Pharmaceutical Research, Vol. 23, No. 8, August 2006 (© 2006)
DOI: 10.1007/s11095-006-9029-y

Research Paper

Applicability of an Ultrasonic Nebulization System for the Airways Delivery of Beclomethasone Dipropionate in a Murine Model of Asthma

Boška Hrvačić,[1,3] Berislav Bošnjak,[1] Marijan Tudja,[2] Milan Mesić,[1] and Mladen Merćep[1]

Received June 6, 2005; accepted April 7, 2006

Purpose. We have assessed the use of an ultrasonic nebulization system (UNS), composed of ultrasonic nebulizer and diffusion dryer filled with charcoal, for the effective delivery of beclomethasone to the airways in a murine asthma model.
Methods. Solution of beclomethasone in ethanol was aerosolized using an ultrasonic nebulizer. Passage of the aerosol through a drying column containing charcoal and deionizer produced dry beclomethasone particles. Particles were delivered to BALB/c mice placed in a whole-body exposition chamber 1 h before intranasal challenge with ovalbumine. Efficacy of beclomethasone delivery was evaluated by examining bronchoalveolar lavage fluid (BALF) cytology.
Results. Effect of three UNS system parameters on aerosol particle size was investigated. The critical parameter affecting the size of dry particles was beclomethasone concentration in aerosolized solution and solution flow rate while power level of ultrasonic nebulizer generator had no effect. Administration of beclomethasone at calculated dose of 150 μg/kg to mice significantly decreased total cell number and relative eosinophil number in BALF.
Conclusions. The UNS system produces a monodisperse aerosol that can be used for inhalative delivery of poorly water soluble substances to experimental animals. The UNS system minimizes formulation requirements and allows rapid and relatively simple efficacy and toxicity testing in animals.

KEY WORDS: asthma; beclomethasone dipropionate; dry powder; inhalation delivery; mice; ultrasonic nebulization system.

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are among the most common chronic diseases and the prevalence of both diseases is increasing. It is estimated that asthma and COPD each affects approximately 4 to 10% of the population (1–4) and they represent an important pharmacoeconomic burden to society. In the treatment of asthma and COPD, as well as other pulmonary disorders, localized delivery of drugs to the respiratory tract is an important and effective therapeutic method. Inhalation delivery for the treatment of lung disorders has the clinical advantages over systemic therapy since it requires relatively small doses for effective therapy, minimizes possible side effects, and facilitates delivery of macromolecules that are poorly absorbed from the gastrointestinal tract (5,6). Inhalation delivery is dependent on dispersion of solid and liquid particles suspended in gas, i.e., aerosol generation.

In human medicine, systems used for drug delivery by inhalation include pressurized metered-dose inhalers (pMDI), dry powder inhalers (DPI), and jet pneumatic or ultrasonic nebulizers, as well as recently developed small volume liquid inhalers (7–9). pMDI was the most frequently prescribed aerosol delivery system because they were effective and convenient for a large proportion of patients (10). However, the most widely used propellants for pMDI operation, chlorofluorocarbons, were prohibited in the year 2000 by the Montreal Protocol. Although new pMDIs that use environmentally benign propellants have been developed, alternative devices, such as DPI and ultrasonic nebulizers, have gained in popularity. DPI are breath-activated inhalers that rely on the patient's inspiratory flow to deliver the micronized drug particles to the respiratory tract (10). Dry powder generation is often hindered by aggregation of drug particles, which is exacerbated by the electrostatic charge of micronized particles and the hygroscopic nature of the drug (10). In contrast to DPI, nebulizers produce aerosols from drug solutions. Ultrasonic nebulizers utilize conversion of high frequency electrical pulses to mechanical vibrations which then convert liquid into a fine mist (11). In comparison to pMDI and DPI, nebulizers offer several advantages: they produce narrow-sized particles that enable uniform delivery of drug into the small airways.

[1] PLIVA Research Institute Ltd., Prilaz baruna Filipovića 29, HR-10000, Zagreb, Croatia.
[2] PLIVA Croatia Ltd., Zagreb, Croatia.
[3] To whom correspondence should be addressed. (e-mail:boska.hrvacic@pliva.hr)

ABBREVIATIONS: BALF, bronchoalveolar lavage fluid; beclomethasone, beclomethasone dipropionate; COPD, chronic obstructive pulmonary disease; DPI, dry powder inhaler; FPF(<2.20 μm), fine particle fraction <2.20 μm aerodynamic diameter; GSD, geometric standard deviation; pMDI, pressurized metered-dose inhaler; SD, standard deviation; UNS, ultrasonic nebulization system.

Nebulizers could also be used for administration of biomolecules in aqueous formulations to the respiratory tract (11–13).

Many of new therapeutics for asthma and COPD are intended to be delivered locally to the respiratory tract. In preclinical testing of these compounds drop wise application of solutions or suspensions intranasally (14,15), intratracheal instillation (16,17) and delivery by dry powder or liquid aerosols are used (17,18). It is preferable to use the same mode of respiratory drug delivery in preclinical testing as well as in clinical development. To be effective in animal studies, the system should reproducibly produce and deliver aerosol particles with a well-defined and narrow size distribution over a wide range of concentrations (19,20). Since breathing of conscious animals in animal exposure studies cannot be controlled, drug particle deposition depends almost exclusively on the aerodynamic properties of the aerosol. Restraints imposed by most commonly used dry powder generators for inhalation delivery in various *in vivo* models include the limited choice of possible drug formulations, the requirement for a relative large quantity of substance and the considerable costs associated with drug micronization. Potential new medications could alternatively be tested as aerosols produced by nebulization of their solutions (21,22), but medications that are used for treatment of asthma and COPD (i.e., corticosteroids) are frequently poorly water-soluble and must therefore be dissolved in organic solvents such as methanol or ethanol prior to nebulization (23). It was recently reported that passing an ethanol aerosol stream through a diffusion drier containing an annular ring of charcoal significantly reduces ethanol content and produces dry inhalable particles (23–25). The system composed of an ultrasonic nebulizator and a diffusion dryer was reported to have a constant and controllable substance output (23–25), but was not formally used for efficacy and toxicity testing of drugs in animals.

In this study we compared characteristics of aerosol generation system composed of ultrasonic nebulizer and diffusion dryer to the UNS systems described earlier (23–25) and determined parameters for effective delivery of a drug to mice. Applicability of the system for delivery and efficacy testing of anti-asthma drugs in the murine model of asthma was tested by delivering standard corticosteroid, beclomethasone dipropionate, and measuring the reduction in airway eosinophilia.

MATERIALS AND METHODS

Animals

All studies were performed on 8–12 weeks old male BALB/c mice (Charles River Laboratories Inc., Wilmington, MA, USA). Mice were kept on wire mesh floors with irradiated maize granulate bedding (Scobis Due, Mucedola, Italy) and maintained under standard laboratory conditions (temperature 22 ± 2°C, relative humidity 55 ± 10%, about 20 air changes *per* hour filtered on HEPA 99.97%, artificial lighting cycle of 12 h). Food (Mucedola, Italy) and tap water were provided *ad libitum*.

All procedures on animals were performed in accordance with (a) the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985); (b) the EEC Council Directive 86/609 of 24th November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes; and (c) Statute of Republic Croatia, Animal welfare law, NN 081-99-266/1 of 9th February 1999.

