



Immunopharmacology and inflammation

TAS-203, an oral phosphodiesterase 4 inhibitor, exerts anti-inflammatory activities in a rat airway inflammation model

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ABSTRACT

Cyclic adenosine monophosphate (cAMP) is a key intracellular second messenger, which is degraded by phosphodiesterase 4 (PDE4). PDE4 suppresses cAMP levels, and thus stimulates the activity of inflammatory cells. Therefore, PDE4 has been considered as a therapeutic target for airway inflammatory diseases including asthma and chronic obstructive pulmonary disease (COPD). Roflumilast, an approved PDE4 inhibitor, has been shown to have clinical benefits in COPD. However, central nervous system-related side effects including nausea and vomiting have limited the therapeutic index of roflumilast. Moreover, although airway mucus hypersecretion is the characteristic feature, which is associated with the severity and prognosis, the inhibitory effect of roflumilast on sputum production is limited to a minority of patients. In this study, we demonstrate the inhibitory effects of TAS-203, which is an orally active PDE4 inhibitor associated with a lowered emetic effect, on airway inflammation and mucus hypersecretion. A cell-based assay showed TAS-203 treatment suppressed epidermal growth factor (EGF)-induced mucin MUC5AC expression. TAS-203 also suppressed monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-5 and IL-13 production in a Sephadex-induced airway inflammation model, and the number of infiltrating cells in bronchoalveolar lavage (BAL) fluid. TAS-203 caused marked reduction of goblet cell hyperplasia in a histopathological analysis of airway epithelium. Furthermore, TAS-203 suppressed 5-hydroxytryptamine (5-HT)-induced airway hyperresponsiveness (AHR). In addition, we preliminarily confirmed TAS-203 prevents airway MUC5AC production in BAL fluid, and shows lower specific airway resistance (sRaw) in a cigarette smoke-induced COPD-like model. Our data suggest that TAS-203 might be useful in the treatment of airway inflammatory disease.

1. Introduction

Phosphodiesterase 4 (PDE4) is involved in the modulation of the signal transduction processes via the degradation of cyclic adenosine monophosphate (cAMP), which is a key intracellular second messenger (Bender and Beavo, 2006). PDE4 inhibitors cause an anti-inflammatory effect in leukocytes by increasing cAMP levels. Inhibition of PDE4 particularly suppresses production of pro-inflammatory cytokines, thereby increasing the secretion of anti-inflammatory cytokines (Schett et al., 2010). Therefore, PDE4 inhibitors such as the orally active roflumilast have been developed and demonstrated to be effective as therapeutic agents for inflammatory diseases, e.g., asthma and chronic obstructive pulmonary disease (COPD) (Lipworth, 2005).

However, since roflumilast has a number of side effects related to the central nervous system (nausea and vomiting) and gastrointestinal system (diarrhea and abdominal pain), the approved therapeutic index

may limit the efficacy of roflumilast (Calverley, 2009). To improve the therapeutic index of roflumilast, attempts have been made to improve drug delivery via an inhaled route, along with the development of a nonemetic compound (Page and Spina, 2011). We previously found that TAS-203, which is an oral PDE4 inhibitor, had a characteristic lowered emetic effect in ferrets (Asaka et al., 2010). Furthermore, TAS-203 inhibited the activity of human PDE4, platelet-activating factor-induced eosinophil chemotaxis, and lipopolysaccharide (LPS)-stimulated tumor necrosis factor- α (TNF- α) release from monocytes, respectively. TAS-203 also alleviated ovalbumin-induced airway eosinophilia and LPS-induced airway neutrophilia in rats. These results suggest that TAS-203 has potent anti-inflammatory activities and low emetogenicity in animal studies.

Airway mucus hypersecretion is the characteristic feature of asthma and COPD. This feature is associated with the severity and prognosis, with these symptoms generally difficult to treat (Fahy and Dickey,

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2010; Tamaoki, 2015). Therefore, numerous studies have focused on expectorant therapies targeting mucus hypersecretion, including the use of a PDE4 inhibitor (Zhang and Zhou, 2014). One of these PDE4 inhibitors is roflumilast, which has been shown to decrease mucin MUC5AC expression in the human airway epithelial cells (Mata et al., 2005). However, roflumilast exhibited an inhibitory effect on sputum production in only a minority of patients (Rennard et al., 2011). We consider that this issue of roflumilast might be attributed to the therapeutic index. Thus, we hypothesize that TAS-203, which does have a good balance between anti-inflammatory activities and low emetogenicity, should be able to overcome this issue.

Our present study first investigated the inhibitory effect of TAS-203 on the EGF-induced MUC5AC expression. Subsequently, we then selected the rat Sephadex-induced airway inflammation and guinea pig cigarette smoke-induced COPD-like models to further investigate the *in vivo* efficacy of TAS-203. In the rat model, TAS-203 suppressed monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-5 and IL-13 production, along with the number of infiltrating cells in the bronchoalveolar lavage (BAL) fluid. TAS-203 also suppressed the airway goblet cell hyperplasia and airway hyperresponsiveness (AHR). In the guinea pig model, TAS-203 prevented airway MUC5AC production and specific airway resistance (sRaw). As these data suggest that TAS-203 suppresses goblet cell hyperplasia and MUC5AC production in rodent models, TAS-203 might be an excellent prophylactic agent for airway inflammatory disease.

2. Materials and methods

2.1. Test compounds and reagents

TAS-203 and roflumilast were synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Prednisolone was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The *in vitro* reagents were RPMI-1640, fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA), dimethylsulfoxide (DMSO) (Nacalai Tesque, Inc., Kyoto, Japan), recombinant human EGF (R&D Systems, Minneapolis, MN), MUC5AC monoclonal antibody (45A1) (Thermo Fisher Scientific) and lectin (soybean) from Glycine max (peroxidase labeled) (Merck KGaA, Darmstadt, Germany), respectively. The *in vivo* reagents used included hydroxypropyl methyl cellulose (HPMC) (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan), Sephadex beads (GE Healthcare Life Sciences, Little Chalfont, United Kingdom), rat ELISA kits for MCP-1 (Thermo Fisher Scientific), IL-5 and IL-13 (MyBioSource, Inc., San Diego, CA), Kwik-Diff Staining Kit (Sysmex Co., Hyogo, Japan), 5-hydroxytryptamine (5-HT) (Merck KGaA), and Hi-Lite brand cigarettes (1.4 mg of nicotine and 17 mg of tar, Japan Tobacco Inc., Tokyo, Japan), respectively.

