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Research paper

Inhalable dry-emulsion formulation of cyclosporine A with improved anti-inflammatory effects in experimental asthma/COPD-model rats

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ABSTRACT

The main purpose of the present study was to develop a novel respirable powder (RP) formulation of cyclosporine A (CsA) using a spray-dried O/W-emulsion (DE) system. DE formulation of CsA (DE/CsA) was prepared by spray-drying a mixture of erythritol and liquid O/W emulsion containing CsA, polyvinylpyrrolidone, and glyceryl monooleate as emulsifying agent. The DE/CsA powders were mixed with lactose carriers to obtain an RP formulation of DE/CsA (DE/CsA-RP), and its physicochemical, pharmacological, and pharmacokinetic properties were evaluated. Spray-dried DE/CsA exhibited significant improvement in dissolution behavior with ca. 4500-fold increase of dissolution rate, and then, nanoemulsified particles were reconstituted with a mean diameter of 317 nm. Laser diffraction analysis on the DE/CsA-RP suggested high dispersion of DE/CsA on the surface of the lactose carrier. Anti-inflammatory properties of the inhaled DE/CsA-RP were characterized in antigen-sensitized asthma/COPD-model rats, in which the DE/CsA-RP was more potent than the RP formulation of physical mixture containing CsA and erythritol in inhibiting inflammatory responses, possibly due to the improved dissolution behavior. Pharmacokinetic studies demonstrated that systemic exposure of CsA after intratracheal administration of the DE/CsA-RP at a pharmacologically effective dose (100 μg-CsA/rat) was 50-fold less than that of the oral CsA dosage form at a toxic dose (10 mg/kg). From these findings, use of inhalable DE formulation of CsA might be a promising approach for the treatment of airway inflammatory diseases with improved pharmacodynamics and lower systemic exposure.

1. Introduction

Cyclosporine A (CsA) has been widely used as an immunosuppressive agent [1], and CsA also exhibits a variety of biological activities, including anti-fungal, anti-inflammatory, immunosuppressive, and anti-parasitic properties [2]. Recent outcomes from clinical studies are indicative of the potent therapeutic potential of CsA for chronic asthma and airway inflammation [3]. However, the clinical use of CsA is partly limited because of (i) low oral bioavailability due to its poor solubility, low intestinal permeability, and CYP3A-related biotransformation [4] and (ii) systemic side effects such as renal dysfunction, hypertension, and notably nephrotoxicity [3]. A number of CsA dosage forms have been proposed to overcome these drawbacks, for example, nebulizer formulation and metered-dose inhaler formulation for inhalation therapy [5]. Current development of some inhaled CsA liquid formulations can be directed specifically to lung transplant recipients; however, inhalable formulation of CsA might also provide preferable clinical outcomes from treatment of asthma and other airway inflammatory diseases.

In general, an inhalable formulation can be effective for maximizing drug concentrations in the airway systems while minimizing systemic exposure and associated toxicity. This can be a rationale for the development of an inhalable CsA. The liquid formulations of CsA aerosol for inhalation sometimes contain organic solvents and other solubilizers [6], and inclusion of these ingredients, such as castor oil or ethanol, may result in local irritant potency. This may compromise their uses in chronic treatment. In this context, CsA-based dry powder inhalation (DPI) system with no use of irritant ingredients would be another delivery option for the direct administration of CsA to the respiratory system. For the development of a CsA-based DPI system, improvement in the

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solubility of CsA in water would be a key consideration for acquiring potent pharmacological effects. Previously, our group proposed a novel DPI system of CsA, employing a methylcellulose-based amorphous solid dispersion (ASD) approach for inhalation therapy on airway inflammations [7]. Despite the development of extensive expertise with ASD formulations, they are not widely used in commercial products mainly because drugs in the amorphous state sometimes undergo crystallization during processing or storage, resulting in unstable formulations with lower dissolution rates [8].

