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Pulmonary absorption of aerosolized fluorescent markers in the isolated rabbit lung

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Abstract

For the development of aerosolized controlled release formulations such as liposomes or nanoparticles, the use of suitable model drugs is necessary. This study compared the pulmonary absorption of the three structurally diverse fluorescent markers 5(6)-carboxyfluorescein (CF), 8-methoxypyrene-1,3,6-trisulfonic acid trisodium salt (MPTS) and rhodamine 6G (R6G) after nebulization in an isolated, perfused and ventilated rabbit lung. Aerosol particle size and lung deposition as well as lipophilicity of the fluorescent markers were determined. Dye concentrations were measured in the recirculating buffer and in the bronchoalveolar lavage.

The MMAD of the dye aerosols ranged between 4.70 and 4.88 μm, total lung deposition was 0.40 ± 0.05 ml. The 1-octanol/water partition coefficient as measure for lipophilicity was -3.45 ± 0.16 for CF, -4.95 ± 0.21 for MPTS and 2.69 ± 0.18 for R6G. The perfusate concentration showed an increase to ~400 ng/ml ($53.4 \pm 6.8\%$ of the intrapulmonary deposited dye) for CF and ~230 ng/ml ($29.1 \pm 2.0\%$) for MPTS, respectively; R6G concentration increased in the first 30 min to ~38 ng/ml followed by a gradual decrease to ~26 ng/ml ($3.3 \pm 0.7\%$).

In conclusion, these data suggest that the hydrophilic dye CF is suitable to study drug transport from aerosolized controlled release formulations across the lung barrier. In contrast, the highly water-soluble fluorescent probe MPTS demonstrates insufficient recovery and the lipophilic R6G high accumulation in lung tissue.

Keywords: Isolated lung; Nebulization; Pulmonary absorption; Fluorescent markers; Controlled release formulations

1. Introduction

Due to the large surface area of the alveolar epithelium and the short air to blood pathway, the lung is permeable to many substances, and therefore pulmonary deposition of aerosols represents an efficient, rapid and non-invasive alternative for the delivery of many drugs. A number of agents, which are poorly absorbed enterally or otherwise are well absorbed by the lung (Pohl et al., 1998b; Yamamoto et al., 2004). Moreover, there is an increasing interest in controlled release formulations for aerosol application to treat pulmonary or systemic diseases. A promising approach is the encapsulation of drugs in liposomes. The carrier potential of liposomes is related to their ability to accommodate water-soluble and lipid-soluble substances in their aqueous and lipid phases (Forsgren et al., 1990). However, the use of liposomes as potential drug carrier is limited due to inherent problems, such as low encapsulation efficiency, rapid leakage of water-soluble drugs in the presence of blood components, instability to nebulization and poor storage stability. Recently, biodegradable, polymeric nanoparticles have received much attention as they can effectively deliver the drug to a target site and increase the therapeutic benefit, while minimizing side effects (Brannon-Peppas, 1995; Soppimath et al., 2001). Furthermore, the biological distribution of drugs can be modified, both at the cellular and organ level, using nanoparticulate delivery systems (Jung et al., 2000). Nanoparticles are solid colloidal polymeric carriers (Molpeceres et al., 1996). The drugs can either be entrapped in the polymer matrix, encapsulated in a liquid core, surrounded by a shell-like polymer membrane or bound to the

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particle surface by adsorption (Jung et al., 2000). In order to combine the advantages of the direct access and large surface area of the lung with the properties of nanoparticles, the authors designed and characterized in vitro biodegradable nanoparticles for aerosol application (Dailey et al., 2003a,b, 2006).

For the further investigation of controlled release formulations based on aerosolizable nanoparticles or liposomes in an ex vivo lung model, it is necessary to have easily detectable markers as model drugs. Therefore, this study compared the chemical properties of three structurally diverse fluorescent probe molecules and investigated the pulmonary deposition and absorption following their inhalative intrapulmonary administration in an isolated lung model.

