

Original article

DK-1108 exerts anti-inflammatory activity against phorbol 12-myristate 13-acetate-induced inflammation and protective effect against OVA-induced allergic asthma

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ABSTRACT

There is an increasing interest in natural products and their derivatives with therapeutic benefits and less side effects compared to steroid therapy. Benzofuran derivatives display biological effects including anti-inflammatory effects. The present study aims to investigate whether (3-(7-methoxy-2-p-tolyl benzofuran-5-yl) propan-1-ol) (DK-1108), new synthetic benzofuran compound exerts anti-asthmatic effects *in vitro* and *in vivo*. DK-1108 strongly reduced the production of inflammatory mediators, cytokines and chemokines in RAW264.7 and A549 cells. DK-1108 significantly regulated the levels of AKT/MAPKs/c-Jun activation, AP-1 luciferase activity and ICAM-1 expression. Furthermore, DK-1108 effectively suppressed the adhesion of A549 and EOL-1 cells. In OVA-induced asthmatic mice, DK-1108 decreased the levels of IL-5/IL-13/IgE production, eosinophils/macrophages influx, ICAM-1/MCP-1 expression, mucus secretion and airway hyperresponsiveness (AHR). These effects of DK-1108 were accompanied by downregulation of MAPKs activation. Therefore, we suggest that DK-1108 exerts protective effect against airway inflammation and mucus overproduction, and therefore could be valuable therapeutic agent for treatment in asthma.

1. Introduction

Allergic asthma is one of the most common chronic inflammatory disorders, and its pathogenesis is closely associated with exposure to environmental allergens [1]. Th2 cell-derived cytokines play important roles in airway inflammation, mucus hypersecretion, and airway obstruction by activating eosinophils and mast cells in response to allergens [2]. Airway epithelial cell-derived monocyte chemoattractant

protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and matrix metalloproteinase-9 (MMP-9) contribute to the recruitment of inflammatory cells, including eosinophils [3].

Previous studies have demonstrated an increase in the expression of inducible nitric oxide synthase (iNOS) in the lungs of patients with asthma [4], and the enhanced levels of cyclooxygenase (COX)-2 are closely associated with increased inflammatory molecules in severe asthma [2]. Recent studies reported that the production of inflammatory

Abbreviations: OVA, ovalbumin; PMA, phorbol-12-myristate-13-acetate; NO, nitric oxide; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IL-1 β , interleukin-1 β ; IL-8, interleukin-8; MMP-9, matrix metalloproteinase-9; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; AP-1, activator protein-1; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; IL-5, interleukin-5; IL-13, interleukin-13; IgE, immunoglobulin E; AHR, airway hyperresponsiveness; Penh, enhanced pause; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; MAPKs, mitogen-activated protein kinases.

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molecules is modulated by the activation of transcription factors such as activator protein-1 (AP-1) through protein kinase B (PKB, AKT) and mitogen-activated protein kinase (MAPK)-mediated signaling pathways [3,5–8]. Therefore, it is necessary to develop agents regulating Th2 cytokines and controlling the activity of AP-1/AKT/MAPKs for the prevention and treatment of inflammatory diseases such as asthma.

Corticosteroids are the most potent and effective medications against chronic and acute manifestations of allergic asthma [9]. However, a clinical study reported that administration of higher doses of corticosteroids may be associated with systemic side effects [10]. Therefore, recent studies have focused on natural compounds and their derivatives that exhibit remarkable biological activities and low toxicity in the treatment of asthma [11]. *Styrax japonica*, widely distributed in Korea, has been extensively used in the treatment of inflammatory disorders as a medicinal plant; homoeogonol, the lignan of *S. japonica*, exerts protective effects in ovalbumin (OVA)-induced asthmatic mice [12]. Homoeogonol is a natural product that exhibits biological activity and contains a benzofuran heterocycle as its basic structural unit [13]. Recent *in vitro* and *in vivo* studies have shown that the benzofuran derivative DK-1014 has anti-asthmatic effects [3]. In the present study, we investigated whether the newly synthesized DK-1108 shows inhibitory effects against nitric oxide (NO), tumor necrosis factor (TNF)- α , and interleukin-6 (IL-6), which are biomarkers of asthma, in *in vitro* experiments. Based on the *in vitro* results, we further evaluated the protective effects of DK-1108 using *in vitro* and *in vivo* models of asthma.

2. Methods

2.1. Synthesis of DK-1108

DK-1108 was synthesized using key reactions, such as Corey-Fuchs reaction, Sonogashira coupling, and Horner–Wadsworth–Emmons olefination, with an overall yield of 54 % [3].

2.2. Chromatographic separation and spectral analysis

All commercially available chemicals used in this study were of reagent grade and were used without further purification. Reactions were conducted under an atmosphere of dried argon using flame-dried glassware. The proton (^1H) nuclear magnetic resonance (NMR) spectra were recorded using a Varian (400 MHz) spectrometer (Varian Medical Systems, Inc., Palo Alto, CA, USA). The ^1H NMR data are reported as peak multiplicities: s for singlet, d for doublet, dd for the doublet of doublets, t for triplet, q for quartet, br for broad singlet, and m for multiplet. The ^{13}C NMR spectra were recorded using a Varian (100 MHz) spectrometer. The values of the chemical shifts are expressed in δ values (ppm), and the coupling constants (J) are reported in hertz (Hz).

Mass spectra were recorded using high-resolution mass spectrometry (HRMS)-electrospray ionization (ESI)-MS, using a G2 quadrupole-time-of-flight (QTOF) mass spectrometer (Waters Corp, Milford, MA, USA). Products were purified via column or flash chromatography (Biotage, Sweden) using silica gel 60 (230–400 mesh Kieselgel 60). Thin-layer chromatography (TLC) with 0.25 mm silica plates (E. Merck; silica gel 60 F254) was used to monitor the reactions. Spots were visually detected under ultraviolet (UV) light and colorized by charring after dipping in anisaldehyde or basic potassium permanganate (KMnO_4) solution.

The purity of the final products was determined using reversed-phase high-performance liquid chromatography (RP-HPLC) using a Waters Corp. HPLC system equipped with an ultraviolet (UV) detector set at 254 nm. The mobile phases used were as follows: (A) water containing 0.05 % trifluoroacetic acid and (B) acetonitrile (CH_3CN). The HPLC system included a YMC hydrosphere C18 (HS-302) column (particle size, 5 μm ; pore size, 12 nm; diameter, 4.6 mm $\times$ 150 mm), and the mobile phase was run at a flow rate of 1.0 mL/min. The compound purity was assessed using mobile phase B at a gradient of 25%–100% for 35 min. The purity of all the biologically evaluated compounds was >95 %. Details of the

synthetic scheme, procedures, and spectral characterization of all intermediates and DK-1108 are provided in the supplementary data (Scheme S1).

2.3. Cell viability assay

Murine RAW264.7 macrophages (ATCC® TIB-71™, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM), whereas human A549 airway epithelial cells (ATCC® CCL-185™) were cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10 % fetal bovine serum (FBS) and a 1 % (w/v) antibiotic-antimycotic solution (Invitrogen; Thermo Fisher Scientific, Inc.) in a 5 % CO_2 incubator at 37 °C. Cells used were cultured up to 15 passages. Cell viability was examined using a CytoX cell viability kit (LPSsolution, Korea). The cells (1×10^4 cells/well) were seeded onto a 96-well plate, treated with various concentrations (0–20 μM) of DK-1108 for 20 h; then, water-soluble tetrazolium (WST) solution was added to each well, followed by further incubation for 4 h. The colorimetric signal was measured in triplicates using a Spark™ 10 M multimode microplate reader (Tecan Science and Technology Co., Ltd., Shanghai, China) at 450 nm. The cell viability was calculated and expressed as a percentage of DK-1108-treated cells to vehicle-treated cells.

2.4. NO assay

RAW264.7 cells (5×10^4 cells/well) were treated with DK-1108 (0–10 μM) for 1 h, followed by incubation with 200 ng mL^{-1} lipopolysaccharide (LPS) for 24 h. The levels of NO in the cell culture supernatant were determined using Griess reagent (1 % sulfanilamide and 0.1 % N-[1-naphthyl]-ethylenediamine dihydrochloride in 5 % phosphoric acid, Sigma-Aldrich, St. Louis, MO, USA) [14]. The colorimetric signal was measured at 540 nm using the multimode microplate reader.

2.5. Measurement of inflammatory molecules in A549 and RAW264.7 cells

To determine the levels of inflammatory mediators and cytokines in the RAW264.7 cells, the cells were seeded at 5×10^4 cells/well in a 96-well plate and incubated with various concentrations of DK-1108 (1.25, 2.5, 5, and 10 μM) for 1 h. Then, the cells were incubated with LPS (200 ng mL^{-1}) for 20 h. To evaluate the secretion of inflammatory molecules, the A549 cells were seeded at a density of 2×10^5 cells/well in a 12 well plate and incubated with DK-1108 (2.5, 5, 10, and 20 μM) for 1 h, after which they were incubated with phorbol 12-myristate 13-acetate (PMA, 10 nM) for 5 h. The cell culture supernatants were collected and stored at -80°C until use. The supernatants were assayed for various cytokines and chemokines such as TNF- α , IL-6, IL-1 β , IL-8, MCP-1, and ICAM-1 using enzyme-linked immunosorbent assay (ELISA) kits (BD biosciences & R&D systems).