2.2. Animals

Male Lewis rats (6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). Male Hartley guinea pigs (4 weeks old) were purchased from SLC (Shizuoka, Japan). All animals were housed in a controlled environment ($24 \pm 1^\circ\text{C}$, 12-h light 12-h dark cycle) with free access to food and water. The animal experiments were performed in compliance with the guidelines established by the Institutional Animal Ethics Committee of Taiho Pharmaceutical Co., Ltd.

2.3. EGF-induced MUC5AC production in NCI-H292 cells

NCI-H292 cells were washed, and then resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Cells were plated on 12-well tissue culture plates (2×10^5 cells/well) and incubated at 37°C for 48 h. After washing with the medium, cells were then added to 0.2% heat-inactivated FBS-containing RPMI 1640, and incubated at 37°C for 48 h. After incubation, the medium was removed,

cells were stimulated by EGF (25 ng/ml final concentration) with vehicle or TAS-203 at 37°C for 48 h. MUC5AC protein in cell lysates and supernatant were measured by ELISA. Recombinant MUC5AC for use in calibration was purified from the NCI-H292 cell culture medium developed by Shin et al. (1998), using gel filtration chromatography. Ninety-six-well microtiter plates were coated with the MUC5AC monoclonal antibody (45A1) during an overnight incubation at 4°C . Plates were washed with phosphate-buffered saline (PBS) and blocked with 2% bovine serum albumin for 1 h at room temperature. After washing the plates with PBS, cell lysates, supernatant and standard MUC5AC protein (3.91 ng/ml to 500 ng/ml: 8-point standard curve) were then added to the wells. Subsequently, the plates were incubated at room temperature for 2 h. After the plates were washed, soybean lectin from Glycine max (peroxidase labeled) was added to each well followed by an incubation at 37°C for 1 h. The plates were washed with PBS, with the color reaction developed with 3,3', 5,5'-Tetra-methylbenzidine (TMB) substrate and then stopped with H_2SO_4 . The absorbance was measured at 450 nm with the reference at 540 nm.

2.4. Sephadex-induced airway inflammation in Lewis rats

This experiment was conducted according to the method published by Matsubara et al. (1998). A suspension of Sephadex beads (particle size 20–50 μm) in sterile saline was prepared and stored for at least 48 h at 4°C with gentle stirring. Rats were administered 1 ml of suspension of Sephadex beads or saline via the tail vein. For the cytokine/chemokine assay, rats were injected with 250 μg beads on days 1 and 3. For infiltrated cells count, rats were injected with 250 μg beads on days 1, 3, and 6. As for morphometric analysis of the goblet cell hyperplasia and AHR induced by 5-HT, rats were injected with 100 μg beads on days 1, 3, and 6. For each administration, TAS-203 and prednisolone were suspended in 0.5% HPMC and then orally administered at a volume of 5 ml/kg to rats. The sham and vehicle groups were treated with 0.5% HPMC only starting from day 1. TAS-203 was administered at single daily doses of 3, 10, and 30 mg/kg, while prednisolone was administered at single daily doses of 10 mg/kg starting from day 1.

2.5. Analysis of BAL fluid in Sephadex-induced airway inflammation model

Rats were killed with an overdose of pentobarbital sodium on day 4 for the cytokine/chemokine assay, on day 8 for the infiltrated cells count, with the trachea then cannulated and the lungs lavaged with Hank's balanced salt (HBSS, volume 2×3 ml/rat). The BAL fluid was collected in a tube, and then centrifuged at 400 g for 10 min at 4°C . The supernatant was collected and stored at -80°C for further ELISA (MCP-1, IL-5 and IL-13). To count the number of infiltrated cells, the pellet was immediately resuspended in HBSS with 5 U/ml heparin, with the number of total cells counted using a multiple automatic blood cell counting device (F-800, Sysmex Co.). Differential cells were counted using a Shandon Cytospin 3 Cytocentrifuge (Thermo Fisher Scientific) preparation stained with the Kwik-Diff Staining Kit. A minimum of 500 cells were counted and differentiated as mononuclear cells, eosinophils which has red granules, or segmented neutrophils using the standard morphological criteria. The concentration of differential cell was calculated by each ratio and the concentration of total cells. The numbers of lymphocytes and monocytes were counted using a flow cytometric hematology system (XT-2000iV, Sysmex Co.).

2.6. Morphometric analysis of goblet cell hyperplasia

Rats were killed by pentobarbital sodium anesthesia and exsanguination on day 8. The lungs were isolated from the saline- or Sephadex-treated rats. Isolated lungs were fixed in 10% neutralized buffered formalin for 48 h. Paraffin-embedded sections of the left lung lobe were stained with periodic acid-Schiff (PAS), and observed using a light microscope. The percentage of the PAS-stained area in the

epithelium was determined using a semi-automatic image analysis system (HC-2500, Fujifilm Co., Tokyo, Japan).

2.7. Measurement of AHR induced by 5-HT

To evaluate AHR after Sephadex injection, rats were anesthetized by pentobarbital sodium on day 9, with all measurements performed using the method by Konzett and Rössler (Matsubara et al., 1998). Briefly, tracheal intubation was performed under anesthesia and rats were mechanically ventilated (Model-683, Harvard Apparatus, Holliston, MA) at a frequency of 80 breaths/min and at a constant tidal volume of 6 ml/kg. Spontaneous respiration was then stopped with pancuronium (0.8 mg/kg i.v.). The changes in AHR were measured by a differential pressure transducer (7020, Ugo Basile S.R.L., Gemonio, Italy) with a DC amplifier (6L06, Nippon Avionics Co., Ltd.) connected to the tracheal cannula. The increase in overflow volume induced by 5-HT (5–160 µg/kg i.v.) was expressed as the percent change from baseline to maximum bronchoconstriction obtained by clamping off the trachea.