Chemicals

Beclomethasone dipropionate (≥99%; beclomethasone), phosphate buffered saline (PBS) and chicken egg ovalbumine grade VI (~99%; OVA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Charcoal, 4- to 8-mesh, was obtained from ALDRICH Chemical Company, Inc. (Milwaukee, WI, USA), ethanol and toluene were from Merck (Darmstadt, Germany) and Alu-Gel-S was from SERVA Electrophoresis GmbH (Heidelberg, Germany). Diff-Quick staining set was purchased from Dade Behring Inc. (Newark, DE, USA).

Murine Asthma Model

To develop pulmonary eosinophilia, mice were sensitized with OVA on experimental days 0 and 14. Sensitization was done by intraperitoneal application of 10 μg of OVA dissolved in 2% Alu-Gel-S in a total volume of 0.2 ml/mouse. On experimental day 20, mice, under light anesthesia (combination of ketamine hydrochloride [2 mg/mouse; Narketan® Vetoquinol, Bern, Switzerland) and xylazine-hydrochloride (0.07 mg/mouse; Rompun®, Bayer, Leverkusen, Germany)], were intranasally instilled with 25 μg of OVA dissolved in PBS in a total volume of 50 μl. A control group of mice received 50 μl of PBS (negative control group). One group of mice was challenged with OVA and left untreated or treated with vehicle (positive control group). All aerosol treatments with beclomethasone were done also on experimental day 20, ending 1 h before the challenge with OVA. Conscious mice were placed into the exposure chamber of the UNS system and simultaneously exposed to beclomethasone aerosol (treated group). On experimental day 22, approximately 48 h after OVA instillation, mice were euthanized, tracheas were cannulated and lungs were lavaged three times with PBS in a total volume of 1 ml (0.4, 0.3 and 0.3 ml, respectively). Bronchoalveolar lavage fluid (BALF) was cytocentrifuged for 10 min at 250 rpm (Cytospin-3, Shandon Instruments, UK). Percentage of eosinophils was determined by morphological examination of at least 200 cells on smears differentially stained with Diff Quick. In one experiment total number of cells in BALF was counted in a hemocytometer (Sysmex SF 3000).

In order to combine results from different experiments, percent inhibition of eosinophil accumulation in BALF induced by beclomethasone was calculated with the formula:

$$\%\ eosinophil\ inhibition\ in\ BALF = 100\% - \left(\frac{\%\ BALF\ eosinophil\ in\ aerosolized\ group}{\%\ BALF\ eosinophil\ in\ positive\ control\ group} \times 100\%\right)$$

Ultrasonic Nebulization System (UNS)

The ultrasonic nebulization system (UNS) was constructed as described previously (19,23,24) with several modifications (Fig. 1). The system consisted of an ultrasonic nebulizer, drying column, deionizer and animal exposure chamber. Solutions were pumped into the nebulizer with a syringe pump (Model 74900, Cole-Parmer Instrument Co., Vernon Hills, IL, USA). The nebulizer, an ultrasonic spray nozzle system (Sono-Tek Corp., Milton, NY, USA) was driven by an ultrasonic generator operating at a fixed frequency of 125 kHz. The nebulizer was attached to an air supply device that injects a stream of air (3 l/min) around nozzle of the nebulizer. The nebulizer and air shaped device were mounted on the upper conical portion of a 500 ml round-bottom flask. On the side arm of the round-bottom flask a 45 cm long drying column (TSI Inc., Shoreview, MN) was connected using the tygon tube. Spray drying of the aerosol was attained by passing of aerosol through the inner cylinder of drying column surrounded by charcoal. The outlet of the drying column was connected through the deionizer (TSI Inc.) to an animal exposure chamber (chamber for guinea pig, cat. no. PLY3215, Buxco Electronics, Inc., Wilmington, NC, USA). Air pumps (Buxco Electronics, Inc.) attached to the exit of the exposure chamber (flow 6 l/min) supplied a continuous flow of fresh air and supported passing of the aerosol through the system.

Determination of the Dry Powder Particle Properties

To determine number of particles, their aerodynamic diameter, width of distribution (expressed as geometric standard deviation, GSD) and fine particle fraction with aerodynamic diameter of <2.20 μm [FPF(<2.20 μm)] the aerosol was delivered to an Aerosizer 3220 (TSI Inc.) attached to the UNS instead of the animal exposure chamber. The Aerosizer is a time-of-flight particle sizer spectrometer that automatically measures number of particles and their aerodynamic diameter and calculates GSD and FPF(<2.20 μm) (19,26,27).

To determine the morphology of the produced particles, they were collected on a microscopic cover glass exposed to the aerosol stream in the exposure chamber. Samples of dry particles were coated with gold in S150 Sputter coater (Edwards) and examined by a scanning electron microscope (JSM-5800, Jeol).

Determination Ethanol Removal by Drying Column

Concentration of ethanol in aerosol generated by UNS was determined in vapors present before and after drying column by using gas chromatography coupled with mass detector (GC-MS, Varian Saturn 2000). Aerosol that contains ethanol vapors produced by UNS system had constant flow which was maintained by UNS. After the system was stably equilibrated for about 5 min, the exact volume (10 ml) of the aerosol was captured by syringe that did not produce resistance or disturbance in overall pressure of the system before and after the drying column. After the sample was collected, additional 1 ml of toluene was drown into the syringe and stopper was closed. Syringe was vigorously shaken for 20 s, placed in the upright position and content transferred

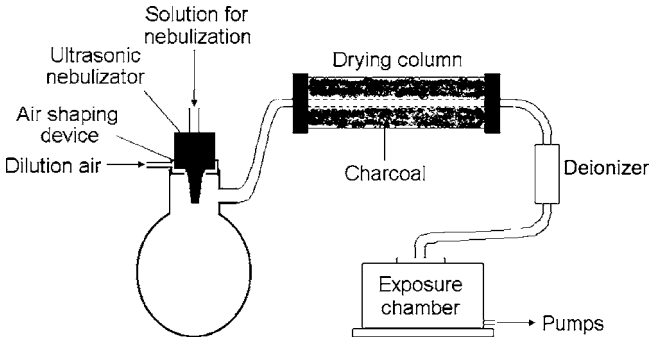

Fig. 1. Schematic diagram of the ultrasonic nebulization system (*UNS*) used for inhalation drug delivery. For details see text.

to the 2 ml glass vial through the needle attached to the syringe. By performing the transfer in this manner, the toluene solution was placed first in the vial and then remaining syringe volume was emptied by slowly bubbling it through the toluene. Amount of ethanol was determined by GC-MS analysis and on the basis of standard calibration curve. Efficacy of drying column was expressed as % ethanol removal and was calculated according to the formula: % ethanol removal = (1-(concentration of ethanol in vapors after the drying column/concentration of ethanol in vapors before the drying column)) × 100%.

Calculation of Beclomethasone Dose

The dose administered to the mice was estimated according to the formula described in Wattenberg *et al.* (21):

$$D_{\mathrm{L}} = \frac{C \times MV \times T}{BW} \tag{1}$$

where D_{L} is the estimated dose level of substance (mg/kg body weight), C is the substance dry powder concentration in the exposure chamber (mg/l), T is the duration of exposure (min), BW is the mean group body weight (kg) and MV is the minute volume (l/min) estimated according to the formula of Guyton (28): $MV = BW^{0.75} \times 2.10$.

The concentration of beclomethasone dry powder in the exposure chamber (mg/l) was calculated according to the formula

$$C = \frac{4 \times \rho \times N \times r^3 \times \pi}{3 \times V_{\mathrm{a}}} \tag{2}$$

where ρ is the estimated substance density (1.2 kg/dm^3), N is the number of dry powder particles *per* minute, r is the substance dry powder particle diameter (m) and V_{a} is the air volume pumped into the system *per* minute (3 l).

