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Formulation design and *in vivo* evaluation of dry powder inhalation system of new vasoactive intestinal peptide derivative ([$R^{15, 20, 21}$, L^{17}, $A^{24,25}$, des-N^{28}]-VIP-GRR) in experimental asthma/COPD model rats

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ABSTRACT

Vasoactive intestinal peptide (VIP) has been considered as a promising drug candidate for asthma and COPD because of its potent immunomodulating and anti-inflammatory activities. Recently, our group developed a new VIP derivative, [$R^{15, 20, 21}$, L^{17}, $A^{24,25}$, des-N^{28}]-VIP-GRR (IK312548), with improved chemical and metabolic stability. In the present study, a dry powder inhaler system of IK312548 was designed for inhalation therapy with minimal systemic side effects, the physicochemical properties of which were also evaluated with a focus on morphology, particle size distribution, inhalation performance, and peptide stability. Laser diffraction and cascade impactor analysis suggested high dispersion and deposition in the respiratory organs with a fine particle fraction of 31.2%. According to UPLC/ESI-MS and circular dichroic spectral analyses, no significant changes in the purity and structure of VIP derivative were observed during preparation of respirable formulation. Anti-inflammatory properties of IK312548 respirable powder (RP) were characterized in antigen-sensitized asthma/COPD-model rats. There were marked inflammatory cells infiltrated into the lung tissues of experimental asthma/COPD-model rats; however, intratracheal administration of IK312548-RP led to significant reductions of recruited inflammatory cells in lung tissues and BALF by 72 and 78%, respectively. Thus, respirable powder formulation of IK312548 might be a promising medication for asthma, COPD, and other airway inflammatory diseases.

1. Introduction

Vasoactive intestinal peptide (VIP), a member of the glucagon–secretin superfamily, has been identified as a potential drug candidate for several chronic diseases such as diabetes, asthma, impotence, and rheumatism (Abad et al., 2006; Gozes and Furman, 2004; Pozo, 2003; Said, 1991). VIP and its specific receptors are abundantly present in normal human lung (Paul and Said, 1987) and monocyte-derived macrophages (Burian et al., 2010). Deficiency of VIP in the respiratory system is considered to be a pathogenetic factor in airway inflammatory disease (Said, 1989). The therapeutic potential of VIP for airway inflammatory disease including asthma and chronic obstructive pulmonary disease (COPD) has drawn considerable attention recently because of its potent anti-inflammatory efficacy in respiratory tissues (Groneberg et al., 2006). In spite of its potent pharmacological activities, the clinical application of VIP has been limited since VIP is susceptible to proteolytic degradation *in vivo* after systemic or topical administration, resulting in low potency and short duration of action (Goetzl et al., 1989). To improve the metabolic and chemical stability of VIP, a number of structure–activity relationship studies on VIP were carried out with different techniques (Onoue et al., 2008; Stiuso et al., 2006). In our previous study, VIP derivative, [$Arg^{15, 20, 21}$, Leu^{17}]-VIP-GRR (IK312532), exhibited high metabolic stability and prolonged duration of action com-

Abbreviations: Alum, aluminum hydroxide; ANOVA, a one-way analysis of variance; BALF, bronchoalveolar lavage fluid; CD, circular dichroism; COPD, chronic obstructive pulmonary disease; DAB, 3,3′-diaminobenzidine; DPI, dry powder inhaler; FPD, fine particle dose; FPF, fine particle fraction; HPMC, hydroxypropyl methylcellulose; MeOH, methanol; OCT, Optimal cutting temperature; OVA, ovalbumin; PBS, phosphate buffered saline; NaPB, sodium phosphate buffer; RP, respirable powder; SEM, scanning electron microscopy; TFA, trifluoroacetic acid; UPLC/ESI-MS, ultra-performance liquid chromatography equipped with electrospray ionization mass spectrometry; USP, United States Pharmacopeia; VIP, vasoactive intestinal peptide.

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pared with VIP (Onoue et al., 2007), although long-term stability testing demonstrated the poor chemical stability of IK312532 in the solution state. On the basis of structural elucidation with major degradants, deamination of Asn residue at position 24, by direct hydrolysis or via an acyclic imide intermediate, was deduced to be responsible for degradation of IK312532 (Onoue et al., 2009b). Further chemical modification study led to successful development of [$R^{15, 20, 21}$, L^{17}, $A^{24,25}$, des-N^{28}]-VIP-GRR (IK312548) with improved chemical stability, and the new VIP analog demonstrated the potent anti-apoptotic effects in rat alveolar L2 cells exposed to cigarette smoke extract (Misaka et al., 2011).