2.8. Cigarette smoke-induced airway inflammation in Hartley guinea pigs

Guinea pigs were placed into an inhalation chamber (flow-past type nose-only inhalation chamber, CR Equipment S.A., Geneva, Switzerland). Cigarette smoke was diluted with fresh air (cigarette smoke: fresh air = 25 ml/s: 140 ml/s) with exposure conducted using a cigarette smoke generator (Smoke 2000, Itec Ltd., Osaka, Japan) for 1 h in a conscious and restrained state. Animals were repeatedly exposed to cigarette smoke, 5 consecutive days a week, during 4 weeks. On the other hand, the fresh air control animals were repeatedly exposed to fresh air instead of cigarette smoke. For the drug administration, TAS-203 and roflumilast were suspended in 0.5% HPMC and orally administered at a volume of 5 ml/kg from day 1. The fresh air control and vehicle groups were treated with 0.5% HPMC only. TAS-203 was administered at single daily doses of either 10 or 25 mg/kg. Roflumilast was administered at single daily doses of 1 mg/kg. sRaw was measured using a double-flow plethysmograph (Pulmos-I, MIPS Co., Osaka, Japan) on days 1, 6, 13, 20, and 26. Individual data were measured with the mean of 100 consecutive breaths. Bronchoconstriction was expressed as the percent change from baseline. After the animals were killed by urethane anesthesia and exsanguination, the trachea was cannulated and the lungs were lavaged with saline (volume 5 ml/animal). The BAL fluid was collected in a tube, centrifuged at 230 g for 10 min at 4 °C. The supernatant was collected and stored at –80 °C for MUC5AC ELISA.

2.9. Statistical analysis

Data are represented as the mean + standard deviation (S.D.) for the in vitro experiments or the mean + standard error of the mean (S.E.M.) for the in vivo experiments. Statistical analysis was performed by Student's *t*-test or Dunnett's test using EXSUS system version 8.0.0 (CAC Croit, Japan, Tokyo). *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Inhibitory effect of TAS-203 on EGF-induced MUC5AC expression

In addition to its important role in inflammation, PDE4 inhibitors, including roflumilast, decrease the MUC5AC expression induced by EGF in human airway epithelial cells (Mata et al., 2005). Therefore, we also examined whether TAS-203 could suppress MUC5AC expression in NCI-H292 cells. As shown in Fig. 1, EGF stimulation significantly increased MUC5AC expression compared with the no stimulus group for both the cell lysate and supernatant (Fig. 1A, B). Using these experimental conditions, we showed that TAS-203 treatment prevented EGF-induced

intracellular and extracellular MUC5AC expression, respectively (Fig. 1A, B).

3.2. Effect of TAS-203 on Sephadex-induced airway inflammation

To investigate the in vivo efficacy of TAS-203, we evaluated its effects in a Sephadex-induced airway inflammation model. Rats administered Sephadex via the tail vein showed airway inflammation, such as infiltration of leukocytes, goblet cell hyperplasia, and AHR (Matsubara et al., 1998). The pathogenic mechanism for this model is thought to be due to damage of the arterial cells by Sephadex beads that results in the release of MCP-1, thereby attracting macrophages. Macrophages produce a number of eosinophil chemoattractants that can subsequently increase inflammatory cells in the airway (Matsubara et al., 1998).

Based on these findings, we investigated the effects of TAS-203 on Sephadex-induced MCP-1, IL-5 and IL-13 in BAL fluid. As shown in Fig. 2, the levels of MCP-1, IL-5 and IL-13 in Sephadex-treated rats were significantly increased compared with saline-treated rats, whereas the levels for IL-1β, IL-4, IFN-γ and TNF-α were undetectable. In the prednisolone-treated rats, the levels of MCP-1, IL-5 and IL-13 were significantly lower than those found in the vehicle-treated rats (Fig. 2B–D). Treatment of TAS-203 also significantly inhibited the Sephadex-induced MCP-1 and IL-5 production at a dose of 30 mg/kg (Fig. 2B, C). Furthermore, TAS-203 at doses higher than or equal to 10 mg/kg significantly suppressed the increase of the IL-13 production (Fig. 2D).

After confirming the inhibitory effects of TAS-203 on the production of MCP-1, IL-5 and IL-13, we then investigated the effects of TAS-203 on the infiltration of inflammatory cells in the BAL fluid. A previous study also reported that there was an increase in the numbers of inflammatory cells including mononuclear cells, eosinophils, and neutrophils in the BAL fluid when using this model (Matsubara et al., 1998). In our current study, our results additionally showed that the numbers of total cells, mononuclear cells, eosinophils and neutrophils in the BAL fluid of Sephadex-treated rats were significantly increased compared with the saline-treated rats (Fig. 3A–D). Although there was no significant improvement in the 3 mg/kg TAS-203 group, treatment with TAS-203 at doses of either 10 or 30 mg/kg resulted in significant reduction of mononuclear cells (Fig. 3B). In addition, TAS-203 treatment significantly inhibited Sephadex-induced airway eosinophilia in a dose-dependent manner (Fig. 3C). Furthermore, prednisolone also inhibited the infiltration of inflammatory cells, including mononuclear cells and eosinophils (Fig. 3B, C). However, TAS-203 and prednisolone did not affect the number of neutrophils in BAL fluid (Fig. 3D). To further assess the fraction of mononuclear cells, isolated cells were analyzed by a flow cytometric hematology system. Results are shown in Supplemental Fig. 1, the lymphocyte numbers of the Sephadex-treated group significantly increased compared with the saline-treated group in BAL fluid. Treatment with TAS-203 tended to inhibit lymphocytes accumulation although treatment with prednisolone did not (Supplemental Fig. 1A). The number of monocytes in BAL fluid showed a tendency to increase after the Sephadex injection. However, neither TAS-203 or prednisolone had an effect (Supplemental Fig. 1B).

3.3. Effect of TAS-203 on Sephadex-induced goblet cell hyperplasia

Compounds that inhibit eosinophilic function have been found to suppress goblet cell hyperplasia in this model (Matsubara et al., 1998). As TAS-203 showed stronger inhibition of eosinophil infiltration induced by Sephadex, we evaluated the effect of TAS-203 on goblet cell hyperplasia. As shown in Fig. 4A, the airway epithelium of Sephadex-treated rats showed a marked increase of PAS-positive goblet cell hyperplasia compared with that of the saline-treated rats. However, prednisolone or TAS-203 treatments at doses higher than or equal to 10 mg/kg significantly suppressed the increase of PAS-positive goblet cell hyperplasia (Fig. 4B). Subsequently, we then tried to examine the