2.6. Adhesion assay of A549 and EOL-1 cells

To evaluate the adhesion of A549 and EOL-1 human eosinophilic leukemia cells (Cat. No. 94042252, ECACC, Salisbury, Wiltshire, UK), the A549 cells were seeded at 1×10^4 cells/well in a 96-well plate and cultured overnight. After a 1-h incubation with DK-1108, the cells were further incubated with PMA for 5 h. Then, EOL-1 cells were stained with Calcein AM. Next, 2×10^4 cells/well were transferred to the 96-well plate containing A549 cells. After co-culturing for 1 h, the cells were washed, observed under a fluorescence microscope (Eclipse Ti-U, Nikon, Japan), and the cell adhesion was quantitated by measuring the fluorescence intensity using the multimode plate reader.

2.7. Luciferase reporter assays

RAW264.7 and A549 cells were transfected with pGL4.14 (luc/MMP-

9/Hygro) and pGL4.14 (luc/AP-1/Hygro; Promega, WI, USA) plasmids using Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After refreshing the media, the cells were treated with DK-1108 for 1 h, followed by LPS or PMA for the MMP-9 or AP-1 luciferase assay, respectively, for 16 h. The cells were subsequently lysed and assessed for luciferase activity using a ONE-Glo™ luciferase assay kit (Promega, WI, USA). The luciferase signal of each sample was measured using the multimode microplate reader, and the relative luciferase activity (RLA) was expressed as fold induction relative to that of the control.

2.8. Semi-quantitative reverse transcription-polymerase chain reaction

RNA extraction from A549 cells was done using Trizol reagent (Invitrogen, CA, USA). NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific, Inc., Waltham, MA) was used to measure the concentration and purity of the RNA. Equal amounts of mRNA were then reverse transcribed into cDNA using the Omniscript RT kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) analysis was performed using PCR premix (Fermentas, Canada) and specific primers (Supplementary Table 2) according to the manufacturer's instructions, with the following PCR conditions: pre-denaturation at 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s for 30 cycles, followed by the final extension phase at 72 °C for 10 min. GAPDH was included as an internal standard. The PCR products were electrophoresed on 1.5 % agarose gel and normalized to GAPDH amplification. The relative intensities of RT-PCR bands were analyzed using ImageJ software (Java 1.8.0_172, NIH, <https://imagej.nih.gov/ij/download.html>).

2.9. Preparation of nuclear and cytosolic proteins

A549 cells were treated with DK-1108 for 1 h and then stimulated with 10 nM PMA for 1 h to assess the translocation of the proteins. The cells were washed with phosphate-buffered saline (PBS), and the nuclear and cytosolic proteins were extracted using NE-PER kits (Pierce, Rockford, IL, USA) containing protease inhibitors (Roche, Basel, Switzerland). All the proteins were quantitated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Illinois, USA).

2.10. Animals

Six-week-old female Balb/c mice (provided by Koatech Co. Pyeongtaek-si, Korea) were allowed to acclimatize to an air-conditioned room (approximately 22–23 °C and 55–60 % humidity) with free access to food and water for 7 days prior to experimentation. All experimental procedures were performed in accordance with the approved guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (Chungbuk, Korea; KRIBB-AEC-18054) and in compliance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals and Korean national laws for animal welfare.

2.11. Mouse models of OVA-induced allergic asthma and drug treatment

The mice were randomly divided into five groups (n = 7 each) and treated as indicated: normal (NC), vehicle; OVA, OVA plus aluminum hydroxide (Alum; OVA + Alum); DEX, dexamethasone: OVA + DEX (2 mg kg⁻¹, orally [p.o.]); and DK-1108 5 and DK-1108 15: OVA + DK-1108 (5 and 15 mg kg⁻¹, p.o., respectively). The mice were sensitized twice intraperitoneally (i.p) on day 0 and 14 with 200 µL of the vehicle containing 20 µg OVA (Sigma-Aldrich, St. Louis, MO, USA) and 2 mg Alum (Thermo Scientific, Rockford, IL, USA). To induce symptoms of asthma, the mice received an airway challenge of 1 % (w/v) OVA using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) for 1 h daily on days 21, 22, and 23. DK-1108 and DEX were solubilized using 1 % dimethyl sulfoxide (DMSO) with 1 % Tween-20 in PBS and

administered daily on days 18–23 by oral gavage using a feeding cannula. DEX was used as a positive control.

2.12. Assessment of airway hyperresponsiveness (AHR)

The airway hyperresponsiveness (AHR) was measured according to a previous protocol [3]. In brief, 24 h after the final OVA challenge, the mice were exposed to methylcholine aerosols (Sigma-Aldrich, St. Louis, MO, USA; 5–20 mg/mL in PBS) for 3 min. The AHR to aerosolized methylcholine was measured as the increasing value of the enhanced pause (Penh) for 3 min using a whole-body plethysmograph chamber (OCP3000; Allmedicus, Seoul, Korea).

2.13. Bronchoalveolar lavage fluid (BALF) collection and measurement of inflammatory molecules and cells

The mice were anesthetized using Zoletil and Xylazine 48 h after the final administration of DK-1108 as described previously [15]. Bronchoalveolar lavage fluid (BALF) was collected twice 48 h after the last OVA inhalation via tracheal cannulation with 700 µL of ice-cold PBS as previously described [3]. The cell number was measured using a hemocytometer (Paul Marienfeld GmbH & Co.). 100 µL of BALF was centrifuged onto a glass slide using a Cytospin (Hanil Science Industrial, Seoul, Korea) for 5 min at 200 g. To distinguish the differential cells, cells were fixed with a fixative solution and stained with Diff-Quik solution I and II, according to the manufacturer's instructions (B4132-1A; IMEB Inc., Deerfield, IL, USA). The levels of IL-5 and IL-13 in BALF were measured using ELISA kits, according to the manufacturer's protocol (R&D System, China). The absorbance was measured at 450 nm using a multimode microplate reader (Tecan System Inc., CA, USA).

2.14. Serum collection and measurement of IgE

Blood samples were collected 48 h after the final oral administration of DK-1108 and DEX, and the serum was separated. The serum total IgE and OVA-specific IgE levels were determined using ELISA as previously described [16]. The absorbance of the resultant reaction solution was measured at 450 nm using a multimode microplate reader.

2.15. Western blot analysis

The RAW264.7 and A549 cell proteins were lysed using cold RIPA buffer (Elpis biotech) containing 1 % Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Lung tissues of OVA-exposed mice were homogenized with a cell lysis reagent (Sigma Aldrich, St. Louis, MO, USA), containing phosphatase and protease inhibitors (Roche, Basel, Switzerland). In order to separate proteins, SDS-PAGE was performed, and the proteins were then transferred to PVDF membranes (Millipore Corp., Bedford, MA). The membranes were incubated in a blocking buffer containing 5% skim milk in TBST for 1 h at RT. Next, the membranes were incubated with primary antibodies (1:1000 dilution), including p-AKT (4060 s, Lot 23), AKT (4691 s, Lot 20), p-JNK (4668 s, Lot 20), JNK (9252 s, Lot 11), p-ERK (9101 s, Lot 31), ERK (9102 s, Lot 27), p-p38 (9211 s, Lot 20), p38 (9212 s, Lot 16), and c-Jun (9165 s, Lot 8) procured from Cell signaling (MA); GAPDH (sc-20357, Lot D0115), COX-2 (sc-1747, Lot B0315), PCNA (sc-56, Lot B2515), and ICAM-1 (sc-7891, Lot L048) procured from Santa Cruz (TX), and iNOS (ADI-905-431, Lot 0805 1101; Enzo life science, NY), overnight at 4 °C. Further they were incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at RT. The specific proteins were visualized by a Clarity™ Western ECL substrate (Bio-Rad, CA), and the band intensities were measured using a chemiluminescence analysis system (Amersham Imager680, GE Healthcare). The band intensities were analyzed using ImageJ, and the relative density was calculated using phosphor/total protein or target protein/internal standard.

2.16. Tissue histopathology and immunohistochemistry

Lung tissue was detached from the mice and fixed with 10 % (v/v) neutral formaldehyde solution (BBC Biochemical, Seattle WA, USA) for 24 h at room temperature (RT). For the histological examination, fixed tissues were embedded in paraffin, cut into 4 μm sections using a rotary microtome (Microm HM325, Thermo Scientific, Walldorf, Germany), and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, MO) to determine inflammatory cell influx, and with periodic acid-Schiff (PAS) to evaluate mucus production.

