

Effects of ONO-6950, a novel dual cysteinyl leukotriene 1 and 2 receptors antagonist, in a Guinea pig model of asthma.

Yasuo Yonetomi, Tomohiko Sekioka, Michiaki Kadode, Tetsuya Kitamine, Akihiro Kamiya, Atsuto inoue, Takafumi Nakao, Hiroaki Nomura, Masayuki Murata, Shintaro Nakao, Fumio Nambu, Manabu Fujita, Shinji Nakade, Kazuhito Kawabata



PII: S0014-2999(15)30215-6
DOI: <http://dx.doi.org/10.1016/j.ejphar.2015.08.041>
Reference: EJP70189

To appear in: *European Journal of Pharmacology*

Received date: 14 May 2015
Revised date: 3 August 2015
Accepted date: 24 August 2015

Cite this article as: Yasuo Yonetomi, Tomohiko Sekioka, Michiaki Kadode, Tetsuya Kitamine, Akihiro Kamiya, Atsuto inoue, Takafumi Nakao, Hiroaki Nomura, Masayuki Murata, Shintaro Nakao, Fumio Nambu, Manabu Fujita, Shinji Nakade and Kazuhito Kawabata, Effects of ONO-6950, a novel dual cysteinyl leukotriene 1 and 2 receptors antagonist, in a Guinea pig model of asthma, *European Journal of Pharmacology* <http://dx.doi.org/10.1016/j.ejphar.2015.08.041>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

TITLE:

Effects of ONO-6950, a novel dual cysteinyl leukotriene 1 and 2 receptors antagonist, in a guinea pig model of asthma.

AUTHORS:

Yasuo Yonetomi, Tomohiko Sekioka, Michiaki Kadode, Tetsuya Kitamine, Akihiro Kamiya, Atsuto inoue, Takafumi Nakao, Hiroaki Nomura, Masayuki Murata, Shintaro Nakao, Fumio Nambu, Manabu Fujita, Shinji Nakade and Kazuhito Kawabata

LABORATORY OF ORIGIN:

Minase Research Institute, Ono Pharmaceutical Co., Ltd.

ADDRESS:

3-1-1 Sakurai, Shimamoto, Mishima, Osaka 618-8585, Japan

Tel: +81-75-961-1151, Fax: +81-75-962-9314

CORRESPONDING AUTHOR AND ADDRESS:

Yasuo Yonetomi

Minase Research Institute, Ono Pharmaceutical Co., Ltd., 3-1-1 Sakurai, Shimamoto-cho
Mishima-gun, Osaka 618-8585, Japan

Tel: +81-75-961-1151, Fax: +81-75-962-9314, yonetomi@ono.co.jp

Abstract

We assessed in this study the anti-asthmatic effects of ONO-6950, a novel cysteinyl leukotriene 1 (CysLT₁) and 2 (CysLT₂) receptors dual antagonist, in normal and S-hexyl glutathione (S-hexyl GSH)-treated guinea pigs, and compared these effects to those of montelukast, a CysLT₁ selective receptor antagonist. Treatment with S-hexyl GSH reduced animals LTC₄ metabolism, allowing practical evaluation of CysLT₂ receptor-mediated airway response. ONO-6950 antagonized intracellular calcium signaling via human and guinea pig CysLT₁ and CysLT₂ receptors with IC₅₀ values of 1.7 and 25 nM, respectively (human receptors) and 6.3 and 8.2 nM, respectively (guinea pig receptors). In normal guinea pigs, both ONO-6950 (1 or 0.3 mg/kg, p.o.) and the CysLT₁ receptor antagonist montelukast (0.3 or 0.1 mg/kg, p.o.) fully attenuated CysLT₁-mediated bronchoconstriction and airway vascular hyperpermeability induced by LTD₄. On the other hand, in S-hexyl GSH-treated guinea pigs ONO-6950 at 3 mg/kg, p.o. or more almost completely inhibited bronchoconstriction and airway vascular hyperpermeability elicited by LTC₄, while montelukast showed only partial or negligible inhibition of these airway responses. In ovalbumin sensitized guinea pigs, treatment with S-hexyl GSH on top of pyrilamine and indomethacin rendered antigen-induced bronchoconstriction sensitive to both CysLT₁ and CysLT₂ receptor antagonists. ONO-6950 strongly inhibited this asthmatic response to the level attained by combination therapy with montelukast and BayCysLT₂RA, a selective CysLT₂ receptor antagonist. These results clearly demonstrate that ONO-6950 is an orally active dual CysLT₁/LT₂ receptor antagonist that may provide a novel therapeutic option for patients with asthma.

KEY WORDS:

Asthma, CysLTs, CysLT₂ receptor, CysLT₁/LT₂ receptor antagonist, ONO-6950, Glutathione

1. Introduction

Asthma is a chronic inflammatory disease characterized by reversible airway constriction, airway inflammation, and airway hyperresponsiveness. Currently, inhaled corticosteroids are the first-line therapy for control of airway inflammation in patients with asthma (Martin, 2003). On the other hand, despite of inhaled corticosteroids treatment, some of the patients still remain asthmatic symptoms (FitzGerald ~~J.M.~~ and Shahidi, 2010). Those patients were prescribed with cysteinyl leukotriene 1 receptor antagonists (LTRA) or long-acting β 2-agonists in addition to inhaled corticosteroids.

Cysteinyl leukotrienes (CysLTs: i.e.; LTC₄, LTD₄ and LTE₄) are known to induce airway smooth muscle constriction, enhance vascular permeability, promote mucus secretion, and recruit eosinophils, all of which contribute to asthma symptoms (Samuelsson, 1983; Nakagawa et al., 1992; Marom et al., 1981; Hemelaers et al., 2006). Over the past 3 decades, a number of new drugs known to modulate leukotriene pathway have been developed. Among them leukotriene receptor antagonists (LTRAs) are now commonly used in combination with inhaled corticosteroids to manage asthma symptoms (Montuschi and Peters-Golden, 2010). However, 35 to 78 % of patients receiving LTRAs therapy remain classified as non-responders (Lima et al., 2006). Two human CysLTs receptors, i.e. cysteinyl leukotriene 1 (CysLT₁) and cysteinyl leukotriene 2 (CysLT₂) receptors, have been identified and cloned (Lynch et al., 1999; Heise et al., 2000). Like CysLT₁ receptor, CysLT₂ receptor is expressed on airway smooth muscle cells (Heise et al., 2000), inflammatory cells (Figueroa et al., 2003; Mita et al., 2001; Mellor et al., 2003; Gauvreau et al., 2005) and vascular endothelial cells (Sjöström ~~M~~ et al., 2003, Duah et al., 2013). In addition, it has been shown that the CysLT₁ receptor antagonist ICI 198,615 does not completely inhibit high LTD₄ concentrations-induced contractions of guinea pig tracheal spiral preparations and that such contractions are inhibited by the CysLT₁/LT₂

receptor antagonist BAY u9773 in vitro (Bäck et al., 2001). These findings raise the possibility that CysLTs-induced asthmatic responses are mediated via both CysLT₁ and CysLT₂ receptors, and that a dual antagonist for CysLT₁/LT₂ receptors would be more useful for the treatment of asthma than current CysLT₁ specific LTRAs. Using CHO-K1 cells expressing human and guinea pig CysLT₂ and CysLT₁ receptors, we have found a series of indole derivatives with potent dual CysLT₁/LT₂ receptors antagonistic activity.

