

*Short Communication***Effects of SA13353, a Transient Receptor Potential Vanilloid 1 Agonist, on Leukocyte Infiltration in Lipopolysaccharide-Induced Acute Lung Injury and Ovalbumin-Induced Allergic Airway Inflammation**Fumio Tsuji^{1,*}, Masaaki Murai¹, Kenji Oki¹, Hiroyuki Inoue¹, Minoru Sasano¹, Hiroyuki Tanaka², Naoki Inagaki², and Hiroyuki Aono¹¹Research and Development Center, Santen Pharmaceutical Co., Ltd.,
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Abstract. We recently demonstrated that SA13353, a transient receptor potential vanilloid 1 (TRPV1) agonist, reduced the severity of the symptoms of kidney injury, arthritis, and encephalomyelitis in disease models. Here, we investigated the effects of orally administered SA13353 on leukocyte infiltration in lipopolysaccharide (LPS)-induced acute lung injury and ovalbumin-induced allergic airway inflammation. In LPS-induced lung injury, SA13353 attenuated neutrophil infiltration and the increase of TNF- α and CINC-1 levels. In allergic airway inflammation, SA13353 tended to inhibit leukocyte infiltration and attenuated the increase of IL-4 and IL-12p40. These results suggest that somatosensory TRPV1 may play an anti-inflammatory role in lung inflammation.

Keywords: SA13353, transient receptor potential vanilloid 1, lung inflammation

The role of transient receptor potential (TRP) channels in pain and sensation was first proposed in 1997, when the vanilloid receptor-1 was identified at the genetic and functional level (TRPV1, ref. 1). TRPV1 is a non-selective cation channel activated by noxious heat, protons, and vanilloids, such as capsaicin, as well as a range of putative endogenous mediators (2). TRPV1 channels play a major role in the integration of afferent noxious signals generated by inflammatory mediators (3).

It has been reported that TRPV1 is located in the airways and plays an important role in cough reflexes (4). Furthermore, there is an increasing amount of evidence showing that TRPV1 activation may contribute to the respiratory symptoms of asthma and chronic obstructive pulmonary disease (COPD). On the other hand, we have demonstrated that orally administered TRPV1 agonists reduce the severity of the symptoms of kidney injury, arthritis, and encephalomyelitis models (5–7). In the

present study, we investigated the effects of an orally administered TRPV1 agonist on leukocyte infiltration in lipopolysaccharide (LPS)-induced acute lung injury and ovalbumin-induced allergic airway inflammation using SA13353.

For this study, male Lewis rats and female BALB/c mice were obtained from Charles River Japan, Inc. (Yokohama) and housed in a room maintained at $23 \pm 1^\circ\text{C}$ under a 12-h light – 12-h dark cycle with free access to food and water. Experimental procedures were carried out with the approval of the Santen Animal Experimental Ethics Committee. For LPS-induced acute lung injury (8), water for injections containing 200 $\mu\text{g}/\text{mL}$ of LPS (*E. coli* 0111:B4; Sigma, St. Louis, MO, USA) was aerosolized in a nasal inhalation exposure chamber using an ultrasonic nebulizer (NE-U17; Omron, Kyoto). Rats, 8 weeks in age, were fasted for 18 h before LPS inhalation exposure for 60 min. Four hours after the end of the LPS inhalation, the rats were thoracotomized and exsanguinated under sodium pentobarbital anesthesia (50 mg/kg, i.p.). After inserting and fixing a tube into the airway, 5 mL of ice-cooled physiological saline solution