According to the clinical observations, systemic dosing of VIP and its derivatives might lead to moderate hypotension and/or other adverse effects (Morice et al., 1983). Previously, our group demonstrated that the combined use of VIP and a pulmonary delivery system would be advantageous for the therapy of pulmonary inflammation diseases (Onoue et al., 2009a). Rationales for developing dry powder inhaler (DPI) formulation of VIP were to maximize pharmacological effects and to minimize systemic exposure. Although physicochemical and biochemical properties of IK312548 were well clarified, the feasibility of developing a DPI system for IK312548 and its therapeutic potential have not been fully elucidated.

The present study was aimed to develop a respirable powder (RP) formulation of IK312548 (IK312548-RP) for the treatment of airway inflammatory diseases. For comparison purposes, further chemical and metabolic stability testing was carried out on VIP and its derivatives. The newly prepared RP formulation was characterized in terms of its morphology by electron microscopic analysis, particle size distribution by laser diffraction analysis, and inhalation performance by cascade impactor analysis. Peptide stability during the formulation process was also assessed by UPLC/ESI-MS and circular dichroism (CD) spectral analyses. With the use of experimental asthma/COPD-model rats, anti-inflammatory properties of IK312548-RP were assessed with a focus on the infiltration of inflammatory cells in pulmonary tissues.

2. Materials and methods

2.1. Chemicals

VIP and its derivatives (Table 1) were chemically synthesized by a solid-phase strategy employing optimal side-chain protection as reported previously (Merrifield, 1969). Peptides were removed from the resin by HF treatment, and the cleaved products were purified using column chromatography over Chromatorex ODS (Fuji Silysia, Aichi, Japan) with CH_3CN–H_2O system mobile phase. The purity (>98%) was checked by reverse-phase HPLC using a TSK-gel ODS-120T column (Tosoh, Tokyo, Japan). Their molecular weights were confirmed on a matrix-assisted laser desorption ionization-time of flight mass spectrometer (Kratos, Manchester, UK).

Respitose® SV-003 was supplied from DMV (Veghel, The Netherlands). OVA and aluminum hydroxide (alum) gel were purchased from Sigma–Aldrich (St. Louis, MO). Trypan blue and 10% formalin neutral buffer solution were bought from Wako Pure Chemical Industries (Tokyo, Japan). 3,3′-Diaminobenzidine (DAB) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Erythritol was supplied by Nikken Chemicals (Tokyo, Japan) and all other reagents were obtained from commercial sources.

2.2. Stability testing on VIP and derivatives in bronchoalveolar lavage fluid (BALF)

Male Sprague–Dawley rats were exsanguinated via the descending aorta under anesthesia and perfused with 30 mL of saline. The

Table 1
Sequence of VIP and its derivatives.

	5	10	15	20	25	30	Ki (nM) [a]	Chemical and metabolic stability	
								Degradation constant in	
								pH7.4 (day^{-1}; 25°C) [b]	BALF (h^{-1}; 37°C)
VIP	H S D A V F T D N Y	T R L R K Q M A V K	K Y L N S I L N				2.6±0.9	4.7×10^{-2}	0.58±0.03
[$R^{15,20,21}$, L^{17}]-VIP-GRR (IK312532)	– – – – – – – – – –	– – – – R – L – – R	R – – – – – – – G R	R			2.7±0.6	3.5×10^{-2}	0.34±0.01
[$R^{15,20,21}$, L^{17}, $A^{24,25}$; des-N^{28}]-VIP-GRR (IK312548)	– – – – – – – – – –	– – – – R – L – – R	R – – A A – – G R	R			3.1±0.4	4.7×10^{-3}	0.30±0.01

All peptides were amidated at their C-termini.
[a] Ki values for inhibition by VIP and its derivatives of specific [^{125}I]VIP binding in rat alveolar L2 cells (Onoue et al., 2009b). Each value represents mean ± SE of 4 determinations.
[b] Degradation kinetics of neuropeptides dissolved in 20 mM NaPB (pH 7.4) (Onoue et al., 2009b).

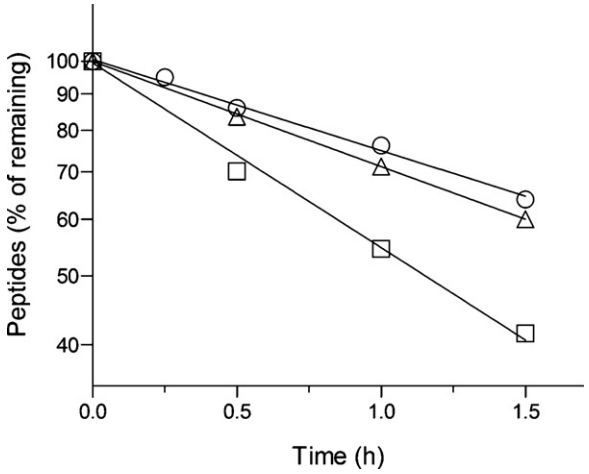

Fig. 1. Degradation of VIP and its derivatives in BALF. Each peptide (0.1 mg/mL) in BALF was stored at 37 °C for the indicated periods and subjected to UPLC/ESI-MS analysis. □, VIP; △, [$R^{15,20,21}$, L^{17}]-VIP-GRR (IK312532); and ○, [$R^{15,20,21}$, L^{17}, $A^{24,25}$, des-N^{28}]-VIP-GRR (IK312548). Data represent the mean of three experiments.