Recently, attention has been drawn to dry emulsion (DE) as a potential oral drug delivery system for improving solubility and dissolution of poorly soluble drugs [9]. DEs are regarded as lipid-based powder formulations obtained by spray-drying, which can immediately reform the original O/W emulsion after rehydration. The DE approach might also be a viable formulation option for inhalation therapy although no DE-based respirable powder (RP) formulation of CsA has ever been prepared or characterized. The purpose of the present study was to develop a novel RP formulation of CsA, employing a DE approach, for treatment of asthma and other airway inflammatory diseases. A DE formulation of CsA (DE/CsA) was prepared by spray-drying and then mixed with a lactose carrier to produce an RP formulation of CsA (DE/CsA-RP). The DE/CsA-RP was characterized in terms of its morphology, particle size distribution, crystallinity, solubility, and dispersibility. The therapeutic potential of the DE/CsA-RP was assessed using experimental asthma/chronic obstructive pulmonary disease (COPD)-like rats, and pharmacokinetic profiling of inhaled CsA was also undertaken to predict the systemic toxic potential of DE/CsA-RP.

2. Materials and methods

2.1. Chemicals

Cyclosporine A (CsA) was supplied by the Department of Product Development, Ito Life Sciences (Ibaraki, Japan). Respitose® and Polyvinylpyrrolidone (PVP) K-25 were supplied by DMV (Veghel, The Netherlands) and BASF Japan (Tokyo, Japan), respectively. Erythritol was supplied by Nikken Chemicals (Tokyo, Japan). Neoral® capsules (10 mg) were purchased from Novartis Pharma (Tokyo, Japan). Acetonitrile (HPLC grade) was bought from Kanto Chemical (Tokyo, Japan). Ammonium acetate and glyceryl monooleate were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were purchased from commercial sources.

2.2. Inhalable dry-emulsion formulation of cyclosporine A

2.2.1. Preparation

To prepare O/W emulsion, 500 mg of CsA, 500 mg of glyceryl monooleate, and 1 g of PVP K-25 were added to 150 mL of distilled water and dispersed by magnet stirring at 400 rpm for 30 min. The emulsion was mixed with 8 g of erythritol, and the mixture was spray-dried using a Buchi mini spray dryer B-290 apparatus (Buchi, Switzerland) under the following conditions: inlet temperature, 140 °C; outlet temperature, 70–80 °C; aspiration, 100%; drying air flow, 35 m^3/h; spraying air flow, 538 L/h; and feeding rate of the emulsion mixture, 2 mL/min. The spray-dried powders were mixed with a 10-fold amount of carrier particle (Respitose®), providing a DE/CsA-RP. In addition to the DE/CsA-RP, an RP formulation of physical mixture (CsA-RP), containing only CsA and erythritol, was also prepared for comparison. The RP formulations were stored in vacuum desiccators until tested.

2.2.2. UPLC/ESI-MS analysis of CsA

The amounts of CsA in the formulations were determined by an absolute calibration curve method using the Waters Acquity UPLC system (Waters, Milford, MA), which included a binary solvent manager, sample manager, column compartment and SQD connected with MassLynx software. An Acquity UPLC BEH C 18 column (particle size: 1.7 μm, column size: 2.1 mm × 50 mm; Waters) was used, and column temperature was maintained at 65 °C. Samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 5 mM ammonium acetate (B) with a flow rate of 0.25 mL/min, and the retention time of CsA was 2.0 min. The gradient condition of the mobile phase was 0–1.0 min, 80% A; 1.0–2.5 min, 80–95% A; and 2.5–4.0 min, 95% A. Analysis was carried out using selected ion recording (SIR) for specific *m/z* 1203 for CsA $[M + H]^+$.

2.3. Dissolution test

Dissolution tests were carried out in 900 mL of distilled water with constant stirring of 50 rpm in a dissolution test apparatus NTR 6100A (Toyama Sangyo, Osaka, Japan) at 37 °C. Crystalline CsA, amorphous CsA, or DE/CsA was weighed to keep the total amount of CsA in the dissolution vessel constant at 27 mg. The collected samples were centrifuged at 15,000 rpm for 5 min, and the supernatants were diluted with 10-fold volume of methanol. The concentrations of CsA were determined by Waters UPLC/ESI-MS as described in Section 2.2.2.

2.4. Electron microscopy

2.4.1. Transmission electron microscopy (TEM)

For the TEM experiment, 1 mg of DE/CsA was dispersed in 1 mL of distilled water, and an aliquot (2 μL) was placed on a carbon-coated Formvar 200 mesh nickel grid. The sample was allowed to stand for 15–30 s, and then, any excess solution was removed by blotting. The samples were negatively stained with 2% (w/v) uranyl acetate and allowed to dry. The samples were then visualized under an H-7600 transmission electron microscope (Hitachi, Tokyo, Japan) operating at 75 kV.