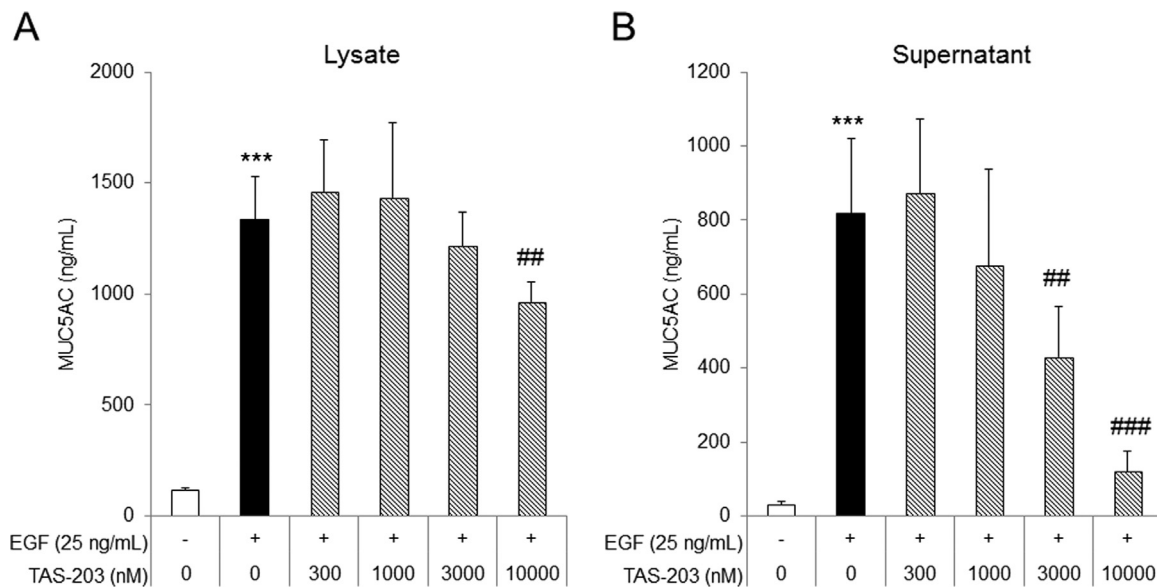


Fig. 1. Inhibitory effects of TAS-203 on EGF-induced MUC5AC expression. NCI-H292 cells were stimulated by EGF with vehicle or TAS-203 for 48 h. MUC5AC protein in cell lysate (A), and supernatant (B) were assessed by ELISA. Data are expressed as mean + S.D. (n = 7 or 8/group). *** $P < 0.001$ when comparing the vehicle group against the non-stimulus control group, ## $P < 0.01$ and ### $P < 0.001$ when comparing the TAS-203 group against the stimulus control group.

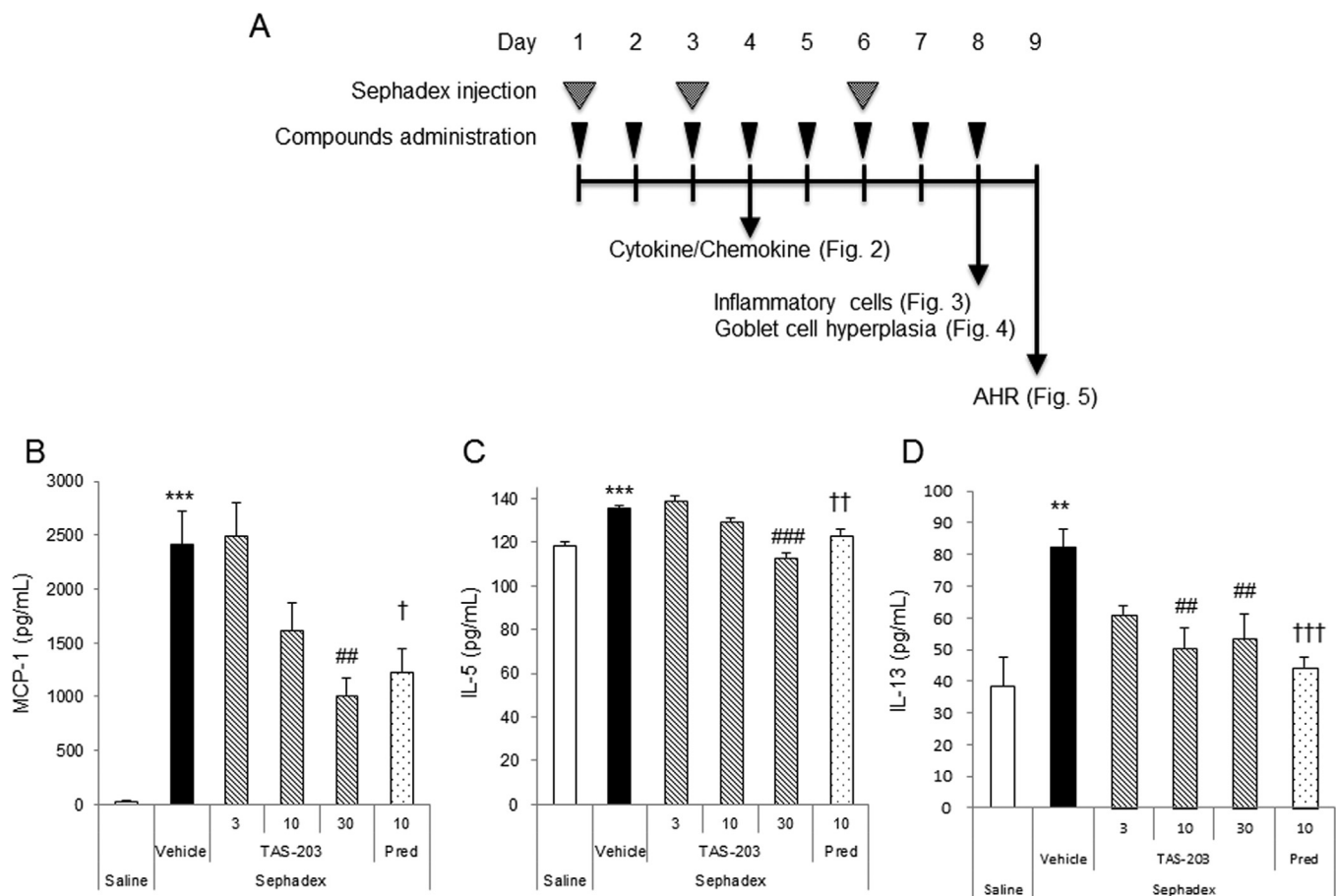


Fig. 2. TAS-203 suppresses production of MCP-1, IL-5 and IL-13 in the BAL fluid of the rat Sephadex-induced model. (A) Experimental protocol for Sephadex-induced airway inflammation in Lewis rats. Rats were treated with TAS-203 (3, 10, and 30 mg/kg) and prednisolone (Pred) (10 mg/kg) on days 1, 2, and 3. On days 1 and 3, rats were intravenously injected with Sephadex beads (250 μ g/rat). BAL fluid was collected on day 4, with the levels of MCP-1 (B), IL-5 (C) and IL-13 (D) detected by ELISA. Data are shown as mean + S.E.M. n = 3 or 8 rats in the saline group; n = 6–8 rats each in the vehicle and treatment groups. ** $P < 0.01$, and *** $P < 0.001$ when comparing the vehicle group against the saline group, ## $P < 0.01$ and ### $P < 0.001$ when comparing the TAS-203 group against the vehicle group, + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$ when comparing the prednisolone group against the vehicle group.

