and 16HBE14o- cells,[27] cultured under ALI conditions. The A549 cells represent alveolar epithelial type II cells[24] and the 16HBE14o- cells represent human airway (bronchial) epithelial cells[25,26] [for a review see Rothen-Rutishauser et al.[27]]. However, many studies indicate that the A549 might not be a good representative for human alveolar epithelium [for a review, see de Souza et al.[54]]. In this study, both cell lines were grown under well-defined and controlled culture conditions, revealing a confluent and dense monolayer as shown by the Dextran Blue translocation assay as well as by the LSM images of the spatial cell morphology.

Moreover, the initial L-CsA dose rates across the epithelial barrier (Table 2) and the asymptotic, compartment-specific L-CsA concentrations (Table 3) are similar for both cell types. Previously, we also reported an apparent permeability coefficient (P_{app}) of the paracellular marker ^{14}C-mannitol in A549 cells similar to that of Madine-Darby canine kidney (MDCK) cells, which reflects a tight epithelial barrier.[55] In addition, A549 cells grown at ALI release surfactant and develop an even denser monolayer compared to cells in suspension.[56] Thus, in summary, the reported data justify the use of both cell types to study the biokinetic distribution of apically delivered drugs.

By measuring the L-CsA dose in six-well plates after nebulization of various volumes of the 4 mg/mL CsA stock suspension, a reproducible and linear deposition of L-CsA was observed resulting in 1.5, 3.3, and 6.6 $\mu g/cm^2$ for 1, 2, and 4 mg of nebulized L-CsA, respectively. These results indicate a deposition factor of 0.626 ± 0.028 (SD), that is, 62.6% of the invested amount of L-CsA is deposited on the bottom plate of the ALICE system, which is in excellent agreement with the deposition factor of 0.566 ± 0.067 (SD) reported by Lenz et al. for various types of nanoparticles and molecular substances.[22]

For the biokinetic studies, the L-CsA dose of 3.3 $\mu g/cm^2$ was chosen, since it is low enough to avoid cytotoxic effects and high enough for accurate analytical detection of L-CsA in all three compartments. For comparison, in a recent preclinical toxicological study with molecular ciclosporin (not a liposomal formulation), up to 281 mg/day was applied

by nose-only inhalation in beagle dogs (24 mg/kg/day for up to 11.7 kg dogs).[11] This corresponds to a surface-specific dose of 562 ng/cm^2 assuming a lung surface area of 50 m^2 for beagle dogs,[57] which is of the same order of magnitude as, although about sixfold lower, the dose applied in our *in vitro* study. In a recent clinical study, inhalation of nebulized molecular ciclosporin in human lung transplant patients has resulted in an average pulmonary dose of 31.8 mg (SD 16.3 mg),[12] which corresponds to about 22.7 ng/cm^2 assuming a lung surface area of 140 m^2[58] and hence a ca. 100-fold lower dose than our *in vitro* dose.

In contrast to the previous studies,[11,12] which used molecular ciclosporin, the clinical inhalation study by Behr et al. delivered about 10-fold lower peripheral (alveolar) lung dose of 1.8–3.4 mg L-CsA (liposomal ciclosporin) to lung transplant patients with a single- or double-sided lung transplant.[5] In this study, the clinically applied peripheral doses are ca. 1000-fold lower (1.6–4.4 ng/cm^2) than the doses applied in our *in vitro* setup. However, the situation is different for the proximal (bronchial) region. The proximally delivered dose of 1.8–3.1 mg L-CsA corresponds to a tissue-specific dose of 0.18–0.56 μg/cm^2 (bronchial surface area: 1 m^2), which can locally be even 10-fold higher (1.8–5.6 μg/cm^2) due to hotspot regions at bronchial bifurcations.[59] Hence, the selected *in vitro* dose of L-CsA is within the clinically relevant dose range of the proximal region, but about 1000-fold higher than the peripheral lung dose.

