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Research paper

Comparative permeability and diffusion kinetics of cyclosporine A liposomes and propylene glycol solution from human lung tissue into human blood *ex vivo*

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ABSTRACT

Aerolized cyclosporine A (CsA) has been successfully used for prevention of organ rejection in lung transplant recipients. Various formulations of CsA are available and so far no direct comparison of their pharmacokinetics has been performed. Since clinical studies are elaborate, we sought a way to predict the kinetic behaviour of a propylene glycol solution of CsA (CsA-PG) and a liposomal formulation (L-CsA). The permeability across the human bronchial cell line Calu-3 revealed low permeability for CsA with the apparent permeability for CsA-PG being twice as high as for L-CsA. Employing a previously described dialysis model, the diffusion of CsA from human lung tissue into human blood was determined *ex vivo*. Consistent with the cell culture model results, we observed that the degree and rate of drug transfer into human blood was more pronounced for CsA-PG than for L-CsA with the area under the curve (AUC) of CsA-PG being about 1.6 times higher than for the L-CsA formulation. The diffusion rate was more than 50% higher from CsA-PG than from the liposomes. To conclude, both model systems consistently revealed that L-CsA displayed clearly a prolonged release effect and favourable longer tissue retention than CsA-PG.

1. Introduction

Inhalation of drugs offers several advantages for the treatment of pulmonary diseases. High local drug concentrations can be achieved in lung tissue without high systemic concentrations. Thus, adverse effects are often less severe and less frequent after delivery of locally acting drugs by the pulmonary route [1].

An interesting example of drug targeting by inhalation is the local immunosuppression by cyclosporine A (CsA) aerosols in lung transplant recipients. Acute and especially chronic organ rejection remains a continuous problem in this patient group. Chronic rejection is the leading cause of mortality after lung transplantation [2] and evinces histologically as bronchiolitis obliterans (BO) and clinically as irreversibly loss in pulmonary function, named bronchiolitis obliterans syndrome [3,4]. The aerosolized CsA was administered on top of base medication with systemic immunosuppressants and this additional therapy showed a positive effect on inflammatory and lung function parameters such as FEV_1 [5]. Recently, a randomised, double-blind clinical trial with 58 lung transplant patients revealed a statistically significant advantage in terms of BO-free survival and overall survival of aerosolized CsA therapy versus placebo [6].

Different CsA formulations suitable for pulmonary administration of the drug have been described. The aerosol delivery devices used ranged from nebulizers over metered dose inhalers (MDI) to dry powder dispersing systems (dry powder inhalers, DPI).

In early trials, the highly lipophilic CsA was dissolved in ethanol to allow nebulization [4]. This cell-irritating formulation was subsequently replaced by a CsA solution in propylene glycol (CsA-PG) [5–10]. Since this preparation still caused significant irritation of the airways, a pre-treatment of the patients with an aerosolized local anesthetic and a bronchodilator was required [6,9]. Another formulation administered by nebulization was CsA encapsulated in different liposome systems, including the liposomal CsA (L-CsA) formulation used in this study [11–13]. This unilamellar liposomal CsA formulation was well tolerated upon inhalation by lung transplant patients [12].

Metered dose inhalers delivered CsA as respirable particles [14]. When hydrofluoroalkane (HFA) was used as propellant a CsA solution aerosol was obtained [15]. Dry powder dispersing systems were used to deliver CsA complexed with cyclodextrins [16,17] or as solid dispersion [18].

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It is obvious that the formulation has a significant influence on the drugs' pharmacokinetics. So far, however, no direct *in vivo* comparisons of the pharmacokinetics of different CsA formulations have been performed yet. Since these studies are elaborate, we sought a way to predict the pharmacokinetic behaviour of different CsA formulations. Based on the availability of data of clinical studies for the nebulization of CsA-PG [5–10] and L-CsA [11–13], we chose these two formulations for our experiments. For compounds meant to exert local effects, a high retention in the lung tissue is highly favourable for prolonged pharmacological effect. In case of inhaled CsA, high local concentrations and a low pulmonary absorption from the lung tissue into blood should be the basis for a pronounced immunosuppressive action in the transplanted organ.

In this study, we pursued a dual approach to obtain information about the comparative uptake of L-CsA and CsA-PG. We analyzed the *in vitro* permeability of both formulations in a cell culture model employing Calu-3 cells and elucidated the absorption process of CsA from human lung tissue into human blood *ex vivo*.