2. Materials and methods

2.1. Fluorescent dyes

The pharmacologically inert material carboxyfluorescein (Babcock, 1983; Weinstein et al., 1986; Woolfrey et al., 1986; Pohl et al., 1998a,b; Yamamoto et al., 2004) has been extensively used to characterize the pulmonary absorption as well as the effects of liposome encapsulation on drug disposition. Therefore, 5(6)-carboxyfluorescein (CF) was chosen as model of a water-soluble compound. The other dyes investigated in this study were the highly water-soluble superpolar fluorescent probe 8-methoxypyrene-1,3,6-trisulfonic acid trisodium salt (MPTS) (Kondo et al., 1982; Sunamoto et al., 1983; Marhold et al., 1990; Becker et al., 1995), and the lipophilic amine dye, rhodamine 6G (R6G) (Duvvuri et al., 2004; Roerig et al., 2004; Roy et al., 2004). Dye solutions containing CF (Fluka, Buchs, Switzerland) and R6G (Sigma–Aldrich, Taufkirchen, Germany) were prepared in isotonic phosphate-buffered saline (PBS) at pH 7.4 to yield a final concentration of 500 μg/ml, whereas MPTS (Invitrogen, Karlsruhe, Germany) at the same concentration was dissolved in NaCl 0.9%. The chemical structure of the three fluorescent dyes is depicted in Fig. 1.

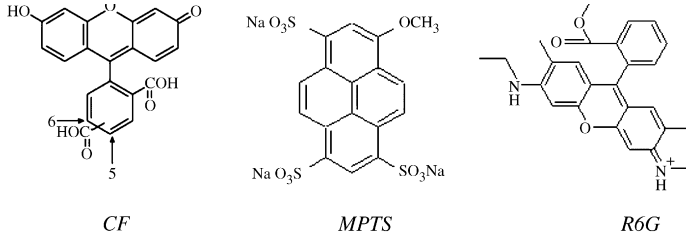
2.2. Octanol/water partition coefficient

The 1-octanol/water partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system containing 1-octanol and water (in this study PBS buffer/NaCl 0.9% was used instead of water) and is usually given in the form of its logarithm to base 10 ($\log P$):

$$P = \frac{C_{\text{octanol}}}{C_{\text{water}}}$$

where C_{octanol} is the dye concentration in the octanol phase and C_{water} is the dye concentration in the water phase.

The measurements of the 1-octanol/water partition coefficients were carried out using the shake-flask method according to the OECD Guideline 107 (OECD, 1995). The amount of test substance required for the analytical procedure ranged between the minimum concentration which was detectable in each phase and a maximum concentration of 0.01 mol/l. In order to assure the accuracy of the partition coefficient, duplicate determinations with various concentrations were prepared. The hydrophilic fluorescent dyes CF and MPTS were dissolved in PBS (CF) and NaCl 0.9% (MPTS) at a concentration of 3, 2 and 1 mg/ml (CF; $n = 6$) and 4 and 2 mg/ml (MPTS; $n = 4$) and mixed with the same volume of 1-octanol. In case of the lipophilic dye R6G 3.0, 1.5 and 0.6 mg/ml were dissolved in 1 ml 1-octanol and then the same volume of PBS was added ($n = 6$). All samples were shaken by hand for more than 5 min, centrifuged to separate the two phases, and then the fluorescence intensity of the phase with the lower dye concentration was measured. The amount of fluorescent dye in the other phase was calculated by subtraction of the measured concentration from the initial concentration.



CF *MPTS* *R6G*

Fig. 1. Chemical structures of the three fluorescent dyes.

2.3. Isolated lung model

The isolated perfused and ventilated lung model has been previously described (Seeger et al., 1986; Walmrath et al., 1992, 1997; Schermuly et al., 1997, 2000, 2001a,b, 2003).