2.17. Data presentation and statistical analysis

All values shown in the results are reported as the mean $\pm$ standard deviation (SD) obtained from at least three independent experiments. The statistical significance was determined using one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test, with $p < 0.05$ as the standard for statistical significance.

3. Results

3.1. Effects of DK-1108 on the LPS-induced inflammatory response in RAW264.7 macrophages

In the present study, based on the CytoX assay results

(Supplementary Fig. S1a) showing that DK-1108 did not significantly affect RAW264.7 cell viability at concentrations $\leq 10 \mu\text{M}$ with or without LPS, we evaluated the anti-inflammatory effects of DK-1108 at a concentration below $10 \mu\text{M}$. As shown in Fig. 1, LPS stimulation induced the generation of NO, TNF- α , IL-6, and IL-1 β in RAW264.7 macrophages (Fig. 1b-e). However, pretreatment with DK-1108 strongly reduced the levels of these molecules. Additionally, LPS significantly upregulated MMP-9 luciferase activity, which was decreased by pretreatment with DK-1108 (Fig. 1f) in a concentration-dependent manner. In addition, LPS-induced upregulation of iNOS and COX-2 expression was attenuated by DK-1108 pretreatment in a concentration-dependent manner (Fig. 1g).

3.2. Effects of DK-1108 on PMA-induced inflammatory molecules in A549 airway epithelial cells

In the present study, we examined the anti-inflammatory effects of DK-1108 in PMA-induced A549 airway epithelial cells after confirming the non-toxic concentration ($\leq 20 \mu\text{M}$) of DK-1108 using a CytoX assay (Supplementary Fig. S1b). Consistent with previous results, we confirmed that exposure of A549 cells to PMA resulted in the notable production of IL-6, IL-8, and MCP-1 at 142.92 ± 4.03 , 108.49 ± 6.19 , and $107.12 \pm 10.11 \text{ pg mL}^{-1}$, respectively (Fig. 2a-c). However, the pretreatment of $20 \mu\text{M}$ DK-1108 significantly decreased the levels of IL-6 ($39.75 \pm 8.41 \%$), IL-8 ($59.50 \pm 1.81 \%$), and MCP-1 ($88.58 \pm 6.25 \%$)

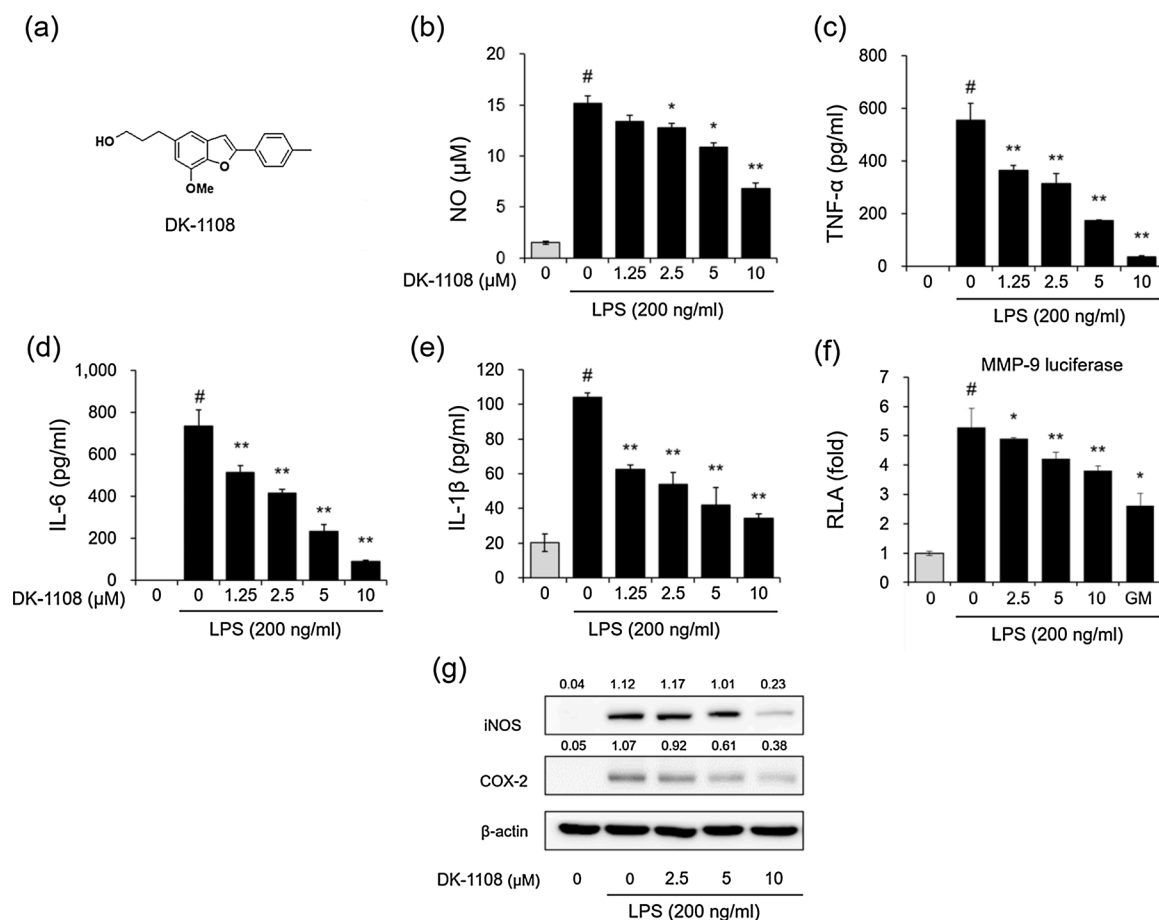


Fig. 1. Effects of DK-1108 on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. (a) Chemical structure of (3-(7-methoxy-2-p-tolyl benzofuran-5-yl) propan-1-ol) (DK-1108). (b) RAW264.7 cells were pre-treated with DK-1108 (1.25–10 μM) for 1 h, followed by 200 ng·mL $^{-1}$ LPS for 20 h. The level of nitric oxide (NO) was measured using NO assay. (c–e) Amounts of tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β was measured using enzyme-linked immunosorbent assay (ELISA). (f) Matrix metalloproteinase (MMP)-9 level was determined using luciferase reporter assay with 2.5 μM of GM (GM6001) as positive control. (g) Levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression were determined using western blot analysis. Data represent the mean SD. # $P < 0.005$ compared to control; * $P < 0.05$ or ** $P < 0.005$ compared to LPS alone.

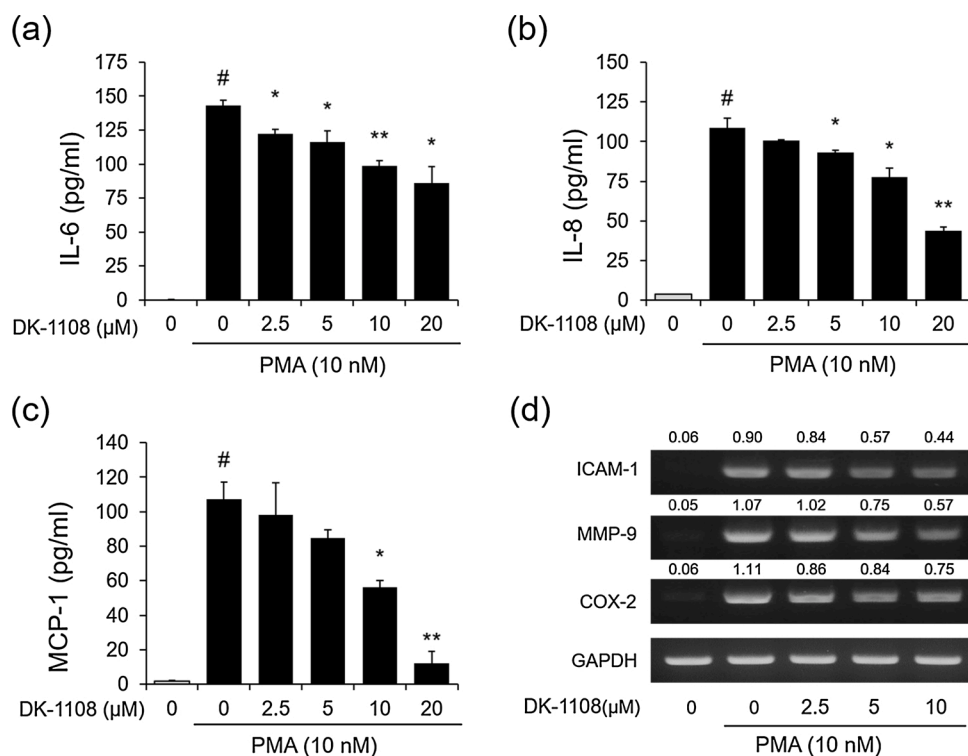


Fig. 2. Effects of DK-1108 on phorbol 12-myristate 13-acetate (PMA)-stimulated A549 airway epithelial cells. (a-c) A549 cells pre-treated with DK-1108 (2.5–20 μM) for 1 h, followed by 10 nM PMA for 5 h. Interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1 levels in the supernatant were measured using ELISA. (d) Levels of intercellular adhesion molecule (ICAM)-1, matrix metalloproteinase (MMP)-9, and cyclooxygenase (COX)-2 mRNA expression was determined using reverse transcription-polymerase chain reaction (RT-PCR). Data represent the mean SD. [#] $P < 0.005$ compared to control; ^{*} $P < 0.05$ or ^{**} $P < 0.005$ compared to PMA alone.

compared with PMA-stimulated cells. Furthermore, DK-1108 also dose-dependently downregulated the mRNA expression of ICAM-1, MMP-9, and COX-2 in PMA-induced A549 cells (Fig. 2d).