2.4.2. Scanning electron microscopy (SEM)

Representative scanning electron microscopic images of DE/CsA and DE/CsA-RP were taken using a VE-7800 scanning electron microscope (Keyence Corporation, Osaka, Japan) without Au or Pt coating. For the SEM observations, each sample was fixed on an aluminum sample holder using double-sided carbon tape.

2.5. Particle size analysis

2.5.1. Dynamic light scattering (DLS)

Mean particle size of the DE/CsA suspended in distilled water at a final concentration of 0.1 mg/mL was measured in triplicate by dynamic light scattering (DLS) using a Zetasizer Nano ZS (MALVERN, Worcestershire, UK). All measurements were performed at 25 °C at a measurement angle of 90°.

2.5.2. Laser diffraction

The particle size of DE/CsA-RP was measured by a laser diffraction scattering method using an LMS-300 (Seishin Enterprise, Tokyo, Japan). The particle size distribution is expressed as the volume median diameter.

2.6. X-ray powder diffraction (XRPD)

The XRPD pattern was collected with a Rigaku RINT-ultra III X-ray diffractometer (Rigaku, Tokyo, Japan) with Cu-Kα radiation

generated at 40 mA and 40 kV. Data were obtained from 3° to 40° (2θ) at intervals of 0.01° and a scanning speed of 2°/min.

2.7. Thermal analysis

Differential scanning calorimetry (DSC) was performed using a DSC Q1000 (TA Instruments, New Castle, DE, USA). The DSC thermograms were collected in an aluminum close-pan system using a sample weight of ca. 3 mg and a heating rate of 5 °C/min with nitrogen purge at 70 mL/min. The temperature axis was calibrated with indium (ca. 5 mg, 99.999% pure, onset at 156.6 °C).

2.8. Animals and drug inhalation

Male Sprague–Dawley rats (8–11 weeks of age; Japan SLC, Shizuoka, Japan), weighing 376 ± 25 g, were housed three per cage in the laboratory with free access to food and water and maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1 °C) and humidity (55 ± 5%). Animals were fasted for 12 h before experiments. To establish experimental asthma/COPD-model rats, rats were sensitized by the intraperitoneal injection of 100 μg of ovalbumin (OVA) with 5 mg of alum on days 0, 7, and 14 as reported previously [10]. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and received intratracheal administration of OVA (100 μg/rat)-RP at 24 h after the last OVA sensitization. At 1 h before the OVA-RP challenge, CsA-RP (100 μg CsA/rat), DE/CsA-RP (100 μg CsA/rat), or control-RP (micronized excipient and carrier powder) was administered via intratracheal insufflation using PennCentury insufflation powder delivery device (DP-4, INA Research Inc., Nagano, Japan). A bolus (2 mL) of air from an attached syringe was used to deliver the preloaded powder from the chamber of the insufflator into the rat airway system. At 24 h after OVA-RP challenge, rats were exsanguinated via the descending aorta under anesthesia with sodium pentobarbital, and the lungs were removed. All procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of University of Shizuoka.

2.9. Total cell count in bronchoalveolar lavage fluid (BALF)

At 24 h after intratracheal administration of OVA-RP, rats were exsanguinated via the descending aorta under anesthesia. A bronchoalveolar lavage was performed immediately using 3 mL of PBS five times. Recovery rate of BALF was approximately 70%. The number of cells in BALF was counted using a Burker–Turk counting chamber after the addition of an equal amount of 0.2% trypan blue. The collected BALF samples were subjected to a flow cytometric hematology system (XT-2000iV, Sysmex Corporation, Kobe, Japan) to classify the type of inflammatory cells.