We note that these dose ranges correspond to an aerosol-induced liquid layer on the epithelial cells, which is ca. 8 μm and 8 nm thick in the bronchial (hotspot) and alveolar region, respectively (5 mg/mL L-CsA suspension was applied), compared to 8 μm of liquid layer thickness for our ALI *in vitro* conditions. Despite the significant differences between *in vitro* and clinical dosing, we have shown that the applied *in vitro* doses were highly reproducible using the ALICE system and did not produce impaired barrier integrity or toxicological effects, which is consistent with the absence of pulmonary side effects in clinical studies, although at much lower doses in the peripheral lung region.[5] Therefore, we conclude that the dose used in this *in vitro* study is acceptable for the investigation of biokinetics of L-CsA.

Our *in vitro* data show that the biokinetic behavior of L-CsA is very similar for the bronchial and alveolar pulmonary epithelial cell lines applied in this study. There is rapid transport of L-CsA into the cell compartment, reaching constant dose (relative) levels of 18.7% ± 0.7% (A549) and 26.1% ± 2.3% (16HBE16o-) within 0.5 hours after exposure. This is a crucial finding for pulmonary drug delivery, since it suggests that aerosolized L-CsA can rapidly attain (<1 hour) and maintain high and constant L-CsA doses (20%–25% of the delivered dose) for at least 24 hours in the lung tissue. This is a favorable dose profile for most clinical applications.

It is also noteworthy that the apical dose is already below 60% at 0 hour, implying that substantial transport had already occurred during the 20 minutes of aerosol exposure time in the ALICE system. This is consistent with the rapid aerosol-cell delivery rate reported for the ALICE by Lenz et al. indicting that 95% of the total dose is delivered within the first 5 minutes of exposure.[22] Expressed in absolute doses, the difference between intracellular L-CsA dose is even lower for A549 and 16HBEo- cells (2.8 and 3.4 μg, respectively), suggesting the existence of a rapidly attained (within 20–50 minutes after aerosol-cell contact) and, for at least 24 hours, maintained intracellular (and apical) L-CsA level of about 1000 μg/mL independent of cell type (Fig. 3 and Table 3). The fact that this level is about 150-fold higher than the L-CsA concentration in the basal medium (Table 3) indicates that both the alveolar (A549) and the bronchial epithelial cell model (16HBE14o-) represent a tight barrier for L-CsA allowing for high tissue retention of L-CsA for at least 24 hours. This is in agreement with other *in vitro* studies reporting high tissue retention of L-CsA also for Calu-3 lung cells.[43]

Moreover, rapid cellular uptake of liposomes was also reported *in vitro* for various cell types such as lung cells, monocytes, and colon cells (Caco-2).[60,61] The interaction of liposomes with cell membranes depends on the lipid composition and size, as well as on the cell type, and can include the exchange of lipids or proteins with cell membranes, adsorption or binding of liposomes to cells, internalization of liposomes by endocytic pathways, and fusion of bound liposome bilayer with the cell membrane.[62] For L-CsA, the exact route of transport or uptake mechanism is, however, unknown. Interestingly, in many of these studies, the cellular uptake ceased within 1 hour, which is also consistent with our results.[60]

However, for liposomes in the size range of 50–100 nm, as were used in this study, asymptotic cellular dose levels were observed only after extended time periods of 24 hours or more.[60] This is likely an artifact of the nonphysiological exposure conditions (submerged cell culture), especially the large medium layer covering the cells. Small particles with densities near 1 g/cm^3 (as is the case for liposomes) experience mitigated cell contact under submerged conditions as Brownian diffusion and buoyancy keep smaller liposomes afloat longer in the thick medium layer (typically at least 5 mm thick) and hence away from the cells than larger ones, which experience faster gravimetric settling.[48]