The inherent assumptions of this approach are that the produced particles are spherical and that all the inhaled particles are completely deposited in the lung. It was assumed that particles from UNS are spherical since it was reported that spray-drying technique produces small spherical particles with narrow size distribution (29).

Data Analysis and Statistical Evaluation

Properties of the measured dry powders produced by the UNS system were compared by one-way ANOVA, followed by the Tukey–Kramer multiple comparison test. Relative eosinophil number in BALF from the positive control and individual beclomethasone-treated groups were compared by unpaired *t*-test. Statistical analysis was performed using the statistical program GraphPad InStat (version 3.05, GraphPad Software Inc., San Diego, CA, USA). Level of significance was set at $p < 0.05$ in all cases.

RESULTS

Previous investigations indicated that dry powder particle size produced by UNS depends not only on the intrinsic properties of the solute and test compound, but is also determined by parameters such as solution flow rate, power level and frequency of the ultrasonic nebulizer. Since the frequency of the ultrasonic nebulizer in the system employed is fixed, the effects of changes in (a) concentration of beclomethasone solution used for nebulization, (b) solution flow rate, and (c) power level of ultrasonic nebulizer generator on aerosol particle properties were tested prior to animal studies in order to determine parameters which ensure production of drug particles optimal for lung deposition in mice. Particle properties were measured by time of flight particle sizer spectrometer through determination of dry particle number, aerodynamic diameter (size), fine particle fraction [FPF(<2.20 μm)] and width of distribution described by geometric standard deviation (GSD).

Effect of Liquid Flow Rate on Dry Powder Particle Properties

The effect of liquid flow rate on the properties of the dry powder was measured using a constant concentration of beclomethasone solution (1 mg/ml) with the power level of ultrasonic nebulization generator set at 2.8 arbitrary units. Small, non-significant changes in the number of produced particles, their size and GSD were observed as liquid flow rate increased from 0.3 to 1.2 ml/min (Fig. 2). However, increasing liquid flow rate above 1.2 ml/min markedly decreased the number of particles produced, as well as their size, with a concomitant increase in GSD (Fig. 2). Since increase in liquid flow rate above 1.2 ml/min led to deterioration of UNS performance, all subsequent measurements were done at this flow rate.

Effect of Ultrasonic Nebulizer Generator Power Level on Dry Powder Particle Parameters

Effect of ultrasonic nebulizer generator power level on particle parameters was measured using a constant concentration of beclomethasone dipropionate solution in ethanol (1 mg/ml) and the liquid flow rate at 1.2 ml/min. In contrast to effects of liquid flow rate, variation of ultrasonic nebulization generator power level in the available range had no significant effect on number, aerodynamic diameter or GSD of dry particles (Table I).

Although the difference was not significant, the aerosol with the narrowest particle distribution (GSD) was produced when the power level of the ultrasonic nebulizer generator was set at 2.8 arbitrary units. Therefore, a power level of 2.8 was used for subsequent testing of the UNS system.

Effect of Nebulized Beclomethasone Solution Concentration on Dry Powder Particle Properties

To test the effect of the concentration of the nebulized solution on dry powder particle properties, solutions with increasing concentrations of beclomethasone in ethanol were nebulized with the ultrasonic nebulization generator power level set at 2.8 arbitrary units and at a constant liquid flow rate of 1.2 ml/min. An increase in the concentration of the nebulized beclomethasone solution increased number and

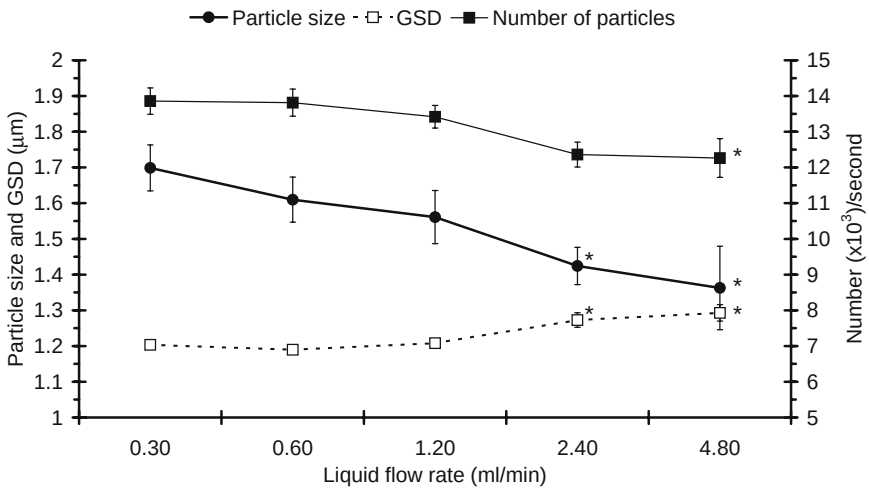

Fig. 2. Effect of liquid flow rate on aerosol particle size, geometric standard distribution (*GSD*) and their number. All measurements were done by nebulizing 1 mg/ml of beclomethasone dipropionate solution in ethanol with the power level of the ultrasonic nebulizer generator set at 2.8 arbitrary units. All data are means ± SD ($n = 5$). Significant differences ($p < 0.05$) *versus* measurements taken at liquid flow rate of 0.3 ml/min are marked with an asterisk (*).

Table I. Effect of Ultrasonic Nebulizer Generator Power Level on Number of Particles, Mean Particle Size and Geometric Standard Deviation (GSD)

	Power Level Setting (Arbitrary Units)		
	1.4	2.8	4.0
Number of particles/s	13,282.4 ± 318.3	13,418.9 ± 316.3	13,265.5 ± 210.7
Particle size (μm)	1.536 ± 0.07	1.561 ± 0.07	1.485 ± 0.03
GSD (μm)	1.223 ± 0.011	1.208 ± 0.013	1.216 ± 0.006

All measurements were obtained after nebulization of a solution of beclomethasone dipropinate (1 mg/ml) in ethanol at a liquid flow rate of 1.2 ml/min. Data are means ± SD (n = 5).

aerodynamic diameter of the dry particles (Fig. 3). The smallest GSD, although not significantly different, was recorded after nebulization of 1 mg/ml beclomethasone solution (Fig. 3). Thus, the concentration of nebulized solution seems to be an important parameter that can be used to obtain an aerosol with different particle number, size and width of distribution.

Use of UNS to Deliver Beclomethasone Dipropionate in a Murine Asthma Model

The particle size that is optimal for lung deposition in mice is approximately 1.5 μm (30,31 and references therein). The results from UNS characterization experiments indicated that for optimal delivery of dry particles to mice, the following parameters should be used: beclomethasone solution concentration of 1 mg/ml, liquid flow rate of 1.2 ml/min and nebulizer power level set at 2.8 arbitrary units. Particle aerodynamic diameter distribution from UNS system set as described was determined by time-of-flight analyzer (Fig. 4a). Produced particles had normal and well-defined distribution with 90% of the produced particles in the range from 0.89 to 1.67 μm, and FPF(<2.20 μm) was 98.95 ± 1.88%. Examination of the particle morphology by scanning electron microscopy revealed that particles produced by UNS are spherical (Fig. 4b).