3.3. Effects of DK-1108 on PMA-induced activation of AKT/MAPKs/AP-1 in A549 cells

We used PMA to activate AKT, MAPKs, and AP-1 in the airway cells

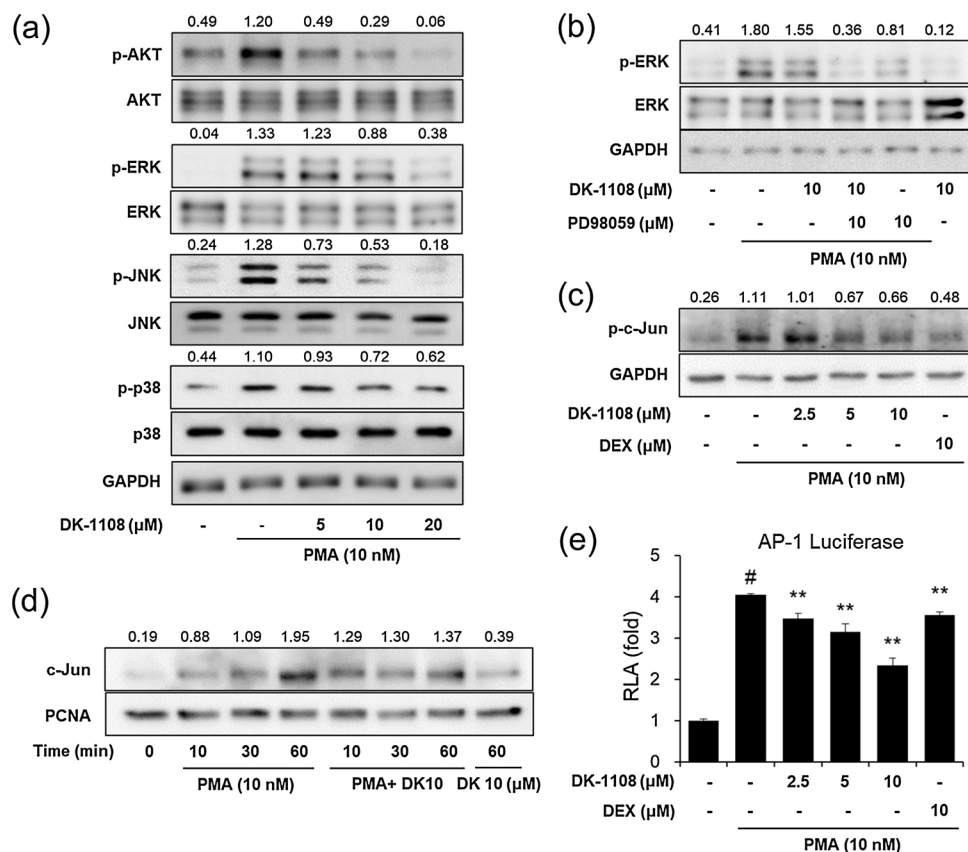


Fig. 3. Effects of DK-1108 on mitogen-activated kinases (MAPKs), AKT and activator protein (AP)-1 activation in phorbol 12-myristate 13-acetate (PMA)-stimulated A549 cells. (a) Levels of phosphorylation or total AKT and MAPKs were analyzed using western blotting. A549 cells were pre-treated with DK-1108 (5, 10, 20 μM) for 1 h and then treated with 10 nM PMA for 30 min. The levels of phospho or total AKT, MAPKs were analyzed by Western blotting with specific antibodies. (b) A549 cells were pre-treated with 10 μM DK-1108 or/and 10 μM PD98059 for 1 h and then treated with 10 nM PMA for 30 min. (c) A549 cells were pre-treated with DK-1108 (2.5, 5, 10 μM) or 10 μM DEX for 1 h and then treated with 10 nM PMA for 30 min. (d) A549 cells were pre-treated with 10 μM DK-1108 for 1 h and then then treated with 10 nM PMA. After 10, 30 and 60 min of 10 nM PMA exposure, the cells pellet was collected and nuclear extracts were isolated from the cells using a Pierce kit. The levels of c-Jun translocation were determined by western blot analysis. (e) A549-AP-1 cells were pre-treated with DK-1108 (2.5, 5, 10 μM) for 1 h and then treated with 10 nM PMA for 16 h. AP-1 promoter activity was detected using luciferase assay. Data represent the mean SD. [#] $P < 0.005$ compared to control; ^{*} $P < 0.05$ or ^{**} $P < 0.005$ compared to PMA alone.

based on previous reports of PMA activation of these molecules [3]. As shown in Fig. 3a, PMA administration activated AKT in A549 cells, whereas the pretreatment with DK-1108 significantly decreased these effects. There was also an obvious upregulation of extracellular signal-regulated kinase (ERK), c-Jun-N terminal kinase (JNK), and p38 phosphorylation in cell lysates of PMA-treated A549 cells (Fig. 3a). The phosphorylation of these molecules (ERK, p38, and JNK) was down-regulated upon pretreatment with DK-1108. In particular, the results showed that the co-treatment of DK-1108 and PD98059, ERK inhibitor, suppressed the ERK phosphorylation to a greater extent than DK-1108 or PD98059 alone (Fig. 3b). Additionally, as MAPKs are related to the translocation of transcriptional factors, we next investigated the activation of c-Jun and AP-1. As shown in Fig. 3, pretreatment with DK-1108 remarkably attenuated the phosphorylation and nuclear translocation of c-Jun in PMA-induced A549 cells (Fig. 3c and d). Furthermore, the AP-1 luciferase activity induced by PMA was suppressed by DK-1108 in a concentration-dependent manner (Fig. 3e).

3.4. Effects of DK-1108 on adhesion of A549 and EOL-1 cells

In this study, the effect of DK-1108 on airway epithelial cells-eosinophils interaction was evaluated by the co-culture of A549 and EOL-1 cells. As shown in Fig. 4, the fluorescence intensity, which reflects the interaction between A549 and EOL-1 cells, was upregulated in the PMA-treated group, whereas pretreatment with DK-1108 effectively inhibited this effect in a concentration-dependent manner (Fig. 4a and b). The inhibitory effect of 10 μ M DK-1108 on fluorescence intensity was

similar to that of 10 μ M DEX, the positive control. Similar to the mRNA analysis results (Fig. 2d), the levels of ICAM-1 protein expression and production were notably upregulated by PMA administration (Fig. 4c and d). However, pretreatment with DK-1108 effectively downregulated PMA-induced ICAM expression and production.

3.5. Effects of DK-1108 on Th2 cytokines and IgE levels in OVA-exposed asthmatic mice

Exposure to allergens causes airway inflammation by the activation of the Th2 cells aggravated by the activation of eosinophils and mast cells [17]. To induce airway inflammatory response, ovalbumin (OVA) sensitization and inhalation were performed as presented in Fig. 5. In the present study, OVA inhalation remarkably increased the releases of the Th2 cytokines, IL-5 and IL-13, in the BALF of sensitized mice compared to the NC group (Fig. 6a and b). In contrast, treatment with DK-1108 significantly decreased the levels of these cytokines in OVA-challenged mice. Notably, 15 μ M DK-1108 inhibited IL-5 secretion to a greater extent than 2 mg kg⁻¹ DEX. The rate of inhibition 15 mg kg⁻¹ DK-1108 on IL-5 and IL-13 was 89.40 $\pm$ 11.01 % and 69.33 $\pm$ 15.52 %, respectively. Based on these results, we examined whether treatment with DK-1108 induces lower serum release of IgE than OVA stimulation. The results showed that the serum levels of total and OVA-specific IgE were attenuated in the OVA group upon treatment with DK-1108 (Fig. 6c and d).