However, in vivo evaluation of antagonists targeting the CysLT₂ receptors is not an easy task, since guinea pig CysLT₂ receptors, unlike human CysLT₂ receptors, are preferentially stimulated by LTC₄ (not LTD₄), which is rapidly metabolized to LTD₄ in the body. It is therefore difficult to detect CysLT₂ receptor-mediated biological responses in guinea pigs (Orning and Hammarström, 1980; Ito et al., 2008). By inhibiting LTC₄ metabolism using S-hexyl glutathione (S-hexyl GSH), a GSH derivative (Bäck et al., 2001), we succeeded in establishing a guinea pig model of asthma in which bronchoconstriction and airway vascular hyperpermeability are mediated not only via CysLT₁ receptors but also via CysLT₂ receptors. Inhibition of LTC₄ metabolism increases tissue LTC₄ concentration in guinea pigs, which in turn results in a CysLT₂ receptor dependent biological response as evidenced by inhibition of LTC₄-induced bronchoconstriction by a specific antagonist for CysLT₂ receptors (Yonetomi et al., 2015).

Using the above established guinea pig model of asthma, we first show in this study that ONO-6950, an indole derivative, inhibits CysLT₁ and CysLT₂ receptor-mediated airway responses in vivo and then evaluate the effects of this compound on antigen-induced bronchoconstriction in sensitized guinea pigs, and compared these effects to those of montelukast, CysLT₁ selective receptor antagonist.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Japan SLC, Shizuoka, Japan) aged 6-7 weeks were used in this study. They were housed in an air-conditioned room maintained at 24 ± 2 °C and 55 ± 15 % relative humidity with alternating 12 h light/dark cycles and provided with food (LRC4, Oriental Yeast Co., Ltd., Japan) and tap water ad libitum. All animal experiments were approved by the Animal Ethical Committee of Ono pharmaceutical Co, Ltd., and performed in accordance with the institutional animal care guidelines.

2.2. Drugs and materials

ONO-6950 (Fig. 1), 4,4'-[4-Fluoro-7-(2-{4-[4-(3-fluoro-2-methylphenyl)butoxy]phenyl}ethynyl)-2-methyl-1H-indole-1,3-diyl]dibutanoic acid was synthesized in our laboratories. Montelukast was purchased from Sequoia Research Product Inc. (Pangbourne, United Kingdom). BayCysLT₂RA [1-(5-carboxy-2-{3-[4-(3-cyclohexylpropoxy)phenyl]propoxy}benzoyl)-4-piperidinecarboxylic acid] (Harter-M et al., 2006) was synthesized in our laboratories. Other materials used in this study were purchased commercially: LTC₄ and LTD₄ (Cayman Chemical Company, MI, USA), ovalbumin (OVA, grade V), S-hexylglutathione (S-hexyl GSH), indomethacin, pyrilamine maleate and fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA, Sigma-Aldrich, MO, USA), Formalin treated *Pseudomonas pertucinogena* Suspension (LSL Co., Ltd., Japan).

ONO-6950 (0.03 to 10 mg/kg) and montelukast (0.1 or 0.3 mg/kg), both suspended in 0.5 w/v% methylcellulose solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), were orally administered to guinea pigs 24 h before challenge. BayCysLT₂RA (1 mg/kg)

dissolved in physiological saline containing 1 vol% WellSolve (Celeste Corporation, Tokyo, Japan) was intravenously administered to guinea pigs approximately 1 min or immediately before challenge.

2.3. Preparation of cells stably expressing human and guinea pig CysLT₁ and CysLT₂ receptors

Full-length cDNAs encoding human CysLT₁ or CysLT₂ receptor were amplified from human spleen poly (A) + RNA by RT-PCR. A Kozak consensus sequence was artificially introduced, and PCR fragments encoding CysLT₁ receptor were subcloned into the mammalian expression vector pCI-neo (Promega Corporation). PCR fragments encoding CysLT₂ receptor were cloned into the mammalian expression vector pIRES-neo (Clontech Laboratories) with a Kozak consensus sequence and myc epitope tag sequence at the N-terminal end. Receptor sequences in the expression vectors were verified by sequencing and confirmed to be identical to GenBank accession number AF119711 for CysLT₁ receptor or AB038269 for CysLT₂ receptor. Both constructs were transfected by lipofection into CHO-K1 cells. After selection with geneticin, positive clones were identified by LTD₄-stimulated intracellular calcium response. Cell-lines expressing guinea pig CysLT₁ and CysLT₂ receptors were prepared as described previously (Yonetomi et al., 2015).

2.4. Calcium response assay

Intracellular calcium response was measured using Fura 2-AM (Dojindo) according to the manufacture's protocol. Briefly, CHO-K1 cells stably expressing human or guinea pig CysLT₁ and CysLT₂ receptors were cultured for at least 24 h (3×10^4 cells/well). After removal of the culture medium (Ham's F-12 supplemented with 10% fetal bovine serum

and 500 µg/ml geneticin), a loading medium containing 5 µM Fura 2-AM was added to each well. After 1 hour incubation at 37°C, the loading medium was removed, and the cells were further incubated at room temperature for 1 h in the assay buffer (Hanks' Balanced Salt Solution containing 20 mM HEPES). The cells were then alternately irradiated at two excitation wavelengths (340 and 380 nm) using FDSS-3000 (Hamamatsu Photonics Co., Ltd., Shizuoka, Japan) to measure the ratio of fluorescence intensity (f340/f380) at 500 nm. To evaluate test-compounds antagonism of CysLT₁/CysLT₂ receptors, ONO-6950 or montelukast was added 30 min before addition of the ligand, i.e. 100 nM LTD₄ for human CysLT₁ receptor, 0.3 nM LTD₄ for human CysLT₂ receptor, 10 nM LTD₄ for guinea pig CysLT₁ receptor and 3 nM LTC₄ for guinea pig CysLT₂ receptor.

2.5. Measurement of LTD₄ and LTC₄-induced bronchoconstriction

Ventilation pressure was measured using the method of Konzett & Rössler (Konzett and Rössler, 1940). Briefly, guinea pigs were anesthetized with intraperitoneal injection of pentobarbital sodium (75 mg/kg). One end of a polyethylene cannula was inserted into the trachea, and the other end was connected to a volume-limited ventilator (Model SN-480-7, Shinano Manufacturing Co., Ltd., Tokyo, Japan). Artificial ventilation was provided at a rate of 4 ml/stroke with 70 strokes/min. Another catheter was inserted into the jugular vein, securing the route for administration. Ventilation pressure was measured via a pneumotachometer (M·I·P·S Co., Ltd., Osaka, Japan) connected to a lateral port of the tracheal cannula, using a Win-PULMOS-III system (Version 3.6, M·I·P·S Co., Ltd., Osaka, Japan). After stabilization of basal ventilation pressure, LTD₄ (0.3 µg/kg) or LTC₄ (15 µg/kg) was intravenously administered via the catheter secured in the jugular vein.

For assessment of LTC₄-induced bronchoconstriction, S-hexyl GSH (15 mg/kg) was intravenously administered 10 s prior to LTC₄ injection. Ventilation pressure was measured for at least 10 min after LTD₄ or LTC₄ injection with the trachea completely blocked thereafter to obtain maximum ventilation pressure. After measurement of maximum ventilation pressure, artificial ventilation was stopped, and the animal was euthanized. The area under the percentage bronchoconstriction curve (AUC) from 0 to 10 min after LTD₄ or LTC₄ injection was calculated by the trapezoidal method, and mean percentage bronchoconstriction (%) was calculated by dividing AUC by measurement time.