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was injected into the lungs and removed by aspiration, three times (total volume: 15 mL), using the recovered liquid as the bronchoalveolar lavage fluid (BALF). The BALF was centrifuged, and the pellet was used for the determination of the total cell count. Then, smear samples were made and stained with May-Grunwald and Giemsa (Muto Pure Chemicals, Tokyo). A binocular microscope was used to count 500 cells for calculating the ratios of neutrophils, eosinophils, and lymphocytes compared with the total cell count; and based on these ratios, the cell counts for each type of cell were calculated. We measured BALF TNF- α and CINC-1 levels by ELISA using a commercial kit (R&D Systems, Minneapolis, MN, USA). To investigate the effects of SA13353 (Santen Pharmaceutical Co., Ltd., Osaka) and dexamethasone (Sigma) on LPS-induced acute lung injury, we administered drugs orally in a 1% methylcellulose solution (vehicle) 30 min before LPS inhalation. We also prepared LPS- and drug-untreated animals (normal).

For ovalbumin-induced allergic airway inflammation (9), mice, 6 weeks in age, were injected intraperitoneally with 100 μ L of 0.01% ovalbumin solution containing 15 mg/mL Alum (Wako Chemicals, Osaka) on day 1. On days 14 and 21, booster injections of 100 μ L of 0.01% ovalbumin solution were administered intraperitoneally. On day 40 to 44 and day 47 to 50, PBS for injection containing 10 mg/mL of ovalbumin was aerosolized in a nasal inhalation exposure chamber using an ultrasonic nebulizer (NE-U17). The animals were exposed to 20 min of inhalation for elicitation of allergic reactions. One day after the last ovalbumin inhalation, the mice were thoracotomized and exsanguinated under sodium pentobarbital anesthesia (40 mg/kg, i.p.). After inserting and fixing a tube into the airway, 0.5 mL of ice-cooled physiological saline solution was injected into the lungs and removed by aspiration, three times (total volume: 1.5 mL), using the recovered liquid as BALF. The BALF

was centrifuged, and the pellet was used for the determination of the total cell count. Then, smear samples were made and the number of neutrophils, eosinophils and lymphocytes were calculated as described for the LPS model. The levels of BALF IL-4 and IL-12(p40) were measured using a Bio-Plex suspension system (Bio-Rad, Hercules, CA, USA). To investigate the effects of SA13353 (Santen Pharmaceutical Co., Ltd.) and prednisolone (Sigma) on ovalbumin-induced allergic airway inflammation, we administered drugs orally in a 1% methylcellulose solution (vehicle) 30 min before ovalbumin inhalation on day 40 to 44 and day 47 to 50. We also prepared ovalbumin- and drug-untreated animals (normal).

Results were statistically evaluated using by Student's *t*-test or Dunnett's test (EXSAS; Arm, Osaka).

In LPS-induced acute lung injury (Table 1), the mean values for neutrophil, eosinophil, and lymphocyte cell counts in the BALF of the vehicle control group 4 h after LPS inhalation were 47.82 ± 5.02 , 0.07 ± 0.03 , and 0.04 ± 0.03 ($\times 10^6$ cells/lung, mean \pm S.E.M.), respectively. In normal rats, neutrophil infiltration was scarcely observed. Compared with the vehicle control, SA13353 significantly attenuated neutrophil infiltration at doses of 10 and 30 mg/kg. Cytokine analysis of the BALF showed that LPS inhalation led to an increase in TNF- α and CINC-1 levels. SA13353 also attenuated TNF- α and CINC-1 levels at doses of 10 and 30 mg/kg. On the other hand, dexamethasone significantly suppressed neutrophil infiltration and the increase of TNF- α and CINC-1 levels after LPS inhalation. Capsaicin, a TRPV1 agonist that is weaker than SA13353 in rats and mice (5–7), also showed a statistically significant 35% inhibition of leukocyte infiltration and a trend towards attenuation of TNF- α and CINC-1 levels, although this was not significant at a dose of 30 mg/kg under similar experimental conditions (Table 2).