Assessment of transbarrier transport is particularly important as a measure of inadvertent extrapulmonary exposure and potential adverse side effects. As mentioned above, our ALI *in vitro* study has shown that both alveolar (A549) and bronchial cells (16HBE14o-) represent a tight, but not impermeable barrier for L-CsA (ca. 150-fold higher L-CsA concentration in cells compared to basal medium). As expected for aerosolized drug delivery to ALI cell cultures, there is an initially very high, but rapidly decreasing concentration at the epithelial barrier, which results in an initial burst of L-CsA transbarrier transport followed by a rapidly decreasing transport rate (ca. 40-fold reduced within 4 hours) ceasing almost completely after about 4 hours (slopes in Fig. 4). This burst-like behavior is similar to clinical L-CsA pharmacokinetic data after inhaled application of L-CsA in lung transplant patients.[5]

It is difficult to compare absolute values of *in vitro* and *in vivo* pharmacokinetic data, since the former is driven by absorption (transepithelial barrier transport) only, while the latter depends on absorption into and clearance from the blood by liver and kidney, the main filtration organs.[16]

Behr et al. reported rapidly declining L-CsA blood levels between 2 and 24 hours after L-CsA inhalation. This implies that the maximal L-CsA concentration in the blood (c_{max}) has occurred significantly before 2 hours and the L-CsA absorption kinetic rate is rapidly decreasing within the first

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

few hours after L-CsA inhalation, reaching transport rates close to zero at about 24 hours. Interestingly, in a pharmacokinetic study in a dog model, c_{max} was reached 15 minutes after aerosolized L-CsA application, providing further support for the burst-like absorption profile of L-CsA.[16] As mentioned above, this pharmacokinetic profile is very similar to that observed under ALI *in vitro* conditions.

In contrast, the kinetics observed for submerged cell exposure conditions are completely different. Trammer et al. performed pharmacokinetic studies with CsA ladenliposomes (L-CsA), which were in the same size range as ours (ca. 50 nm).[43] They incubated Calu-3 cells (human airway epithelial cell line) with 133 μg/mL L-CsA suspended in 0.5 mL medium (i.e., 66 μg L-CsA on the apical side of 12-well transwell insert) and observed a transport rate of 8.4 ng/($cm^2 \cdot$min) or 0.013%/($cm^2 \cdot$min), which was not only constant for more than 24 hours (no burst-like transport profile) but was also 15- to 30-fold lower than the initial transport rate of 0.22%–0.36%/(min$\cdot cm^2$) measured under ALI conditions in this study.

Similar, but less pronounced differences in transport rate are expected for soluble drugs. This lack of a burst-like pharmacokinetic profile under submerged cell culture conditions is at least partially due to the large volume (column height) of the apical donor compartment compared to submerged culture conditions the cells are typically covered with, about 0.5 mL medium per cm^2 of cell-covered area.[43,63] This corresponds to a 5 mm (= 5000 μm) thick liquid column, which implies that initial transport of L-CsA across the cell barrier will not substantially reduce the L-CsA concentration in the donor compartment resulting in an initially constant transport rate. In contrast, under ALI conditions as described in this study, the cells are covered with ca. 8 μm of L-CsA suspension, which results in a rapidly decreasing concentration in the apical donor compartment and thus burst-like systemic pharmacokinetics.

As mentioned above, under clinical conditions, the L-CsA dose per tissue area (and hence the layer thickness, as similar L-CsA concentrations were nebulized) in the bronchial and alveolar region was approximately identical to and 1000-fold lower compared to ALI *in vitro* conditions, respectively. Hence, at least in the alveolar region, which is most relevant for the systemic absorption kinetics, the currently available ALICE conditions are not an ideal match with clinical conditions, although much more realistic than submerged cell culture conditions as evidenced by the burst-like pharmacokinetic profile of L-CsA observed only under ALI conditions.

Consequently, the method for aerosolized drug delivery to ALI-cultured cells presented in this study can conceivably contribute to answering the U.S. FDA's aforementioned call for the characterization of the performance of inhalation products under physiological conditions.[4] As an example of how this request could be addressed, we investigated the biokinetics of two different L-CsA formulations. It is evident from Figure 4 that the liposomes produced by high-pressure homogenization (formulation 1; presented in Figs. 2 and 3) and extrusion (formulation 2) show similar biokinetic behavior.