2.10. Plasma concentration of CsA after intratracheal or oral administration

Blood samples were taken in a volume of 200 μL from the tail vein in the indicated periods after oral administration of Neoral® (10 mg/kg CsA) or intratracheal administration of DE/CsA-RP (100 μg CsA/rat). The blood samples were centrifuged at 10,000*g* to prepare plasma samples which were then kept frozen at below −20 °C until they were analyzed. CsA concentrations in plasma were estimated by an internal standard method using UPLC/ESI-MS. Briefly, 100 μL of acetonitrile and 5 μL of internal standard (tamoxifen, 500 ng/mL) were added to 50 μL of plasma sample and centrifuged at 2000*g* for 5 min. The supernatants were filtrated through a 0.20-μm filter and then analyzed by UPLC/ESI-MS to determine the plasma concentration of CsA. Column temperature was maintained at 65 °C, and the standard and samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 5 mM ammonium acetate (B) with a flow rate of 0.25 mL/min. The gradient condition of mobile phase was 0–1.0 min, 60–70% A; 1.0–2.0 min, 70% A; 2.0–3.0 min, 84% A; 3.0–3.5 min, 95% A; and 3.5–4.0 min, 60% A. Peaks for internal standard and CsA were detected at the retention times of 2.25 and 3.02 min, respectively. The newly developed UPLC/ESI-MS method for determination of CsA was validated in terms of linearity, accuracy, precision, and assay recovery according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines "Q2B Validation of Analytical Procedures: Methodology". At the concentrations of 100 and 500 ng/mL, the observed recovery of CsA was 97.3–100.9%. The pharmacokinetic parameters for CsA were calculated by means of noncompartmental methods using the WinNonlin® program (Ver. 4.1, Pharsight Corporation, Mountain View, CA).

2.11. Statistical analysis

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by Fisher's least significant difference procedure was used. A P value of less than 0.05 was considered significant for all analyses.

3. Results and discussion

3.1. Spray-dried O/W emulsion of CsA

In the present study, the DE formulation of CsA was prepared by spray-drying of lipid–CsA mixture, and the recovery of the DE formulation was found to be 31.5%, possibly due to too small-scale manufacturing. Theoretically, powders produced by spray-drying can be predominantly amorphous in nature [11], and, overall, the SEM images indicated that the powders were amorphous in nature as there were no clear crystalline particles visible (Fig. 1A). Morphology of re-suspended DE/CsA was also characterized by TEM observation, indicating that all the nanoemulsion particles were evenly distributed and spherical (Fig. 1B). The DE/CsA re-suspended in water formed a nanoemulsion where particle sizes were less than 500 nm. In addition, re-suspended DE/CsA was subjected to DLS analysis, and the average particle size of CsA emulsions was found to be 317 nm (data not shown). The particle size was approximately the same as the diameters observed by TEM. Thus, a nanoemulsified dispersion of CsA can be reconstituted in the aqueous layer.

In the XRPD analysis (Fig. 1C), crystalline CsA powders exhibited a number of intense peaks, the patterns of which were indicative of a tetragonal crystal form [12]. In contrast, a halo diffraction pattern was observed in both amorphous CsA and DE/CsA, suggesting amorphization of CsA during preparation of DE formulation. For further characterization, the thermal behaviors of CsA formulations were also clarified using DSC (Fig. 1D). As we reported previously [13], crystalline CsA produced a melting endotherm at 115 °C. Amorphous CsA exhibited thermal events at 128 °C, and the transition during the endothermic events might be attributable to a solid-to-liquid transition at over 120 °C. There was no thermal event in the DSC thermograms of DE/CsA within the examined temperature range, suggesting that CsA in the DE/CsA might exist in a high-energy amorphous state with a homogeneous molecular interaction between CsA and the lipidic ingredient. In addition to the DE approach, amorphization of CsA might be effective for improvement of wettability through reduction in particle size and the absence of crystallinity.

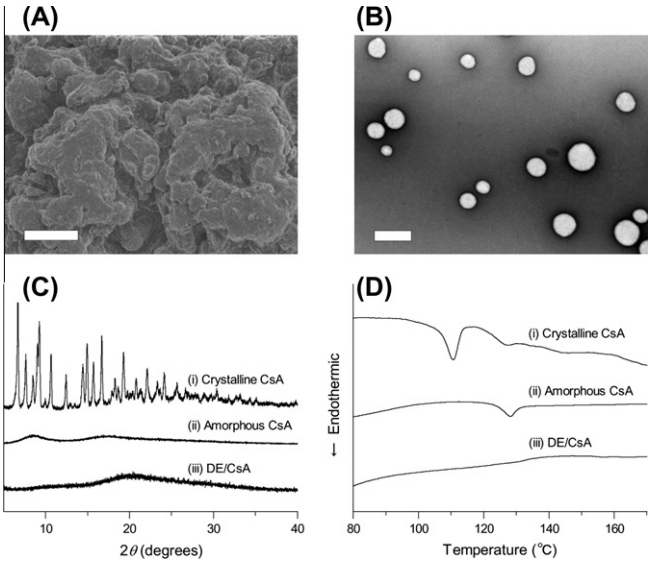

Fig. 1. Physicochemical characterization of CsA samples. (A) Scanning electron microscopic images from DE/CsA. Bar represents 5 μm. (B) Transmission electron microscopic image of the DE/CsA re-dispersed in water. Bar represents 500 nm. (C) Powder X-ray diffraction and (D) differential scanning calorimetry. (i) Crystalline CsA, (ii) amorphous CsA, and (iii) DE/CsA.