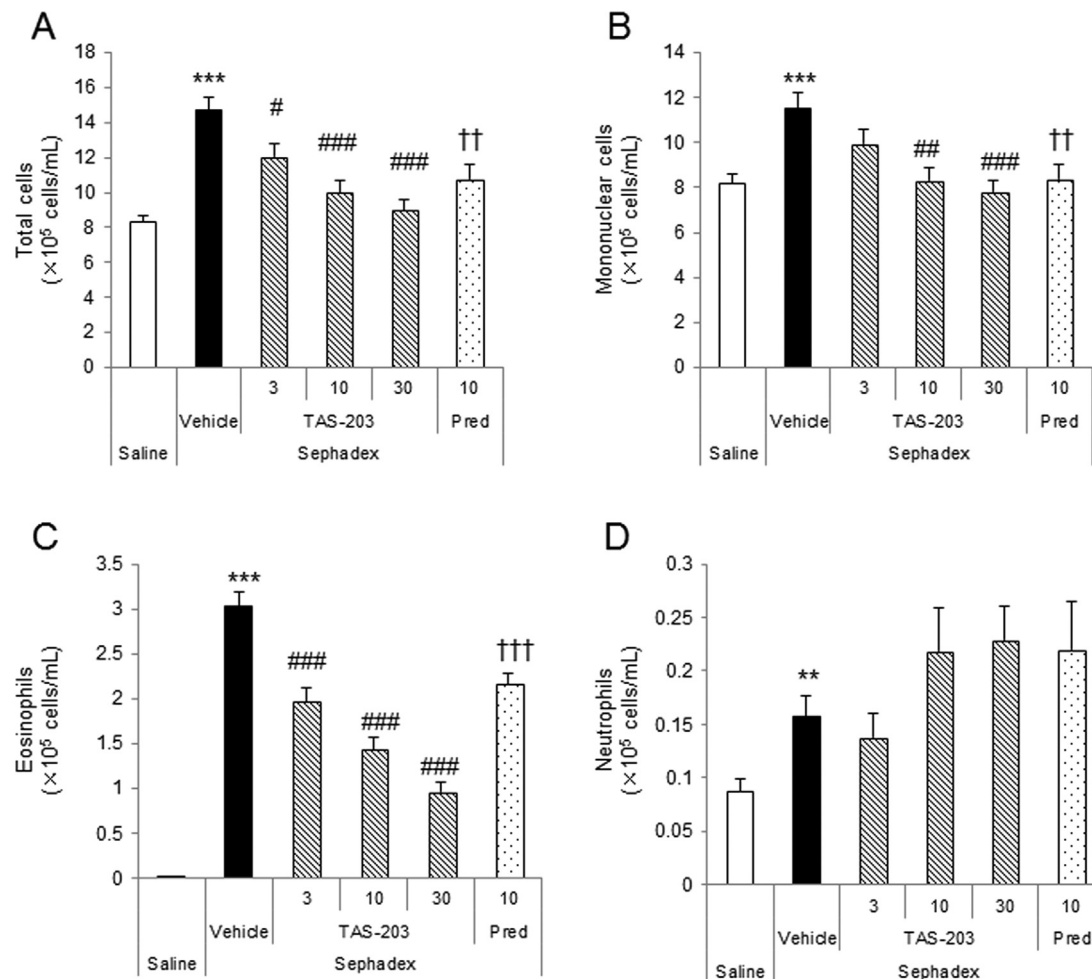


Fig. 3. TAS-203 attenuates infiltration of inflammatory cells in the BAL fluid of the rat Sephadex-induced model. Rats were treated with TAS-203 (3, 10, and 30 mg/kg) and prednisolone (Pred) (10 mg/kg) from day 1 and continued for 6 days. On days 1, 3 and 6, rats were intravenously injected with Sephadex beads (250 μ g/rat). BAL fluid was collected on day 8, and then cells underwent Kwik-Diff staining. The numbers of total cells (A), mononuclear cells (B), eosinophils (C), and neutrophils (D) were counted under a light microscope. Data are expressed as mean $\pm$ S.E.M. ($n = 12$ /group). *** $P < 0.001$ when comparing the vehicle group against the saline group, ## $P < 0.01$ and ### $P < 0.001$ when comparing the TAS-203 group against the vehicle group, ++ $P < 0.01$ and +++ $P < 0.001$ when comparing the prednisolone group against the vehicle group.

inhibitory effect of TAS-203 on MUC5AC production, as TAS-203 inhibited goblet cell hyperplasia in this model. However, as our established ELISA analysis did not detect MUC5AC protein in all animal groups, it remains unclear whether TAS-203 has an inhibitory effect on MUC5AC production in this model.

3.4. Effect of TAS-203 on 5-HT-induced AHR in the Sephadex-induced model

It had been reported that rats treated with Sephadex showed increased AHR in response to intravenous injection of 5-HT (Matsubara et al., 1998), and that roflumilast significantly decreased histamine-induced AHR in ovalbumin-sensitized guinea pigs (Mokry et al., 2017). Based on these findings, we decided to examine the effect of TAS-203 on 5-HT-induced AHR using the rat Sephadex-induced airway inflammation model. Our results showed that there was a significant increase of the AHR of Sephadex-treated vehicle rats in response to a 5-HT injection compared to that observed in the saline-treated rats (Fig. 5). However, TAS-203 treatment significantly suppressed 5-HT-induced AHR in a dose-dependent manner (Fig. 5). Moreover, prednisolone also inhibited the AHR induced by 5-HT (Fig. 5).

3.5. Effect of TAS-203 on the cigarette smoke-induced COPD-like model

Finally, in order to investigate the efficacy of TAS-203 on COPD, we preliminarily examined the effects of TAS-203 on the cigarette smoke-induced COPD-like model as compared to that for roflumilast. Repeated exposure of guinea pigs to cigarette smoke resulted in COPD-like symptoms, including mucus hypersecretion and airway resistance (Domínguez-Fandos et al., 2014; Kubo et al., 2005). Furthermore, roflumilast at a dose of 1 mg/kg resulted in significant anti-inflammatory effects in this model (Kubo et al., 2011).