Briefly, male rabbits weighing 2.5–3.5 kg were gradually anesthetized with a mixture of ketamine (Ketavet®, Pharmacia, Erlangen, Germany) and xylazine (Rompun®, Bayer Vital, Leverkusen, Germany) in a ratio of 3–2 and anticoagulated with heparin (1000 U/kg). After achieving a deep anesthetization the animals were ventilated with room air via a tracheal cannula using a Harvard respirator (cat/rabbit Ventilator; Hugo Sachs Elektronik, March Hugstetten, Germany). Catheters were inserted into the left atrium and the pulmonary artery after a midsternal thoracotomy. Perfusion with ice-cold Krebs–Henseleit hydroxyethylamylopectine buffer (Serag-Wiessner, Naila/Bayern, Germany) with a pulsatile flow of 10–20 ml/min was immediately started. The lungs were removed from the thorax and placed in a temperature-equilibrated chamber at 4 °C, freely suspended from a force transducer for monitoring the lung weight. Then the perfusion flow was slowly increased to 100 ml/min. At the same time the temperature of the perfusion fluid and the housing chamber were raised to 39 °C. After a 30 min steady-state period, the perfusion fluid was exchanged once by fresh buffer (total volume 300 ml). With the onset of artificial perfusion an air mixture of 21% O_2, 5.3% CO_2, and 73.7% N_2 was used for ventilation (tidal volume 30 ml, frequency 30 strokes/min). A positive end-expiratory pressure of 1 cm H_2O was used throughout. Pulmonary arterial

pressure (PAP), pulmonary venous pressure (PVP, measured in the left atrium), inflation pressure and the weight of the isolated lung were registered continuously. Left atrial pressure was set 1.5 mmHg in all experiments. Only those lungs were selected for the study that fulfilled three criteria: homogeneous white appearance with no signs of haemostasis, oedema or atelectasis; pulmonary artery and ventilation pressure in the normal range; and no weight gain during the steady-state period. During the experiments, there was loss of perfusate due to sampling, evaporation and fluid dripping (in total ~65 ml/300 min). The perfusate drops which escaped over the complete surface of the lungs (~8 ml/300 min) were collected and analysed.

2.4. Particle size determination by laser diffraction

For aerosolization of the dye solutions a piezoelectric nebulizer (Aeroneb® Professional, Aerogen, Ireland) was employed. In order to compare particle size distribution of the three aerosolized dyes and NaCl 0.9%, the mass median aerodynamic diameters (MMAD) of the aerosol droplets were determined using laser light scattering (Sympatec, Clausthal-Zellerfeld, Germany). The measurements (six runs of 100 × 50 ms duration each) were performed with an additional air flow of 10 l/min through the T-piece of the Aeroneb® Professional nebulizer, and the data were analyzed in MIE mode. The density of the nebulized solution was set equal to unit density and thus the measured volume median diameter (VMD) equalled the mass median aerodynamic diameter. The geometric standard deviation (GSD) was calculated from the laser diffraction values according to the following equation:

$$\mathrm{GSD} = \sqrt{\frac{84\%\ \text{undersize}}{16\%\ \text{undersize}}}$$

Statistical analysis of the MMAD values was performed using a nonparametric analysis of variance (Kruskal–Wallis test, SPSS.12.0.1 for windows, SPSS Software GmbH, München, Germany). After significance was found, post hoc analysis was performed with the Tukey-HSD test. Statistical significance was considered to exist at $P < 0.05$.

2.5. Aerosol output and lung deposition

The aerosol output was determined by weighing the nebulizer before and after nebulization. The amount of aerosol deposited in the isolated rabbit lung was examined in separate experiments using ^{99m}Tc-enriched saline ($n = 4$). ^{99m}Tc (11–16 MBq) (Department of Nuclear Medicine, Universitätsklinikum Giessen und Marburg GmbH, Germany) was dissolved in 6 ml saline and 3 ml of this solution was nebulized for ~8 min into the lung. In order to prevent diffusion of deposited ^{99m}Tc into the perfusate, the lung was clamped and perfusion was stopped during aerosol delivery. After nebulization, the lung was immediately removed from the system and the radioactivity of the lung (lung deposition) and the expiratory filter (exhaled fraction) were measured by a gamma-counter (Raytest, Straubenhardt, Germany). The deposition-fraction (DF) was calculated as follows:

$$\mathrm{DF} = \frac{\mathrm{LD}}{\mathrm{IF}} = \frac{\mathrm{LD}}{\mathrm{LD} + \mathrm{EF}}$$

where LD is the lung deposition, EF the exhaled fraction, and IF is the inhaled fraction = LD + EF.