To clarify possible enhancement of dissolution behavior by DE approaches, dissolution testing for CsA formulations was carried out in distilled water. Poor dissolution behaviors were seen in both crystalline and amorphous CsA powders at dissolution rates of 4.7×10^{-3} and 6.3×10^{-3} h^{-1}, respectively (Fig. 2). In contrast, DE formulation of CsA showed fast dispersion with formation of nanoemulsion as determined by DLS, and the dissolution rate of DE/CsA was estimated to be 28.6 h^{-1} (95% confidence interval: 6.5–50.7). No precipitation was observed at room temperature for at least 24 h after re-suspension of DE/CsA (data not shown). These observations were consistent with previous reports, showing that formulation of poorly soluble drugs as DE could lead to marked improvement in the dissolution properties [14].

3.2. Inhalable dry-emulsion formulation of CsA

Treatment of asthma/COPD with inhaled drugs would offer several advantages over systemic therapy, including more rapid onset and reduced adverse effects, because of direct targeting of the airway systems [15]. In this context, combined use of inhalation system and DE approach might be efficacious for enhancing therapeutic potential of CsA for asthma/COPD therapy. In most dry powder formulations, a carrier has been used to improve the flowability and dispersibility of fine particles in order to facilitate the drug release from the inhaler and to enable deep lung deposition of the drug. Therefore, Respitose®, the sieved lactose carrier, with a diameter of ca. 50 μm, was mixed with spray-dried DE/CsA, providing DE/CsA-RP. The morphology and surface structure of the inhalable formulation (DE/CsA-RP) were analyzed by SEM (Fig. 3A). The spray-dried DE/CsA adhered to the surface of the lactose carriers, and no significant agglomeration of spray-dried particles was observed, possibly due to stabilization of spray-dried particles by Respitose®.

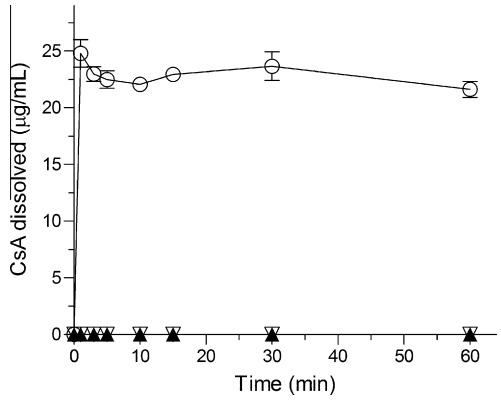

Fig. 2. *In vitro* dissolution behavior of CsA samples in water (37 °C). ▲, Crystalline CsA; ▽, amorphous CsA; and ○, DE/CsA. Data represent mean ± SE of three experiments.

Particle size is a key parameter in defining the deposition pattern and bioavailability of drug material delivered to the respiratory system using inhalers. To determine the particle size and re-dispersibility of spray-dried particles, laser diffraction analysis was carried out on the DE/CsA-RP. According to the size-distribution diagram (Fig. 3B), the DE/CsA-RP could be readily dispersed into a cloud of fine particles and carrier at a pressure of 0.20 MPa. There were mainly two peaks for the spray-dried DE/CsA particles and lactose carrier, ranging from 0.2 to 10 and 20 to 140 μm, d_{50} values of which were calculated to be 2.9 and 55 μm, respectively. The micronized DE/CsA with this size diameter would be of a suitable size to avoid deposition by inertial impaction in the oropharyngeal cavity, possibly leading to effective delivery of the inhaled particles to the airway system [16].