lungs were exposed and lavaged five times using 5 mL of phosphate buffer saline (PBS). The solution with lavage material was immediately aspirated back after each aliquot. The BALF was centrifuged at 4 °C for 10 min at 500 rpm, and the supernatants were gently aspirated and stored at −80 °C until use. Each peptide solution (50 μL, 1 mg/mL) was added into 0.45 mL of BALF, and mixed well. Each solution was kept at 37 °C and subjected to UPLC/ESI-MS analysis at the indicated periods (Fig. 1). All analyses were performed on Waters Acquity UPLC/MS system (Waters, Milford, MA), which includes the binary solvent manager, sampler manager, column compartment, Acquity TUV detector with a detection wavelength of 220 nm, and a single quadrupole mass detector (mass range: *m*/*z* 300–2000), connected with Waters MassLynx software. The mass spectrometer instrument was operated in positive electrospray ionization mode using a cone voltage of 50 mV. The source and desolvation temperatures were set at 120 and 400 °C, respectively. An Acquity UPLC BEH C18 column (particle: size 1.7 μm, column size: *Φ*2.1 mm × 50 mm; Waters) was used. Column temperature was maintained at 40 °C. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA)–acetonitrile 75:25 (v/v) with a flow rate of 0.25 mL/min.

2.3. Preparation of respirable powder formulation

OVA aerosol has been used as a potent tool for establishing experimental asthma/COPD model, since repeated exposure of actively immunized animals to OVA aerosol induced severe inflammatory events (Misaka et al., 2009). Dry powder formulation of OVA and IK312548 was prepared as described previously (Misaka et al., 2009; Onoue et al., 2009c). Briefly, OVA or IK312548 was firstly ground with excipient to fine powder using a pestle and mortar and then milled using an A-O JET MILL (Seishin Enterprise, Tokyo, Japan) at a pusher nozzle pressure and grinding nozzle pressure of 0.60 and 0.55 MPa, respectively. The ratio of the compounds to excipient was 1:9 (w/w). The micronized materials were decompounded with 10-fold carrier particles (Respitose® SV-003), and the obtained dry powders of OVA and IK312548 were stored in a vacuum desiccator until testing.

2.4. Scanning electron microscopy (SEM)

Representative SEM images of inhalable formulation were taken using a scanning electron microscope, VE-7800 (Keyence Corporation, Osaka, Japan), without Au or Pt coating as previously reported (Onoue et al., 2009c). For the SEM observations, each sample was fixed on an aluminum sample holder using double-sided carbon tape.

2.5. Particle size distribution

The particle size of IK312548-RP was measured by a laser diffraction scattering method using an LMS-300 (Seishin Enterprise, Tokyo, Japan).

2.6. Circular dichroic spectral analysis

For CD analysis, each peptide was dissolved in 50% methanol (MeOH)/20 mM sodium phosphate buffer (NaPB). The solvent system of water/alcohol, including methanol and trifluoroethanol, has often been used as a membrane-mimetic medium, and secondary structures existing in methanol/water reflect those in the lipidic milieu (Kobayashi et al., 2000; Onoue et al., 2004a). CD spectra were baseline-corrected and smoothed using the algorithm provided by the manufacturer, and they were recorded at room temperature in a Jasco model J-720 spectropolarimeter (Jasco, Tokyo, Japan) with a cell path length of 10 mm. Each sample was scanned five times in the wavelength range of 200–400 nm (scan speed: 10 nm/min; sensitivity: 20 mdeg). Ellipticity was calculated as mean residue ellipticity [θ] (degrees cm^2 $dmol^{-1}$).