To ensure that ethanol is efficiently removed and does not unduly interfere with determination and interpretation of beclomethasone efficacy, removal of ethanol by the drying column was tested both analytically and functionally. To measure the efficacy of ethanol removal by drying column in the vapors before and after the column, ethanol quantity was determined by GC-MS analysis. Obtained results indicate that the drying column removes 66% of ethanol initially present in the aerosol. In functional experiments, mice were exposed to 60 or 100 ml of nebulized ethanol, corresponding to volumes of ethanol used for dissolution and delivery of two highest doses of beclomethasone, 1 h prior to antigen challenge (Table II). OVA sensitized and challenged groups treated with ethanol had practically identical relative number of eosinophils in BALF (32 ± 9 and 47 ± 11) as the non-treated positive control group (33 ± 8 and 48 ± 11), respectively. Obtained results demonstrate that remaining ethanol even at the highest tested dose had no effect on eosinophilia in BALF. However,

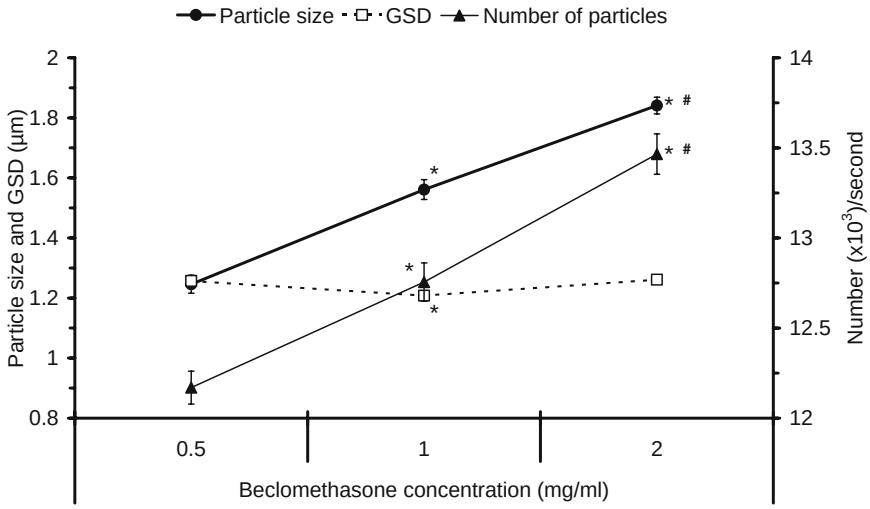

Fig. 3. Effect of concentration of nebulized beclomethasone dipropionate (beclomethasone) solution in ethanol on aerosol particle size, geometric standard distribution (*GSD*) and their number. Liquid flow rate was set at 1.2 ml/min and the power level of ultrasonic nebulizer generator was set at 2.8 arbitrary units for all measurements. Data are means ± SD ($n \geq 5$). Significant differences ($p < 0.05$) *versus* measurements taken with 0.5 mg/ml beclomethasone solution are marked with an asterisk (*) and *versus* measurements taken with 1 mg/ml beclomethasone solution with sign (#).

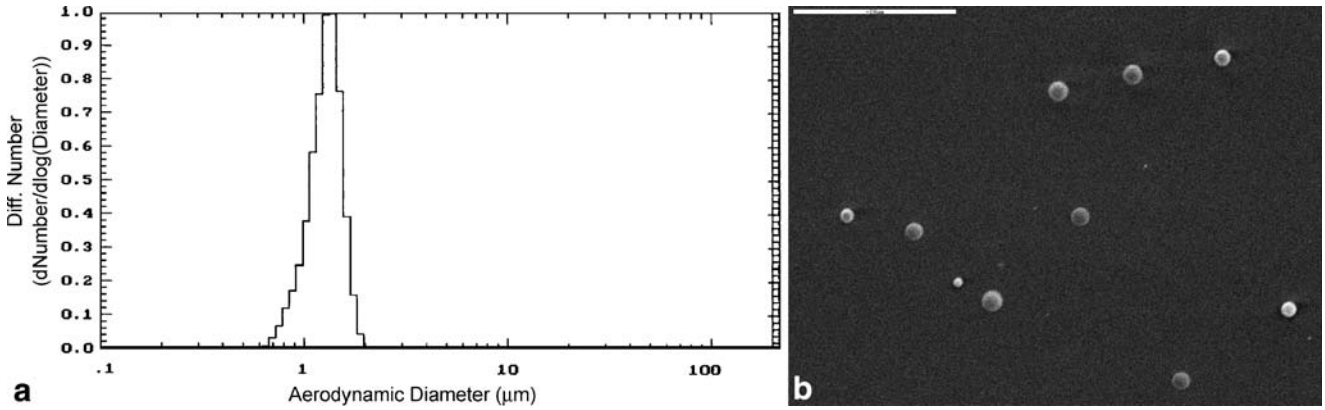

Fig. 4. Size distribution of dry particles produced by UNS and their morphology. Dry powder aerosol was produced by nebulizing solution of beclomethasone dipropionate in ethanol (1 mg/ml) with liquid flow rate at 1.2 ml/min and the power level of ultrasonic nebulizer generator set at 2.8 arbitrary units. **a** Size distribution was determined by Aerosizer 3220 (TSI Inc.). **b** Scanning electron microscopy picture showing the morphology of dry particles produced by UNS.

prolonged nebulization of ethanol (nebulization of volumes ≥200 ml, or for ≥140 min) induced initial symptoms of ethanol intoxication in animals, including irritation of the nose and throat, dizziness and confusion (data not shown).

Subsequently, the UNS system was used to deliver beclomethasone to OVA-sensitized mice. Calculation of the approximate dose delivered to the animals according to formulas (1) and (2) indicated that nebulization of 15 ml of beclomethasone solution over 12.5 min delivers ~22.5 μg/kg (b.w.) to mice. To increase the delivered dose nebulization of larger volumes of beclomethasone solution was required, which prolonged exposure time. To test various beclomethasone doses in OVA induced eosinophilia in mice a series of consecutive experiments was conducted. Relative number of eosinophils in BALF is a good indicator of asthma severity (32–35) and was scored as the principal experimental parameter in this model. In total, ten different experiments were performed and experimental data are shown in Table III. In order to compare effects of beclomethasone from different experiments, inhibition of pulmonary eosinophilia was calculated as described in Materials and Methods and graphically depicted in Fig. 5. When lower doses of beclomethasone (22.5 or 45 μg/kg (b.w.)) were administered, only slight inhibition (<20%) of eosinophil accumulation in BALF was detected. Delivery of beclomethasone by the UNS system at doses larger than 67.5 μg/kg (b.w.) inhibited accumulation of eosinophils in BALF by more than 60%, indicating a relatively steep dose response that is characteristic for the effect of steroids in asthma. The 150 μg/kg dose was found to be the most effective in decreasing relative number of eosinophil and the effect of this dose on BALF cytology was evaluated in detail (Fig. 6). Beclomethasone treatment significantly decreased both the total cell number (Fig. 6a), eosinophil percentage in BALF (Fig. 6b) and number of macrophages (Fig. 6c). In contrast, treatment with ethanol, applied in the same volume as beclomethasone, did not significantly change total cell number or percentage of individual cell populations in BALF (Fig. 6).