Calu-3 is a human bronchial adenocarcinoma cell line, which forms tight, polarized and well-differentiated monolayers with apical microvilli and tight junctional complexes [19]. Monolayers of Calu-3 cells represent a suitable *in vitro* model to study the pulmonary drug delivery [20–22]. In contrast to other respiratory cell lines, Calu-3 cells express physiological features of the airway epithelium such as generation of extracellular mucus and cilia when grown under air interface conditions. Calu-3 cells have been shown to express functional active drug efflux transporters such as P-glycoprotein (P-gp) in their apical membrane [23]. Moreover, they possess multidrug resistance-associated protein 1 (MRP1) functional activity, which is located in the basolateral membrane and is subordinate to Pgp efflux [24]. This is of importance since these proteins are also highly expressed in bronchial epithelia and can significantly affect absorption and bioavailability [25–27], tissue distribution [25,28], excretion [25], and pharmacodynamic effects of drugs [29]. Many drugs have been identified as P-gp inhibitors including CsA [29–31]. Most importantly, however, an *in vitro–in vivo* correlation of drug absorption has been shown for these cells [32].

We recently demonstrated in a model system that one glucocorticoid delivered by two different HFA-propelled MDIs revealed different absorption rates from human lung tissue into plasma due to different drug particle sizes [33]. This difference in pulmonary absorption was also seen in a clinical trial [34]. Thus, we chose the previously employed dialysis system to assess the absorption process of L-CsA and CsA-PG from a human lung tissue preparation into human blood *ex vivo*.

2. Materials and methods

2.1. Chemicals and reagents

N-(2-Hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES) was purchased from GERBU (Heidelberg, Germany) and a stock solution containing 10,000 IU/mL penicillin and 10,000 μg/mL streptomycin in 0.9% NaCl from Biochrom AG (Berlin, Germany). All other chemicals were obtained from E. Merck (Darmstadt, Germany). Water was obtained from a Millipore™ water purification unit.

2.2. Preparation of drug formulations

CsA-PG and L-CsA were a generous gift from PARI Pharma (Munich, Germany). The detailed composition and preparation procedure of the liposomes are described in a patent held by PARI Pharma (WO 2007/065588). Briefly, unilamellar liposomes of about 50 nm in diameter with 4 mg/mL CsA and a phosphatidylcholine content of 41.5 mg/mL were produced by high pressure homogenization followed by sterile filtration. The liposomes revealed a uniform size distribution (polydispersity index < 0.3). The resulting formulation of pH 4.9 was free of irritant organic solvents and showed a dynamic viscosity of 1.4 mN/m and a surface tension of 33.8 mPa. Upon storage at 5 °C the formulation displayed satisfying stability over 9 months with all physicochemical parameters remaining unchanged.

2.3. Cell culture, qualification of monolayer batches, and cells' tolerance towards the test compounds

Calu-3 cells (HTB-55, ATCC, Manassas, VA, USA) of passage 4 were used for the transport experiments. Cells were grown under liquid conditions and were maintained at 37 °C, 5% CO_2 and 90% relative humidity (CO_2 incubator 20022640, Heraeus, Germany) in 75 cm^2 culture flasks with supplemented Minimal Essential Medium (MEM). The cells were passaged once a week using Trypsin/EDTA solution. The culture medium was changed three times a week.

For the transport experiments, Calu-3 cells were seeded with a density of 100,000 cells per cm^2 on Transwell™ filter inserts, which were placed into 12-well flat bottom cluster plates. The inserts (apical compartments) were supplied with 0.5 mL and the outer wells (basal compartments) with 1.5 mL of MEM culture medium. The cells were cultured at 37 °C, 5% CO_2 and 90% relative humidity in MEM culture medium and they formed confluent monolayers after 7–8 days. The culture medium was replaced every 2–3 days. Confluency and tightness of the cell monolayer was routinely checked by measuring the transepithelial electrical resistance using an epithelial tissue voltohmmeter (EVOM™, World Precision Instruments). For the experiments, confluent monolayers were used within 8–30 days after seeding if they passed the quality control.