High levels of tissue retention of L-CsA (in this study: 25% of cell-delivered dose) are rapidly attained (<1 hour) and retained at a constant level for at least 24 hours. After incubation times longer than 4 hours, there is a somewhat lower, but still substantial enrichment of L-CsA in the cell compartment over the medium of 75 compared to 100 for formulation 1 (based on 52.9% ± 2.7% compared to 39.0% ± 1.3% of asymptotic dose in the medium). Both values are within the range required to guarantee a high local dose in the target tissue (lung) and a low dose in the blood stream, which maximizes efficacy, while minimizing systemic side effects. Thus, the biokinetic behavior, and hence the bioavailable dose, can be considered equivalent for both formulations.

Conclusions

The combination of lung epithelial cells cultured at the ALI and aerosolized drug delivery with the ALICE system offers an excellent tool to study the effects of inhalable substances under the physiological conditions experienced during inhalation therapy. This method allows detailed investigation of the biokinetic and toxicodynamic events at the air-blood barrier that are difficult and time-consuming to study *in vivo*. This method is particularly useful for nanocarrier-based drug formulations, since direct application of the nanocarriers to the cells provides control over the cell-delivered dose and avoids biases due to interactions between medium and nanocarriers as potentially encountered in submerged cell assays.

As an example of nanocarrier-based drug formulations, we have shown that the aerosolization of different volumes of the L-CsA stock suspension using the ALICE system leads to a highly reproducible and dose-controlled deposition of L-CsA onto ALI cells, which can be varied linearly by increasing the nebulized volume of L-CsA suspension from 0.5 to 1.0 mL. The concentrations applied in this study did not induce any toxicologically adverse effects and allowed the study of the biokinetic behavior of L-CsA at the lung epithelial tissue barrier *in vitro*. The alveolar (A549) and bronchial (16HBEo-) epithelial cells used in this study represent a tight barrier for L-CsA as evidenced by high (ca. 150-fold) enrichment of CsA in the apical/cell compartment (target tissue) compared to that in the basal compartment.

Moreover, the asymptotic cellular dose level was reached rapidly (<1 hour) and remained constant for up to 24 hours independent of cell type, which is conducive for targeted pulmonary drug delivery by inhalation therapy. The observed pharmacokinetic profile with an initial burst-like transbarrier transport of L-CsA is a hallmark of inhalation drug delivery. Our ALI *in vitro* results are in general agreement with this clinically observed pharmacokinetics profile, while—according to literature—the observed biokinetic behavior of L-CsA is very different for submerged cell culture conditions (initially constant followed by slowly decreasing transport rate). The flat pharmacokinetic profile under submerged conditions is at least, in part, due to the large liquid volume in the donor compartment (ca. 0.5 mL/cm^2).

In contrast, aerosolized drug delivery with the ALICE results in a ca. 8 μm thin liquid layer, which is about 1000-fold lower than typical media heights under submerged cell culture conditions, and it resembles the clinical conditions in the bronchial regime. Hence, we argue that for *in vitro* biokinetic studies of inhaled drug formulations, aerosolized drug delivery to ALI cells is crucial, but the currently applied *in vitro* aerosol liquid layer and tissue-specific dose are

often considerably higher, although nontoxic, compared to clinical conditions in the alveolar region.

No substantial differences in biokinetics or toxicodynamics were observed for two L-CsA preparations with similar physicochemical characteristics, confirming the bioequivalence of these two formulations prepared according to two different generation protocols. To the best of our knowledge, the study presented here is the first *in vitro* translocation biokinetic study for aerosolized drug-loaded nanocarriers delivered to pulmonary cells cultured under physiological ALI conditions.

Acknowledgments

This work was supported by PARI Pharma GmbH, the Swiss National Science Foundation (grant 310030_159847/1), and the Adolphe Merkle Foundation.