DISCUSSION

Preclinical animal experiments constitute specific challenges and requirements on the system that is intended for inhalation drug delivery. This type of delivery to the respiratory tract is influenced by inhaled particles properties (i.e., diameter and size distribution) as well as on breathing patterns of experimental animals (i.e., frequency, tidal volume and flow) (10). As the respiration of experimental animals is species-dependent and cannot be controlled, ability of the system to efficiently deliver drug to the animal respiratory tract depends very much on inhaled particle properties. Therefore, such system should be able to controllably and reproducibly produce aerosols that have particles with precisely defined size, narrow width of distribution and should require minimal amount of test substances. This is of critical importance in efficacy testing

Table II. Effect of Ethanol Delivered by the UNS System on the Relative Number of Eosinophils in the Murine Model of Pulmonary Eosinophilia

Experiment Number	Pos. Control (% Eosinophils)	Percent Eosinophils	
		60 ml Ethanol Administered[b]	100 ml Ethanol Administered[c]
1	33±8 [8][a]	32±9 [9]	
10	48±11 [6]		47±11 [6]

Data are given as mean percentage eosinophils in bronchoalveolar lavage fluid ± SD.
[ ][a] Indicates number of mice *per* experimental group.
[b] Corresponds to the volume of ethanol given at a beclomethasone dipropionate dose of 90 μg/kg.
[c] Corresponds to the volume of ethanol given at a beclomethasone dipropionate dose of 150 μg/kg.

Table III. Effect of Beclomethasone Dipropionate Delivered by the UNS System on Pulmonary Eosinophilia in the Murine Model Asthma

Experiment Number	Pos. Control (% Eosinophils)	Percent Eosinophils at Different Beclomethasone Doses (μg/kg)				
		22.5	45	67.5	90	150
1	33±8 [8][a]	25±6 [8]			6±5*[9]	
2	32±12 [7]	28±11 [9]	27±9 [9]			
3	56±16 [8]			20±17* [5]		
4	37±16 [7]				17±11* [7]	
5	43±11 [9]					4±7* [9]
6	46±13 [9]					8±8* [9]
7	46±10 [9]				26±16* [9]	
8	46±8 [10]					31±16* [8]
9	51±10 [8]		51±15 [8]	37±17 [8]		
10	48±11 [6]					4±9* [6]

Data are given as mean percentage eosinophils in bronchoalveolar lavage fluid ± SD.
[ ][a] Indicates number of mice *per* experimental group.
*Indicates significant differences ($p < 0.05$) *versus* appropriate positive control group.

of new chemical entities, especially in the discovery phase when drug supply is limited.

Ultrasonic nebulizers described in the literature produce aerosols with narrow-sized particles from drug solutions and produce aerosol with suitable characteristics for animal testing. However, as many medications are poorly water soluble, organic solvents are required for their dissolution. Before such aerosol is applied to experimental animals organic solvent should be removed as much as possible. It was shown recently that dry and inhalable drug particles could be produced by aerosolizing organic solution of drug and passage of aerosol stream through a drying column filled with charcoal (23,24). With the use of scanning electron microscopy we demonstrated that spray drying of such droplets in the drying column surrounded by charcoal results in small spherical dry particles with uniform size distribution. This is in good agreement with earlier investigation (29). Therefore, system composed of ultrasonic nebulizator and drying column could fulfill requirements for inhalation drug delivery to experimental animals (23,24). This led us to test whether UNS system could indeed be applicable for delivery and efficacy testing of poorly water-soluble compounds such as corticosteroids and its representative beclomethasone in the murine model of asthma.

The UNS systems described in the literature have a very similar design and are composed of basic components such as syringe pump, ultrasonic nebulizer, drying column, deionizer and air pumps. However they may differ in their particle generation properties since components could be provided by various suppliers (23,24,36). Therefore, each UNS system should

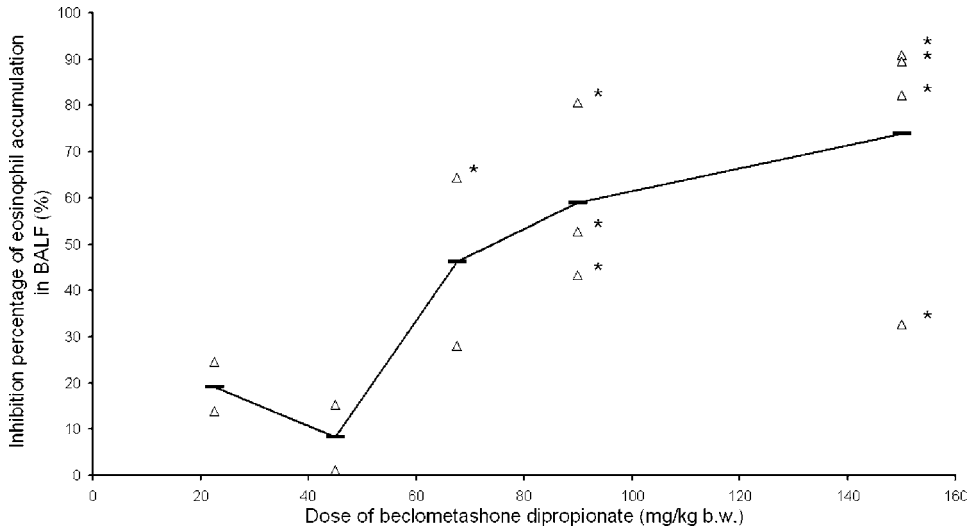

Fig. 5. Effect of beclomethasone dipropionate (beclomethasone) delivered by ultrasonic nebulization system on the relative number of eosinophils in the bronchoalveolar lavage fluid (*BALF*) in the murine asthma model. Data are represented as percent eosinophil inhibition calculated in each experiment according to the formula described in Materials and Methods is labeled as (△) and mean percent inhibition observed with the same beclomethasone dose from different experiment is marked as (—). *Asterisk* indicates significant differences ($p < 0.05$) between beclomethasone treated groups and appropriate positive control groups (untreated mice challenged with ovalbumine).