Before transport experiments were initiated, Calu-3 monolayer batches were qualified based on selected transport markers. Experiments were performed in triplicate for each transport condition. The following qualification parameters were checked before a monolayer batch was used for permeability studies: apparent permeability P_{app} apical–basolateral with test compound fluorescein (P_{app} fluorescein (ab), test for low permeability); P_{app} [^{3}H]-Propranolol (ab) (test for high permeability) with the P_{app} fluorescein required to be at least 100-fold higher than P_{app} propranolol; P_{app} rhodamine 123 (ab/ba) (test for P-gp expression); transepithelial electrical resistance (TEER) >300 Ω cm^2 (test for tightness of barrier) after pre-incubation (30–45 min) as well as after the transport study. This TEER value (after subtraction of the blank filter value of 100 Ω cm^2) was chosen based on the previously published finding that TEER values of 400 Ω cm^2 indicate tight barriers [22].

A preliminary test was performed to assess the tolerance of the Calu-3 cell monolayers towards the test compounds. For this purpose the cells were incubated with two different test concentrations (0.266 and 0.133 mg/mL) within the designated range to be applied in the transport studies. After incubation periods of 4, 12, 14, 18, 22, and 24 h at 37 °C the TEER was checked to assess the integrity of the cell monolayers and to choose a suitable concentration. All experiments were performed as triplicates.

2.4. Transport experiments

[^{3}H]-CsA was spiked to the liquid formulations containing CsA obtained from PARI Pharma. Therefore, 2 mL of CsA (0.133 mg/mL) in MEM, L-CsA (0.133 mg/mL), and CsA-PG (0.133 mg/mL) was mixed with 10 μL [^{3}H]-CsA (1 mCi/mL, activity 37 MBq) result-

ing in 11,044,776 dpm/mL. The spiked test solutions were shaken at 200 rpm (KS-15, Edmund Bühler, Germany) for at least 24 h.

Calu-3 monolayers were rinsed once with fresh MEM. The spiked test solutions containing [^{3}H]-CsA were applied to the donor compartments. Subsequently, the cells were equilibrated in a CO_2 incubator for 30 min. After this pre-incubation, the transport experiment was started by taking samples from the donor and acceptor compartments. The concentration of CsA found in the donor was taken as initial donor concentration (c_{D0}). At 4, 12, 14, 18, 22, and 24 h, samples of 300 μL were taken from the acceptor compartments and immediately replaced by fresh pre-warmed MEM. At the end of the study, additional samples were taken from the donor compartments. Between the sampling points, the monolayers were incubated at 37 °C in a CO_2 incubator. All experiments were performed in triplicate. The TEER was controlled at the first and the last sampling time.

To identify any loss of test compounds during the transport experiments due to metabolism, absorption by the cells or adsorption to the plastic material, the recovery of the test compounds was calculated after the pre-incubation phase and at the end of the experiment. As [^{3}H]-CsA is a very poor soluble, lipophilic substance, the cells and the wells were washed after the study with MEM to remove [^{3}H]-CsA absorbed to the cells and the plastic material. The wash solutions were analyzed.

The P_{app} was calculated according to

$$P_{app} = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{m_0} \cdot \frac{1}{A} V_D \ [\mathrm{cm \cdot s^{-1}}], \quad (1)$$

where $\Delta Q/\Delta t$ is the permeability rate (steady state transport rate) obtained from the profile of the transported amount of substrate versus time [dpm s^{-1}], calculated by the linear regression of time and concentration; A is the area of the exposed cell monolayer [cm^2]; m_0 is the initial mass of test compound in the donor compartment [dpm]; and V_D is the buffer volume of donor compartment [cm^3].

The TEER was calculated according to

$$\mathrm{TEER} = R_{c(A)} = (R_{c+f} - R_f) \cdot A \ [\Omega\ \mathrm{cm^{-2}}], \quad (2)$$

where $R_{c(A)}$ is electrical resistance of the monolayer with the area A [Ω cm^2]; R_{c+f} is electrical resistance of the monolayer including the filter [Ω]; R_f is electrical resistance of the filter without cells [Ω]; and A is the area of monolayer [cm^2].

The electrical resistance of a cell-free filter with an area of 1.13 cm^2 is 100 Ω.

Experiments with CsA were performed in triplicate in apical to basolateral direction for the lipid formulations containing CsA, and in both directions (apical to basolateral and basolateral to apical) for CsA in MEM.

[^{3}H]-CsA was quantified by liquid scintillation counting (Microbeta Wallac™, Perkin–Elmer, Germany). The samples (300 μL) were transferred into 24 well plates and mixed with 500 μL liquid scintillation cocktail (Optiphase™ Supermix, Perkin Elmer, Germany). After an equilibration period of at least 1 h, radiation was measured for 2 min per well. The recorded counts in a blank sample were subtracted from the value of each test sample.