3.3. Anti-inflammatory effects of inhaled CsA formulation

To elucidate the therapeutic potential of DE/CsA-RP, anti-inflammatory effects of inhaled DE/CsA-RP were assessed using experimental asthma/COPD-model rats that we developed previously [10]. Both COPD and asthma can be defined as airway inflammation with some differences in inflammatory cells, mediators, response to inflammation, anatomical distribution, and BALF being frequently used as a biological source for clinical investigation of these inflammatory lung diseases [17]. In the present study, BALF was obtained at 24 h after the last antigen (OVA) challenge, and the inflammatory cells in BALF were counted (Fig. 4). The antigen challenge caused marked recruitment of inflammatory cells in BALF as evidenced by a ca. 7-fold increase of cell numbers. Treatment of antigen-exposed rats with CsA- and DE/CsA-RP (100 μg CsA/rat) resulted in significant reductions of inflammatory cells in BALF by 63% ($P < 0.01$) and 98% ($P < 0.01$), respectively. There was no significant difference in the numbers of granulocytes between control and antigen-exposed rats with DE/CsA-RP treatment, suggesting a better anti-inflammatory effect of inhaled DE/CsA-RP compared with CsA-RP.

For further characterization of anti-inflammatory effects of the inhaled DE/CsA-RP in these experimental model rats, types of recruited cells in BALF were analyzed using a Sysmex XT-2000iV system as reported previously [18]. DIFF scattergram of BALF from the antigen-sensitized rats suggested that the recruited cells mainly consisted of monocytes and neutrophils, and a smaller number of eosinophils were also observed (Fig. 5B). Activated macrophages tend to produce and secrete matrix metalloproteases, and activated neutrophils also produce matrix-degrading proteases and reactive oxygen free radicals, leading to damage of the epithelium and underlying basement membrane in pulmonary tissues [19]. Interestingly, intratracheal administration of the DE/CsA-RP in the antigen-sensitized rats resulted in decreases of both macrophages and eosinophils (Fig. 5D), while treatment with CsA-RP was likely to be less effective (Fig. 5C). The improved dissolution behavior of DE formulation might contribute to the marked differences in anti-inflammatory effects between DE/CsA- and CsA-RPs.

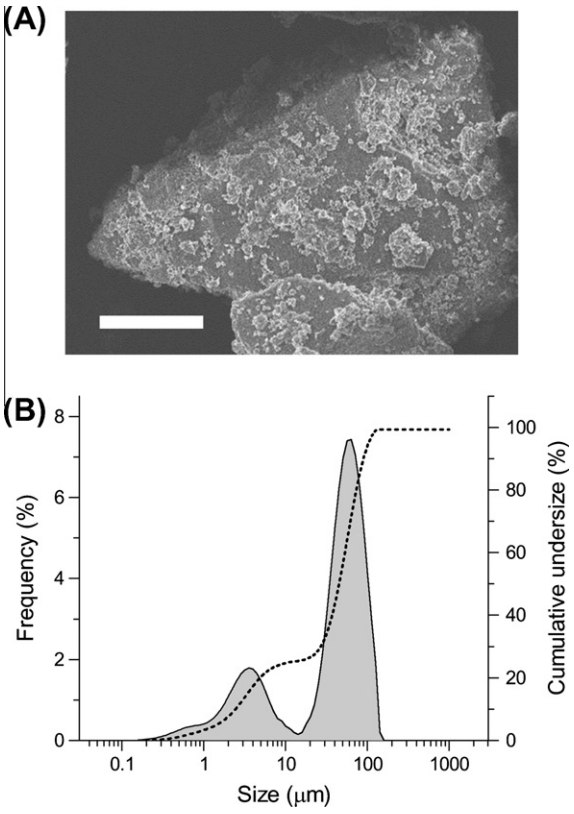

Fig. 3. Morphological characterization on inhalable dry-emulsion formulation of CsA. (A) Scanning electron microscopic images from DE/CsA-RP. Bar represents 20 μm. (B) Particle size and aerosolization efficiency of DE/CsA-RP as determined by laser diffraction particle size analysis. The DE/CsA-RP was dispersed by dry air at a pressure of 0.2 MPa, and the size distribution and mean particle size were estimated.

The present observation would be indicative of the therapeutic potential of inhaled DE/CsA-RP for treatment of asthma, COPD, and other airway inflammatory diseases.