2.6. Absorption characteristics of nebulized dyes in the isolated lung model

For aerosol delivery a piezoelectric nebulizer (Aeroneb® Professional, Aerogen, Ireland) was connected to the inspiratory tubing between the ventilator and the rabbit lung to be passed through by the inspiration gas. Three millilitres of the dissolved fluorescent dye were nebulized for ~8 min ($n = 4$ for each dye). In order to determine the CF, MPTS and R6G perfusate concentrations, 700 μl samples were taken from the venous part of the perfusion system. The sampling interval was every 5 min for the first 30 min and every 10 min thereafter up to a total of 300 min. Additionally, the dye concentrations in the collected perfusate drops and the amount of fluorescent dye remaining in the lung lining fluid were determined. Therefore, the lung was lavaged with a total of 150 ml of fresh perfusion fluid at the end of the experiment. A 50 ml fraction was intratracheally instilled and reaspirated three times. The total recovery of lavage fluid was ~80–95%. An additional lavage with 50 ml ethanol was carried out for the lipophilic dye R6G. Before measurement, the lavage and the dripping fluid were centrifuged at 300 × g for 10 min to remove cells. The fluorescence in the samples was measured by a fluorescence plate reader (FL600, Bio-Tek, USA) using following filters: (1) CF—λ_{ex}: 485/20, λ_{em}: 530/25; (2) MPTS—λ_{ex}: 360/40, λ_{em}: 460/40; (3) R6G—λ_{ex}: 530/25, λ_{em}: 590/35. In a 96-well plate, 100 μl (CF) or 200 μl (MPTS) of each sample were pipetted; in case of the lipophilic dye R6G 100 μl ethanol were added to the 100 μl sample fluid.

In order to consider loss of perfusate during the experiments the measured fluorescent concentration was corrected by the following formula:

$$C_{\mathrm{corr}}(t) = \frac{C(t) \times V_{\mathrm{p}}(t) + (V_{\mathrm{p}}(0) - V_{\mathrm{p}}(t)) \times C(t)/2}{V_{\mathrm{p}}(0)} = \frac{C(t)}{2} \times \frac{V_{\mathrm{p}}(t)}{V_{\mathrm{p}}(0) + 1}$$

where $C_{\mathrm{corr}}(t)$ is the corrected dye concentration in perfusate after time (t), $C(t)$ the measured dye concentration in perfusate after time (t), $V_{\mathrm{p}}(t)$ the perfusate volume after time (t), and $V_{\mathrm{p}}(0)$ is the perfusate volume at the beginning.

2.7. Experimental pulmonary hypertension

In order to examine the effect of pulmonary hypertension (PH) on the absorption of the inhaled fluorescent dye CF, an increase of pulmonary artery pressure (PAP) was provoked by a continuous infusion of the stable thromboxane A_2 mimetic U46619 (Paesel-Lorei, Frankfurt, Germany). Individual titration

Table 1
Chemical properties of the fluorescent dyes

Fluorescent dye	Molecular formula	Molecular weight (g/mol)	Fluorescence	pK_a	Octanol/water partition coefficient
CF	$C_{21}H_{12}O_7$	376.32	λ_{ex} 492 nm; λ_{em} 517 nm (0.1 M Tris)	6.5	$\log P = -3.45 \pm 0.16$
MPTS	$C_{17}H_9Na_3O_{10}S_3$	538.41	λ_{ex} 404 nm; λ_{em} 431 nm (H_2O)	n.a	$\log P = -4.95 \pm 0.21$
R6G	$C_{28}H_{31}ClN_2O_3$	479.02	λ_{ex} 528 nm; λ_{em} 551 nm (MeOH)	7.5	$\log P = 2.69 \pm 0.18$

The log P values are expressed as the mean ± S.D. λ_{ex} = laser excitation wavelength; λ_{em} = fluorescent emission wavelength; pK_a = extent of protonation; n.a = not available.

of U46619 at a concentration of 2.5 μg/ml and infusion rate of 1 to 2.5 ml/h induced an increase in PAP to ~25 mmHg within 10 min. After achieving a stable PAP plateau, 3 ml of the CF-solution were nebulized for ~8 min into the lung ($n = 4$). The samples were taken during a reduced time interval of 140 min as the rise in PAP was usually accompanied by lung oedema formation and weight gain.