Author Disclosure Statement

D.M., A.B., F.G., O.D., and R.E. are working for PARI Pharma GmbH (Starnberg, Germany). All other authors have no competing financial interests.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

References

1. Bozzuto G, and Molinari A: Liposomes as nanomedical devices. Int J Nanomedicine. 2015;10:975–999.
2. Bolukbas DA, and Meiners S: Lung cancer nanomedicine: Potentials and pitfalls. Nanomedicine (Lond). 2015;10:3203–3212.
3. Mueller L, Lehmann AD, Johnston BD, Blank F, Wick P, Fink A, and Rothen-Rutishauser B: Inhalation pathway as a promising portal of entry: What has to be considered in designing new nanomaterials for biomedical application? In: SC Sahu and DA Casciano (eds). *Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology*. John Wiley & Sons, Ltd., Chichester; 2014, pg. 205.
4. U.S. Food and Drug Administration: *Critical Path Initiative on ''Transforming the Way FDA-Regulated Products Are Developed, Evaluated, Manufactured, and Used*. U.S. Food and Drug Administration; 2009. Washington, DC.
5. Behr J, Zimmermann G, Baumgartner R, Leuchte H, Neurohr C, Brand P, Herpich C, Sommerer K, Seitz J, Menges G, Tillmanns S, and Keller M: Lung deposition of a liposomal cyclosporine A inhalation solution in patients after lung transplantation. J Aerosol Med Pulm Drug Deliv. 2009;22:121–130.
6. Gilbert BE, Knight C, Alvarez FG, Waldrep C, Rodarte JR, Knight V, and Eschenbacher WL: Tolerance of volunteers to cyclosporine A-dilauroylphosphatidylcholine liposome aerosol. Am J Respir Crit Care Med. 1997;156:1789–1793.
7. Yusen RD, Edwards LB, Kucheryavaya AY, Benden C, Dipchand AI, Dobbels F, Goldfarb SB, Levvey BJ, Lund LH, Meiser B, and Stehlik J: The registry of the International Society for Heart and Lung Transplantation: Thirty-first adult lung and heart-lung transplant report—2014; focus theme: Retransplantation. J Heart Lung Transplant. 2014;33:1009–1024.
8. Scheffert JL, and Raza K: Immunosuppression in lung transplantation. J Thorac Dis. 2014;6:1039–1053.
9. Corcoran TE: Aerosol drug delivery in lung transplant recipients. Expert Opin Drug Deliv. 2009;6:139–148.
10. Sato M: Chronic lung allograft dysfunction after lung transplantation and late onset non-infectious pulmonary complications after hematopoietic stem cell transplantation: Are they mirror images? Clin Res Pulmonol. 2014;2:1014.
11. Niven R, Lynch M, Moutvic R, Gibbs S, Briscoe C, and Raff H: Safety and toxicology of cyclosporine in propylene glycol after 9-month aerosol exposure to beagle dogs. J Aerosol Med Pulm Drug Deliv. 2011;24:205–212.
12. Corcoran TE, Niven R, Verret W, Dilly S, and Johnson BA: Lung deposition and pharmacokinetics of nebulized cyclosporine in lung transplant patients. J Aerosol Med Pulm Drug Deliv. 2014;27:178–184.
13. Johnson BA, Zamora MR, Budev MM, Kotloff RM, Iacono A, Dilly SG, Verret WJ, and Golden JA: Cyclosporine inhalation solution does not improve bronchiolitis obliterans syndrome-free survival following lung transplant: Results from the CYCLIST trial. J Heart Lung Transplant. 2012;31(4 Suppl):S66.
14. Klyashchitsky BA, and Owen AJ: Nebulizer-compatible liquid formulations for aerosol pulmonary delivery of hydrophobic drugs: Glucocorticoids and cyclosporine. J Drug Target. 1999;7:79–99.
15. Liu H, Wang Y, and Li S: Advanced delivery of ciclosporin A: Present state and perspective. Expert Opin Drug Deliv. 2007;4:349–358.
16. Letsou GV, Safi HJ, Reardon MJ, Ergenoglu M, Li Z, Klonaris C, Baldwin JC, Gilbert BE, and Waldrep JC: Pharmacokinetics of liposomal aerosolized cyclosporine A for pulmonary immunosuppression. Ann Thorac Surg. 1999;68:2044–2048.
17. Bur M, and Lehr CM: Pulmonary cell culture models to study the safety and efficacy of innovative aerosol medicines. Expert Opin Drug Deliv. 2008;5:641–652.
18. Darquenne C, Fleming JS, Katz I, Martin AR, Schroeter J, Usmani O, Venegas J, and Schmid O: Bridging the gap between science and clinical efficacy: Physiology, imaging, and modeling of aerosols in the lung. J Aerosol Med Pulm Drug Deliv. 2016;29:107–126.
19. Limbach LK, Li Y, Grass RN, Brunner TJ, Hintermann MA, Muller, Gunther D, and Stark WJ: Oxide nanoparticle uptake in human lung fibroblasts: Effects of particle size, agglomeration, and diffusion at low concentrations. Environ Sci Technol. 2005;39:9370–9376.
20. Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, and Pounds JG: Particokinetics in vitro: Dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicol Sci. 2007;95:300–312.
21. Paur HR, Cassee FR, Teeguarden JG, Fissan H, Diabate S, Aufderheide M, Kreyling W, Hänninen O, Kasper G, Riediker M, Rothen-Rutishauser B, and Schmid O: In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung—A dialog between aerosol science and biology. J Aerosol Sci. 2011;42:668–692.
22. Lenz AG, Karg E, Lentner B, Dittrich V, Brandenberger C, Rothen-Rutishauser, Schulz H, Ferron GA, and Schmid O: A dose-controlled system for air-liquid interface cell exposure and its application to zinc oxide nanoparticles. Part Fibre Toxicol. 2009;6:32.
23. Lieber M, Smith B, Szakal A, Nelson-Rees W, and Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer. 1976;17:62–70.