Table 1. Effects of SA13353 and dexamethasone on inflammatory cell infiltration and cytokine concentrations in the bronchoalveolar cavity in LPS-exposed rats

Group	Dose (mg/kg)	Neutrophil $\times 10^6$ cells/lung	Eosinophil $\times 10^6$ cells/lung	Lymphocyte $\times 10^6$ cells/lung	TNF- α pg/mL in BALF	CINC-1 pg/mL in BALF
Normal	0	0.32 ± 0.12	0.06 ± 0.03	0.00 ± 0.00	15.25 ± 2.00	31.03 ± 3.99
Control	0	47.82 ± 5.02	0.07 ± 0.03	0.04 ± 0.03	220.92 ± 35.35	1199.90 ± 46.58
SA13353	3	55.43 ± 5.02	0.06 ± 0.03	0.03 ± 0.02	202.25 ± 33.49	1228.72 ± 74.87
SA13353	10	$23.04 \pm 3.80^{**}$	0.01 ± 0.01	0.02 ± 0.01	182.44 ± 16.17	$937.03 \pm 79.29^*$
SA13353	30	$10.75 \pm 2.10^{**}$	0.00 ± 0.00	0.02 ± 0.01	$114.99 \pm 10.12^*$	$707.54 \pm 54.82^{**}$
Dexamethasone	10	$9.45 \pm 1.62^{##}$	0.02 ± 0.01	0.01 ± 0.01	$23.71 \pm 1.99^{##}$	$697.79 \pm 79.26^{##}$

Each value represents the mean \pm S.E.M. of 5–8 animals. * $P < 0.05$, ** $P < 0.01$: significant difference from the control group (Dunnett's test). ## $P < 0.01$: significant difference from the control group (Student's *t*-test).

Table 2. Effects of capsaicin on inflammatory cell infiltration and cytokine concentrations in the bronchoalveolar cavity in LPS-exposed rats

Group	Dose (mg/kg)	Leukocyte ^a ×10 ⁶ cells/lung	TNF- α pg/mL in BALF	CINC-1 pg/mL in BALF
Control	0	32.17 ± 2.29	179.18 ± 16.14	825.99 ± 15.06
Capsaicin ^b	30	22.09 ± 1.01**	154.21 ± 16.23	785.86 ± 46.80

Each value represents the mean ± S.E.M. of 6–7 animals. ^aPredominantly neutrophils, but also including eosinophils and lymphocytes. ^bThe experimental method for capsaicin is a little different from the described method for SA13353 in the text. ** $P < 0.01$: significant difference from the control group (Student's *t*-test).

Table 3. Effects of SA13353 and prednisolone on inflammatory cell infiltration and cytokine concentrations in the bronchoalveolar cavity in antigen-exposed mice

Group	Dose (mg/kg)	Neutrophil ×10 ⁶ cells/lung	Eosinophil ×10 ⁶ cells/lung	Lymphocyte ×10 ⁶ cells/lung	IL-4 pg/mL in BALF	IL-12(p40) pg/mL in BALF
Normal	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.01	18.45 ± 1.19
Control	0	0.85 ± 0.13	10.42 ± 1.33	1.28 ± 0.26	2.80 ± 0.18	316.33 ± 29.89
SA13353	10	0.48 ± 0.09	6.43 ± 1.87	0.86 ± 0.28	2.06 ± 0.23	236.89 ± 18.77*
SA13353	30	0.79 ± 0.14	6.68 ± 1.44	0.94 ± 0.34	2.26 ± 0.29	211.81 ± 11.36**
SA13353	100	0.61 ± 0.14	5.72 ± 0.96	0.40 ± 0.10	1.78 ± 0.16**	108.39 ± 10.61**
Prednisolone	3	0.59 ± 0.11	2.37 ± 0.36 ^{###}	0.35 ± 0.12 ^{###}	1.29 ± 0.10 ^{###}	167.05 ± 19.74 ^{###}

Each value represents the mean ± S.E.M. of 9–10 animals. * $P < 0.05$, ** $P < 0.01$: significant difference from the control group (Dunnett's test). ^{###} $P < 0.01$: significant difference from the control group (Student's *t*-test).