Our results confirmed that the MUC5AC production in animals repeatedly exposed to cigarette smoke was significantly elevated as compared with the fresh air control group (Supplemental Fig. 2B). TAS-203 treatments at doses of either 10 or 25 mg/kg significantly prevented cigarette smoke exposure-induced MUC5AC production in BAL fluid (Supplemental Fig. 2B). In contrast, there was no significant suppression in the roflumilast-treated group (Supplemental Fig. 2B). Furthermore, repeated cigarette smoke exposure gradually increased sRaw (Supplemental Fig. 2C). On day 26, there was a tendency for TAS-203 at a dose of 10 mg/kg to suppress sRaw, while the 25 mg/kg TAS-203-treated group exhibited a significant reduction compared with the vehicle-treated group (Supplemental Fig. 2C). In addition, roflumilast inhibited sRaw compared with the vehicle-treated group on day 13,

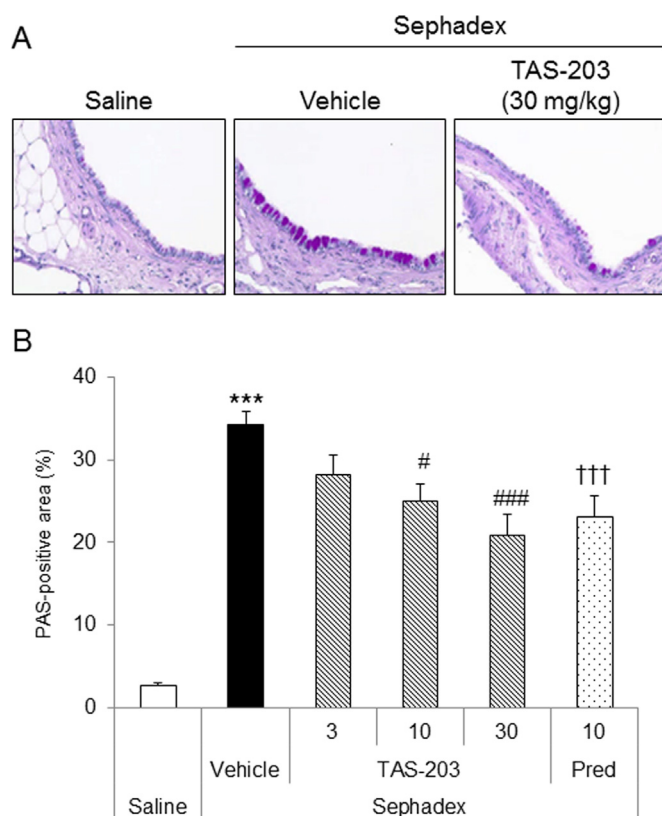


Fig. 4. TAS-203 blocks goblet cell hyperplasia in the airway epithelium of Sephadex-treated rats. Rats were treated with TAS-203 (3, 10, and 30 mg/kg) and prednisolone (Pred) (10 mg/kg) from day 1 and continued for 6 days. On days 1, 3 and 6, rats were intravenously injected with Sephadex beads (100 μ g/rat). The lungs were collected on day 8, with the fixed left lung lobes stained with PAS. (A) The representative histological PAS staining sections are magnified 24 $\times$. (B) PAS-positive area was calculated by an image analysis system. Data are presented as mean $\pm$ S.E.M. (n = 11 or 12/group). *** $P < 0.001$ when comparing the vehicle group against the saline group, # $P < 0.05$ and ### $P < 0.001$ when comparing the TAS-203 group against the vehicle group, + + + $P < 0.001$ when comparing the prednisolone group against the vehicle group.

although it had no effect on days 20 and 26 (Supplemental Fig. 2C).

4. Discussion

Roflumilast has anti-inflammatory effects and clinical benefits in asthma and COPD. However, the therapeutic index is limited due to the central nervous system-related side effects, which include nausea and vomiting (Calverley, 2009). Therefore, we attempted to find a compound with a low blood-brain-barrier permeability in order to minimize these side effects. As a result, we discovered a novel PDE4 inhibitor, TAS-203, which is an orally active compound with a low emetogenic effect in ferrets (Asaka et al., 2010). Airway mucus hypersecretion is a problem in asthma and COPD, and thus PDE4 inhibitors have been anticipated as expectorant therapies targeting mucus hypersecretion (Fahy and Dickey, 2010; Tamaoki, 2015; Zhang and Zhou, 2014). However, the inhibitory effect of roflumilast on sputum production is limited to a minority of patients (Rennard et al., 2011). Therefore, we examined the effects of TAS-203 on mucus hypersecretion, as we hypothesized that TAS-203, which has a good balance between anti-inflammatory activities and low emetogenicity, would be able to overcome the outstanding issues associated with roflumilast.

In the first step, we confirmed TAS-203 treatment suppressed EGF-induced MUC5AC expression. Using the rat Sephadex-induced airway inflammation model, we then confirmed the in vivo efficacy of TAS-

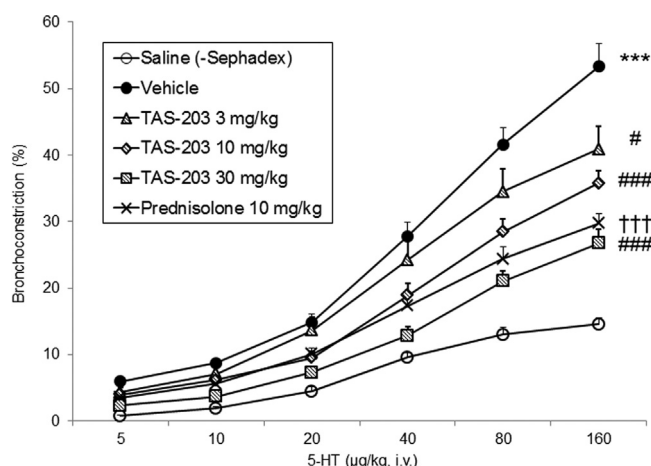


Fig. 5. TAS-203 protects the Sephadex-treated rat from 5-HT-induced AHR. Rats were treated with TAS-203 (3, 10, and 30 mg/kg) and prednisolone (10 mg/kg) from day 1 and continued for 7 days. On days 1, 3 and 6, rats were intravenously injected with Sephadex beads (100 μ g/rat). AHR to 5-HT was measured using the method by Konzett and Rössler on day 9. Data are shown as mean $\pm$ S.E.M. (n = 8/group). *** $P < 0.001$ when comparing the vehicle group against the saline group, # $P < 0.05$ and ### $P < 0.001$ when comparing the TAS-203 group against the vehicle group, + + + $P < 0.001$ when comparing the prednisolone group against the vehicle group.