2.5. Source and handling of human specimen

Human lung tissue specimen was obtained from patients with bronchial carcinomas who gave informed consent. Only cancer-free tissue was used for the experiments. The precise location of the lung tumor was pre-operatively identified by CT. The resected lung lobe underwent visual inspection by the surgeon who excised cancer-free tissue samples distant from the tumor. The completeness and integrity of the tumor tissue were subsequently confirmed during histological tumor examination by a pathologist.

Tissue samples were shock frozen in liquid nitrogen after resection and stored at −80 °C until usage. To collect sufficient material for the experiments, tissue samples of three patients were pooled. Tissue was thawed and cut into small pieces. One part of the tissue pieces was homogenized in two parts of Krebs–Ringer–HEPES buffer (118 mM NaCl, 4.84 mM KCl, 1.2 mM KH_2PO_4, 2.43 mM $MgSO_4 \cdot 6H_2O$, 2.44 mM $CaCl_2 \cdot 2H_2O$, and 10 mM HEPES; pH 7.4). Penicillin–streptomycin solution was added to the buffer achieving a final concentration of 10 IU/mL penicillin and 10 μg/mL streptomycin. Homogenization was performed under continuous cooling using an Ultraturrax (Janke and Kunkel, Staufen, Germany). Before starting the series of dialysis experiments, the required amount of human lung tissue homogenate was estimated and subsequently a sufficient amount was prepared and divided into aliquots. Since all aliquots descended from this preparation, protein content and enzymatic activity of the homogenate were identical for each experiment.

Human blood and plasma were obtained from healthy volunteers who gave informed consent. Blood samples were purchased from the DRK-Blutspendedienst, Baden-Württemberg, Germany, and plasma samples from the Department for Transfusion Medicine and Immune Haematology, Würzburg, Germany. Blood samples were used within 5 days after blood donation. Plasma samples were shock frozen in liquid nitrogen and stored at −80 °C until usage.

To ensure a constant hematocrit-rate for each dialysis experiment, the hematocrit–plasma ratio was determined by centrifugation of a blood sample with the following analysis of the contained plasma-volume for each blood batch. Plasma was added, when necessary, to achieve a hematocrit–plasma ratio of 40:60.

2.6. Dialysis experiments

Dialysis was performed with a dialysis unit as described in detail previously [33]. Briefly, the dialysis unit (designed by our working group) consisted of two individual tightly fitting Teflon chambers separated by a dialysis membrane. One chamber was prepared for lung tissue homogenate, the other chamber was supposed to be filled with human blood. The chamber for blood had two apertures, one for obtaining dialysis samples and the other for addition of fresh blood.

To ensure accuracy and reproducibility for the volume of the samples, the mass of a defined volume of each blood batch was determined experimentally using an electronic precision balance from Kern and Sohn GmbH (Balingen-Frommern, Germany). Afterwards, the required setting for the pipette was calculated by using the particular density. Thus, the samples drawn had a constant volume for each experiment.

A 5 g aliquot of pre-warmed human lung homogenate (37 °C) was filled into the chamber with less internal depth. 250 μL of either the CsA-PG solution (concentration 4 mg/mL) or the L-CsA formulation (concentration 4 mg/mL) was added to the lung homogenate and mixed gently for a fixed period of time. Accordingly, the CsA-dose applied to the lung homogenate was 1 mg. The dose was chosen due to experiments of other research groups who administered up to 300 mg CsA-PG to lung transplant patients using nebulization [6,8,9]. Subsequently, the dialysis membrane was placed on the homogenate and the dialysis unit was closed by attaching the second chamber. The second chamber was filled with blood of 37 °C. The drug transfer from the lung tissue into human blood was monitored over 420 min at 37 °C by obtaining samples (0.5 mL) at the specific time points from the dialysis chamber filled with blood. The blood volume withdrawn was replaced with fresh blood of 37 °C.

Three dialysis experiments with blood from three different donors were performed for each CsA preparation. The samples were frozen immediately and stored at −20 °C until analysis.

For each preparation, one blank experiment was performed to obtain blank matrix samples for the calibration curves. Therefore, either 250 μL propylene glycol solution or 250 μL plain liposomes was added to lung tissue homogenate, and the samples (0.5 mL) were obtained analogous to the verum-experiments. The samples were treated as described above.