2.6. Measurement of airway vascular hyperpermeability

Immediately after intravenous injection of FITC-BSA (2 mg/kg), the guinea pigs were intravenously given LTD₄ (1 µg/kg) or S-hexyl GSH (60 mg/kg) followed by LTC₄ (2 µg/kg). Fifteen minutes later, pentobarbital sodium (Somnopentyl®: 19.4 mg/animal) was intravenously administered, and the animals were exsanguinated by cutting first the femoral artery and vein and then the abdominal aorta and vena cava. The animals were then thoracotomized, and the trachea was cannulated. The whole lung was gently lavaged 5 times with 10 ml of ice-cold physiological saline containing 3.8 mg/ml sodium citrate acid via the tracheal cannula to collect bronchoalveolar lavage fluid (BALF). The BALF was centrifuged at 1870g for 5 min at 4°C (Hitachi Himac Centrifuge, Rotor RPRS3, Tokyo, Japan), and 200 micro liter of the resultant supernatant was transferred into a 96-well plate (Corning Incorporated) under protection from light. Fluorescence intensity (Ex: 485 nm/Em: 538 nm) was measured for each supernatant and calibration sample, using a microplate reader (Fmax, Nihon Molecular Devices Corporation, Tokyo, Japan) and its bundled software (SOFTmax PRO version 1.3.1f, Nihon Molecular Devices

Corporation).

2.7. Measurement of OVA-induced bronchoconstriction mediated by endogenous cysteinyl leukotrienes

On Day 0, guinea pigs were actively sensitized by intraperitoneal injection of saline containing 1 mg OVA and approximately 5×10^9 dead *Bordetella pertussis*. On Day 12, 20 μ l of a 30 mg/ml OVA solution was instilled in both eyes, and ocular symptoms were monitored for approximately 30 min to confirm sensitization. Animals with unilateral or bilateral hyperemia or eyelid swelling or both were considered to have been sensitized and used for measurement of bronchoconstriction.

On Day 15, 20 and 21, the sensitized animals were anesthetized, and ventilation pressure was measured according to the procedure described in 2.5. After confirmation of a stable basal ventilation pressure, a mixture of pyrilamine and indomethacin (1 and 5 mg/kg, respectively) was administered via the venous catheter to eliminate any contribution of endogenous histamine, prostaglandins or thromboxane A₂. Approximately 3 min later, the animals were intravenously treated with S-hexyl GSH (15 mg /kg), and then challenged by intravenous administration of OVA (0.2 mg/kg). Ventilation pressure was measured for at least 30 min after OVA injection with the trachea completely blocked thereafter to obtain maximum ventilation pressure. After measurement of maximum ventilation pressure, artificial ventilation was stopped and the animal was euthanized. The AUC from 0 to 30 min after OVA injection was calculated by the method described in 2.5.

2.8. Statistical analysis

Mean percentages of bronchoconstriction and FITC-BSA levels in the BALF are

expressed as mean $\pm$ S.E.M. Differences in mean values between 2 groups (normal vs. control, control vs. montelukast, control vs. BayCysLT₂RA and montelukast vs. montelukast + BayCysLT₂RA) were evaluated by t-test. Dunnett's test was used for evaluation of differences between ONO-6950 groups and the control group. To confirm the dose-dependency of ONO-6950 effect, test for slope of regression line was performed. For assessment of differences between montelukast and ONO-6950 groups, t-test was performed with a step-down closed testing procedure, starting from the high-dose ONO-6950 group. Data were statistically analyzed using SAS 9.1.3 Service Pack 4 (SAS Institute Japan, Tokyo, Japan) and the linked system EXSAS Version 7.5.2 (CAC EXICARE Corporation, Tokyo, Japan). Significance level was set at 0.05. IC₅₀ values are shown as estimated values with the corresponding 95% confidence intervals. Nonlinear regression analysis for estimation of the IC₅₀ values was performed by a 4-parameter logistic model using GraphPad Prism Ver. 5.01 (GraphPad Software. Inc., CA, USA).

3. Results

3.1 Calcium response assay

LTC₄, LTD₄ and LTE₄ elicited a concentration-dependent increase in intracellular calcium in CHO-K1 cells expressing CysLT₁ or CysLT₂ receptors with different potency. In addition, LTD₄-induced activation of CysLT₂ receptors was species-dependent. LTD₄ was rather more potent than other CysLTs in stimulating both human and guinea pig CysLT₁ receptors. On the other hands, LTD₄ stimulation of human CysLT₂ receptors was equivalent to that of LTC₄. LTC₄ was the only potent stimulant of guinea pig CysLT₂ receptors. Based on these results, a sub-maximum concentration of LTD₄ was selected to stimulate human CysLT₁, human CysLT₂, and guinea pig CysLT₁ receptors for evaluation of test-compounds antagonistic activity. LTC₄, on the other hand, was used to activate

guinea pig CysLT₂ receptors (data not shown).

Both ONO-6950 and montelukast inhibited human CysLT₁ receptor-mediated calcium response with IC₅₀ values of 1.7 and 0.46 nM, respectively (Table 1). ONO-6950 also inhibited human CysLT₂ receptor-mediated calcium response with an IC₅₀ value of 25 nM, whereas montelukast inhibition of this receptor was 3 orders less potent than that of human CysLT₁ receptors with an IC₅₀ value of 1800 nM. As observed with human CysLT₁ and CysLT₂ receptors, ONO-6950 potently inhibited both guinea pig CysLT₁ and CysLT₂ receptors with IC₅₀ values of 6.3 and 8.2 nM, respectively. Montelukast, on the other hand, preferentially inhibited guinea pig CysLT₁ receptors with an IC₅₀ value of 1.6 nM compared to 1800 nM for inhibition of guinea pig CysLT₂ receptors.

3.2. CysLT-induced bronchoconstriction

In the absence of S-hexyl GSH, LTD₄ (0.3 µg/kg) elicited bronchoconstriction in anesthetized guinea pigs. Montelukast (0.01 to 1 mg/kg, p.o.) dose-dependently attenuated LTD₄-induced bronchoconstriction with almost complete inhibition at 0.3 mg/kg or more. In contrast, the selective CysLT₂ receptor antagonist BayCysLT₂RA at 1 mg/kg, i.v. did not inhibit LTD₄-induced bronchoconstriction (data not shown), indicating that LTD₄-induced bronchoconstriction in normal guinea pigs depends on a CysLT₁-mediated pathway. ONO-6950 (0.03 to 3 mg/kg, p.o.) dose-dependently inhibited LTD₄-induced bronchoconstriction with maximum effect at 1 mg/kg (Fig. 2).

In the presence of S-hexyl GSH (15 mg/kg), LTC₄ (15 µg/kg) elicited bronchoconstriction in anesthetized guinea pigs. Treatment with montelukast or BayCysLT₂RA partially inhibited this bronchoconstriction, while combination of montelukast and BayCysLT₂RA fully abrogated animals' spasmogenic response (Fig.3). These findings indicate that LTC₄-induced bronchoconstriction in S-hexyl GSH-treated

guinea pigs involves both CysLT₁ and CysLT₂ pathways. ONO-6950 at 0.03 to 10 mg/kg, p.o. dose-dependently attenuated LTC₄-induced bronchoconstriction with almost complete inhibition at 3 mg/kg. The inhibitory effect of ONO-6950 on LTC₄-induced bronchoconstriction was significantly stronger than that of montelukast at the dose of 1 mg/kg or more (Fig. 3).

3.3. CysLT-induced airway vascular hyperpermeability

In the absence of S-hexyl GSH, LTD₄ (1 µg/kg) induced airway vascular hyperpermeability in conscious guinea pigs. Montelukast (0.03 mg/kg, p.o.) completely inhibited this hyperpermeability. ONO-6950 (0.03 to 1 mg/kg, p.o.) dose-dependently attenuated LTD₄-induced airway vascular hyperpermeability with complete inhibition at 0.3 mg/kg (Fig. 4).