203. TAS-203 suppressed MCP-1, IL-5 and IL-13 levels in BAL fluid. Furthermore, TAS-203 treatment also inhibited the number of infiltrating mononuclear cells and eosinophils in BAL fluid. Moreover, TAS-203 significantly suppressed goblet cell hyperplasia of the airway epithelium and 5-HT-induced AHR. In the final step, we preliminarily demonstrated the inhibitory effects of TAS-203 on cigarette smoke-induced COPD-like symptoms, such as MUC5AC production and sRaw.

PDE4 inhibitors, including roflumilast, have been previously shown to suppress MUC5AC expression in addition to having anti-inflammatory effects (Mata et al., 2005). A wide variety of stimuli (e.g., allergens, bacteria, mechanical injury, cytokines, and cigarette smoke) were shown to cause the airway epithelium of patients with asthma and COPD to differentiate into goblet cells via activation of the EGF receptor cascade (Nadel, 2001). Therefore, we examined the inhibitory effect on EGF-induced MUC5AC expression. Consistent with findings for other PDE4 inhibitors, TAS-203 also blocked EGF-induced MUC5AC expression by NCI-H292 cells in our present study.

Using the rat Sephadex-induced airway inflammation model, we evaluated the inhibitory effects of TAS-203 on Sephadex-induced MCP-1, IL-5 and IL-13 in BAL fluid. Bronchial samples from patients with asthma and/or COPD have elevated levels of these factors (Hamid et al., 1991; Saha et al., 2008; Sousa et al., 1994; Traves et al., 2002). Furthermore, IL-5 and its receptor have attracted the attention of pharmaceutical industries as a possible target in the treatment of eosinophilic asthma, with mepolizumab subsequently approved as an anti-IL-5 therapy for the treatment of patients with severe asthma (Varricchi et al., 2017). Based on these previous findings, we propose that the inhibition of MCP-1, IL-5 and IL-13 production by TAS-203 would be beneficial for treating airway inflammatory disease. Subsequently, we then examined whether infiltration of inflammatory cells, such as mononuclear cells and eosinophils, was suppressed as a result of inhibition of the cytokine/chemokine production. However, it has also been shown that TAS-203 and other PDE4 inhibitors directly suppressed eosinophil chemotaxis in an in vitro assay (Alves et al., 1996; Asaka et al., 2010). Thus, it is likely that the inhibitory effect of TAS-203 on eosinophil infiltration in this model can be attributed to both an indirect and direct mechanism.

In asthma and COPD, goblet cell hyperplasia and subsequent mucus overproduction are remarkable pathological findings (Fahy and Dickey,

2010; Tamaoki, 2015). The primary cause of death in asthma patients is suffocation due to widespread mucus plugging (Kuyper et al., 2003). Furthermore, mucus accumulation in COPD patients affects exacerbation, hospitalization, and mortality (Ramos et al., 2014). Therefore, targeting goblet cell hyperplasia and mucus overproduction might be useful in preventing the disease symptoms (Morcillo and Cortijo, 2006; Tamaoki, 2015). Our PDE4 inhibitor, TAS-203, exhibited an inhibitory effect on Sephadex-induced goblet cell hyperplasia of airway epithelium. Although the precise mechanisms for goblet cell hyperplasia in this model remain unclear, compounds that inhibit eosinophilic function have been found to suppress Sephadex-induced goblet cell hyperplasia (Matsubara et al., 1998). It has also been demonstrated that IL-13 directly drives goblet cell metaplasia (Atherton et al., 2003). These previous reports suggest that TAS-203 might ameliorate the Sephadex-induced goblet cell hyperplasia via blockade of eosinophil infiltration and IL-13 production.

AHR is a functional hallmark of asthma and COPD, and reflects the risk for disease development as well as the disease progression (Postma and Kerstjens, 1998). Previous studies have shown that the inhibitory effect of roflumilast on AHR induced by ovalbumin was associated with a lower production of IL-4, IL-5 and IL-13, thereby decreasing the numbers of circulating eosinophils (Kim et al., 2016; Mokry et al., 2017). Since TAS-203 inhibits the production of IL-5 and IL-13, eosinophil infiltration in the Sephadex-induced model, we hypothesize that TAS-203 indirectly suppresses the Sephadex-induced AHR by inhibiting IL-5 and IL-13, eosinophil infiltration.

The last part of our study examined the inhibitory effects of TAS-203 on COPD-like symptoms, such as MUC5AC production and sRaw, using the guinea pig cigarette smoke-induced model. TAS-203 at a dose of 25 mg/kg and roflumilast at a dose of 1 mg/kg were the maximum tolerated doses that did not affect body weight gain in this model. Although TAS-203 at doses of 10 and 25 mg/kg significantly attenuated MUC5AC production in BAL fluid, roflumilast exhibited no significant suppression as compared with the vehicle group. Using the same model, a report by the US Food and Drug Administration also showed that a 4 mg/kg dose of roflumilast had no effects on mucin in BAL fluid, and on the mRNA level of MUC5AC in lung tissue (CENTER FOR DRUG EVALUATION AND RESEARCH, 2010). While it remains unknown why TAS-203 exhibits a greater inhibitory effect on MUC5AC production compared to roflumilast, our preliminary data showed that the inhibitory effect of TAS-203 on MCP-1 production from human monocyte-derived macrophages was more potent than that of roflumilast (unpublished data). Moreover, MCP-1 was shown to function as a positive regulator of MUC5AC production in human airway epithelium (Monzon et al., 2011). In addition, there was a significant increase in the mRNA expression of MCP-1 compared with the fresh air control animals in the lung tissue of this model (Kubo et al., 2005). Based on these findings, we hypothesize that the difference in the MCP-1 inhibition between TAS-203 and roflumilast plays an important role in the MUC5AC expression. However, since it remains unknown whether elevated MCP-1 directly contributes to MUC5AC production in this model, additional studies will need to be undertaken in order further analyze the MUC5AC expression. In addition, while TAS-203 at a dose of 25 mg/kg significantly repressed cigarette smoke exposure-induced sRaw on day 26, roflumilast at a dose of 1 mg/kg had no effect compared with the vehicle-treated group. As airway mucus hypersecretion is associated with the severity of COPD (Ramos et al., 2014), we hypothesize that the inhibitory effect of TAS-203 on sRaw partially contributes to inhibition of MUC5AC production.