2.7. Analysis of CsA by LC–MS/MS

All samples were shipped frozen at −20 °C to A&M GmbH, Kopernikusstrasse 25, 50126 Bergheim (Germany) and analyzed by LC–MS/MS after ESI ionization. The method was based on the CsA assay previously described by Keevil et al. [35]. Briefly, a 10 μL blood sample was mixed with 50 μL of 100 mM zinc sulphate solution, and proteins were precipitated with 100 μL acetonitrile containing 50 ng/mL ascomycin (BioTrend, Cologne, Germany) as internal standard. The LC–MS/MS system was an Agilent 1100 HPLC system (Agilent technologies, Waldbronn, Germany) consisting of a binary pump, a vacuum degasser, and a thermostatted autosampler coupled with a TSQ Quantum Ultra mass spectrometer (Thermo Electron). An ESI interface was used in the positive ionization mode. Analysis was performed on a Phenomenex Security Guard Cartridge C18 (ODS) 4 × 3.0 mm at 60 °C. A gradient elution with 50–95% methanol containing 0.1% (v/v) formic acid and 50–5% of 2 mM ammonium acetate containing 0.1% (v/v) formic acid was employed at a flow rate of 0.3 mL/min. A total sample volume of 10 μl was injected. The mass spectrometer was operated in selective reaction monitoring, observing the transitions from 1220 m/z to 1203 m/z for CsA and 809 m/z to 756 m/z for ascomycin. Method validation included the determination of intra- and inter-assay precision and accuracy of the method, the quenching rate, the extraction recovery, dilution impact, the specificity and the lower limit of quantification (LLOQ). Stability of the analyte in matrix and pure solution as well as the stability of processed samples was checked. The LLOQ was 1 ng/mL.

2.8. Calculation of kinetic parameters

The area under the curve (AUC) and the diffusion rate (DR) were calculated using EXCEL spreadsheets. The AUC was calculated using the trapezoidal rule and the DR was derived from the slope of the regression line underlying a linear correlation.

3. Results

3.1. Qualification of the Calu-3 monolayers and cells' tolerance towards the test compounds

The permeability of the lowly permeable test compound fluorescein was within the range of previously reported values [36,37] and it was more than 100-fold lower than the permeability of the highly permeable test compound [^{3}H]-propranolol, demonstrating that the cell monolayers were able to differentiate between highly and lowly permeable test compounds (Table 1). The apical to basolateral (ab) permeability of the P-gp substrate rhodamine 123 was lower than the permeability from the basolateral to the apical (ba) side, demonstrating a functional expression of P-gp for this passage. Though the apparent permeability of rhodamine 123 in this study was lower than that reported previously [23], the efflux ratio which is concentration dependent [38] was sufficiently high with a ratio of 5. These results demonstrated that the cells formed tight and functional monolayers and the monolayer batch was suitable for the transport studies with the test compounds.

The cells' tolerance towards the test compounds revealed that CsA in MEM did not damage the cells' monolayer. In both concentrations of CsA in MEM, there was an initial decrease of TEER in ab direction during the studies, but after 24 h the cells were able to recover 70% and 80% of the value at time t_0 (data not shown). The addition of L-CsA in MEM to the cells decreased the TEER in both concentrations continuously to a value of about 20% of t_0 and lower after 24 h. The higher concentration had a more pronounced effect than the lower concentration. An initial decrease of TEER was also observed after addition of CsA-PG, though values of about 50% of t_0 continuously increased again after 14 h. Thus, for the transport experiment the lower concentration of each compound was chosen for which TEER was always >300 Ω cm^2 which was the defined pre-set threshold value for qualification of the monolayer.

3.2. Permeability experiments

Drug transfer of CsA from different formulations across Calu-3 monolayers (passage 4) was determined and apparent permeability (P_{app}) was calculated. CsA revealed a low permeability in all the tested formulations as the P_{app} values range under 5×10^{-6} cm s^{-1}, (Fig. 1). The P_{app} for CsA in MEM from apical to basolateral side was $2.30 \pm 0.05 \times 10^{-6}$ cm s^{-1}, from basolateral to apical side it was $1.95 \pm 0.05 \times 10^{-6}$ cm s^{-1}. Thus, the efflux ratio (P_{app}ba/P_{app}ab) of CsA in MEM showed no directionality. P_{app} for CsA-PG was $2.18 \pm 0.28 \times 10^{-6}$ cm s^{-1} and lowest for L-CsA with $0.96 \pm 0.04 \times 10^{-6}$ cm s^{-1}. Consequently, the flux for the transport of CsA across the monolayers was highest for CsA in MEM from apical to basolateral side (1.069 ± 0.045 μg/h cm^2), from basolateral to apical side (0.95 ± 0.041 μg/h cm^2). The Flux of CsA-PG was within the same range (1.068 ± 0.187 μg/h cm^2), while the flux was considerably lower for L-CsA (0.505 ± 0.042 μg/h cm^2).