3. Results

3.1. Octanol/water partition coefficient

The measured log P values of the dyes and their basic chemical properties are shown in Table 1.

3.2. Particle size distribution

The mean MMAD (±S.D.) of the aerosolized saline and dyes are presented in Fig. 2. MMAD was 4.73 μm (±0.05 μm) for saline, 4.88 μm (±0.06 μm) for CF, 4.81 μm (±0.05 μm) for MPTS and 4.70 μm (±0.06 μm) for R6G with a statistically significant difference between saline and CF only. The mean (±S.D.) geometric standard deviation (GSD) for each aerosol was 2.00 (±0.05).

3.3. Aerosol output and lung deposition

The aerosol output, calculated by weighing the nebulizer before and after 8.0 ± 0.2 min (mean ± S.D.) of nebulization, amounted to 2.65 ± 0.15 ml (mean ± S.D.). Lung deposition, as assessed by nebulization of ^{99m}Tc-enriched saline, was 0.4 ± 0.05 ml (mean ± S.D.). In the expiratory filter 0.38 ± 0.02 ml (mean ± S.D.) were found and therefore the deposition fraction was calculated to 51 ± 3% (mean ± S.D.). Consequently, the lung deposition of nebulized dyes with a concentration of 500 μg/ml filled in the nebulizer was 200 ± 25 μg (mean ± S.D.).

3.4. Absorption characteristics of nebulized dyes in the isolated lung model

Fig. 3 shows the concentration–time profiles in the perfusate of the three dyes following pulmonary administration by inhalation. After a continuous increase during the first 160 min, the CF-concentration reached a stable plateau at approximately 400 ng/ml. MPTS showed a similar increase during the first 30 min reaching a steady state concentration of ~230 ng/ml after 120 min. The lipophilic R6G demonstrated the lowest perfusate concentrations with a slow increase in the first 30 min to ~38 ng/ml, followed by a gradual decrease to ~26 ng/ml.

Fig. 4 illustrates the perfusate concentrations of CF in the isolated rabbit lung with normal PAP (as described before) and with experimental pulmonary hypertension (CF + PH). During the sampling interval of 140 min there were no significant differences between the concentration–time profiles.

The dye distribution within the experimental system is shown in Table 2.

After CF administration a total of 74.9 ± 7.3% of the aerosolized dye was recovered with 53.4 ± 6.8% in the perufsate and 14.8 ± 2.1% in the lavage fluid and no significant changes in the pulmonary hypertension experiments (49.9 ± 4.1% in the perfusate, 22.8 ± 6.6% in the lavage fluid). A lower recovery

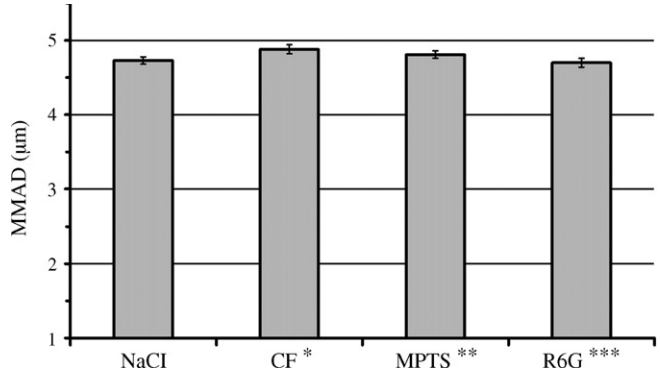


Fig. 2. Particle sizes of the aerosolized fluorescent dyes in comparison to NaCl 0.9%. Results are expressed as mean ± S.D. ($n = 6$). $^{*}P = 0.001$, compared with NaCl 0.9%. $^{**}P = 0.073$, compared with NaCl 0.9%. $^{***}P = 0.744$, compared with NaCl 0.9%.