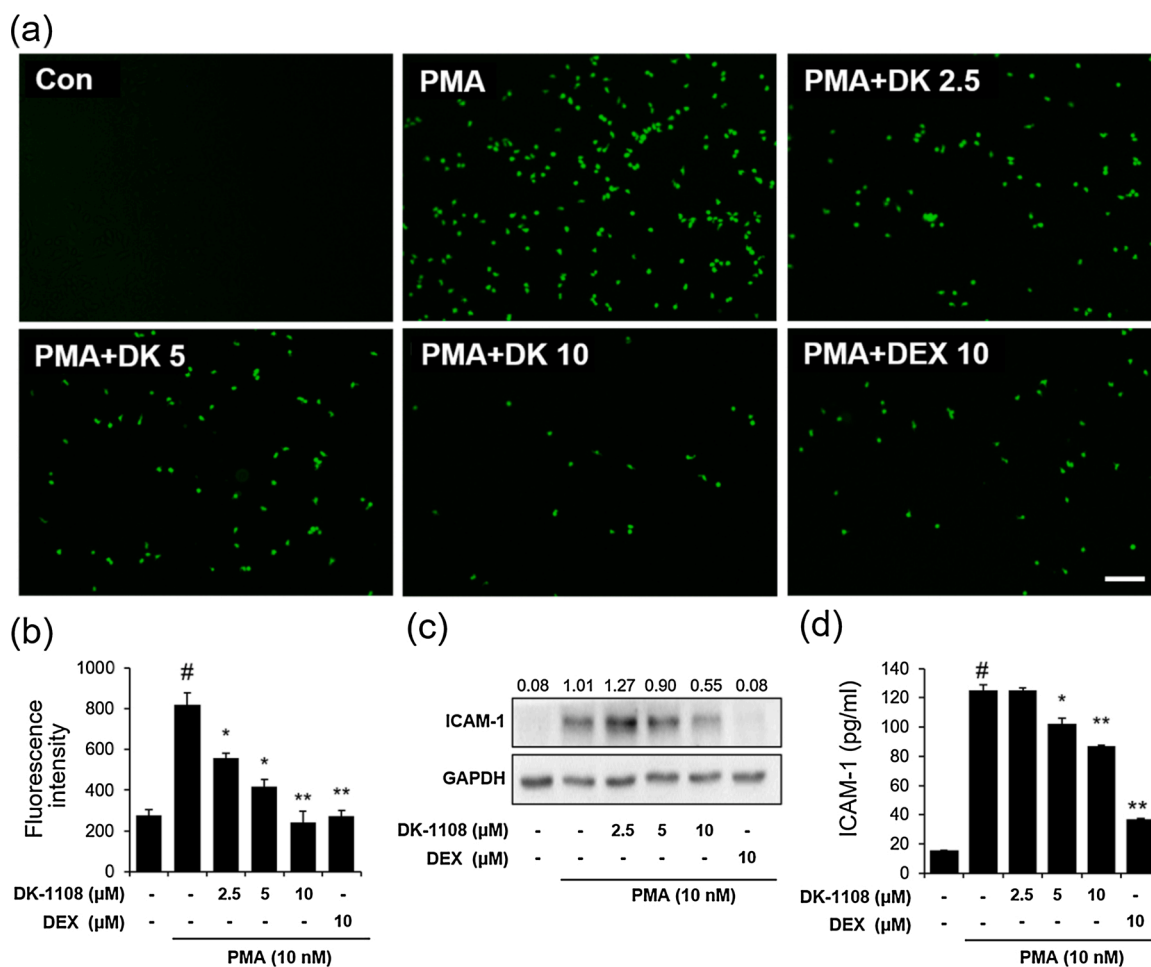


Fig. 4. Effects of DK-1108 on adhesion of EOL-1 and A549 cells. (a) Cells were observed at magnification $\times 100$ using a fluorescence microscope. Adherence of EOL-1 cells to A549 cells was quantified by measuring the (b) fluorescence and performing (c) western blot and (d) enzyme-linked immunosorbent assay (ELISA). [#]P < 0.005 compared to control; ^{*}P < 0.05 or ^{**}P < 0.005 compared to PMA alone.

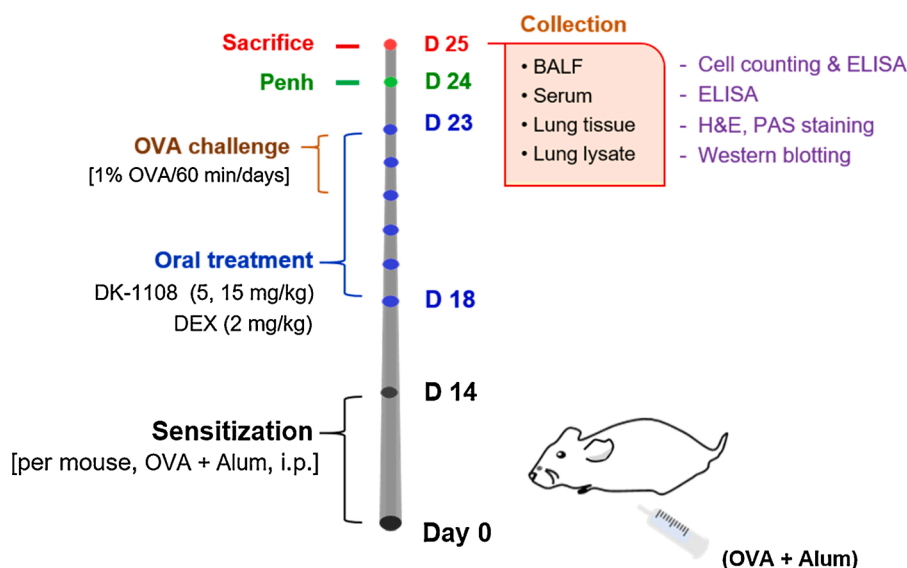


Fig. 5. Experimental procedure. Female BALB/c mice were five groups ($n = 7$ in each group). The mice were sensitized by OVA and Alum injection (i.p.) on days 0 and 14 and were orally administered with DK-1108 ($5, 15 \text{ mg.kg}^{-1} \text{ p.o.}$) or DEX ($2 \text{ mg.kg}^{-1} \text{ p.o.}$) for days 18 to 23. The mice were challenged by a 1% OVA aerosol on days 21 to 23. On day 24, the value of enhanced pause (Penh) was analyzed by plethysmography. On day 25, the mice were sacrificed, and the BALF, serum and lung tissues were acquired. OVA, ovalbumin; DEX, dexamethasone; BALF, bronchoalveolar lavage fluid.

3.6. Effects of DK-1108 on the recruitment of inflammatory cells in OVA-exposed asthmatic mice

In this study, the regulatory effects of DK-1108 on ICAM-1, MCP-1, and IL-5 were investigated and confirmed through *in vitro* and *in vivo* experiments (Figs. 2, 4 and 6). Therefore, DK-1108 was expected to regulate the recruitment of inflammatory cells, including eosinophils. The results shown in Fig. 6 indicated that OVA inhalation significantly increased eosinophil numbers in BALF and levels of ICAM-1 protein expression in the lung of mice (Fig. 6e, g, and h). Interestingly, these cells and ICAM-1 expression were significantly reduced upon treatment with DK-1108. The inhibitory effects of 15 mg kg^{-1} DK-1108 were similar to those of DEX, the positive control. The histological examination also showed that OVA aerosol challenge induces a notable influx of inflammatory cells into the peribronchial lesion (Fig. 6f). Consistent with the results shown in Fig. 6e, the increased influx of inflammatory cells was effectively reduced by DK-1108. Furthermore, OVA-induced upregulation of macrophage numbers and MCP-1 production was also confirmed to be notably downregulated by treatment with DK-1108 (Fig. 6e, g, and i).

3.7. Effects of DK-1108 on mucus secretion and AHR in OVA-exposed asthmatic mice

In our study, DK-1108 effectively ameliorated the levels of IL-13, which is strongly associated with mucus hypersecretion in the pathogenesis of asthma [18]. Based on the importance of mucus inhibition and the inhibitory effects of DK-1108 on IL-13 secretion (Fig. 6b), we investigated whether DK-1108 has an inhibitory activity on mucus secretion. As presented in Fig. 7, mouse lungs exposed to OVA showed mucus overproduction in the epithelium. In contrast, DK-1108-treated mice exhibited a notable reduction in mucus (Fig. 7a). To investigate whether DK-1108 has any activity against AHR, the value of enhanced pause (Penh) was determined via plethysmography. As shown in Fig. 7b, exposure to methylcholine increased the Penh value, whereas treatment with DK-1108 significantly decreased this value compared to the OVA group. These results suggested that this inhibition of AHR by DK-1108 might be associated with a reduction in Th2 cytokines, such as IL-13, and mucus secretion (Fig. 7b).

3.8. Effects of DK-1108 on the expression of inflammatory mediators and the activation of MAPKs in OVA-exposed asthmatic mice

The regulation of iNOS and COX-2, as important inflammatory molecules, is important in asthma [2,4]; therefore, we examined the regulatory effect of DK-1108 on the expression of these molecules in the lungs of OVA-exposed asthmatic mice. Western blot analysis results showed that OVA inhalation upregulated the expression of iNOS and COX-2 in the lungs of sensitized mice compared to the NC group (Fig. 8a), whereas treatment with DK-1108 reduced this effect. We already confirmed the inhibitory effect of DK-1108 on MAPK activation in PMA-stimulated A549 cells (Fig. 3a); therefore, we next evaluated its regulatory effects on MAPK activation in the lungs of asthmatic mice. As shown in Fig. 8b, increased levels of ERK, p38, and JNK phosphorylation were observed in the lungs of OVA-exposed mice, whereas treatment with DK-1108 effectively inhibited this increase. These results revealed that the anti-asthmatic effects of DK-1108 are closely related to the inhibition of the MAPK signaling pathway (Fig. 9).