The cumulative apical to basolateral and basolateral to apical transport of CsA in MEM, apical to basolateral transport of L-CsA and in CsA-PG at Calu-3 cell monolayers revealed a more pronounced transport of CsA in MEM (ab and ba) and CsA-PG, while again the transport of L-CsA was considerably lower, about only half of CsA-PG (Fig. 2).

3.3. Dialysis experiments

The drug transfer from the lung tissue into human blood was monitored over 7 h at 37 °C. The blood concentrations of CsA in

Table 1
Qualification procedure results for Calu-3 cells

Sample name	Concentration (μM)	Test for	Direction	$P_{app} \times 10^{-6}$ (cm/s)	Efflux ratio P_{app}ba/P_{app}ab
Fluorescein	26.6	Low permeability	ab	0.17 ± 0.02	–
[^{3}H]-Propranolol	10	High permeability	ab	26.51 ± 0.42	–
Rhodamine 123	5	P-gp expression	ab	0.55 ± 0.04	5
Rhodamine 123	5		ba	2.82 ± 0.34	

Experiments were performed in triplicate for each transport condition.
P_{app}, apparent permeability; ab, apical–basolateral; ba, basolateral–apical.

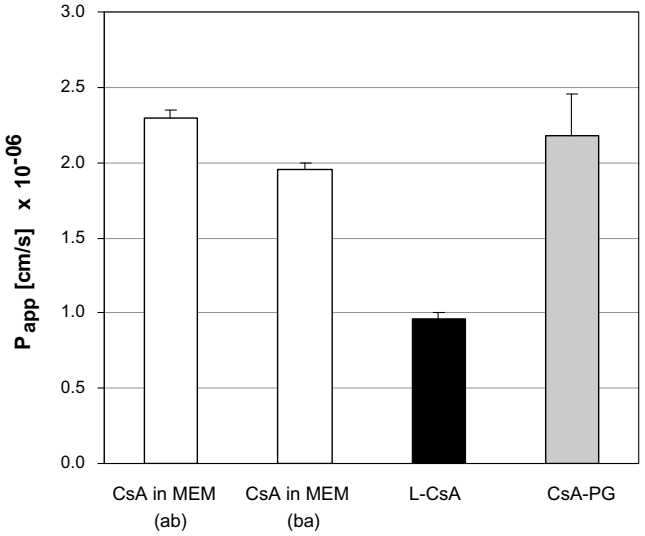

Fig. 1. Apparent permeability (P_{app}) for the transport of CsA from different formulations across Calu-3 monolayers passage 4. The values are the mean values ± SD from experiments performed in parallel (n = 3).

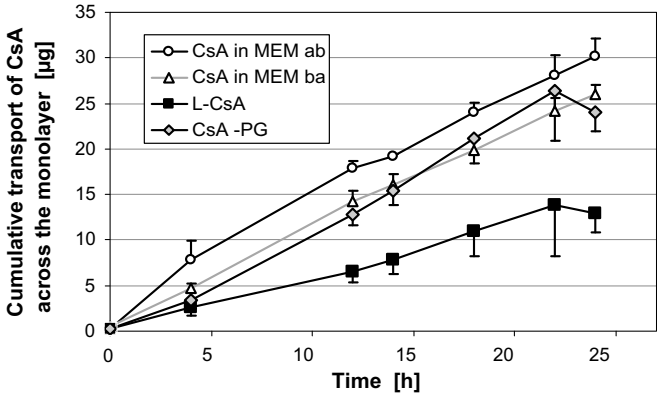

Fig. 2. Apical to basolateral (ab) and basolateral to apical (ba) transport of CsA in MEM, apical to basolateral transport of L-CsA and CsA-PG in Calu-3 cell monolayers over 24 h. The values are the mean values ± SD from experiments performed in parallel (n = 3).