3.4. Systemic exposure of CsA after inhalation of dry-emulsion formulation

In humans, oral CsA at a dose of 3–5 mg/kg/day has been shown to be effective in asthma treatment, but its use is also limited by severe nephrotoxicity and hepatotoxicity [3,20]. In this context, excess systemic exposure to CsA after inhalation might be associated with serious side effects, especially nephrotoxicity and hepatotoxicity. In general, the lung offers a large surface area for drug absorption, and the alveolar epithelium is very thin (approximately 0.1–0.5 μm thick), thereby permitting rapid drug absorption [21]. Therefore, the bioavailability of therapeutic peptides and proteins is 10–200 times greater by the pulmonary route than other noninvasive routes. In addition, the majority of emulsified formulations increase surface area of the drugs to improve solubilization behavior and membrane permeation, which might also result in bioavailability enhancement. In our study, the inhaled DE/CsA-RP at a dose of 100 μg of CsA was found to be effective to suppress antigen-induced inflammatory responses of respiratory systems in rats; however, far less is known as to whether DE/CsA-RP has any strong toxic risk after systemic exposure. To assess the systemic toxic potential of inhaled DE/CsA-RP, an *in vivo* test in rats was carried out with DE/CsA-RP and Neoral®, a commercially available CsA formulation for oral administration. A previous study demonstrated that oral administration of CsA at a dose of 10 mg/kg or higher resulted

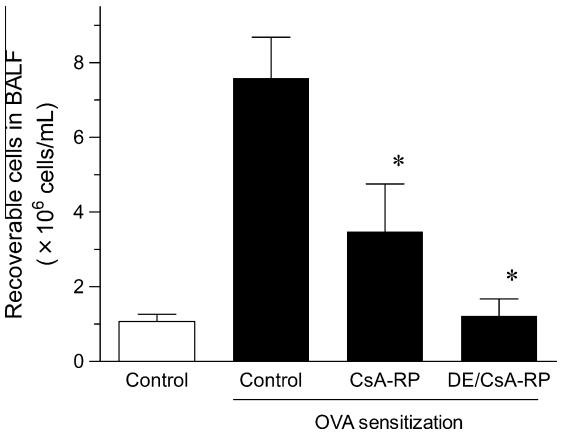

Fig. 4. Anti-inflammatory effects of inhaled dry-emulsion formulation of CsA in antigen-sensitized rats. Inflammatory cells recruited in BALF were counted in antigen-sensitized rats with or without inhalable CsA formulation. Data represent the mean ± SE of 6–7 rats. *, $p < 0.01$ with respect to antigen-sensitized rats with inhaled control-RP.

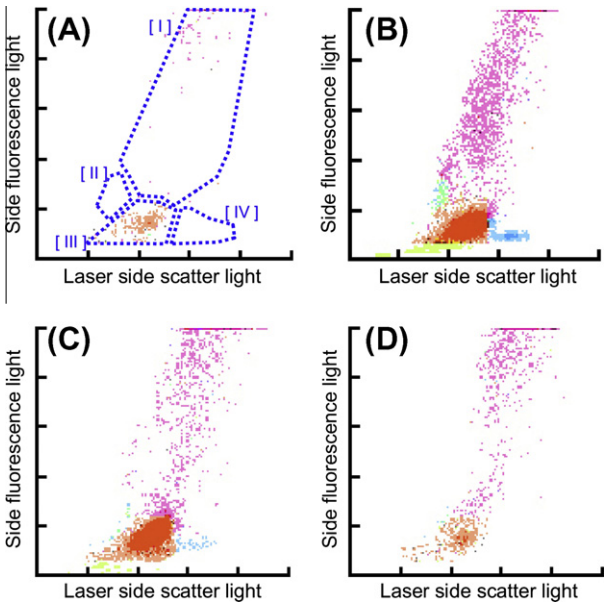

Fig. 5. Cytogram of BALF from antigen-sensitized rats. (A) Control (non-sensitized); (B) OVA-sensitized rat; (C) OVA-sensitized rat with pretreatment of CsA-RP; and (D) OVA-sensitized rat with pretreatment of DE/CsA-RP. Cells in BALF were classified as monocytes (I), lymphocytes (II), neutrophils (III), and eosinophils (IV). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in significant nephrotoxic responses in rats [22]; therefore, CsA was orally administered at a dose of 10 mg/mL in the present investigation. The plasma concentration–time profiles of CsA in rats after intratracheal administration of DE/CsA-RP (100 μg CsA) and oral administration of Neoral® (10 mg/kg CsA) were determined by UPLC/ESI-MS analysis (Fig. 6), and relevant pharmacokinetic parameters, including C_{max}, T_{max}, $T_{1/2}$, and AUC_{0-inf}, are listed in Table 1. Although both formulations showed rapid absorption with a relatively short T_{max} of around 2–4 h, orally administered Neoral® at a toxic dose exhibited a significantly higher mean C_{max} of ca. 3.2 μg/mL compared with that of the inhaled DE/CsA-RP (50 ng/mL). The plasma concentration of CsA decreased gradually with an elimination half-life of ca. 6–8 h. Consistently, the AUC value of the DE/CsA-RP at a pharmacologically effective dose (100 μg