4. Discussion

Macrophages perform a variety of functions in the host defense systems; they act as antigen-presenting cells (APCs) producing various molecules such as enzymes, cytokines, and chemokines in the pathogenesis of asthma [19]. There is evidence that the increased generation of macrophage-derived NO reflects the severity of asthma in patients [4]. A previous study showed that the macrophages in patients with asthma produce higher levels of TNF- α , IL-6, and IL-1 β than those in healthy individuals [20]. In addition, MMP-9 plays a pivotal role in airway remodeling and AHR, and macrophages are a major source of MMP-9 [21,22]. Therefore, these reports indicated that macrophages-derived NO or inflammatory cytokines and chemokines could serve as valuable markers for asthma; moreover, inhibitors of these molecules may be effective in the treatment of asthma. In the present study, DK-1108 exerted excellent anti-inflammatory effects by suppressing NO, TNF- α , IL-6, IL-1 β , MMP-9, iNOS, and COX-2 in activated murine macrophages (Fig. 1). Furthermore, the IC₅₀ value of DK-1108 against TNF- α and IL-6 was much better than that of the previously developed DK-1014, which is a derivative of homoegonol (Table. 1). Therefore, we expected DK-1108 to exert strong anti-inflammatory effects in asthma.

Airway epithelial cells generate inflammatory cytokines, chemokines, and mediators, which induce the recruitment of various

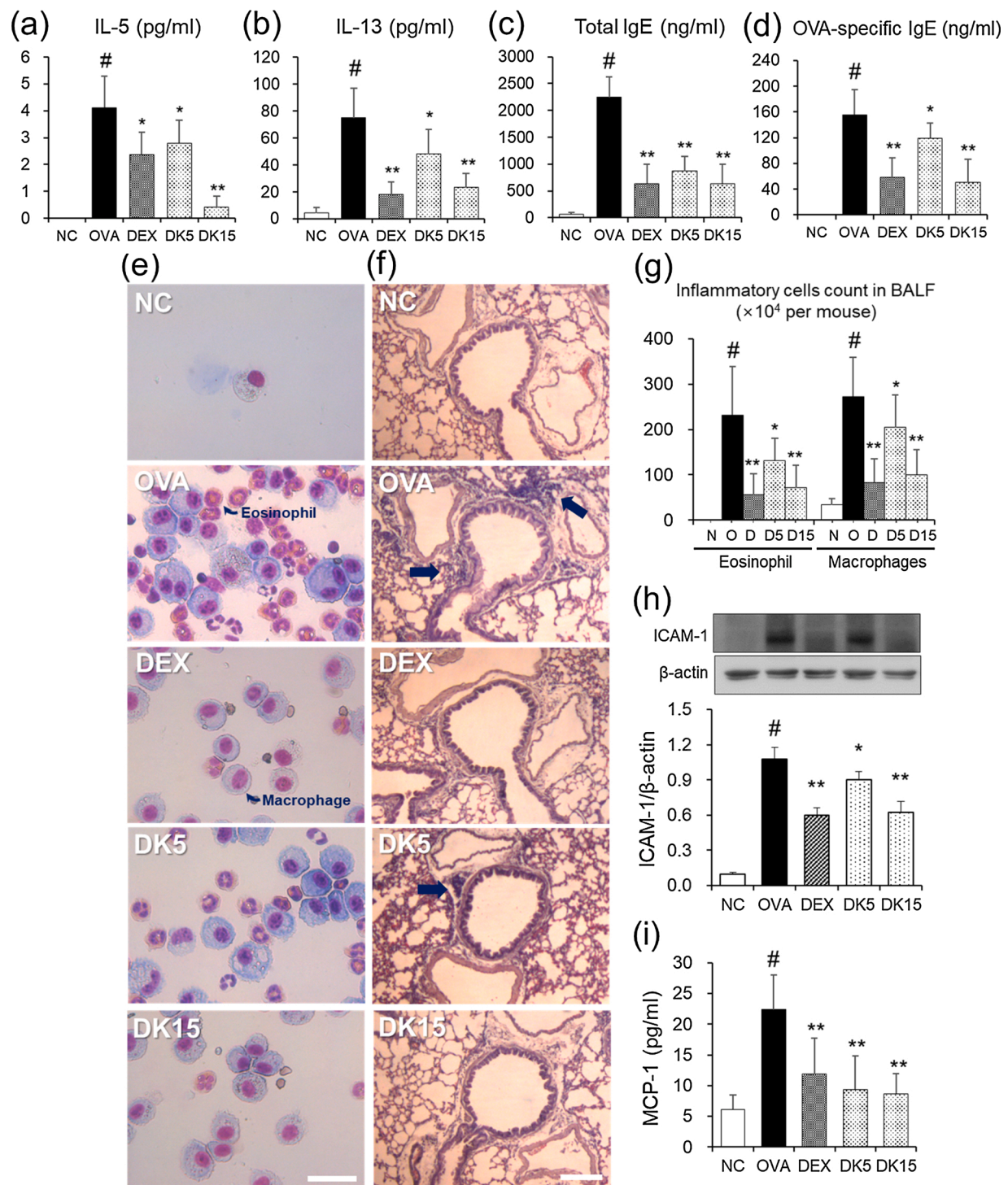


Fig. 6. Effects of DK-1108 on Th2 cytokines, immunoglobulin E (IgE), and inflammatory cells recruitment in the lung. Levels of (a) interleukin (IL)-5 and (b) IL-13 in the bronchoalveolar lavage fluid (BALF). (c) Total IgE and (d) ovalbumin (OVA)-specific IgE in the serum were determined using enzyme-linked immunosorbent assay (ELISA). (e) Inflammatory cells in the BALF were stained with Diff Quik® staining solution (magnification, $\times 400$, scale bar 25 μ m). (f) Peribronchial lesion in the lung tissue were examined using hematoxylin and eosin (H&E) (magnification, $\times 100$, scale bar 100 μ m). Black arrows indicate the influx of inflammatory (g) Inflammatory cell count of BALF. (h) Intercellular adhesion molecule (ICAM)-1 expression was determined in the lung tissue using western blot analysis. (i) Production of monocyte chemoattractant protein (MCP)-1 was measured in BALF using ELISA. [#]P < 0.05 compared to control; ^{*}P < 0.05 or ^{**}P < 0.01 compared to OVA group alone.

inflammatory cells in the lung along with the secretion of adhesion molecules [23]. A previous study reported that bronchial epithelial cells act as the main source of IL-6, which is elevated in asthma, indicating that IL-6 may be a sensitive and useful indicator for assessing bronchial asthma [24]. Chemoattractant cytokines, such as IL-8 and MCP-1, have

been suggested to regulate the recruitment of inflammatory cells, including eosinophils, at the airway inflammatory response sites [25, 26]. It is increasingly apparent that airway epithelial cells are a source of MCP-1, ICAM-1, and MMP-9, which play a pivotal role in promoting the adsorption of leukocytes into the lungs [3,27,28]. This sustained