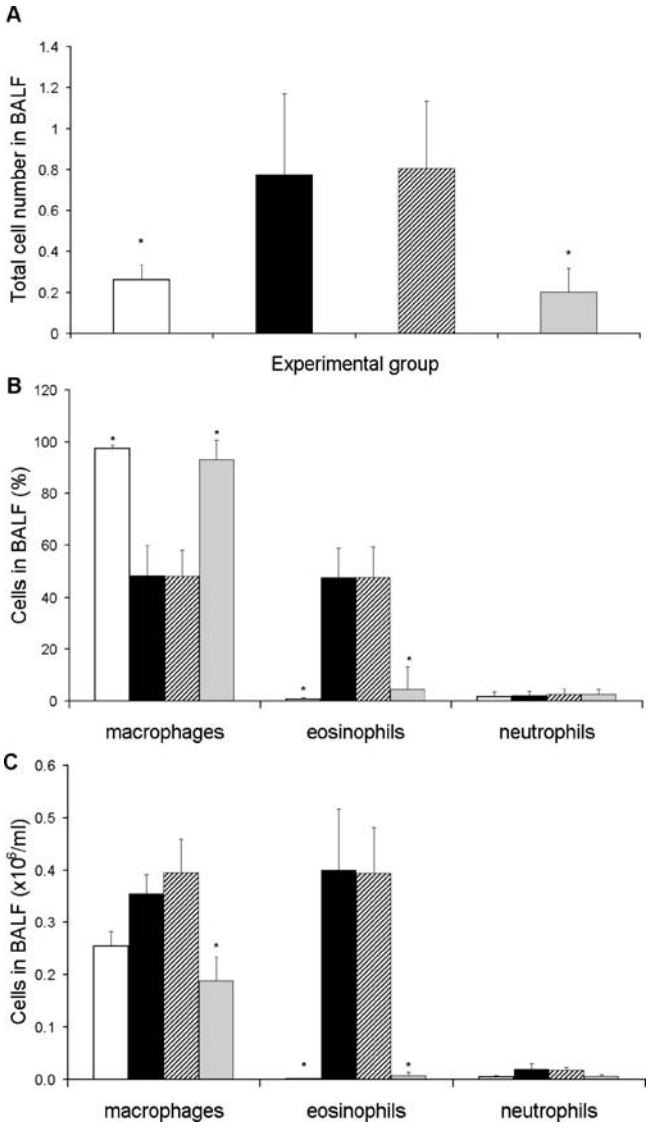

Fig. 6. Effects of beclomethasone administered by UNS system on BALF cytology. Beclomethasone solution in ethanol (~150 μg/kg) or the same volume of vehicle (100 ml of ethanol) was applied to groups of six mice by nebulization. Experimental groups are labeled with *clear bars* (negative control—sensitized, PBS challenged mice), *solid bars* (positive control—sensitized, OVA challenged mice), *hatched bars* (vehicle control—sensitized, OVA challenged mice exposed to nebulized ethanol) and *gray bars* (beclomethasone—sensitized, OVA challenged mice exposed to nebulized beclomethasone solubilized in ethanol). **A** Total number of cells in BALF (10^6/ml). **B** Macrophage, eosinophil and neutrophil percentage in BALF. **C** Total number of macrophages, eosinophils and neutrophils in BALF. Data are given as mean ± SD (n = 6 mouse *per* group). Significant differences ($p < 0.05$) *versus* positive control group [sensitized and ovalbumine (*OVA*) challenged mice] are marked with an asterisk (*).

be carefully tuned for production of applicable dry particles for delivery of effective dose to experimental animals. To obtain particles with suitable properties, parameters such as nebulizer frequency, solvent with its surface tension and density properties, flow rate of liquid solution through nebulizer, concentration of drug in the nebulized solution and power level of ultrasonic nebulizer generator should be evaluated and accordingly adjusted when possible.

The size of particle after drying is directly correlated to the initial aerosol particle size produced by ultrasonic nebulizator, which in turn are a function of the nebulizer frequency and the properties of the solvent used in nebulization (surface tension and density). It would be ideal to have ultrasonic nebulizer with adjustable frequency, but nebulizer frequency is usually predetermined and cannot be changed.

Although earlier studies used various organic solvents (methanol, ethanol, ethyl acetate) for aerosol production (23–25), we had used ethanol as the solvent of choice since it has excellent liquid properties for ultrasonic nebulization (23), is already present in therapeutic inhalation preparations, it generally facilitates dissolution of poorly water soluble compounds and in this particular case because steroids, including beclomethasone, dissolve well in it.

Changing of liquid flow rate from 0.3 to 1.2 ml/min did not significantly affect number of dry particles, their size or GSD. These findings are in good agreement with earlier investigation (24). Increasing of liquid flow rate over 1.2 ml/min deteriorated dry particle properties (decreased number of particles and their size and increased GSD), most probably since the drug solution was passing too fast through the nebulizer and was not nebulized efficiently.

In our experiments changes in the UNS power level have no significant effect on investigated dry powder properties. The exact value of power required for nebulization depends on the (a) nozzle type, (b) liquid characteristics (i.e., viscosity), and (c) flow rate (37). Therefore, it is possible that in other investigations, using different nozzle, solvent and/or flow rate this parameter would significantly affect dry powder properties.

The critical parameter affecting particle size and delivery of dry powder to the animals seems to be concentration of the drug in nebulized solution. Increase of solute concentration increases particle size, which is in good agreement with previous studies (23,24,36).

Monodisperse aerosols, defined as aerosols with well-defined and narrow distribution of particle sizes (GSD ≤ 1.2 μm) (38), are preferred for inhalation delivery of drugs. In human medicine, application of monodisperse aerosols was found to increase efficacy of inhalation therapy and eliminates side effects attributed to particles that are either too large or too small (39,40). Application of monodisperse aerosols of appropriate size in animal exposure studies is even more critical since breathing of conscious animals cannot be controlled. However, the means to produce monodisperse were hitherto restricted to complicated laboratory equipment, like spinning top, vibrating orifice and Sinclair–LaMer generators, and by recently described system based on electrohydrodynamic atomization (19,38). Our UNS system produces dry particles with GSD (~1.2 μm) that achieve the limit of monodisperse aerosol.

The dose of aerosolized drug applied to animals depends on the concentration of dry particles and on exposure time (21). The number of dry particles produced by UNS system and, consequently, their concentration is largely dependent on liquid flow rate while particle size is a function of substance concentration. Therefore, beclomethasone dose applied through the UNS system can be varied by changing

either exposure time or air flow rate and thereby increasing or decreasing dry powder particle concentration.

Efficacy of UNS System in Delivery of Beclomethasone in Mice Asthma Model

Several earlier investigations have described a UNS system with a drying column filled with charcoal and proposed its use for the delivery of dry powders to experimental animals (23,24,36). However, in none of these reports was the UNS system actually used for delivery of dry powders to experimental animals, and to the best of our knowledge, this is the first description of the use of a UNS system to deliver a poorly water-soluble compound (beclomethasone) to the airways in a murine asthma model. Another previously described system that used an ultrasonic nebulizer to deliver beclomethasone and budesonide to mice to prevent lung carcinogenesis used a series of condensers heated to 60°C to remove the ethanol from dry particles (21,22).

As expected, nebulization of pure ethanol in volumes equivalent to those used in the beclomethasone solution administration (nebulization of volumes ≤100 ml up to 70 min) had no effect on relative number of eosinophils in BALF.

Previous reports indicated that the drying column efficiently removes ethanol even after 3 h of nebulization (23–25). In our system, prolonged nebulization of ethanol for more than 2 h induced initial symptoms of ethanol intoxication in animals. This discrepancy could be attributed to the differences in the UNS systems used. One of the parameters is an air flow rate that propels the aerosol through UNS system. Pham and Wiedmann (23–25) showed that the solvent removal efficiency decreases as the air flow rate increases. In our experiments relatively large air flow rate (3 l/min) was used, and it is possible that this parameter decreased efficiency of ethanol removal. However, air flow rate of 3 l/min was necessary to ensure enough air for normal breathing of animals in the exposure chamber. More efficient ethanol removal could be achieved by increasing the total length of charcoal exposed to ethanol, either by using a single longer drying column or serially connecting two shorter columns. Further, our measurements indicated that the increase in liquid flow rate from 0.3 to 1.2 ml/min induces only a small and non-significant decrease in dry particle number, their size and GSD (Fig. 2). Therefore, a lower liquid flow rate could be tested in animal exposure studies to deliver the same beclomethasone dose while reducing the amount of ethanol vapors produced. By increasing the length of the charcoal column and reducing the liquid flow rate, further improved delivery of ethanol-dissolved substances could be achievable.

The results of present study demonstrate that the UNS system can efficiently and reproducibly deliver beclomethasone to mice and inhibit accumulation of eosinophils in BALF induced by ovalbumin challenge. The shape of dose-response curve for beclomethasone delivered through the UNS system (Fig. 6) is similar to data obtained for inhaled corticosteroids in human asthma (41,42). Although it may be argued that the shown effects could be attributed to swallowed drug absorbed from the gastrointestinal tract, we consider this explanation highly unlikely. Previous studies employing whole-body exposure of mice to various aerosols (thorium and plutonium oxides or ricin) indicated that only minimal amounts of the substance (1–3%) are detected in the stomach (43,44). Furthermore, clinical studies had shown that the fraction of an oral dose of beclomethasone that reaches the systemic circulation was only 0.26 (45). Therefore, we do not suppose that observed anti-inflammatory effect could be attributed to small amounts of beclomethasone that possibly reach the gastrointestinal tract.