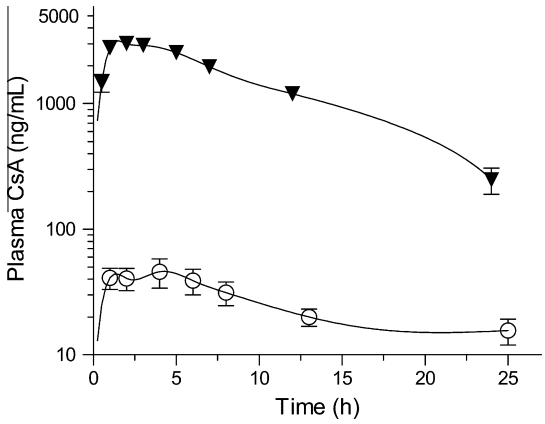

Fig. 6. Systemic exposure of CsA in rats after oral and intratracheal administration of CsA formulations. ○, intratracheal administration of DE/CsA-RP (100 μg CsA/rat); and ▼, oral administration of Neoral® (10 mg CsA/kg body weight of rat). Data represent mean ± SE of 6–7 experiments.

Table 1
Pharmacokinetic parameters of CsA formulations.

	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-inf} (ng h/mL)
DE/CsA-RP (100 μg CsA/rat, intratracheal)	50 ± 11	4.2 ± 0.75	8.3 ± 0.61	764 ± 184
Neoral® (10 mg CsA/kg, oral)	3200 ± 170	2.3 ± 0.61	5.8 ± 0.59	35,500 ± 3200

C_{max}: maximum concentration; T_{max}: time to maximum concentration; $T_{1/2}$: half-life; and AUC_{0-inf}: area under the curve of blood concentration vs. time from $t = 0$ to $t = \infty$ after administration. Values are expressed as means ± SE from 6–7 experiments.

CsA/rat) was approximately 50-fold lower than the oral dosage form at a toxic dose (10 mg CsA/kg). Therefore, inhalation of CsA might be a simple and safe method to achieve optimal local pharmacological action of CsA on the respiratory systems with minimal systemic side effects.

The CsA-induced nephrotoxicity is generally reversible; however, chronic treatment with CsA may result in irreversible morphological changes, leading to progressive loss of renal functions. Nebulized CsA at a dose of up to 4.2 mg/kg has been administered to lung transplant patients for 6 months with no reports of nephrotoxicity or hepatotoxicity [23], while oral CsA at a dose of 3–5 mg/kg/day for asthma treatment led to severe nephrotoxicity and hepatotoxicity [3,20]. These clinical studies demonstrated that steady-state trough concentrations greater than 200 ng/mL are generally associated with nephrotoxicity, and the inhalation system of CsA might be effective for suppressing excess systemic exposure of CsA. On the basis of the lower systemic CsA concentration after inhalation of the DE/CsA-RP, there might be a lower incidence of adverse events, and inhalable DE/CsA-RP might provide an interesting alternative to oral therapy with a better safety margin for treatment of asthma. However, the potential for local toxicity in chronic use is still uncertain, so further safety assessment is required before clinical use.

4. Conclusion

In the present study, a novel inhalable formulation of CsA employing a spray-dried emulsion system was designed for treatment of respiratory inflammation. There was marked improvement in dissolution behavior of the DE/CsA compared with that of an amorphous CsA, and a nanoemulsified dispersion of CsA can be reconstituted in the aqueous media. The DE/CsA-RP exhibited high dispersibility and suitable particle distribution for inhalation therapy. *In vivo* experiments demonstrated that inhaled DE/CsA-RP attenuated inflammatory symptoms in experimental asthma/COPD-model rats as evidenced by a decrease of infiltrated granulocytes, and there was no excessive increase in systemic exposure of CsA at a pharmacologically effective dose, possibly leading to reduced systemic side effects. These findings indicated that the inhalable DE formulation might be an efficacious delivery option for CsA, possibly providing an optimal local pharmacological action of CsA in the airway systems.

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