In conscious guinea pigs treated with S-hexyl GSH (60 mg/kg), LTC₄ (2 µg/kg) induced airway vascular hyperpermeability. Montelukast at 0.1 mg/kg did not inhibit this hyperpermeability, although at the same dose this drug fully abrogated LTD₄-induced airway vascular hyperpermeability in S-hexyl GSH untreated guinea pigs. BayCysLT₂RA at 1 mg/kg, i.v. inhibited LTC₄-induced hyperpermeability to a level comparable to that of normal guinea pigs (no LTC₄ treatment) (Fig.5). ONO-6950 at 0.1 to 3 mg/kg, p.o. dose-dependently inhibited LTC₄-induced airway vascular hyperpermeability with statistical significance at 3 mg/kg.

3.4. OVA-induced bronchoconstriction involving endogenous cysteinyl leukotrienes

All experiments were conducted under anesthesia. OVA-sensitized guinea pigs were treated with pyrilamine and indomethacin to elicit endogenous cysLTs-mediated bronchoconstriction and with S-hexyl GSH (15 mg/kg) to suppress metabolism of LTC₄

to LTD₄ before intravenous administration of OVA as antigen challenge. Antigen challenge induced strong bronchoconstriction, as indicated by increased airway resistance. Compared with antigen-induced bronchoconstriction in normal guinea pigs, a slow gradual bronchoconstriction was observed in S-hexyl GSH-treated guinea pigs (data not shown). Montelukast at 0.3 mg/kg, p.o. or BayCysLT₂RA at 1 mg/kg, i.v antigen-induced bronchoconstriction was partially, but significantly, inhibited antigen-induced bronchoconstriction. Combination therapy with both compounds additively inhibited this bronchoconstriction. ONO-6950 at 0.1 to 3 mg/kg, p.o. dose-dependently inhibited OVA-induced bronchoconstriction. The inhibitory effect of ONO-6950 at 3 mg/kg was significantly greater than that of montelukast alone and comparable to that of combination therapy with montelukast and BayCysLT₂RA (Fig. 6).

4. Discussion

In the present study, we demonstrated that ONO-6950 fully suppresses not only LTD₄-induced bronchoconstriction (through the CysLT₁ receptor) in normal guinea pigs, but also LTC₄-induced bronchoconstriction and airway vascular hyperpermeability (mainly through the CysLT₂ receptor) in S-hexyl GSH-treated guinea pigs. In addition, we showed that ONO-6950 has stronger effect on antigen-induced asthmatic response than the selective CysLT₁ receptor antagonist montelukast in S-hexyl GSH-treated guinea pigs. These findings suggest that ONO-6950 is an orally active CysLT₁ and CysLT₂ dual antagonist that may provide a novel therapeutic option for patients with asthma.

Cysteinyl leukotrienes contribute to multiple aspects of asthma pathophysiology (Peters-Golden and Henderson, 2007; Singh et al., 2013). CysLT₁ receptor antagonists are now widely prescribed for the treatment of asthma (Montuschi and Peters-Golden, 2010; Matsuse and Kohno, 2014). However, implication of CysLT₂ receptor in the

pathophysiology of asthma is still unclear despite the fact that CysLT₂ receptors, like CysLT₁ receptors, are expressed on airway smooth muscle cells (Heise et al., 2000), inflammatory cells (Figuroa et al, 2003; Mita et al., 2001; Mellor et al., 2003; Gauvreau et al., 2005), and vascular endothelial cells (Sjöström et al., 2003; Duah-E et al., 2013). Studies on the involvement of CysLT₂ receptors in the pathophysiology of asthma have been hampered by lack of appropriate animal models. However, we have recently reported that exogenously administered LTC₄ can elicit CysLT₂-mediated bronchoconstriction and airway vascular hyperpermeability in S-hexyl GSH-treated guinea pigs (Yonetomi et al., 2015). In this study, antigen challenge induced CysLT₂ receptor-mediated bronchoconstriction in sensitized guinea pigs when S-hexyl GSH was administered before the challenge. These results suggest that S-hexyl GSH may be used to control the metabolism of endogenous leukotrienes and keep LTC₄ concentration high in target organs, allowing investigation of CysLT₂ receptors involvement in asthma pathophysiology.

ONO-6950 antagonized intracellular calcium signaling via human and guinea pig CysLT₁ and CysLT₂ receptors with IC₅₀ values of 1.7 and 25 nM, respectively (human receptors) and 6.3 and 8.2 nM, respectively (guinea pig receptors). ONO-6950 at 1 or 0.3 mg/kg, p.o. fully attenuated LTD₄-induced bronchoconstriction and airway vascular hyperpermeability, respectively. These LTD₄-induced airway responses were mediated via CysLT₁ receptors, because montelukast fully attenuated both responses. ONO-6950 at 3 mg/kg, p.o. or more almost completely inhibited LTC₄-induced bronchoconstriction and airway vascular hyperpermeability in S-hexyl GSH-treated guinea pigs. These LTC₄-induced airway responses were largely mediated via CysLT₂ receptors, because montelukast showed only partial or negligible inhibition of these responses, while the CysLT₂ receptor antagonist BayCysLT₂RA strongly inhibited both events. Although

higher doses were needed to inhibit CysLT₂-mediated response compared to CysLT₁-mediated response, ONO-6950 inhibited both CysLT₁ and CysLT₂ receptor-mediated airway responses at a similar dose range. These results clearly demonstrate that ONO-6950 antagonizes CysLT₁ and CysLT₂ receptors both in vitro and in vivo.

ONO-6950 inhibited OVA-induced bronchoconstriction involving endogenous cysteinyl leukotrienes in sensitized guinea pig treated with S-hexyl GSH. The effect of ONO-6950 was significantly greater than that of montelukast alone and comparable to that of combination therapy with montelukast and BayCysLT₂RA. These results indicate that ONO-6950 inhibits asthmatic responses mediated via both CysLT₁ and CysLT₂ receptors. As previously reported, we found a species difference between human and guinea pig in the reactivity of CysLT₂ receptor to natural ligands. LTC₄ is the only potent stimulator of CysLT₂ receptors in guinea pigs, whereas LTC₄ and LTD₄ are equally potent stimulators of CysLT₂ receptors in human (Ito et al., 2008; Heise et al., 2000). Therefore, it would be reasonable to speculate that both CysLT₁ and CysLT₂ receptors are activated by endogenous CysLTs in the lung of asthmatic patients. If so, ONO-6950 would provide a new therapeutic approach for asthmatic patients.

As mentioned above, CysLT₂ receptors, like the CysLT₁ receptors, can mediate bronchoconstriction and airway vascular hyperpermeability in guinea pigs. However, CysLT₂-mediated airway vascular hyperpermeability seems to be minor compared to the CysLT₁-mediated one, because the maximal response induced by LTC₄ in the presence of S-hexyl GSH was smaller than that induced by LTD₄ or LTC₄ in the absence of S-hexyl GSH (data not shown). On the contrary, CysLT₂-mediated bronchoconstriction seems to be major compared to the CysLT₁ receptor-induced one, because bronchoconstriction induced by LTC₄ in the presence of S-hexyl GSH was more severe and sustained than

that induced by LTC₄ in the absence of S-hexyl GSH (Yonetomi et al., 2015). The results of bronchoconstriction and airway vascular hyperpermeability in S-hexyl GSH-treated animals seem to be controversial. Although the reason for this controversy remain unclear, our observations suggest that CysLT₁ and CysLT₂ receptors contribution to asthmatic responses is triggered via different pathways. With regards to bronchoconstriction, we are currently investigating the possibility that CysLT₂ receptors contribute to peripheral airway obstruction, because activation of CysLT₂ pathway induces more prominent lung collapse in anesthetized guinea pigs (unpublished data). Further studies are needed to elucidate the different action of CysLT₁ and CysLT₂ receptors in various airway responses.