In ovalbumin-induced allergic airway inflammation (Table 3), the mean neutrophil, eosinophil, and lymphocyte cell counts in the BALF of the vehicle control group 24 h after the last ovalbumin inhalation were 0.85 ± 0.13 , 10.42 ± 1.33 , and 1.28 ± 0.26 ($\times 10^6$ cells/lung, mean ± S.E.M.), respectively. In normal mice, leukocyte infiltration was scarcely observed. Compared with the vehicle control, SA13353 tended to inhibit leukocyte infiltration at a dose of 100 mg/kg, but this was not statistically significant (neutrophil: $P = 0.4225$, eosinophil: $P = 0.0592$, lymphocyte: $P = 0.0524$ by Dunnett's test). Ovalbumin inhalation led to an increase in IL-4 and IL-12p40 levels in BALF. SA13353 also attenuated IL-4 and IL-12p40 levels at a dose of 100 mg/kg. On the other hand, prednisolone significantly suppressed eosinophil and lymphocyte infiltrations and the increase of IL-4 and IL-12p40 levels after the last ovalbumin inhalation. Ovalbumin inhalation also led to an increase in IL-5 levels in BALF, which was not attenuated by SA13353 and prednisolone (data not shown). Capsaicin could not be investigated in this model due to its high oral toxicity.

Clinically, both adult respiratory distress syndrome (ARDS) and COPD are respiratory diseases in which pulmonary inflammation is a cause. ARDS is characterized by inflammatory pulmonary edema and alveolar

collapse (10); and COPD is characterized by mucous plugging, epithelial abnormalities, inflammatory cellular infiltrates, fibrosis, and distortion (11), among other varied characteristics. These are thought to be due to inflammatory reactions in the lungs in response to neutrophil-derived inflammatory mediators. Therefore, neutrophil infiltration is thought to play an important role in these diseases.

Exposure of animals to LPS has been reported to induce neutrophil infiltration and inflammatory cytokines, provoking changes similar to those in ARDS and COPD (8). In the present study, SA13353 dose-dependently reduced the number of inflammatory cells in the BALF. Therefore, systemic TRPV1 agonists may be expected to show efficacy in clinical treatment of the pulmonary inflammation found in conditions such as ARDS and COPD.

Asthma is a chronic allergic airway inflammatory disease characterized by increased numbers and activation of inflammatory and immune cells within the airways, including eosinophils, T helper 2 (Th2) lymphocytes, mast cells, neutrophils, and macrophages (12). Cytokines produced by Th2 cells are particularly important in the pathophysiology of asthma; IL-4 promotes Th2 cell differentiation, induces IgE production, and upregulates IgE receptors; IL-5 promotes development,

differentiation, recruitment, activation, and survival of eosinophils; and IL-13 is necessary for allergen-induced airway hyperresponsiveness (13). Therefore, attenuation of IL-4 by SA13353 may lead to the inhibition of allergic reactions. Regarding IL-12p40, IL-23 shares the p40 subunit with IL-12, and IL-23 induces Th17 cells (14). Th17 cells may also contribute to the pathogenesis of classically recognized Th2-mediated allergic disorders (15). Therefore, attenuation of IL-12p40 may also lead to the inhibition of allergic reactions.

Here, we have demonstrated that two orally administered TRPV1 agonists inhibit leukocyte infiltration in LPS-induced acute lung injury and that one orally administered TRPV1 agonist inhibits leukocyte infiltration in ovalbumin-induced allergic airway inflammation. In conclusion, somatosensory TRPV1 may play an anti-inflammatory role in lung inflammation. We believe that TRPV1 agonists modulate cytokine production via neuropeptide release from afferent C fibers (5, 7) and inhibit leukocyte infiltration into the lung. Further studies to clarify the mechanisms behind the modification of cytokine production and leukocyte infiltration by TRPV1 agonists are necessary.

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