ng/mL after addition of the CsA-PG solution and the liposomal formulation (L-CsA), respectively, to the tissue homogenate revealed clear differences (Fig. 3). About 90 min after initiation of the experiment the blood concentrations were distinctly higher with CsA-PG (615 ± 129 ng/mL) than with L-CsA (349 ± 55 ng/mL), which was added to the lung tissue. This more pronounced absorption of CsA from the CsA-PG solution became increasingly evident over the experimental period with final blood concentrations of 2573 ± 79 ng/mL (CsA-PG) versus 1633 ± 145 ng/mL (L-CsA). While the absorption of CsA from the CsA-PG solution steadily increased up to 420 min, the rate of absorption of CsA from L-CsA slowed down between 360 and 420 min and apparently reached a plateau.

The distinctly different degree and rate of absorption of CsA from lung tissue into blood was also reflected by the pharmacokinetic properties that were calculated. Since the drug transfer from lung tissue into blood was not yet completed after 420 min, parameters such as c_{max}, t_{max}, and $t½$ could not be calculated. It had been shown before that this model system mirrors only the absorption phase [33]. However, the area under the curve (AUC) and the diffusion rate (DR) were calculated from the experimental data (Table 2). The AUC of CsA in blood after addition of CsA-PG to

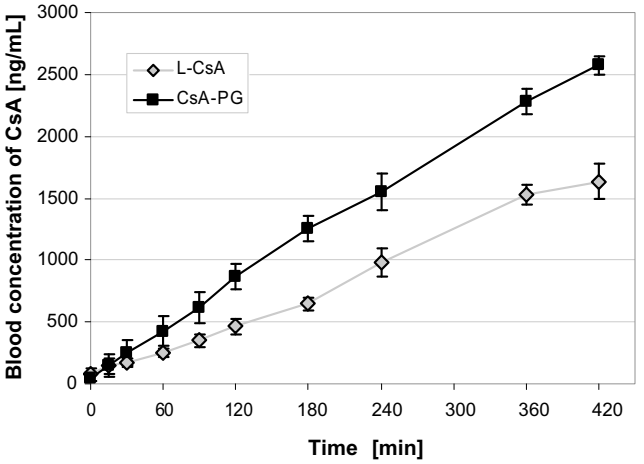

Fig. 3. Time course of cyclosporine A (CsA) transfer from human lung tissue into human blood at 37 °C in a dialysis model. Diffusion kinetics was clearly different after addition of a liposomal CsA preparation (L-CsA) and a CsA propylene glycol solution (CsA-PG) to human lung tissue homogenate. Symbols represent mean and mean deviation of the mean of three independent experiments with blood from three different donors.

Table 2
Area under the curve (AUC) and the diffusion rate (DR) of cyclosporine A (CsA) in blood after addition of CsA liposomes (L-CsA) or CsA propylene glycol solution (CsA-PG), respectively, to human lung tissue homogenate in a dialysis model

	CsA liposomes	CsA propylene glycol solution
AUC	359,094 ± 21,957(ng min)/mL	575,458 ± 30,536(ng min)/mL
DR	3.87 ± 0.46 ng/(mL min)	6.08 ± 0.36 ng/(mL min)

Data represent mean and mean deviation of the mean of three independent experiments with blood from three different donors.

the lung tissue was about 1.6 times higher than for L-CsA. The absorption rate was more than 50% higher with CsA-PG (6.08 ± 0.36 ng/mL min which equals 5.06 ± 0.30 nM/min) than with L-CsA (3.87 ± 0.46 ng/mL min which equals 3.22 ± 0.38 nM/min).

4. Discussion

In this study we compared the diffusion kinetics of two different cyclosporine A (CsA) preparations that have been used for inhalation by lung transplant recipients in clinical trials. Since comparative clinical data for these drug formulations are not available yet, we intended to predict the possible *in vivo* absorption behaviour in an *in vitro* and *ex vivo* setting. We detected significant differences in the *in vitro* permeability of the drug across Calu-3 cell monolayers and in the degree and rate of CsA uptake into human blood with longer *ex vivo* tissue residence times of the liposomal CsA preparation (L-CsA) compared to the CsA propylene glycol solution (CsA-PG).