In conclusion, ONO-6950 is a novel orally active dual CysLT₁/LT₂ receptor antagonist that reduces asthmatic responses mediated by both CysLT₁ receptor and CysLT₂ receptors. More importantly, ONO-6950 suppressed asthmatic responses that were not sufficiently attenuated by treatment with montelukast. These results indicate that ONO-6950 may provide a novel therapeutic option for patients with asthma.

5. References

- Bäck M., Kumlin M., Cotgreave I.A., Dahlén S.E., 2001. An alternative pathway for metabolism of leukotriene D₄: effects on contractions to cysteinyl-leukotrienes in the guinea-pig trachea. *Br J Pharmacol.* 133, 1134-1144.
- Duah E, Adapala RK, Al-Azzam N, Kondeti V, Gombedza F, Thodeti CK, Paruchuri S., 2013. Cysteinyl leukotrienes regulate endothelial cell inflammatory and proliferative signals through CysLT₂ and CysLT₁ receptors., *Sci Rep.* 3, 3274.
- Figuroa D.J., Borish L., Baramki D., Philip G., Austin C.P., Evans J.F., 2003. Expression of cysteinyl leukotriene synthetic and signalling proteins in inflammatory cells in active

seasonal allergic rhinitis. *Clin Exp Allergy*. 33, 1380-1388.

FitzGerald J.M., Shahidi N., 2010. Achieving asthma control in patients with moderate disease. *J Allergy Clin Immunol*. 125, 307-311.

Gauvreau G.M., Plitt J.R., Baatjes A., MacGlashan D.W., 2005. Expression of functional cysteinyl leukotriene receptors by human basophils. *J. Allergy Clin. Immunol*. 116, 80-87.

Heise C.E., O'Dowd B.F., Figueroa D.J., Sawyer N., Nguyen T., Im D.S., Stocco R., Bellefeuille J.N., Abramovitz M., Cheng R., Williams D.L. Jr, Zeng Z., Liu Q., Ma L., Clements M.K., Coulombe N., Liu Y., Austin C.P., George S.R., O'Neill G.P., Metters K.M., Lynch K.R., Evans J.F., 2000. Characterization of the human cysteinyl leukotriene 2 receptor. *J Biol Chem*. 275, 30531-30536.

Harter M., Erguden J., Wunder F., Tinel H., Kobberling J., Becker E.M., Munter K., Schlemmer K.H., Kast R., Kolkhof P., Karig G., Barfacker L., Hubsch W., Schuhmacher J., Zuleger S., Concepcion A., Shimizu H., 2006. Isophthalic acid derivatives. US20060154912, (Example 31).

Hemelaers L., Henket M., Sele J., Bureau F., Louis R., 2006. Cysteinyl-leukotrienes contribute to sputum eosinophil chemotactic activity in asthmatics. *Allergy*. 61, 136-139.

Ito Y., Hirano M., Umemoto N., Zang L., Wang Z., Oka T., Shimada Y., Nishimura Y., Kurokawa I., Mizutani H., Tanaka T., 2008. Guinea pig cysteinyl leukotriene receptor 2 (gpCysLT2) mediates cell proliferation and intracellular calcium mobilization by LTC₄ and LTD₄. *BMB Rep*. 41, 139-145.

Konzett H., Rössler R., 1940. Versuchsanordnung zu untersuchungen an der Bronchial-muskulatur. *Arch Exp Pathol Pharmacol*. 195, 71-74.

Lima J.J., Zhang S., Grant A., Shao L., Tantisira K.G., Allayee H., Wang J., Sylvester J., Holbrook J., Wise R., Weiss S.T., Barnes K., 2006. Influence of leukotriene pathway

polymorphisms on response to montelukast in asthma. *Am J Respir Crit Care Med.* 173, 379-385.

Lynch K.R., O'Neill G.P., Liu Q., Im D.S., Sawyer N., Metters K.M., Coulombe N., Abramovitz M., Figueroa D.J., Zeng Z., Connolly B.M., Bai C., Austin C.P., Chateauneuf A., Stocco R., Greig G.M., Kargman S., Hooks S.B., Hosfield E., Williams D.L. Jr, Ford-Hutchinson A.W., Caskey C.T., Evans J.F., 1999. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature.* 399, 789-793.

Marom Z., Shelharmer J.H., Bach M.K., Morton D.R. and Kaliner M., 1982. Slow-reacting substances of anaphylaxis, leukotrienes C4 and D4 increase the release of mucus from human airways in vitro. *Am. Rev. Respir. Dis.* 126, 449-451.

Martin R.J., 2003. Considering therapeutic options in the real world. *J Allergy Clin Immunol.*, 112, S112-115.

Matsuse H., Kohno S., 2014. Leukotriene receptor antagonists pranlukast and montelukast for treating asthma. *Expert Opin Pharmacother.* 15, 353-363.

Mellor E.A., Frank N., Soler D., Hodge M.R., Lora J.M., Austen K.F., Boyce J.A., 2003. Expression of the type 2 receptor for cysteinyl leukotrienes (CysLT2R) by human mast cells: Functional distinction from CysLT1R. *Proc Natl Acad Sci U S A.* 100, 11589-11593.

Mita H., Hasegawa M., Saito H., Akiyama K., 2001. Levels of cysteinyl leukotriene receptor mRNA in human peripheral leucocytes: significantly higher expression of cysteinyl leukotriene receptor 2 mRNA in eosinophils. *Clin Exp Allergy.* 31, 1714-1723.

Montuschi P., Peters-Golden, M.L., 2010. Leukotriene modifiers for asthma treatment. *Clin. Exp. Allergy.* 40, 1732-1741.

~~Montuschi P., Peters-Golden M.L., 2010. Leukotriene modifiers for asthma treatment. Clin Exp Allergy. 40, 1732-1741.~~

- Orning L., Hammarström S., 1980. Inhibition of leukotriene C and leukotriene D biosynthesis. *J Biol Chem.* 255, 8023-8026.
- Nakagawa N., Obata T., Kobayashi T., Okada Y., Nakagawa N., Terawaki T., Aishita H., 1992. In vivo pharmacologic profile of ONO-1078: a potent, selective and orally active peptide leukotriene (LT) antagonist. *Jpn J Pharmacol.* 60, 217-225.
- Peters-Golden M., Henderson WR Jr., 2007. Leukotrienes., *N Engl J Med.* 357, 1841-1854.
- Samuelsson B., 1983. Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. *Science.*, 220, 568-575.
- Singh R.K., Tandon R., Dastidar S.G., Ray A., 2013. A review on leukotrienes and their receptors with reference to asthma. *J Asthma.* 50, 922-931.
- Sjöström M., Johansson A.S., Schröder O., Qiu H., Palmblad J., Haeggström J.Z., 2003. Dominant expression of the CysLT2 receptor accounts for calcium signaling by cysteinyl leukotrienes in human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol.* 23, e37-41.
- Yonetomi Y., Sekioka T., Kadode M., Kitamine T., Kamiya A., Matsumura N., Fujita M., Kawabata K., 2015. Leukotriene C₄ induces bronchoconstriction and airway vascular hyperpermeability via the Cysteinyl leukotriene receptor 2 in S-hexyl glutathione-treated guinea pigs. *Eur J Pharmacol.* 754, 98-104.

Figures Legends

Fig.1 Chemical structure of ONO-6950

Fig.2 Effects of ONO-6950 and montelukast on LTD₄-induced bronchoconstriction

Anesthetized guinea pigs were challenged intravenously with LTD₄ (0.3 µg/kg). ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before LTD₄ challenge. Vertical axis represents mean percentage bronchoconstriction for 10 min following LTD₄ injection. Each column represents the mean ± S.E.M. for 8 animals.

, P < 0.01, *, P < 0.001: Dunnett's test vs. control group, \$\$\$; P < 0.001: t-test vs. control group.