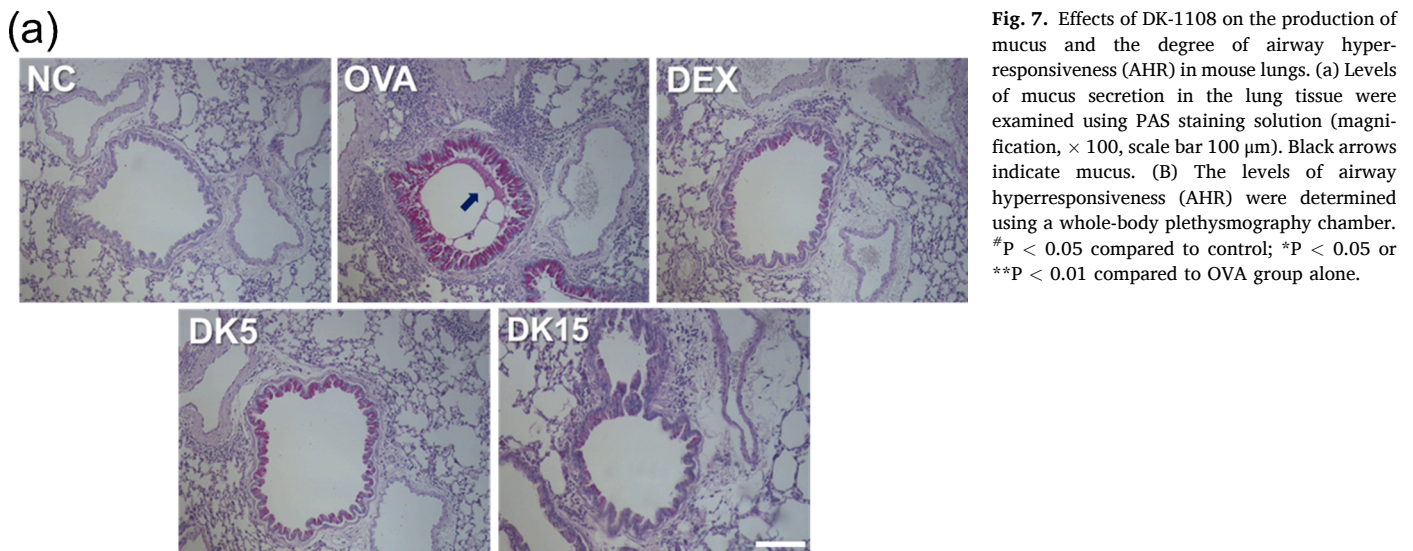


Fig. 7. Effects of DK-1108 on the production of mucus and the degree of airway hyper-responsiveness (AHR) in mouse lungs. (a) Levels of mucus secretion in the lung tissue were examined using PAS staining solution (magnification, $\times 100$, scale bar 100 μm). Black arrows indicate mucus. (b) The levels of airway hyperresponsiveness (AHR) were determined using a whole-body plethysmography chamber. #P < 0.05 compared to control; *P < 0.05 or **P < 0.01 compared to OVA group alone.

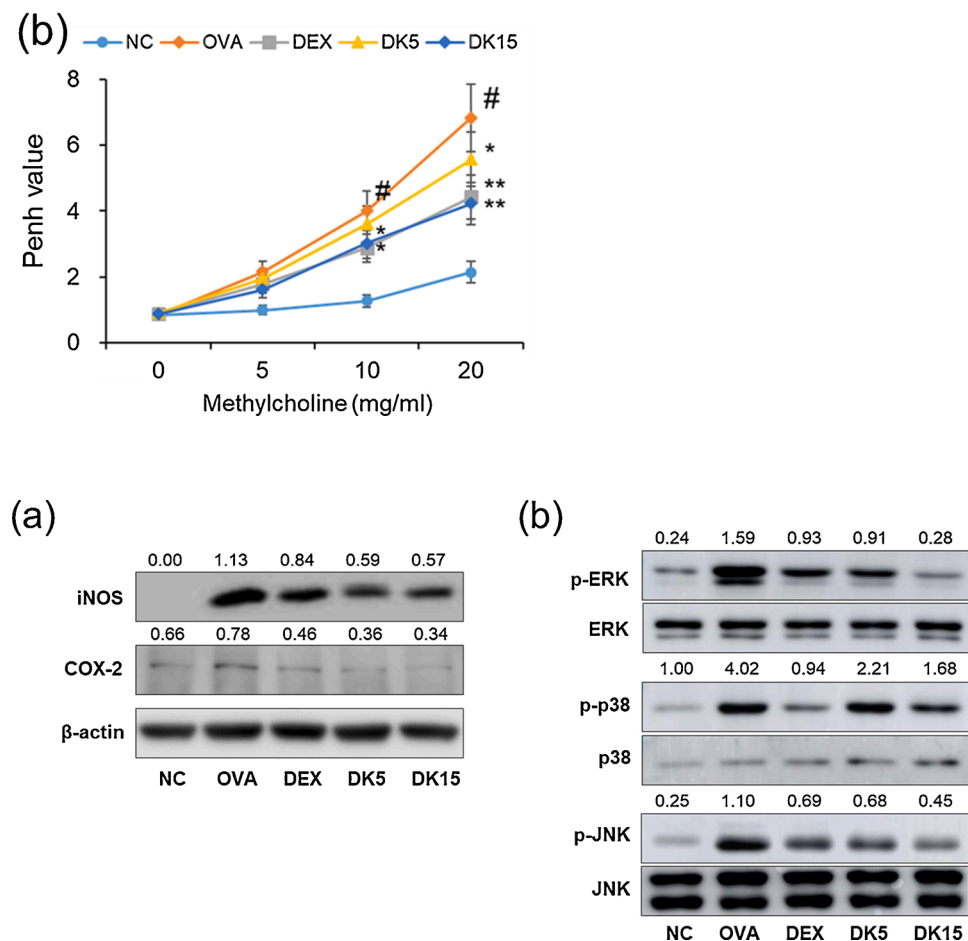


Fig. 8. Effects of DK-1108 on protein expression of inflammatory mediators and activation of mitogen-activated protein kinases (MAPKs) in mouse lungs. The levels of (a) iNOS/COX-2 expression and (b) MAPKs phosphorylation in the lung were determined using western blot analysis.

response leads to hyper-inflammation, resulting in the upregulation of mucus overproduction, airway thickening, and AHR. A previous report indicated that COX-2 is a pivotal regulator of inflammation in airway inflammatory diseases, and IL-1 β increases the mRNA expression of COX-2 in A549 airway epithelial cells [29]. PMA is also well known to produce a variety of inflammatory molecules, such as IL-6, IL-8, MCP-1, ICAM-1, and MMP-9, in the airway epithelial cells [3,22,30]; therefore, PMA is widely used to induce bronchial inflammation in the study of the

effects and mechanism of candidate agents. In the present study, DK-1108 dose-dependently inhibited the production of IL-6, IL-8, and MCP-1 in PMA-stimulated A549 airway cells (Fig. 2A-C). DK also reduced the mRNA expression of ICAM-1, MMP-9, and COX-2 in PMA-stimulated A549 cells. (Fig. 2D). These results, therefore, indicated that DK-1108 could exert a protective effect against asthmatic symptoms.

The AKT pathway is suggested to be important in asthma, and

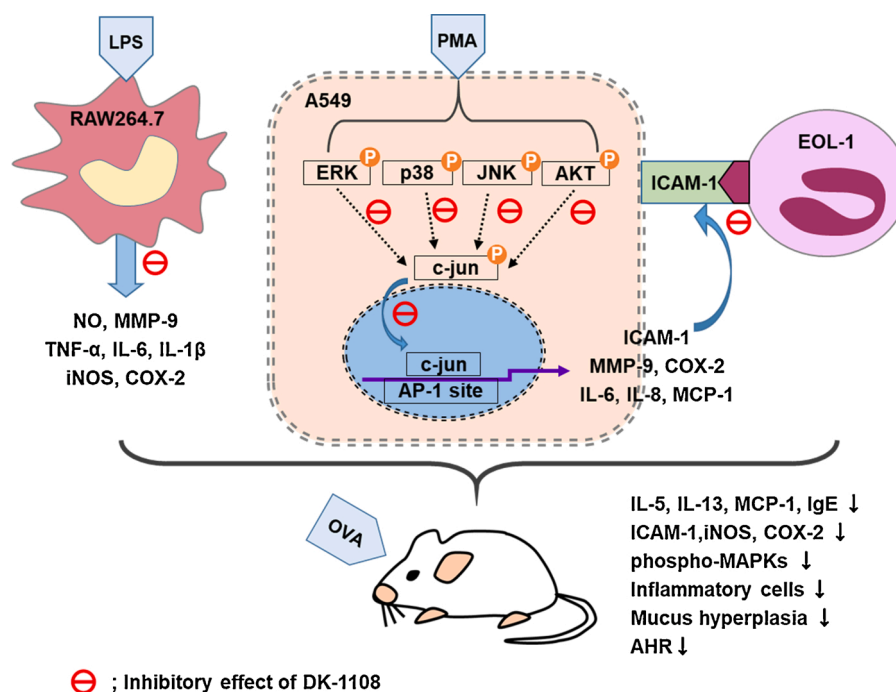


Fig. 9. Schematic illustration of DK-1108 retarding the asthma and inflammation. Treatment of DK-1108 negatively regulated the inflammation in macrophage and epithelial cells. Moreover, the inhibitory mode of DK-1108 in PMA-induced ICAM-1 lead to retarding the adhesion of epithelial cells and eosinophil, thus protecting the mice from OVA-induced asthma.

Table 1

Comparison of IC₅₀ values for cytokine of homoeogonol, DK-1014 and DK-1108.