24. Foster KA, Oster CG, Mayer MM, Avery ML, and Audus KL: Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. Exp Cell Res. 1998;243:359–366.
25. Forbes B, and Ehrhardt C: Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm. 2005;60:193–205.
26. Steimer A, Haltner E, and Lehr CM: Cell culture models of the respiratory tract relevant to pulmonary drug delivery. J Aerosol Med. 2005;18:137–182.
27. Rothen-Rutishauser B, Clift MJD, Jud C, Fink A, and Wick P: Human epithelial cells in vitro–Are they an advantageous tool to help understand the nanomaterial-biological barrier interaction? ENTL. 2012;1:1–20.
28. Lehmann A, Brandenberger Ch, Blank F, Gehr P, and Rothen-Rutishauser B: A 3D model of the human epithelial airway barrer. In: T Maguire and E Novik (eds). *Methods in Bioengineering. Alternative Technologies to Animal Testing*. Artech House, Boston, London; pp. 239–260, 2010.
29. Horvath L, Umehara Y, Jud C, Blank F, Petri-Fink A, and Rothen-Rutishauser B: Engineering an in vitro air-blood barrier by 3D bioprinting. Sci Rep. 2015;5:7974.
30. Lenz AG, Stoeger T, Cei D, Schmidmeir M, Semren N, Burgstaller, Lentner B, Eickelberg O, Meiners S, and Schmid O: Efficient bioactive delivery of aerosolized drugs to human pulmonary epithelial cells cultured in air-liquid interface conditions. Am J Respir Cell Mol Biol. 2014;51: 526–535.
31. Bachler G, Losert S, Umehara Y, von Goetz N, Rodriguez-Lorenzo L, Petri-Fink, Rothen-Rutishauser B, and Hungerbuehler K: Translocation of gold nanoparticles across the lung epithelial tissue barrier: Combining in vitro and in silico methods to substitute in vivo experiments. Part Fibre Toxicol. 2015;12:18.
32. Patton JS, and Byron PR: Inhaling medicines: Delivering drugs to the body through the lungs. Nat Rev Drug Discov. 2007;6:67–74.
33. Dahl R: Systemic side effects of inhaled corticosteroids in patients with asthma. Respir Med. 2006;100:1307–1317.
34. Derendorf H, Nave R, Drollmann A, Cerasoli F, and Wurst W: Relevance of pharmacokinetics and pharmacodynamics of inhaled corticosteroids to asthma. Eur Respir J. 2006;28: 1042–1050.
35. Hewer SL: Inhaled antibiotics in cystic fibrosis: What's new? J R Soc Med. 2012;105 Suppl 2:S19–S24.
36. Pilcer G, Rosiere R, Traina K, Sebti T, Vanderbist F, and Amighi K: New co-spray-dried tobramycin nanoparticles-clarithromycin inhaled powder systems for lung infection therapy in cystic fibrosis patients. J Pharm Sci. 2013;102: 1836–1846.
37. Zarogoulidis P, Chatzaki E, Porpodis K, Domvri K, Hohenforst-Schmidt W, Goldberg E, Karamanos N, and Zarogoulidis K: Inhaled chemotherapy in lung cancer: Future concept of nanomedicine. Int J Nanomedicine. 2012;7: 1551–1572.
38. Kreyling WG, Hirn S, Moller W, Schleh C, Wenk A, Celik G, Lipka J, Chaffler M, Haberl N, Johnston BD, Sperling R, Schmid G, Simon U, Parak WJ, and Semmler-Behnke M: Air-blood barrier translocation of tracheally instilled gold nanoparticles inversely depends on particle size. ACS Nano. 2014;8:222–233.
39. Jud C, Clift MJ, Petri-Fink A, and Rothen-Rutishauser B: Nanomaterials and the human lung: What is known and what must be deciphered to realise their potential advantages? Swiss Med Wkly. 2013;143:w13758.
40. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, and Farokhzad OC: Nanoparticles in medicine: Therapeutic applications and developments. Clin Pharmacol Ther. 2008; 83:761–769.
41. Burckart GJ: Ciclosporin aerosol in lung transplantation. Expert Opin Investig Drugs. 2006;15:981–986.
42. Onoue S, Sato H, Ogawa K, Kojo Y, Aoki Y, Kawabata, Wada K, Mizumoto T, and Yamada S: Inhalable dry-emulsion formulation of cyclosporine A with improved anti-inflammatory effects in experimental asthma/COPD-model rats. Eur J Pharm Biopharm. 2012;80:54–60.
43. Trammer B, Amann A, Haltner-Ukomadu E, Tillmanns S, Keller M, and Hogger P: Comparative permeability and diffusion kinetics of cyclosporine A liposomes and propylene glycol solution from human lung tissue into human blood ex vivo. Eur J Pharm Biopharm. 2008;70:758–764.
44. Poelma DL, Zimmermann LJ, van Cappellen WA, Haitsma JJ, Lachmann B, and van Iwaarden JF: Distinct effects of SP-B and SP-C on the uptake of surfactant-like liposomes by alveolar cells in vivo and in vitro. Am J Physiol Lung Cell Mol Physiol. 2004;287:L1056–L1065.
45. Joshi N, Kaviratna A, and Banerjee R: Multi trigger responsive, surface active lipid nanovesicle aerosols for improved efficacy of paclitaxel in lung cancer. Integr Biol (Camb). 2013;5:239–248.
46. Schottler S, Becker G, Winzen S, Steinbach T, Mohr K, Landfester K, Mailander V, and Wurm FR: Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. Nat Nanotechnol. 2016;11:372–377.
47. Endes C, Schmid O, Kinnear C, Mueller S, Espinosa S, Vanhecke D, Foster JE, Petri-Fink A, Rothen-Rutishauser B, Weder C, and Clift M: An in vitro testing strategy towards mimicking the inhalation of high aspect ratio nanoparticles. Part Fibre Toxicol. 2014;11:40.
48. Hinderliter PM, Minard KR, Orr G, Chrisler WB, Thrall BD, Pounds J, and Teeguarden JG: ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. Part Fibre Toxicol. 2010;7:36.
49. Schmid O, Karg E, Hagen DE, Whitefield PD, and Ferron GA: On the effective density of non-spherical particles as derived from combined measurements of aerodynamic and mobility equivalent size. J Aerosol Sci. 2007;38:431–443.
50. Schreier H, Gagne L, Conary JT, and Laurian G: Simulated lung transfection by nebulization of liposome cDNA complexes using a cascade impactor seeded with 2-CFSME0-cells. J Aerosol Med. 1998;11:1–13.
51. Brandenberger Ch, Rothen-Rutishauser B, Mühlfeld Ch, Schmid O, Ferron GA, Maier K, Gehr P, and Lenz AG: Effects and uptake of gold nanoparticles deposited at the air-liquid interface of a human epithelial airway model. Toxicol Appl Pharmacol. 2010;242:56–65.
52. Herzog F, Clift MJ, Piccapietra F, Behra R, Schmid O, Petri-Fink A, and Rothen-Rutishauser B: Exposure of silver-nanoparticles and silver-ions to lung cells in vitro at the air-liquid interface. Part Fibre Toxicol. 2013;10:11.
53. Chortarea S, Clift MJD, Vanhecke D, Endes C, Wick P, Petri-Fink A, and Rothen-Rutishauser B: Repeated exposure to carbon nanotube-based aerosols does not affect the functional properties of a 3D human epithelial airway model. Nanotoxicology. 2015;9:983–993.