2.7. Cascade impactor analysis

The dispersibility of dry powder was assessed according to USP 29 <601> AEROSOLS using an AN-200 system (Shibata Scientific Technology, Tokyo, Japan), consisting of a vacuum pump, a mass flow meter, and an eight-stage Andersen cascade impactor. Briefly, dry powders were filled into a JP No. 2 hard capsule of HPMC, and the capsule was installed in a JetHaler® (Hitachi Unisia, Kanagawa, Japan) powder inhaler. The dry powder formulations (40 mg) in each capsule were dispersed via the device with an inspiration rate of 28.3 L/min for an inhalation time of 30 s × 10 times, and the collection stages of the impactor (stage 0–7) were washed with purified water. VIP derivative in each solution was determined by UPLC/ESI-MS as described in Section 2.2. The fine particle dose (FPD) was defined as the mass of drug particles measuring less than 5.8 μm (particles deposited at stage 2 and lower). The fine particle fraction (FPF) was calculated as the ratio of FPD to total loaded dose.

2.8. In vivo experiments

2.8.1. Animals and inhalation

Male Sprague–Dawley rats, weighing ca. 400 g (11 weeks of age; Japan SLC, Shizuoka, Japan), were housed two 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%). Rats were sensitized by intraperitoneal injection of 100 μg of OVA with 5 mg of alum on days 0, 7, and 14. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and administered intratracheally with OVA (100 μg/rat)-RP at 24 h after the last OVA sensitization. At 1 h before the OVA-RP challenge, 5 mg of IK312548-RP (50 μg of IK312548/rat) or control-RP (micronized excipient and carrier powder) was administered to rats (n = 8 for each group) via intratracheal insufflation using a 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 airway system of the rats. All procedures used in the present study were conducted in accordance with the guidelines approved by the Institutional Animal Care and Ethical Committee of the University of Shizuoka.

2.8.2. Histochemical studies

Lung left lobes were removed at 24 h after OVA-RP challenge and fixed in 10% formalin neutral buffer solution. Fixed tissues were washed with 10 mL of PBS three times and immersed in 30% sucrose containing 0.1% sodium azide at 4 °C for 24 h. The tissues were embedded in OCT Compound (Sakura Finetek Japan, Tokyo, Japan), frozen in liquid nitrogen, and cut into 12-μm-thick sections in a cryostat. For peroxidase staining to count inflammatory cells, the sections on glass slides were dried and washed with distilled water. The sections were allowed to react for 3 min in a solution of 1 mL of 0.05 M Tris–HCl buffer (pH 7.6) containing 14 mM DAB and 0.005% H_2O_2, and then the sections were stained with hematoxylin for 1 min. The sections were dehydrated and mounted with Mount Quick™ (Daido Sangyo, Saitama, Japan) before examination under a light microscope (Microphot-FXA, Nikon Corporation, Tokyo, Japan) equipped with digital sight DS-Fi1 (Nikon).

2.8.3. Inflammatory cells in BALF

At 24 h after intratracheal administration of OVA-RP, rats were exsanguinated via the descending aorta under anesthesia and perfused with 30 mL of saline. A bronchoalveolar lavage was performed immediately using 5 mL of PBS × 2 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.

2.9. Statistical analysis

For statistical comparisons, a 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. Physicochemical and biochemical properties of VIP derivative

To clarify the possible improvement in metabolic stability of new VIP derivative, VIP, IK312532 and IK312548 were incubated in BALF and subjected to UPLC/ESI-MS analysis. According to UPLC/ESI-MS chromatograms of VIP and its derivatives stored for 1.5 h at 37 °C, all tested peptides were gradually decomposed (data not shown). Decomposition of each neuropeptide in BALF was plotted in a typical logarithm of percent remaining versus time (Fig. 1). A linear relationship was obtained according to the following equation: $\ln A = \ln A_0 - kt$, where A is the remaining peak area of neuropeptide, k is slope, and t is time (h). This indicated that the degradation of VIP and its derivatives in the diluted BALF followed apparent first-order degradation kinetics under the conditions studied, possibly due to low enzymatic activity in the diluted BALF. The stability of each peptide was evaluated on the basis of the kinetic degradation constant k with respect to the initial drug concentration, and the following data were obtained: slope (rate constant) = $0.58 \pm 0.03\ h^{-1}$ for VIP, $0.34 \pm 0.01\ h^{-1}$ for IK312532, and $0.30 \pm 0.01\ h^{-1}$ for IK312548. Half-lives of VIP, IK312532 and IK312548 in the diluted BALF were calculated to be 1.20, 2.05 and 2.33 h, respectively. Thus, VIP exhibited relatively rapid degradation under the present experimental conditions, although the metabolic stability of the VIP derivative was found to be improved. The enhanced metabolic stability of VIP derivatives in pulmonary tissues might contribute to better clinical outcomes from VIP-based therapy for airway inflammatory diseases.