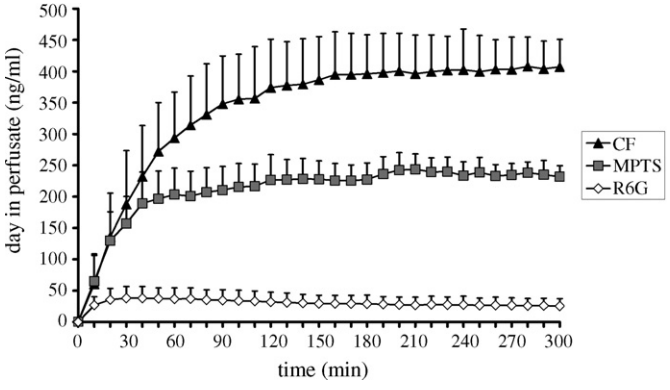


Fig. 3. Perfusate concentration–time profiles after nebulization of 3 ml fluorescent dye (500 μg/ml) with deposition of 0.4 ml (200 μg) into the isolated, perfused rabbit lung. Results are expressed as mean + S.D. ($n = 4$ for each dye).

Table 2
Final distribution of the fluorescent dyes in the different compartments of the system

	Lavage	Perfusate	Dripping fluid	Samples
CF	29.5 ± 4.1 μg *14.8 ± 2.1%*	106.9 ± 13.7 μg *53.4 ± 6.8%*	5.7 ± 4.9 μg *2.9 ± 2.5%*	7.8 ± 1.4 μg *3.9 ± 0.7%*
MPTS	20.0 ± 1.7 μg *10.0 ± 0.9%*	58.2 ± 3.9 μg *29.1 ± 2.0%*	1.1 ± 0.7 μg *0.6 ± 0.4%*	7.4 ± 1.4 μg *3.7 ± 0.7%*
R6G	42.8 ± 9.9 μg *21.4 ± 5.0%*	6.6 ± 1.3 μg *3.3 ± 0.7%*	0.1 ± 0.1 μg 0.1 ± 0.1%	0.7 ± 0.2 μg *0.4 ± 0.1%*
CF + PH	45.5 ± 13.2 μg *22.8 ± 6.6%*	99.8 ± 8.1 μg *49.9 ± 4.1%*	7.7 ± 3.9 μg *3.9 ± 2.0%*	3.1 ± 0.6 μg *1.6 ± 0.3%*

In order to calculate the percentage (in italics) of the dye concentration at each compartment, the deposited amount of 200 μg was set 100%. Results are expressed as mean ± S.D.

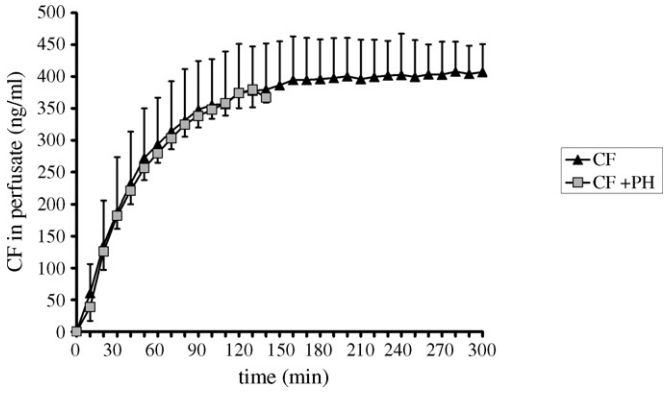


Fig. 4. Perfusate concentration–time profiles after nebulization of 3 ml CF (500 μg/ml) and deposition of 0.4 ml (200 μg) into the isolated rabbit lung with normal PAP (CF) and with experimental pulmonary hypertension (CF + PH). The CF-results are expressed as mean + S.D. ($n = 4$), CF-PH-results as mean − S.D. ($n = 4$).

of only 43.2 ± 2.4% was measured in the MPTS experiments with 29.1 ± 2.0% in the perfusate and 10.0 ± 0.9% in the lavage fluid. After R6G nebulization hardly any dye (3.3 ± 0.7%) was found in the perfusate and 21.4 ± 5.0% in the lavage fluid. However, the additional ethanol lavage removed another 60.6 ± 6.2% of R6G from the lung and thus total recovery was 85.7 ± 9.2%.