Fig.3 Effects of ONO-6950, montelukast, BayCysLT₂RA and combination of montelukast and BayCysLT₂RA on LTC₄-induced bronchoconstriction

Anesthetized guinea pigs treated with S-hexyl GSH (15 mg/kg) were challenged intravenously with LTC₄ (15 µg/kg). ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before LTC₄ challenge. BayCysLT₂RA, a cysLT₂ receptor antagonist, was intravenously administered 1 min before LTC₄ challenge. Vertical axis represents mean percentage bronchoconstriction for 10 min following LTC₄ injection. Each column represents the mean ± S.E.M. for 10 animals.

\$\$\$; P < 0.001: t-test vs. control group, #; P < 0.05, ##; P < 0.01, ###; P < 0.001: t-test vs. montelukast group, **, P < 0.01, ***, P < 0.001: Dunnett's test vs. control group.

Fig.4 Effects of ONO-6950 and montelukast on LTD₄-induced airway vascular

hyperpermeability

LTD₄ (1 µg/kg) was intravenously administered to conscious guinea pigs treated with FITC-BSA, except for the Normal group. Fifteen minutes later, BALF was collected to determine FITC-BSA level. ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before LTD₄ challenge. Each column represents the mean ± S.E.M. for 17-18 animals.

††; P < 0.01: t-test vs. normal group, \$\$; P < 0.01: t-test vs. control group, **; P < 0.01, ***; P < 0.001: Dunnett's test vs. control group.

Fig.5 Effects of ONO-6950, montelukast, BayCysLT₂RA and combination of montelukast and BayCysLT₂RA on LTC₄-induced airway vascular hyperpermeability

LTC₄ (2 µg/kg) was intravenously administered to conscious guinea pigs treated with FITC-BSA and S-hexyl GSH (60 mg/kg), except for the Normal group. Fifteen minutes later, BALF was collected to determine FITC-BSA level. ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before LTC₄ injection. BayCysLT₂RA was intravenously administered just before LTC₄ challenge. Each column represents the mean ± S.E.M. for 18 animals.

†; P < 0.05: t-test vs. normal group, \$; P < 0.05: t-test vs. control group, *; P < 0.05: Dunnett's test vs. control group.

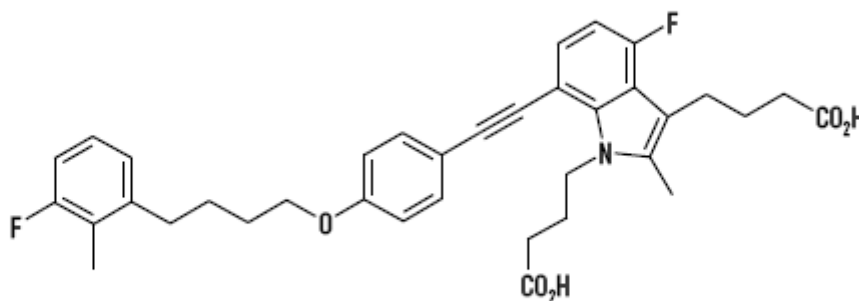
Fig.6 Effects of ONO-6950, montelukast, BayCysLT₂RA and combination of montelukast and BayCysLT₂RA on OVA-induced bronchoconstriction

Sensitized guinea pigs were treated with S-hexyl GSH (15 mg/kg) and challenged intravenously with OVA (0.2 mg/kg). ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before OVA challenge. BayCysLT₂RA, a cysLT₂ receptor

antagonist, was intravenously administered 1 min before OVA challenge. Vertical axis represents mean percentage bronchoconstriction for 30 min following OVA injection. Each column represents the mean $\pm$ S.E.M. for 20 animals.

\$; $P < 0.05$, \$\$; $P < 0.01$: t-test vs. control group. #; $P < 0.05$, ##; $P < 0.01$: t-test vs. montelukast group. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$: Dunnett's test vs. control group.