With respect to the solution-state stability of these peptides in 20 mM NaPB (pH 7.4), degradation kinetics at 25 °C were previously calculated to be $4.7 \times 10^{-2}\ day^{-1}$ for VIP, $3.5 \times 10^{-2}\ day^{-1}$ for IK312532, and $4.7 \times 10^{-3}\ day^{-1}$ for IK312548 (Table 1) (Onoue et al., 2009b). There appeared to be ca. 10-fold improvement in solution-state stability of IK312548 compared with those of VIP and IK312532. In addition, the binding activities of these neuropeptides for VPAC2 receptor expressed in rat alveolar cells were previously examined with a radioligand binding assay using [^{125}I]VIP (Onoue et al., 2009b). VIP and its derivatives such as IK312532 and IK312548 could compete with [^{125}I]VIP for VPAC2 receptor with Ki values of $2.6 \pm 0.9 \times 10^{-9}$ M (VIP), $2.7 \pm 0.6 \times 10^{-9}$ (IK312532), and $3.1 \pm 0.4 \times 10^{-9}$ M (IK312548) (Table 1). Thus, VIP derivatives were found to be equipotent to VIP in terms of receptor binding activity. The present findings, taken together with previous observations (Onoue et al., 2009b), suggested that IK312548 exhibited considerably improved in solution-state stability, although the metabolic stability and receptor-binding activity were almost identical to those of IK312532. These observations were in agreement with the results from previous chemical modification studies, showing that (1) the replacement of Lys residues at positions 15, 20, and 21 by Arg, and Met at position 17 by Leu in VIP resulted in a significant improvement in metabolic stability (Kashimoto et al., 1996); and (2) substitution of amino acid residues at positions 24 and 25 in VIP led to the significant improvement of chemical stability (Onoue et al., 2009b).

3.2. Preparation of respirable powder formulation containing VIP derivative

Treatment of asthma/COPD with inhaled drugs might offer some advantages over systemic therapy, including a more rapid onset and reduced adverse effects, because of direct targeting of the airway systems (Chan, 2003). For the preparation of an RP formulation of IK312548, a mixture of IK312548 and erythritol was micronized with jet mill, and the milled particles were mixed with lactose carriers with a diameter of ca. 50 μm for stabilization. According to the SEM images (Fig. 2A), on the surface of carrier particles, there was no agglomeration of micronized particles with diameter of over 20 μm, possibly due to stabilization of jet-milled particles by lactose carriers. To clarify the re-dispersibility of micronized particles, laser diffraction analysis was carried out on the IK312548-RP (Fig. 2B). Under an air pressure of 0.2 MPa, IK312548-RP could be dispersed well, for which two clear peaks for micronized particles and carriers were observed at ca. 4 and 55 μm, respectively. The result suggested fine dispersibility of micronized particles to generate aerosols for inhalation therapy.

During the formulation process or long-term storage, therapeutic peptides/proteins sometimes undergo structural conversion and/or degradation, followed by a decrease of pharmacological effects (Onoue et al., 2004b). In particular, misfolding of glucagon–secretin family peptides, such as glucagon and glucagon-like peptide-1, led to structural transition from α-helical structure into β-sheet-rich structure, and generation of cytotoxic fibrils was also confirmed (Maji et al., 2009; Onoue et al., 2004b). In the present study, UPLC/ESI-MS and CD spectral analyses were carried out on IK312548-RP to elucidate the peptide stability during