In order to validate the theoretically calculated beclomethasone dose of 150 μg/kg (b.w.) (corresponding to the dose of 15 μg/g lung tissue) we tried to determine its concentration in the lungs immediately following the completion of delivery using the HPLC-MS detection (results now shown). Since beclomethasone dipropionate is a prodrug that is rapidly and extensively converted to an active form, beclomethasone-17-monopropionate (17-BMP) (46,47), both beclomethasone dipropionate and 17-BMP levels were analyzed. We had detected signals corresponding to beclomethasone dipropinate in two of eight lungs, and signals corresponding to 17-BMP in all eight lungs. Unfortunately, all signals were below the limit of quantification of our method (5 and 2.5 μg/g lung tissue for beclomethasone dipropinate and 17-BMP, respectively). Although this experiment demonstrates effective delivery of the compound in the lungs of all mice, due to low exposure levels and limitations of the analytical method we were unable to determine the exact amount of delivered beclomethasone.

The theoretically calculated doses of beclomethasone delivered by UNS are similar to those applied intratracheally (17,48) and are markedly lower than those required for therapeutic effects of intranasally applied drug substance in the form of suspensions or solution (14,15). Additionally, in comparison to intratracheal administration, UNS system offers less invasive and more practical way of substance delivery. Further, in comparison to UNS system dry powder generators require greater amounts of substances to deliver the same dose, due to the micronization process and less optimal particle size distribution. It is worth noting that calculated inhaled dose of budesonide delivered as dry powder aerosol in murine model of asthma was 12.3 mg/kg (49) while effective dose using UNS system is only 100–150 μg/kg (b.w.). Therefore, a properly adjusted UNS system offers significant savings in terms of amount of substance needed formulation development requirements and due to relative simplicity probably represents one of the best means of compound administration for inhalation drug delivery in preclinical drug efficacy testing.

CONCLUSION

A UNS system suitable for delivery and efficiency testing of compounds dissolved in organic solvent is described and tested for inhalation delivery of beclomethasone to the airways in a murine model of asthma. The UNS system produces a monodisperse dry powder particle aerosol. We found that inhalation delivery of beclomethasone aerosol produced by the UNS system markedly inhibited eosinophilia in BALF of the mice. Moreover, the described UNS system can be used for effective delivery of other poorly water soluble substances to the respiratory system of experimental animals where such application is required. Described UNS

system greatly reduces the amount of substance needed for efficacy testing, an important factor at an early stage of drug discovery, minimizes formulation requirements and allows rapid and relatively simple efficacy testing in animals.

ACKNOWLEDGMENTS

This work was supported by PLIVA Research Institute, Inc. Authors wish to thank dr. Michael J. Parnham for critical reading of the manuscript. Authors also wish to thank Ms. Anica Pešut and Milka Horvatinčić and Mr. Željko Osman for their excellent technical assistance.

REFERENCES

1. A. A. Arif, G. L. Delclos, E. S. Lee, S. R. Tortolero, and L. W. Whitehead. Prevalence and risk factors of asthma and wheezing among US adults: an analysis of the NHANES III data. *Eur. Respir. J.* **21**:827–833 (2003).
2. D. M. Mannino, D. M. Homa, C. A. Pertowski, A. Ashizawa, L. L. Nixon, C. A. Johnson, L. B. Ball, E. Jack, and D. S. Kang. Surveillance for asthma—United States, 1960–1995. *MMWR CDC Surveill. Summ.* **47**:1–27 (1998).
3. N. Pearce, J. Sunyer, S. Cheng, S. Chinn, B. Bjorksten, M. Burr, U. Keil, H. R. Anderson, and P. Burney. Comparison of asthma prevalence in the ISAAC and the ECRHS. ISAAC Steering Committee and the European Community Respiratory Health Survey. International Study of Asthma and Allergies in Childhood. *Eur. Respir. J.* **16**:420–426 (2000).
4. R. J. Halbert, S. Isonaka, D. George, and A. Iqbal. Interpreting COPD prevalence estimates: what is the true burden of disease?. *Chest* **123**:1684–1692 (2003).
5. S. Newman and S. Clarke. Aerosols in medicine: principles, diagnosis and therapy. In F. Moren M. Newhouse, and M. Dolovich (eds.), *Aerosol in Therapy*, Elsevier, Amsterdam, 1985.
6. T. J. Clark. Effect of beclomethasone dipropionate delivered by aerosol in patients with asthma. *Lancet* **1**:1361–1364 (1972).
7. A. D. Perera, C. Kapitza, L. Nosek, R. S. Fishman, D. A. Shapiro, T. Heise, and L. Heinemann. Absorption and metabolic effect of inhaled insulin: intrapatient variability after inhalation *via* the Aerodose insulin inhaler in patients with type 2 diabetes. *Diabetes Care* **25**:2276–2281 (2002).
8. J. B. Fink. Aerosol device selection: evidence to practice. *Respir. Care* **45**:874–885 (2000).
9. M. P. Timsina, G. P. Martin, C. Marriott, D. Ganderton, and M. Yianneskis. Drug delivery to the respiratory tract using dry powder inhalers. *Int. J. Pharm.* **101**:1–13 (1994).
10. S. Suarez and A. J. Hickey. Drug properties affecting aerosol behavior. *Respir. Care* **45**:652–666 (2000).
11. P. K. Gupta and A. J. Hickey. Contemporary approaches in aerosolized drug delivery to the lung. *J. Control. Release* **17**: 127–147 (1991).
12. P. Atkins and A. R. Clark. Drug delivery to the respiratory tract and drug dosimetry. *J. Aerosol Med.* **7**:33–38 (1994).
13. M. F. Biddiscombe, R. Melchor, V. H. F. Mak, R. J. Marriot, A. J. Taylor, M. D. Short, and S. G. Spiro. The deposition of salbutamol, directly labelled with technetium-99m, delivered by pressurised metered and dry powder inhalers. *Int. J. Pharm.* **91**:111–121 (1993).
14. T. J. Huang, P. Eynott, M. Salmon, P. L. Nicklin, and K. F. Chung. Effect of topical immunomodulators on acute allergic inflammation and bronchial hyperresponsiveness in sensitised rats. *Eur. J. Pharmacol.* **437**:187–194 (2002).
15. Y. Fujitani and A. Trifilieff. *In vivo* and *in vitro* effects of SAR 943, a rapamycin analogue, on airway inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* **167**:193–198 (2003).
16. K. E. Driscoll, D. L. Costa, G. Hatch, R. Henderson, G. Oberdorster, H. Salem, and R. B. Schlesinger. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol. Sci.* **55**:24–35 (2000).
17. A. Miller-Larsson, H. Mattsson, E. Hjertberg, M. Dahlback, A. Tunek, and R. Brattsand. Reversible fatty acid conjugation of budesonide. Novel mechanism for prolonged retention of topically applied steroid in airway tissue. *Drug Metab. Dispos.* **26**:623–630 (1998).
18. J. L. Rau. The inhalation of drugs: advantages and problems. *Respir. Care* **50**:367–382 (2005).
19. J. C. Ijsebaert, K. B. Geerse, J. C. Marijnissen, J. W. Lammers, and P. Zanen. Electro-hydrodynamic atomization of drug solutions for inhalation purposes. *J. Appl. Physiol.* **91**: 2735–2741 (2001).
20. P. J. Barnes and T. T. Hansel. The need for new therapy. In T. T. Hansel and P. J. Barnes (eds.), *New Drugs for Asthma, Allergy and COPD, Vol. 31, Progress in respiratory research*, Krager, Basel, 2001, pp. 2–5.
21. L. W. Wattenberg, T. S. Wiedmann, R. D. Estensen, C. L. Zimmerman, A. R. Galbraith, V. E. Steele, and G. J. Kelloff. Chemoprevention of pulmonary carcinogenesis by brief exposures to aerosolized budesonide or beclomethasone dipropionate and by the combination of aerosolized budesonide and dietary *myo*-inositol. *Carcinogenesis* **21**:179–182 (2000).
22. L. W. Wattenberg, T. S. Wiedmann, R. D. Estensen, C. L. Zimmerman, V. E. Steele, and G. J. Kelloff. Chemoprevention of pulmonary carcinogenesis by aerosolized budesonide in female A/J mice. *Cancer Res.* **57**:5489–5492 (1997).
23. S. Pham and T. S. Wiedmann. Analysis of a diffusion dryer for the respiratory delivery of poorly water soluble drugs. *Pharm. Res.* **16**:1857–1863 (1999).
24. T. S. Wiedmann and A. Ravichandran. Ultrasonic nebulization system for respiratory drug delivery. *Pharm. Dev. Technol.* **6**:83–89 (2001).
25. S. Pham and T. S. Wiedmann. Production of aerosol particles from organic solutions for respiratory delivery to animals. *Pharm. Res.* **14**:S133 (1997).
26. S. W. Stein, P. B. Myrdal, B. J. Gabrio, D. Obereit, and T. J. Beck. Evaluation of a new Aerodynamic Particle Sizer Spectrometer for size distribution measurements of solution metered dose inhalers. *J. Aerosol Med.* **16**:107–119 (2003).
27. J. P. Mitchell, M. W. Nagel, K. J. Wiersema, and C. C. Doyle. Aerodynamic particle size analysis of aerosols from pressurized metered-dose inhalers: comparison of Andersen 8-stage cascade impactor, next generation pharmaceutical impactor, and model 3321 Aerodynamic Particle Sizer aerosol spectrometer. *AAPS PharmSciTech* **4**:E54 (2003).
28. A. C. Guyton. The measurements of the respiratory values of laboratory animals. *Am. J. Physiol.* **150**:70–78 (1942).
29. J. Broadhead, S. K. Rouan, and C. T. Rhodes. The spray drying of pharmaceuticals. *Drug Dev. Ind. Pharm.* **18**:1169–1206 (1992).
30. D. B. Warheitand and M. A. Hartsky. Species comparisons of proximal alveolar deposition patterns of inhaled particulates. *Exp. Lung Res.* **16**:83–99 (1990).
31. R. B. Schlesinger. Comparative deposition of inhaled aerosols in experimental animals and humans: a review. *J. Toxicol. Environ. Health* **15**:197–214 (1985).
32. M. Synek, R. Beasley, A. J. Frew, D. Goulding, L. Holloway, F. C. Lampe, W. R. Roche, and S. T. Holgate. Cellular infiltration of the airways in asthma of varying severity. *Am. J. Respir. Crit. Care Med.* **154**:224–230 (1996).
33. M. L. Bartoli, E. Bacci, S. Carnevali, S. Cianchetti, F. L. Dente, A. Di Franco, D. Giannini, M. Taccola, B. Vagaggini, and P. L. Paggiaro. Clinical assessment of asthma severity partially corresponds to sputum eosinophilic airway inflammation. *Respir. Med.* **98**:184–193 (2004).
34. W. W. Busse, W. F. Calhoun, and J. D. Sedgwick. Mechanism of airway inflammation in asthma. *Am. Rev. Respir. Dis.* **147**: S20–S24 (1993).
35. J. Bousquet, P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, and P. Godard. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* **323**:1033–1039 (1990).
36. S. Pham and T. S. Wiedmann. Note: dissolution of aerosol particles of budesonide in Survanta, a model lung surfactant. *J. Pharm. Sci.* **90**:98–104 (2001).