The Calu-3 cell line is a useful *in vitro* model for qualitative prediction of *in vivo* pulmonary drug absorption after inhalation [21,22]. Monolayers of the polarized cells differentiate into phenotypes that display physiologic airway epithelium properties as well as transport and metabolic features that are relevant for drug absorption. The Calu-3 monolayers used for the present evaluation were fully functional and represented a tight barrier. In the concentrations used, none of the test formulations of CsA exhibited toxicity to a degree compromising the barrier functionality of the monolayer. CsA-PG revealed a higher permeability across the monolayer and higher flux values than L-CsA which showed a clear prolonged release effect.

The dialysis model had been developed for monitoring the absorption phase of different pharmaceutical drug preparations, and we previously used it to compare the distribution of two different glucocorticoid formulations from human lung tissue into human plasma. The differences that emerged in the absorption rates of these two formulations were similarly seen in a human lung perfusion model [33], which in turn revealed results that were highly consistent with the data from a clinical study employing the same drug formulations [34]. The dialysis model has advantages in ease of use and is suitable for screening the absorption behaviour of different drug preparations [39]. In this context, the drug formulations can be administered directly as they are to the lung tissue so that, if applicable, the dissolution process of a lipophilic drug will be accounted for as well. In this model, a more rapid distribution of CsA-PG from human lung tissue into human blood was observed compared to L-CsA which again revealed prolonged release characteristics.

CsA is a highly lipophilic drug with low water solubility [18,40]. Liposomes offer the opportunity to solubilize poorly water soluble drugs which partition into the phospholipid bilayer [40,41]. Though liposomes may behave comparably to solvent formulations without influencing the drug's pharmacokinetics [40], they may as well prolong local therapeutic drug concentrations [42]. It has been shown that the lipid composition of the inhaled liposome formulation plays a major role in the release rate of drugs [43]. In case of a pulmonary delivery of CsA to lung transplant recipients, prolonged tissue retention due to low pulmonary absorption into systemic circulation would be a favourable attribute along with the fact that liposomes prevent local irritation in the pulmonary tract [42].

Prolonged tissue retention of CsA delivered by inhalation should be reflected in a lower pulmonary absorption rate, a delayed time of maximum blood concentrations (larger t_{max}) and lower blood concentrations (lower c_{max}) over a longer time (longer $t½$).

Most data from clinical studies were obtained with aerosolized CsA-PG [5–10]. In a pharmacokinetic study with lung transplant patients, a single dose of 300 mg inhaled CsA-PG was compared with the intravenous administration of 1 mg/kg (62.7–90.5 mg) CsA [9]. The pulmonary absorption was described as biphasic with absorption half-lives of 0.73 ± 0.38 and 16.2 ± 13.2 h. The t_{max} after inhalation was 0.68 ± 0.30 h and the terminal half-life $t½$ was longer after inhalation (40.7 ± 17.7 h) than after intravenous administration (6.5 ± 2.3 h). Despite this remarkably long terminal half-life after inhalation, the blood concentrations were reported to decline from peak levels of 119–402 ng/mL to a range from 9–48 ng/mL within 24 h.

After administration of 3–20 mg CsA by an MDI human pharmacokinetic, data were determined [14]. No pulmonary absorption rates were reported. After inhalation of a single dose the median t_{max} after inhalation ranged from 0.50 to 0.88 h, the terminal half-life $t½$ ranged from 3.09 to 5.59 h.

Inhaled L-CsA (10–20 mg) administered to lung transplant patients revealed a t_{max} of 2 h and a terminal half-life $t½$ of about 6 h [12,13]. Though the t_{max} appears to be significantly delayed compared to the t_{max} 0.68 h [9] and 0.50–0.88 h [14] reported in the other studies, this has to be regarded with caution since the first blood sample was obtained not until 2 h after inhalation.

Since it is apparently difficult to compare these data directly due to the highly divergent administered doses and the different study population with healthy volunteers and asthmatic patients [14] or lung transplant recipients [9,12,13], a direct comparison of CsA preparations in a model system under simplified experimental conditions is useful.

Based on the results of the permeability across the human bronchial cell line Calu-3, and the absorption behaviour of CsA from human lung tissue into human blood after administration of two different CsA formulations, it can be expected that the liposomal CsA preparation displays a lower pulmonary absorption and longer tissue retention than the propylene glycol solution of CsA. This is not unexpected since prolonged local therapeutic drug concentrations have been reported for liposomes [42,43]. However, finally the predictive potential of our model systems needs to be established in ensuing clinical trials.

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