4. Discussion

The absorption characteristics of the fluorescent dyes CF, MPTS and R6G after intrapulmonary administration in an isolated rabbit lung showed a wide variety of differences.

The amount of dye deposited within the lung depends on aerosol particle size, tidal volume, breathing frequency and ventilatory mode (Forsgren et al., 1990). Tidal volume, breathing frequency and ventilatory mode were identical in all experiments. The differences in particle size distribution and output of the dye containing aerosols were negligible (Fig. 2) and therefore the same total lung deposition for the three model drugs could be achieved. Consequently, there must be other reasons for the disparate absorption characteristics. In general, pulmonary absorption of drugs is influenced by various physicochemical and biological factors, including molecular size, extent of protonation (pK_a), pH of the drug solution and lipophilicity (Anderson et al., 1974; Folkesson et al., 1990; Yamamoto et al., 2004). As shown in Table 1, there are minor differences in molecular weight and pK_a of the fluorescent dyes, but a clearly different lipophilicity. The 1-octanol/water partition coefficient is the most commonly used parameter in medicinal chemistry and gives accurate predictions of activity in a complex biological system such as membranes (Roda et al., 1990). In the present study, the lipophilicity of the dyes was determined by using the 1-octanol/water shake-flask procedure. In contrast to the hydrophilic dyes CF and MPTS ($\log P = -3.45$ and -4.95), the lipophilic amine dye R6G ($\log P = 2.69$) can pass through the lipid region of the membranes.

After aerosolization of the hydrophilic dyes CF and MPTS in the isolated rabbit lung a rapid increase of dye perfusate concentration with a stable plateau after approximately 120 min was observed. In contrast, the perfusate concentration of the lipophilic R6G showed an increase only in the first 30 min after pulmonary administration followed by a gradual decrease. The low recovery of R6G in the perfusate can easily be explained by the hydrophilic character of the perfusate, the significant absorption and the redistribution phenomenon of R6G can be attributed to the presence of P-gp-mediated efflux in the isolated rabbit lung. In fact, Roerig et al. (2004) selected R6G as test substrate based on its reported uptake in cells and developed an experimental and kinetic model to characterize the kinetics of P-glycoprotein (P-gp) mediated efflux of R6G out of the isolated, perfused rabbit lung. In these experiments, known amounts of R6G were added to the recirculating perfusate reservoir and the amount of R6G taken up by the lung was determined by its disappearance from the recirculating reservoir. After 120 min nearly 67% of the perfusate R6G was taken up by the lung. In the presence of P-gp inhibitors, R6G uptake increased significantly, indicating a functional pulmonary P-gp efflux transporter. The majority of P-gp substrates are lipophilic amine drugs which also exhibit rapid extensive accumulation in lung tissue. The presence of the P-gp, which is an ATP-dependent drug efflux transporter involved in multidrug resistance and expressed in lung tissue, could be the main reason in our experiments for the efflux of the nebulized R6G from the lipophilic milieu of the rabbit lung into the aqueous perfusate. The assumption, that after a saturation of the P-gp-mediated efflux the R6G dye was

taken up by the lung again, is consistent with the observed decrease in perfusate concentration–time profile of this dye (Fig. 3).