Fig. 1



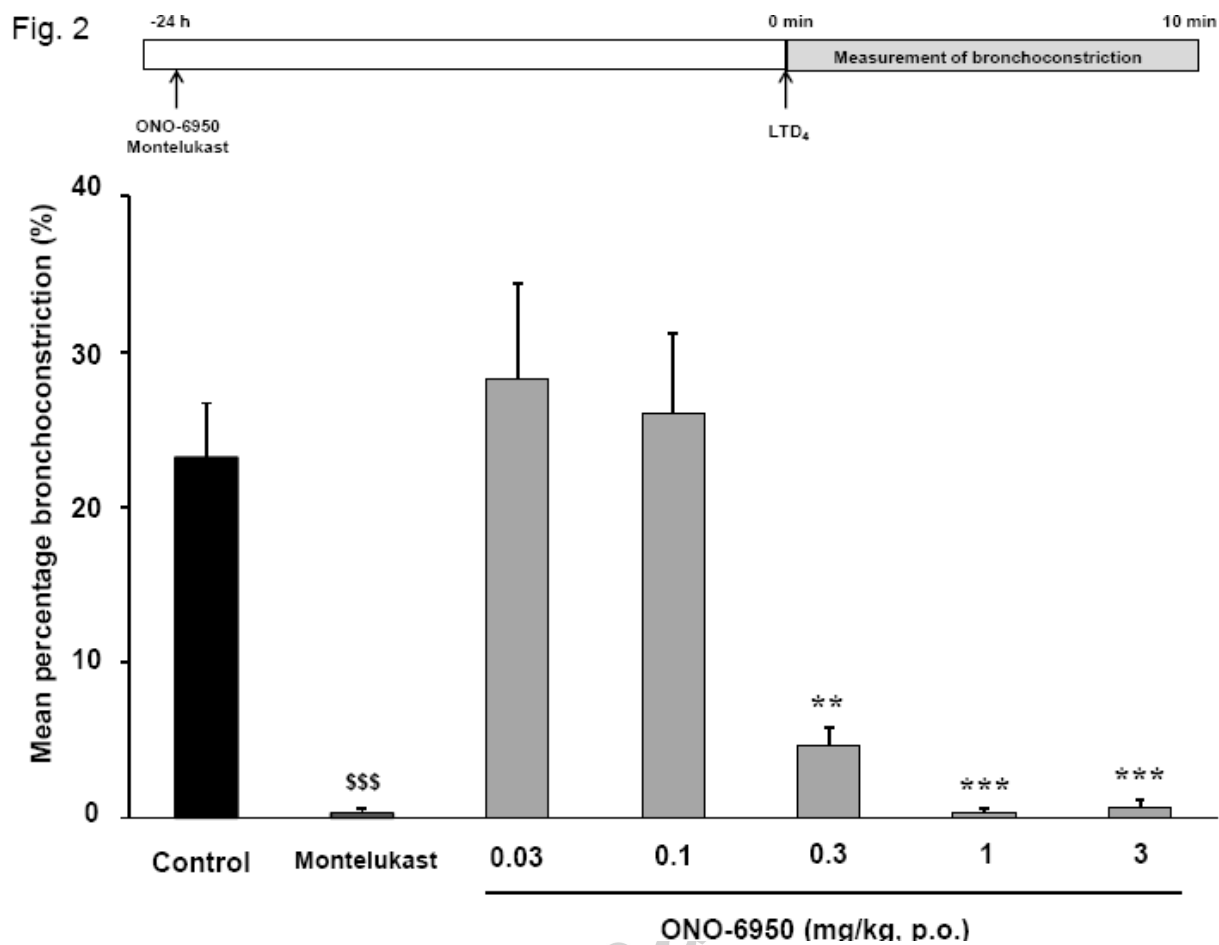


Fig. 3

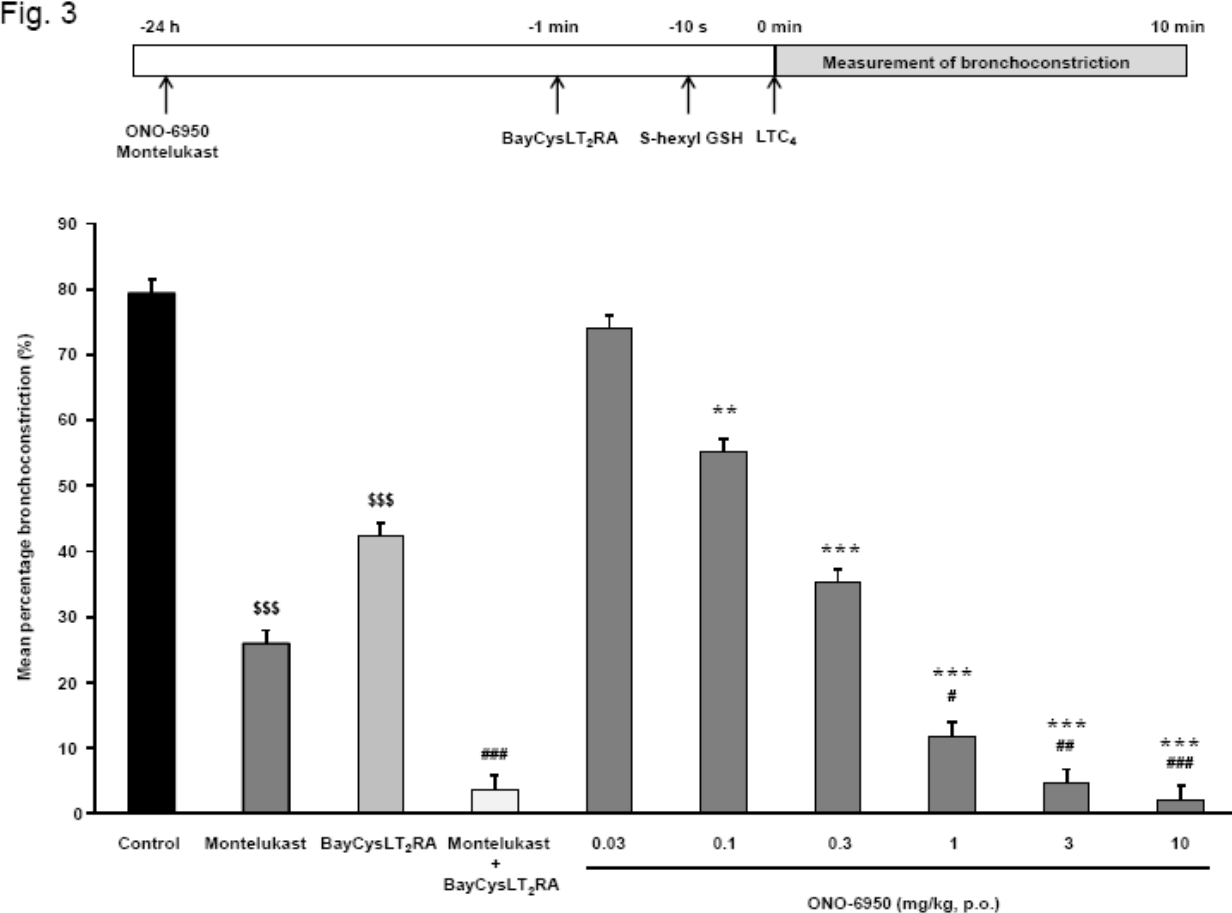


Fig. 4

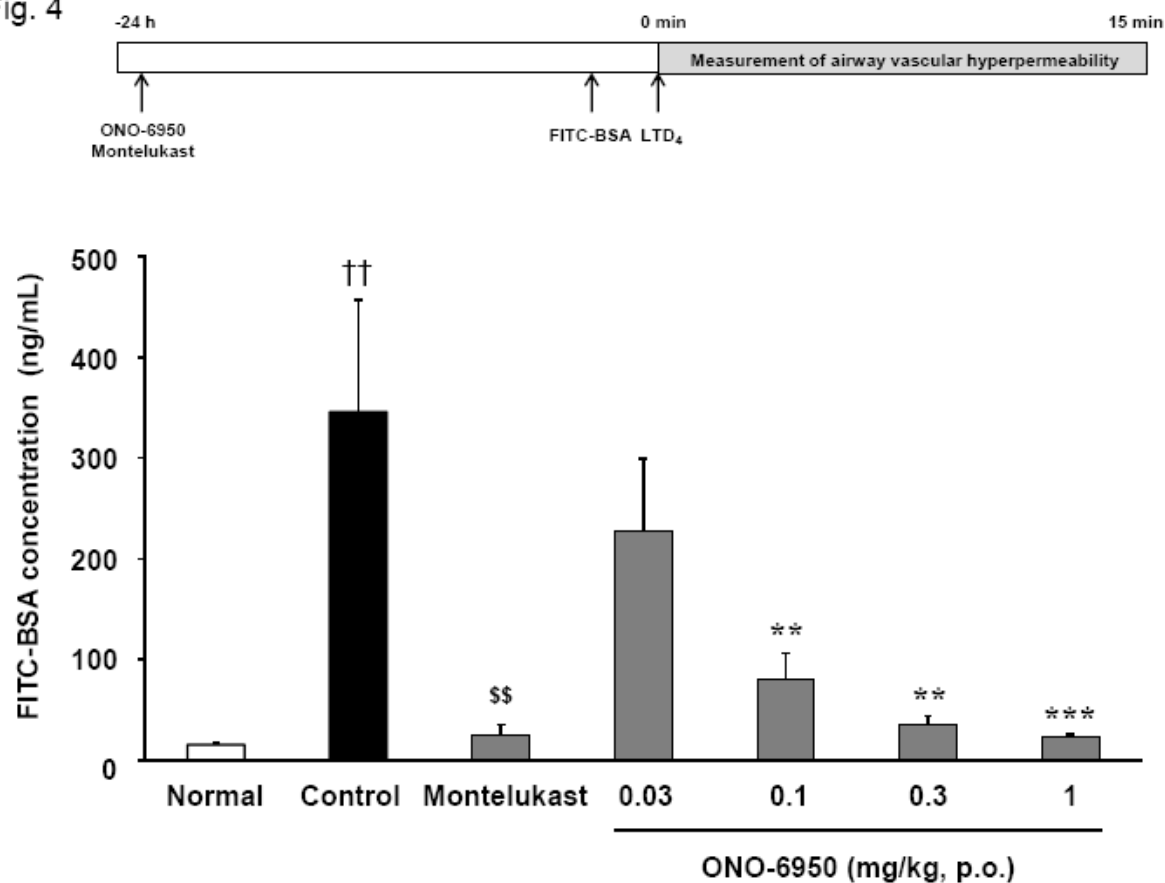


Fig. 5

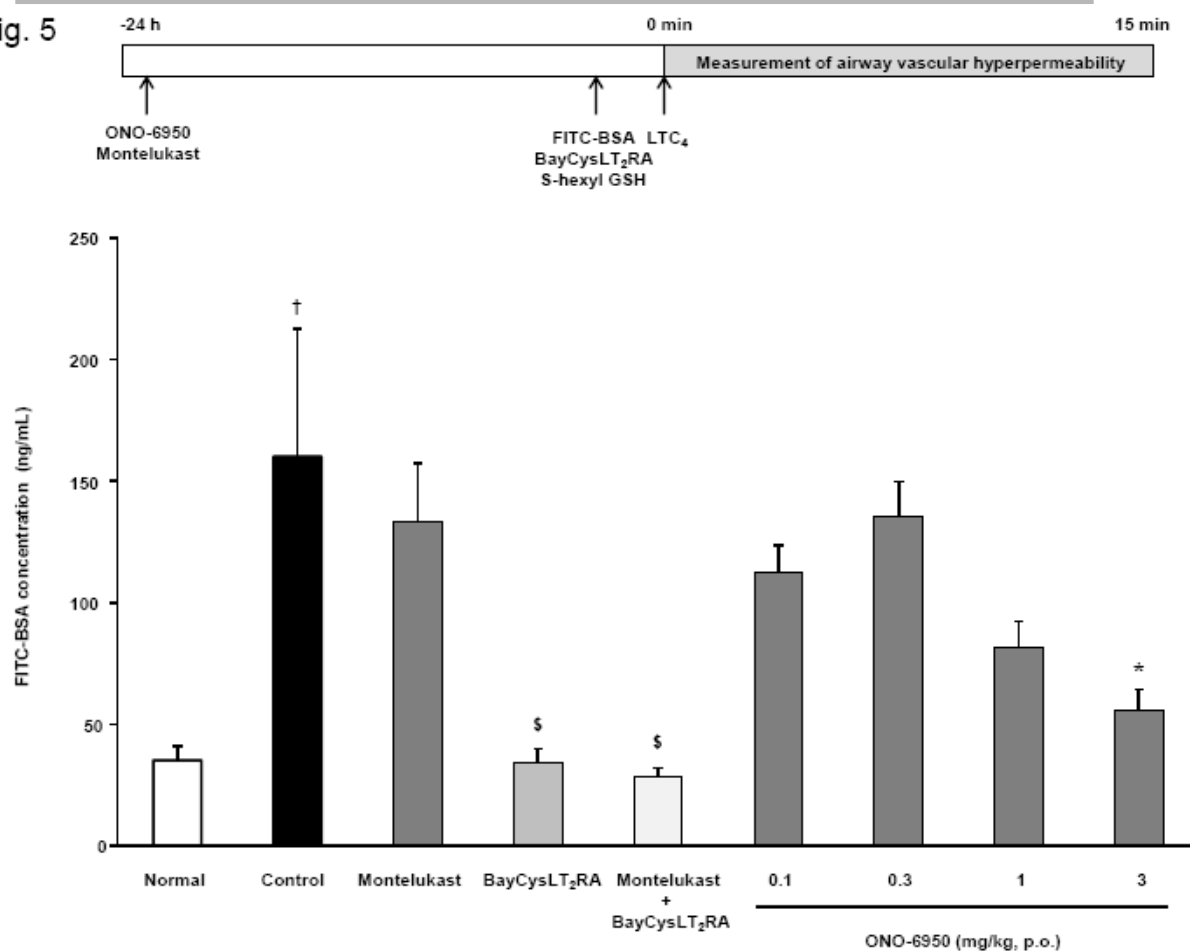


Fig. 6

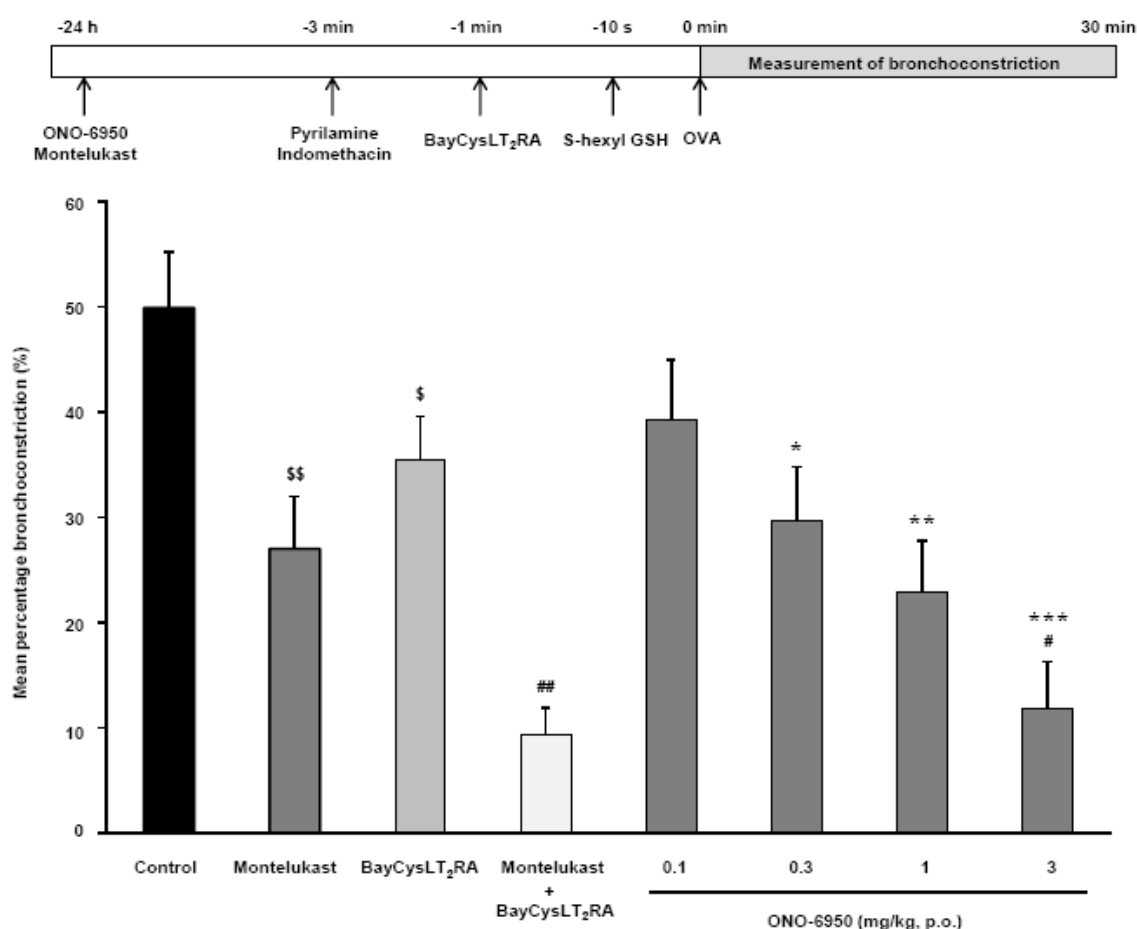


Table 1 IC₅₀ values of ONO-6950 and montelukast for inhibition of CysLT₁ and CysLT₂ receptor-mediated intracellular calcium response

Receptor	IC ₅₀ (nM)	
	ONO-6950	Montelukast
Human CysLT ₁ receptor	1.7 (1.2-2.4)	0.46 (0.37-0.58)
Human CysLT ₂ receptor	25 (19-32)	1800 (1500-2100)
Guinea pig CysLT ₁ receptor	6.3 (5.3-7.5)	1.6 (1.1-2.2)
Guinea pig CysLT ₂ receptor	8.2 (6.8-9.9)	1800 (1600-2000)

Fura 2-AM-loaded CHO-K1 cells expressing human CysLT₁ or CysLT₂ receptor were stimulated with 100 nM LTD₄, for the CysLT₁ receptor, or 0.3 nM LTD₄, for the CysLT₂ receptor. Fura 2-AM-loaded CHO-K1 cells expressing guinea pig CysLT₁ or CysLT₂

receptor were stimulated with 10 nM LTD₄, for the CysLT₁ receptor, or 3 nM LTC₄ for the CysLT₂ receptor. IC₅₀ values were estimated by the 4-parameter logistic model using data from 5 separate experiments. Parameters at the top and bottom were constrained by 100 and 0, respectively. Values indicated in parentheses represent 95% confidence intervals of the IC₅₀ values.

Accepted manuscript