37. Operating instrutions for ultrasonic atomizing nozzle systems v1.2, Sono-Tek Co., 2001.
38. J. D. Brain and P. A. Valberg. Deposition of aerosol in the respiratory tract. *Am. Rev. Respir. Dis.* **120**:1325–1373 (1979).
39. P. Zanen, L. T. Go, and J. W. Lammers. The efficacy of a low-dose, monodisperse parasympathicolytic aerosol compared with a standard aerosol from a metered-dose inhaler. *Eur. J. Clin. Pharmacol.* **54**:27–30 (1998).
40. P. Zanen and J. W. Lammers. Reducing adverse effects of inhaled fenoterol through optimization of the aerosol formulation. *J. Aerosol Med.* **12**:241–247 (1999).
41. S. Holt, A. Suder, M. Weatherall, S. Cheng, P. Shirtcliffe, and R. Beasley. Dose-response relation of inhaled fluticasone propionate in adolescents and adults with asthma: meta-analysis. *BMJ* **323**:253–256 (2001).
42. H. Powell and P. G. Gibson. Inhaled corticosteroid doses in asthma: an evidence-based approach. *Med. J. Aust.* **178**:223–225 (2003).
43. S. R. Moores, A. Black, B. E. Lambert, P. J. Lindop, A. Morgan, J. Pritchard, and M. Walsh. Deposition of thorium and plutonium oxides in the respiratory tract of the mouse. In S. L. Sanders, F. T. Cross, G. E. Dagle, and J. A. Mahaffey (eds), *Proceedings of the Nineteenth Annual Hanford Life Sciences Symposium*, Technical Information Center, Department of Energy, Washington, District of Columbia, 1980, pp. 103–118.
44. C. J. Roy, M. Hale, J. M. Hartings, L. Pitt, and S. Duniho. Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. *Inhal. Toxicol.* **15**:619–638 (2003).
45. I. Soria, L. I. Harrison, J. H. Machacek, A. C. Cline, and P. A. Stampone. Beclomethasone relative availability of oral *versus* inhaled beclomethasone dipropionate from an HFA-134A metered dose inhaler. *Biopharm. Drug Dispos.* **19**:297–302 (1998).
46. L. E. Martin, C. Harrison, and R. J. Tanner. Metabolism of beclomethasone dipropionate by animals and man. *Postgrad. Med. J.* **51** Suppl 4, 11–20 (1975).
47. F. Chanoine, C. Grenot, P. Heidmann, and J. L. Junien. Pharmacokinetics of butixocort 21-propionate, budesonide, and beclomethasone dipropionate in the rat after intratracheal, intravenous, and oral treatments. *Drug Metab. Dispos.* **19**:546–553 (1991).
48. H. Kuss, N. Hoefgen, S. Johanssen, T. Kronbach, and C. Rundfeldt. *In vivo* efficacy in airway disease models of *N*-(3, 5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyo xylic acid amide (AWD 12-281), a selective phosphodiesterase 4 inhibitor for inhaled administration. *J. Pharmacol. Exp. Ther.* **307**:373–385 (2003).
49. R. E. Wiley, M. Cwiartka, D. Alvarez, D. C. Mackenzie, J. R. Johnson, S. Goncharova, L. Lundblad, and M. Jordana. Transient corticosteroid treatment permanently amplifies the Th2 response in a murine model of asthma. *J. Immunol.* **172**: 4995–5005 (2004).