	IC ₅₀ of TNF-α (μM)	IC ₅₀ of IL-6 (μM)
homoeogonol	9.16 ± 1.26	19.94 ± 1.26
DK-1014	16.97 ± 0.15	11.32 ± 1.43
DK-1108	1.11 ± 0.17	3.65 ± 0.96

inhibition of its activation was previously shown to downregulate the expression of IL-6 in airway epithelial cells [31]. Furthermore, activation of the intracellular MAPK signaling pathway underlies the inflammatory processes of asthma. For instance, ERK participates in the regulation of cytokines, including IL-5 [32], whereas JNK has been reported to be involved in IgE switching [33]. In addition, increased p38 activation has been shown to accompany cytokine upregulation in the macrophages of patients with asthma [34]. Current studies have shown that inhibition of the MAPK signaling pathway has potential therapeutic benefits in an allergic asthma model [35]. The MAPK signaling pathway is important in the activation of inflammatory cells, airway epithelial, and smooth muscle cells, and in regulating the expression of cytokines, chemokines, and adhesion molecules [36,37]. Thus, MAPK activation contributes to the exacerbation of the airway inflammatory response by upregulating inflammatory molecules. AP-1 has been identified as an important transcription factor in the expression of inflammatory molecules, and its activation triggers the airway inflammatory response involved in the underlying mechanism of asthma [38]. Xu et al. found that the inhibition of AP-1 effectively ameliorated airway inflammation by reducing eosinophil recruitment, Th2 cytokines, IgE, and mucus hypersecretion in a mouse asthma model [3]. MMP-9 is also well-documented to be regulated by AP-1 through AKT and MAPKs signaling pathways in PMA-stimulated airway epithelial cells [5]. Thus, these reports indicate that suppression of AKT/MAPK/AP-1 activation could be beneficial in the prevention or treatment of asthma. In the present study, we confirmed that DK-1108 effectively downregulated the activation of AKT and MAPKs in activated A549 cells (Fig. 3a). Moreover, co-treatment of 10 μM DK-1108 and 10 μM PD98059, an ERK

inhibitor, notably inhibited ERK phosphorylation more potently than did DK-1108 or 10 μM PD98059 treatment alone (Fig. 3b). DK-1108 also inhibited c-Jun activation and nuclear translocation (Fig. 3c and d) and significantly downregulated the activity of AP-1 luciferase activity (Fig. 4e). Collectively, these results indicate that DK-1108 regulated inflammatory molecules production in airway epithelial cells via AKT/MAPKs/AP-1 signaling pathways in airway epithelial cells.

The interactions between airway epithelial cells and eosinophils lead to airway inflammation and tissue remodeling in the pathogenesis of asthma [39]. Therefore, it is important to regulate this interaction. The results presented in section 3.2 indicate that airway cells derived-MCP-1, ICAM-1, and MMP-9 are closely associated with the recruitment of inflammatory cells, including eosinophils. DK-1108 exerted regulatory effects on these molecules and, as expected, inhibited the interaction between airway epithelial cells and eosinophils. Therefore, the inhibitory effect of DK-1108 on MCP-1, MMP-9, and ICAM-1 might be attributed to the inhibition of airway cell-eosinophils interaction *in vitro* and inflammatory cell influx *in vivo*.

Th2 cell-derived IL-5 plays a crucial role in eosinophil maturation and recruitment [40]. Th2 cell-derived IL-13 leads to B-cell activation, IgE over-secretion, goblet cell metaplasia, and mucus overproduction [18]. IL-13 was reported to induce the expression of iNOS in human bronchial epithelial cells [41,42] and activate macrophages [43]. Therefore, Th2 cytokines are closely related to the major pathophysiological symptoms of asthma. An increased serum IgE level is a well-known key component and an important marker in the pathogenesis of allergic asthma [44]. The pivotal role of IgE involves the initiation and expansion of airway inflammation, and Th2 response has a decisive influence on the regulation of IgE production [45,46]. In the present study, the ELISA results showed that DK-1108 inhibited IL-5, IL-13, and IgE production in asthmatic mice. Especially, 5 mg/kg DK-1108 significantly suppressed OVA-induced IL-5 production and its inhibitory effect also appeared to be similar to that of 2 mg/kg DEX. Interestingly, the inhibitory effect of 5 mg/kg DK-1108 on IL-5 was better than that of the same dose of DK-1014 (Table 2). These results suggest that DK has beneficial effects on the regulation of Th2 cytokines and IgE generation,

Table 2

Comparison of inhibition rate for IL-5 and MCP-1 of DK-1014 and DK-1108.

	Inhibition rate for IL-5	Inhibition rate for MCP-1
5 mg/kg DK-1014	16.49 %	45.82 %
5 mg/kg DK-1108	27.13 %	59.95 %

which are important elements in airway inflammation of allergic asthma.

The recruitment of eosinophils into the airway epithelium is the major characteristic of asthma, and adhesion of eosinophils to epithelial cells is induced by ICAM-1 [47]. IL-5 [40] and macrophages-derived MCP [16] are closely involved in the recruitment of eosinophil in asthma. High levels of MCP-1 have also been found in the BALF of asthmatic patients [48], and its inhibition reduces the influx of eosinophil and T cells with a notable inhibition in AHR [49]. The *in vitro* experiments in this study confirmed the regulatory effect of DK-1108 on ICAM and MCP-1; the subsequent evaluation of the effects on eosinophils and macrophages influx revealed an effective reduction in their numbers in the BALF and the influx of inflammatory cells in the lung tissues. The increased levels of ICAM-1 and MCP-1 were also remarkably attenuated upon treatment with DK-1108. In similar to those of IL-5 results, 5 mg/kg DK-1108 exerted excellent inhibitory activity on OVA-induced MCP-1 production and its effect was similar to that of DEX. Therefore, we further analyzed whether DK-1108 could show a better effect on MCP-1 inhibition compared to DK-1014. As expected, 5 mg/kg DK-1108 had beneficial effects on the suppression of MCP-1 (Table 2). These results suggest that the decreased influx of inflammatory cells may be mediated by a reduction in ICAM-1 and MCP-1 levels.

Mucus has been reported to be predominantly produced in goblet cells and is selectively induced as a biological defense in response to pathogens [50]. However, mucus overproduction can block the airway causing an obstruction [51]. A recent study, focusing on the inhibition of mucus hypersecretion in the treatment of allergic asthma [52], demonstrated an improvement in asthmatic symptoms *via* the inhibition of airway inflammation and multiple intracellular signaling pathways. AHR has been reported to be an important feature of asthma and may be the pathophysiologic consequence of the Th2 cytokines-induced inflammatory response and mucus hypersecretion [53,54]. In our study, treatment with DK-1108 significantly inhibited the levels of Th2 cytokines, eosinophil recruitment, and mucus hypersecretion, which alleviated AHR. These results indicate that DK-1108 may be a potentially useful mucus inhibitor for the prevention or treatment of airway inflammatory diseases, including asthma.

Based on previous reports showing the importance of iNOS and COX-2 regulation [2,4], in this study, we confirmed the *in vitro* and *in vivo* regulatory inhibitory effect of DK-1108 on iNOS, and COX-2.

There is an increasing awareness about the promising role of MAPKs as molecular targets for the treatment of asthma [8]. Our *in vitro* study showed that DK-1108 notably inhibited the activation of ERK. Furthermore, co-treatment of DK-1108 and an ERK inhibitor exhibited outstanding and more potent *in vitro* inhibitory effects on ERK activation than did treatment with DK-1108 or the ERK inhibitor alone. The results presented in section 3.8 indicate that the regulation of IL-5 is associated with ERK signaling [32]. Interestingly, the regulatory effect of DK-1108 on IL-5 was excellent and better than that of the DEX *in vivo* study. Furthermore, DK-1108 exerted inhibitory activity on JNK and p38 activation in both *in vitro* and *in vivo* studies. Based on the results, we propose that the molecular mechanism of the anti-asthmatic effects of DK-1108 is mediated by the downregulation of MAPK activation.

5. Conclusions

In this study, we investigated the *in vitro* and *in vivo* anti-asthmatic effects of DK-1108, a new synthetic benzofuran compound. DK-1108 significantly inhibited inflammatory cytokines/chemokines production

and adhesion of A549 and EOL-1 cells *in vitro*. The favorable pharmacokinetic profile (Supplementary Table S1 and Fig. S2) and toxicity (Supplementary Fig. S3) and *in vivo* inhibitory effect of DK-1108 encouraged further *in vivo* efficacy studies to the pharmacology profile, which revealed its protective effects in the asthma animal model mediated by the reduction of Th2 cytokines, IgE generation, inflammatory cell influx, and ICAM-1/MCP-1 expression. Moreover, DK-1108 effectively ameliorated the overproduction of mucus and increased levels of AHR, which are closely associated with disturbed airflow and poor lung function. The increased expression of iNOS and COX-2 was also downregulated by DK-1108. The effects of DK-1108 were accompanied by the downregulation of MAPKs activation. Therefore, these results provide strong evidence for the usefulness of DK-1108 against OVA-induced allergic asthma and suggest that DK-1108 may be a useful potential candidate for the treatment of allergic asthma in the future.

Author contributions

Kwon OK, Lee JW designed this study, wrote the manuscript and conducted the experiments.

Xu X, Harmalkar DS synthesized DK-1108.

Park JW, Hwang DS and Kim JH conducted the *in vitro* experiments and Min JH conducted the animal studies.

Song JG, Han HK conducted the animal studies and data analysis to examine the pharmacokinetic profiles of DK 1108 in rats.

Jeong HG and Oh SR revised the manuscript.

Ahn KS and Lee K provided financial support for this study and revised the manuscript.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.110950>.

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