In this study, we provide evidence that TAS-203 has inhibitory effects on airway inflammation, goblet cell hyperplasia and AHR in a Sephadex-induced airway inflammation model. Furthermore, we preliminarily confirmed the inhibitory effects of TAS-203 on MUC5AC production and sRaw in a cigarette smoke-induced COPD-like model. These results suggest that TAS-203 has a good prophylactic window of efficacy for preventing emesis compared with roflumilast, thereby

supporting further development of TAS-203 as an agent for treating airway inflammatory disease.

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Declarations of interest

All of the authors state no declarations of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2019.01.068.

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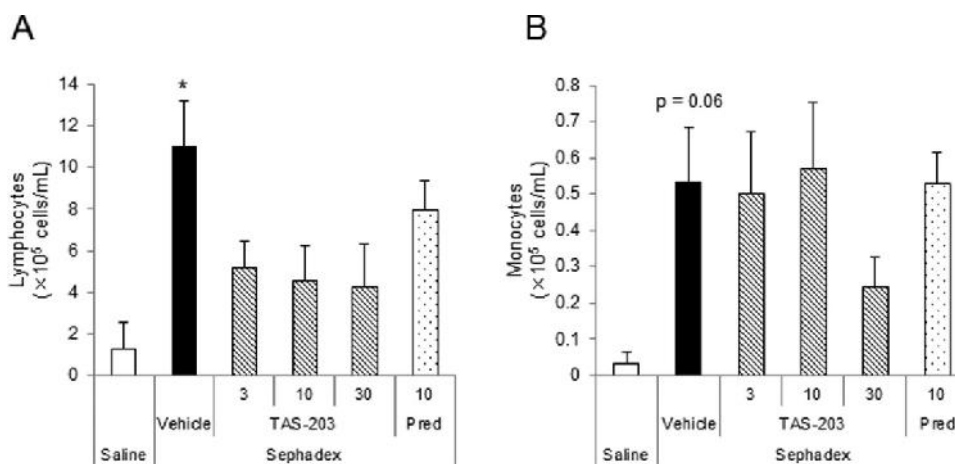


Fig. S1. The effects of TAS-203 on infiltration of lymphocytes and monocytes in the BAL fluid of the rat Sephadex-induced model. Rats were treated with TAS-203 (3, 10, and 30 mg/kg) and prednisolone (Pred) (10 mg/kg) from day 1 and continued for 6 days. On days 1, 3 and 6, rats were intravenously injected with Sephadex beads (250 μ g/rat). BAL fluid was collected on day 8, and the numbers of lymphocytes (A) and monocytes (B) were counted by XT-2000iV. Data are shown as mean + S.E.M. $n = 3$ rats in the saline group; $n = 6$ or 7 rats each in the vehicle and treatment groups. * $P < 0.05$ and $P = 0.06$ when comparing the vehicle group against the saline group.

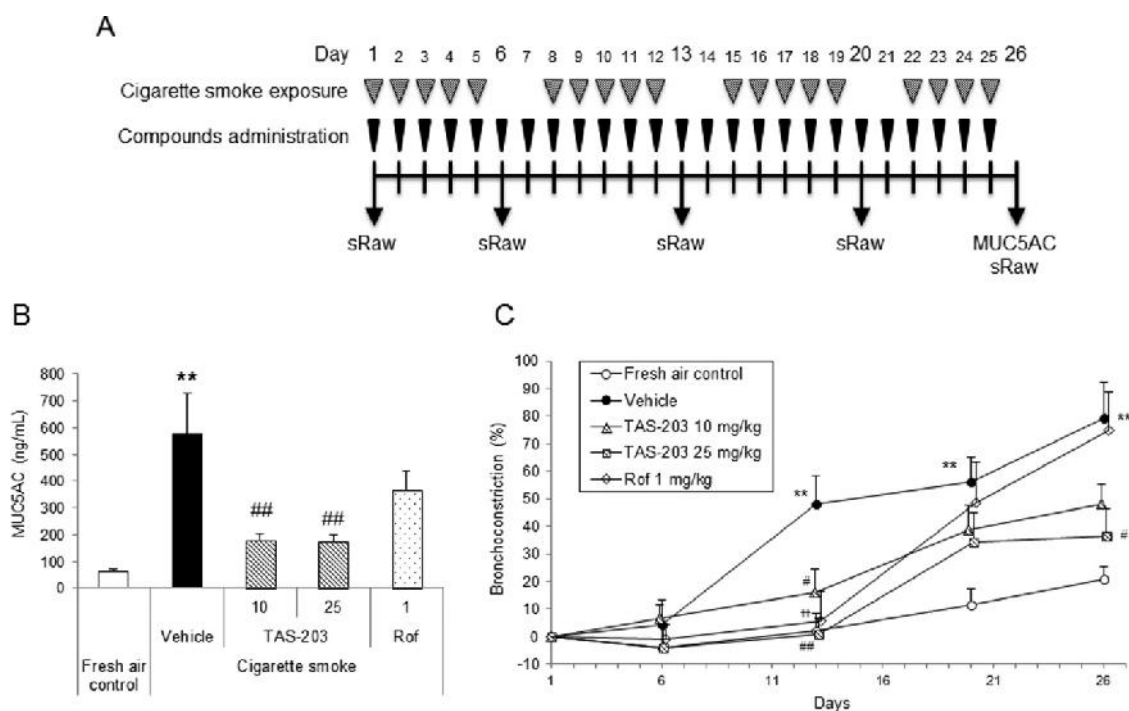


Fig. S2. TAS-203 showed inhibitory effects on COPD-like symptoms such as MUC5AC production and airway resistance. (A) Experimental protocol for cigarette smoke-induced airway inflammation in Hartley guinea pigs. Guinea pigs were repeatedly exposed to cigarette smoke (1 h/day, 5 consecutive days/week) for 4 weeks. TAS-203 (10 and 25 mg/kg) or roflumilast (Rof) (1 mg/kg) were orally administered from day 1 and continued for 25 days. (B) BAL fluid was collected on day 26, with the levels of MUC5AC detected by ELISA.

(C) sRaw was measured using a double-flow plethysmograph on days 1, 6, 13, 20, and 26. Data are shown as mean + S.E.M. n = 8 guinea pigs in the fresh air control group; n = 10 or 11 guinea pigs each in the vehicle and treatment groups. * * P < 0.01 when comparing the vehicle group against the fresh air control group, # P < 0.05 and ## P < 0.01 when comparing the TAS-203 group against the vehicle group, + + P < 0.01 when comparing the roflumilast group against the vehicle group.