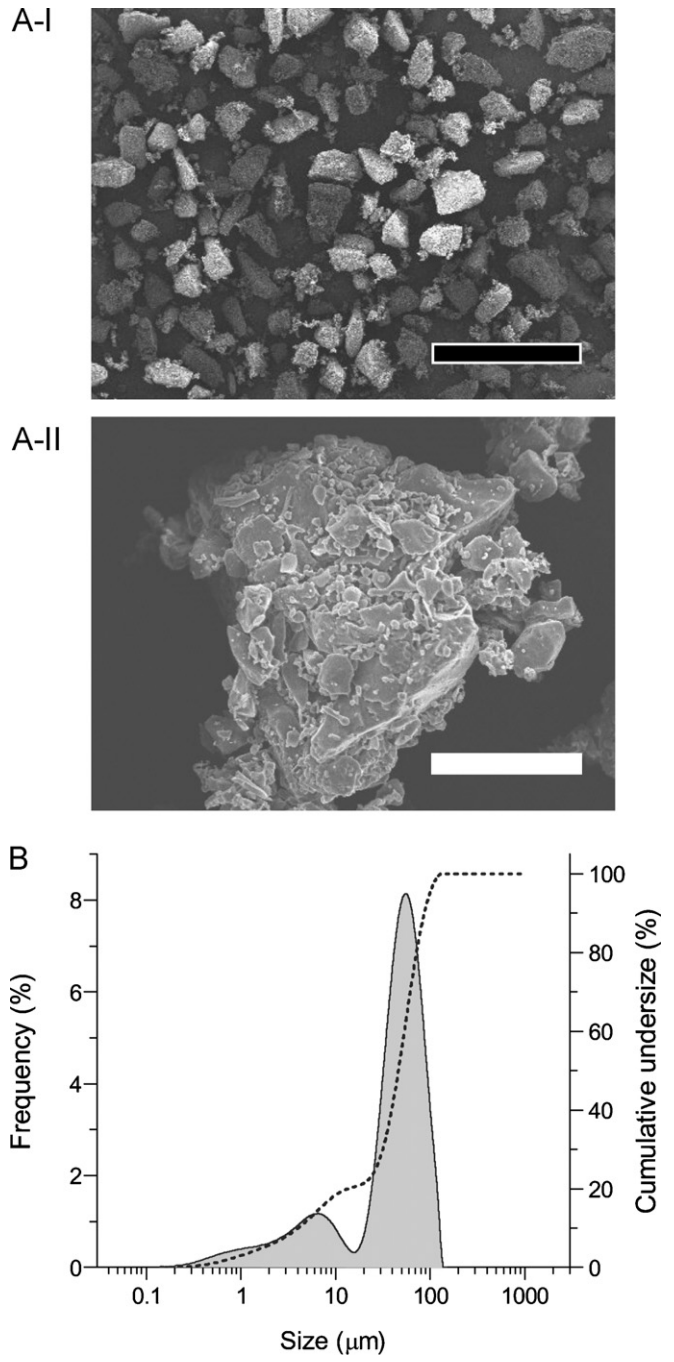

Fig. 2. Appearance and particle size distribution of IK312548-RP. (A-I and II) Scanning electron microscopic images of IK312548-RP. Black and white bars represent 250 and 20 μm, respectively. (B) Particle size distribution of aerosolized IK312548-RP. The IK312548-RP was aerosolized by dry air at a pressure of 0.2 MPa, and the size distribution of dispersed particles was analyzed by laser diffraction. Solid line, frequency; and dotted line, cumulative undersize fraction curve.

the formulation process. In the UPLC/ESI-MS analyses, no peaks (>0.5%) for degradants of IK312548 were seen in the RP formulation prepared (data not shown). The CD spectrum of IK312548 was almost identical to that of VIP, showing two negative peaks at 209 nm and 222 nm (Fig. 3). These spectral patterns were indicative of the typical α-helical structure of neuropeptides in a hydrophobic condition (50% MeOH/20 mM NaPB, pH 7.4) (Onoue et al., 2004a). In contrast, the CD spectrum of a β-sheet structure typically shows an intense positive band at 198 nm and a negative extremal band at 218 nm (Cort et al., 1994), as observed in mis-

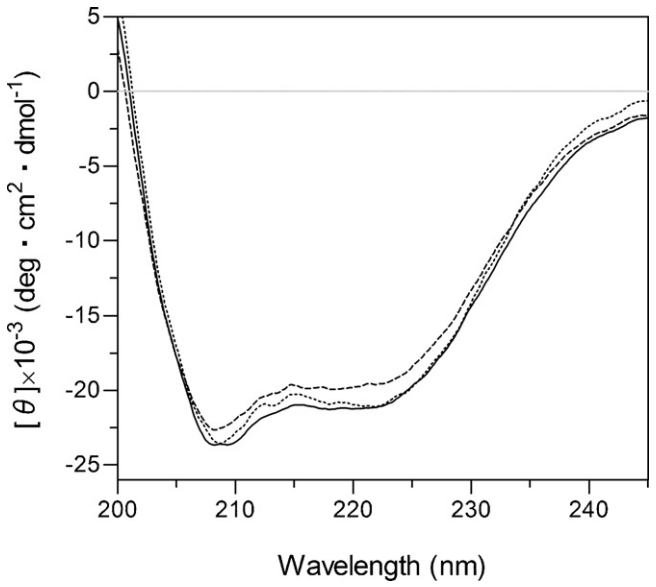

Fig. 3. CD spectra of VIP and IK312548. The measurements were carried out in 50% methanol/20 mM NaPB (pH 7.4). Solid line, VIP; broken line, IK312548; and dotted line, IK312548-RP.

folded glucagon previously. No significant transitions were seen in the conformation of IK312548 in the RP formulation compared to those of VIP and IK312548. According to the calculation established by Greenfield and Fasman (1969), the α-helical contents of VIP, IK312548, and IK312548 in the RP formulation were estimated to be 68, 64 and 66%, respectively. From these findings on purity and secondary structure, IK312548 was found to be stable without degradation and conformational transition during the formulation process.