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.

54. de Souza CC, Daum N, and Lehr CM: Carrier interactions with the biological barriers of the lung: Advanced in vitro models and challenges for pulmonary drug delivery. Adv Drug Deliv Rev. 2014;75:129–140.
55. Rothen-Rutishauser BM, Kiama SG, and Gehr P: A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol. 2005;32:281–289.
56. Blank F, Rothen-Rutishauser BM, Schurch S, and Gehr P: An optimized in vitro model of the respiratory tract wall to study particle cell interactions. J Aerosol Med. 2006;19: 392–405.
57. Stone KC, Mercer RR, Gehr P, Stockstill B, and Crapo JD: Allometric relationships of cell numbers and size in the mammalian lung. Am J Respir Cell Mol Biol. 1992;6:235–243.
58. Gehr P, Bachofen M, and Weibel ER: The normal human lung: Ultrastructure and morphometric estimation of diffusion capacity. Respir Physiol. 1978;32:121–140.
59. Balashazy I, Hofmann W, and Heistracher T: Local particle deposition patterns may play a key role in the development of lung cancer. J Appl Physiol. 2003;94:1719–1725.
60. Epstein-Barash H, Gutman D, Markovsky E, Mishan-Eisenberg G, Koroukhov N, Szebeni J, and Golomb G: Physicochemical parameters affecting liposomal bisphosphonates bioactivity for restenosis therapy: Internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death. J Control Release. 2010;146:182–195.
61. Andar AU, Hood RR, Vreeland WN, Devoe DL, and Swaan PW: Microfluidic preparation of liposomes to determine particle size influence on cellular uptake mechanisms. Pharm Res. 2014;31:401–413.
62. Podlipec R: Interaction of liposomes on endothelial cells; pp.1–15, 2010. http://mafija.fmf.uni-lj.si/seminar/files/2010_2011/seminar2_pop.pdf
63. Lenz AG, Karg E, Brendel E, Hinze-Heyn H, Maier KL, Eickelberg O, Stoeger T, and Schmid O: Inflammatory and oxidative stress responses of an alveolar epithelial cell line to airborne zinc oxide nanoparticles at the air-liquid interface: A comparison with conventional, submerged cell-culture conditions. Biomed Res Int. 2013;2013:652632.

Received on November 27, 2016
in final form, April 25, 2017

Reviewed by:
Timothy Corcoran

Address correspondence to:
Barbara Rothen-Rutishauser, PhD
BioNanomaterials
Adolphe Merkle Institute
University of Fribourg
Chemin des Verdiers 4
CH-1700 Fribourg
Switzerland

E-mail: barbara.rothen@unifr.ch

Downloaded by GSF Munich from online.liebertpub.com at 01/22/18. For personal use only.