Despite the hydrophilic character of CF and the resulting low membrane permeability, a high recovery of this dye in the perfusate was measured suggesting a significant paracellular uptake of small hydrophilic molecules. Pohl et al. (1998a,b) illustrated the distribution of the fluorescent dyes Rhodamine B (RB) and 6-carboxyfluorescein (6-CF) by confocal laser scanning fluorescence microscopy (CLSFM) after intratracheal instillation in intact, fully hydrated, unfixed rat lungs. Similar to R6G, RB is an amphoteric dye with a high partition coefficient ($\log P = 2.44$) and would, therefore, be expected to readily partition into such lipophilic regions as the lamellar bodies of type II cells and the lipid bilayer of cell membranes. In fact, when RB was intratracheally instilled into the airspace it quickly and preferentially illuminated alveolar macrophages and alveolar epithelial surfaces. On the other hand, the majority of 6-CF ($\log P = -3.1$) appeared to occupy interstitial spaces. Cuboidal cells in the corners of alveoli (probably type II cells) which were stained red by RB, did not appear to contain 6-CF within their cytoplasm. 6-CF left the airspaces most rapidly and accumulated in the extracellular regions of the interstitium behind type II epithelial cells, where three alveoli in close apposition formed a "trijunctional pore". In contrast to paracellular diffusion, passage through such pores could involve an osmotically driven convective process wherein water accompanies the movement of 6-CF into interstitial spaces that might distend as a result. This is consistent with the speed of the 6-CF appearance in the interstitium accompanied by extension of the interstitium during the CLSFM-experiments. Woolfrey et al. (1986) proposed that the very short time in which peak blood concentrations were reached after intratracheal instillation of 6-CF, was incongruous with a slow singular first-order absorption process. Therefore, he proposed an absorption model for 6-CF involving two simultaneous inputs into the central compartment by both, active transport and passage through the pores of the epithelium membrane.

In various lung diseases, such as pulmonary hypertension, pulmonary embolism, and adult respiratory distress syndrome (ARDS), pulmonary vascular pressure and resistance are elevated. Therefore, we investigated the influence of an increased pulmonary artery pressure provoked by a continuous infusion of the stable thromboxane A_2 mimetic U46619 on the pulmonary absorption of CF. Despite a marked increase of pulmonary artery pressure accompanied by progressive lung oedema formation and weight gain, there were no changes in CF absorption in the experiments with U46619-induced pulmonary hypertension compared to normal control lungs (Fig. 4). Firstly, we conclude that the amount and deposition pattern of inhaled aerosols is not altered by experimental U46619-induced pulmonary hypertension. This is in line with the observations of Forsgren et al. (1990). They investigated whether the pulmonary dysfunction related to endotoxin infusion influenced the intrapulmonary distribution. Based on analysis of Evans blue dye and FITC-labeled liposomes in different lung-pieces, they did not find any systematic difference in the deposition pattern between the control and endotoxin groups. Furthermore, as demonstrated by Wakerlin et al. and Shibamato et al., the mechanism by which thromboxane produces pulmonary oedema in the isolated lung is pulmonary venoconstriction resulting in increased pulmonary hydrostatic pressure without affecting vascular permeability (Wakerlin et al., 1991; Shibamoto et al., 1995).

Although there were clear differences in absorption between the lipophilic R6G which accumulated in cells and remained in lung tissue, and hydrophilic dyes which passed the lung and appeared in perfusate, this can hardly explain the variation between the two hydrophilic model probes CF and MPTS. However, it is known that the fluorescence of MPTS and related anionic ethers is statically quenched by a number of substances including cationic molecules, halides and various metal ions such as Fe(III) (Marhold et al., 1990; Cordes et al., 2005). Therefore, an interaction of MPTS with Fe(III), contained in the lung tissue, or other substances like cationic surfactant proteins might be the reason for the low concentrations as well in the perfusate as in the lavage fluid, resulting in a low total recovery of MPTS (Table 2).

In conclusion, the present findings suggest that CF is a suitable model drug for the examination of the controlled release effects of aerosolized carrier formulations. After inhalative administration, CF shows a rapid, high and reproducible absorption with a stable concentration plateau in the perfusate independent on elevated pulmonary artery pressure and consecutive vascular leakage and oedema formation. In contrast, the lipophilic dye R6G shows high accumulation in lung tissue and the highly water-soluble fluorescent probe MPTS demonstrates insufficient recovery due to quenching. Therefore, both dyes are unsuitable for the examination of drug transport from the lung compartment into the perfusate.

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