3.3. Inhalation performance of IK312548-based respirable powder formulation

From a theoretical point of view, a drug must be properly delivered to the deep lung tissue for inhalation therapy since its delivery might affect clinical outcomes, therapy cost, and medication compliance (Patton and Byron, 2007). In particular, the lung can be effectively targeted for asthma/COPD treatment by delivering the drug as an aerosol, with an aerodynamic diameter ranging from 1 to 6 μm, and thus most inhaled products are formulated with a high proportion of drug in this size range (Onoue et al., 2009a). In the present study, the *in vitro* inhalation performance of the IK312548-RP was evaluated by cascade impactor analysis in accordance with USP. On the basis of the results from cascade impactor analysis on the IK312548-RP (Fig. 4), the emitted dose of the IK312548-RP from HPMC capsules was calculated to be 99%, and the FPF value was estimated to be 31.2%. These findings suggested that the particles of the IK312548-RP might be fine enough to be inhaled and reach the lung; however, as much as 52% of the IK312548-RP was found to be deposited in stage 0. Generally, inhaled particles with a diameter of over 10 μm are deposited in the oropharynx or large airways, where they have little, if any, systemic therapeutic effects (Labiris and Dolovich, 2003). Before the clinical application, further optimization of RP formulation might be required to improve inhalation performance, with a focus on excipient compatibility, selection of stabilizing carrier, and choice of suitable devices.

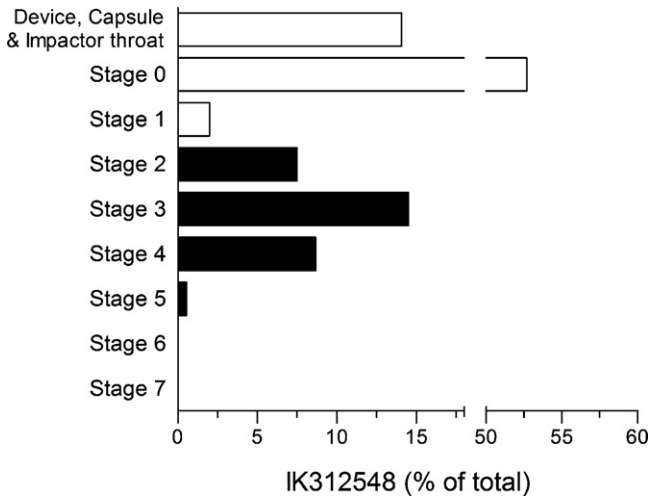

Fig. 4. *In vitro* inhalation property of IK312548-RP. Deposition pattern analysis of IK312548-RP was carried out using a cascade impactor connected with a JetHaler® with an airflow rate of 28.3 L/min. Amount of IK312548 in inhalation device, capsule, throat part and each stage (stage 0–7) was determined by UPLC/ESI-MS analysis.

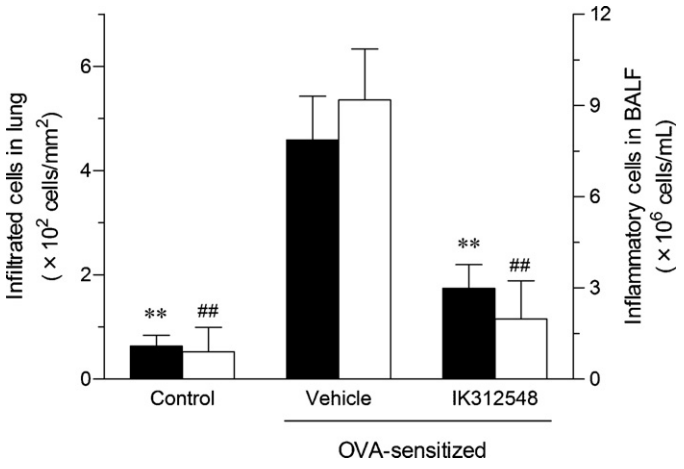

Fig. 5. Anti-inflammatory effects of inhaled IK312548-RP (50 μg of IK312548/rat) in antigen-sensitized rats. Inflammatory cells recruited in the lung (filled bars) and BALF (open bars) were counted. Data represent the mean ± SE of 5–7 rats. $^{**}P < 0.01$ (lung) and $^{\#\#}P < 0.01$ (BALF) vs. antigen-sensitized rats without inhalation of IK312548-RP.

3.4. Pharmacological effects of inhaled VIP derivative

To elucidate the therapeutic potential of IK312548-RP, anti-inflammatory effects of inhaled IK312548 were assessed using experimental asthma/COPD-model rats. At 24 h after OVA challenge, the left lobe of the lung was sectioned and subjected to peroxidase staining using DAB and hematoxylin, to evaluate infiltration of granulocytes into the airway system. As shown in Fig. 5, peroxidase staining revealed that numerous cells infiltrated into the bronchial epithelium and interstitium in the antigen-exposed rats, and they partly consisted of granulocytes, such as neutrophils and eosinophils. There was a ca. 7-fold increase in the number of infiltrated inflammatory cells around the airways of the antigen-exposed group compared with that of control rats. In contrast, rats treated with the IK312548-RP (50 μg of IK312548/rat) showed reduced inflammatory responses of lung, as shown by the 72% decrease of infiltrated granulocytes compared with those of antigen-treated rats. In addition to histochemical analyses of lung tissues, recruitment of inflammatory cells in BALF was also evaluated since BALF has been frequently used as a biological source for clinical investigation of inflammatory lung diseases (Tzortzaki et al., 2006). BALF was obtained at 24 h after the last antigen challenge, and the inflammatory cells in BALF were counted (Fig. 5). As observed in lung tissues, the antigen challenge caused a marked recruitment of inflammatory cells in BALF as evidenced by a ca. 10-fold increase of cell numbers. Treatment of antigen-exposed rats with IK312548-RP (50 μg of IK312548/rat) resulted in as much as a 78% reduction of inflammatory cells in BALF. There was no significant difference in the numbers of granulocytes between control and antigen-exposed rats with IK312548-RP treatment, suggesting a potent anti-inflammatory effect of inhaled IK312548.

For further characterization of anti-inflammatory effects of the inhaled IK312548-RP in these experimental model rats, types of recruited cells in BALF were analyzed using a Sysmex XT-2000iV system as reported previously (Mathers et al., 2007). DIFF scattergram of BALF from the antigen-sensitized rats suggested that the recruited cells were mainly consisted of monocytes and neutrophils, and a smaller number of eosinophils were also observed (Fig. 6A). Activated macrophages, T cells, and mast cells also produce and secrete matrix metalloproteases, resulting in damage to the epithelial barrier. Neutrophils and eosinophils possess granules containing matrix-degrading proteases, and activated neutrophils also produce reactive oxygen free radicals, leading to damage of the epithelium and underlying basement membrane in pulmonary tissues (Barnes, 2004). Interestingly, intratracheal administration of the IK312548-RP in the antigen-sensitized rats resulted in decreases of neutrophils and eosinophils (Fig. 6B). Both COPD and asthma can be defined as airway inflammation; however, marked differences were observed in the nature of the inflammatory process, with differences in inflammatory cells, mediators, response to inflammation, and anatomical distribution (Jeffery,

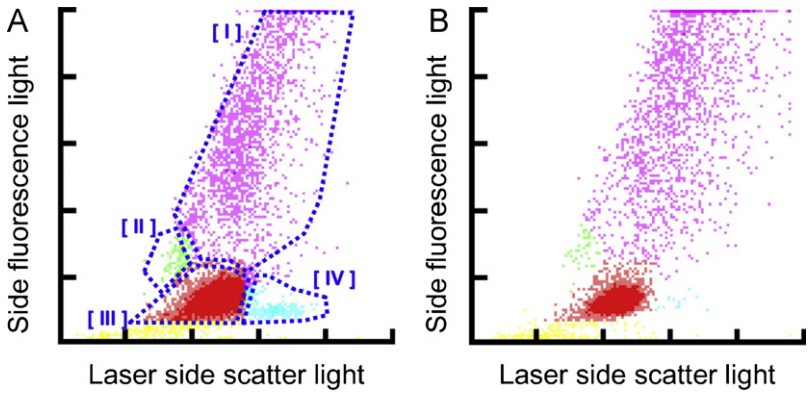

Fig. 6. Cytogram of BALF from antigen-sensitized rats with control-RP (A) and IK312548-RP (50 μg of IK312548/rat) (B). Cells were classified as monocytes (I), lymphocytes (II), neutrophils (III), and eosinophils (IV).

1999). The present results would indicate the therapeutic potential of IK312548-based inhalation therapy for treatment of asthma, COPD, and other airway inflammatory diseases.

4. Conclusion

In the present study, RP formulation of [$R^{15, 20, 21}$, L^{17}, $A^{24,25}$, des-N^{28}]-VIP-GRR (IK312548), a stable VIP derivative, was designed for inhalation therapy. No degradation and conformational changes were observed during the formulation process, and the IK312548-RP exhibited high inhalation performance. *In vivo* experiments demonstrated that inhaled IK312548-RP attenuated inflammatory symptoms in experimental asthma/COPD model rats as evidenced by a decrease of infiltrated granulocytes. Although VIP-based drugs have not yet been used for clinical purposes, the combination of newly developed VIP derivative and DPI system might provide efficacious medication for the clinical treatment of airway inflammatory diseases.

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