Drug Discovery Research of Novel CysLT$_1$/LT$_2$ Receptor Antagonist for the New Treatment of Asthma

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1 CHAPTER I：INTRODUCTION

1.1 General 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 and Shahidi, 2010). Those patients were prescribed with cysteinyl leukotriene 1 receptor antagonists (LTRA) or long-acting β2-agonists in addition to inhaled corticosteroids.

Cysteinyl leukotrienes (CysLTs: i.e.; LTC_4, LTD_4 and LTE_4) 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., 1982; Hemelaers et al., 2006). Over the past 3 decades, a number of new drugs known to modulate leukotriene pathway have been developed. Among them 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 ($CysLT_1$) and cysteinyl leukotriene 2 ($CysLT_2$) receptors, have been identified and cloned (Lynch et al., 1999; Heise et al., 2000). Like $CysLT_1$ receptor, $CysLT_2$ 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 et al., 2003, Duah et al., 2013). In addition, it has been shown that the $CysLT_1$ receptor

antagonist ICI 198,615 does not completely inhibit high LTD_4 concentrations-induced contractions of guinea pig tracheal spiral preparations and that such contractions are inhibited by the $CysLT_1/LT_2$ receptor antagonist BAY u9773 *in vitro* (Bäck et al., 2001). These findings raise the possibility that CysLTs-induced asthmatic responses are mediated via both $CysLT_1$ and $CysLT_2$ receptors, and that a dual antagonist for $CysLT_1/LT_2$ receptors would be more useful for the treatment of asthma than current $CysLT_1$ specific LTRAs. Therefore, we undertook a drug discovery research of novel $CysLT_1/LT_2$ receptor antagonist for the new treatment of asthma.

Using CHO-K1 cells expressing human and guinea pig $CysLT_1$ and $CysLT_2$ receptors, we have found a series of indole derivatives with potent dual $CysLT_1/LT_2$ receptors antagonistic activity and selected ONO-6950 as a candidate compound. However, *in vivo* evaluation of the compounds for their antagonistic activities against $CysLT_2$ receptors was not an easy task in guinea pigs, frequently used animals for development of asthma therapeutic agent. Because guinea pig $CysLT_2$ receptors are preferentially stimulated by LTC_4, but LTC_4 is rapidly metabolized to LTD_4 by metabolizing enzymes, such as gamma-glutamyl transpeptidase (γ-GTP) and gamma-glutamyl leukotrienase in the body (Orning and Hammarström, 1980; Ito et al., 2008; Snyder et al., 1984; Lieberman et al., 1999; Han et al., 2002). In contrast, the human $CysLT_2$ receptors are stimulated by both LTC_4 and LTD_4 (Lynch et al., 1999; Heise et al., 2000). Thus lack of appropriate animal models has hampered so far the study on implication of $CysLT_2$ receptors in asthma. By using S-hexyl GSH, a GSH derivative (Bäck et al., 2001) to suppress LTC_4 metabolism, we addressed establishment of a guinea pig asthma model in which bronchoconstriction and airway vascular hyperpermeability are mediated not only via $CysLT_1$ receptors but also via $CysLT_2$ receptors. Under such conditions we confirmed that exogenously administered LTC_4 induces $CysLT_2$-mediated airway responses in guinea pigs.

In the above guinea pig model of asthma with or without S-hexyl GSH, we evaluated the effects of ONO-6950 on either of $CysLT_1$ and $CysLT_2$ receptor-mediated airway responses and compared them with those of montelukast, a $CysLT_1$ selective receptor antagonist. Additionally, we evaluated the effects of ONO-6950 on antigen-induced bronchoconstriction in S-hexyl GSH-treated guinea pigs, and compared these effects to that of montelukast. Furthermore, in chronic asthma model we examined the effects of ONO-6950 and montelukast on late phase airway resistance characterized by β_2-stimulant-resistant persistent airway inflammation in S-hexyl GSH-treated guinea pigs.

Through these studies we investigated the involvement of $CysLT_2$ receptor in asthma and possibility of $CysLT_1/LT_2$ dual receptor antagonists as a new treatment for asthma.

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2 CHAPTER II：Leukotriene C_4 induces bronchoconstriction and airway vascular hyperpermeability via the Cysteinyl leukotriene receptor 2 in S-hexyl glutathione-treated guinea pigs

2.1 Introduction

Cysteinyl leukotrienes (CysLTs, including LTC_4, LTD_4 and LTE_4) play an important role in both the bronchoconstriction and airway inflammation associated with asthma. Recent advances in molecular biology have shown that CysLTs act via two G protein-coupled receptors known as cysteinyl leukotriene 1 ($CysLT_1$) (Lynch et al., 1999; Sarau et al., 1999) and cysteinyl leukotriene 2 ($CysLT_2$) receptors (Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000) and that both LTD_4 and LTC_4 equally bind to the human $CysLT_2$ receptor (Heise et al., 2000). Like the $CysLT_1$ receptor, the $CysLT_2$ 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., 2000) and vascular endothelial cells (Sjöström et al., 2003), suggesting that this receptor may also be involved in asthma. However, studies showing that a $CysLT_1$ receptor antagonist almost completely inhibits LTD_4- or LTC_4-induced bronchoconstriction in guinea pig (Nakagawa et al., 1992; Muraki et al., 2009) undermine the physiological importance of $CysLT_2$ receptors in asthma.

On the other hand, cDNA encoding the guinea pig $CysLT_2$ receptor has been cloned (Ito et al., 2008), and agonist-stimulated signal transduction has clearly shown species difference in the reactivity of $CysLT_2$ receptor natural ligands between human and guinea pig. Whereas LTD_4 is a potent ligand not only for the $CysLT_1$ receptor, but also for the $CysLT_2$ receptor in humans, it is much less potent than LTC_4 for the guinea pig-$CysLT_2$ receptor (Ito et al., 2008). Since LTC_4 is rapidly transformed to LTD_4 by metabolizing

enzymes, such as gamma-glutamyl transpeptidase (γ-GTP) (Orning et al., 1980; Snyder et al., 1984) and gamma-glutamyl leukotrienase (Lieberman et al., 1999; Han et al., 2002) in the body, it would be very difficult to induce $CysLT_2$ receptor-mediated biological responses in guinea pigs. Such species difference may hamper studies on the role of $CysLT_2$ receptors in asthma and other diseases.

To induce $CysLT_2$ receptor-mediated biological response in guinea pigs, we hypothesized that it is necessary to block LTC_4 metabolism to LTD_4, thereby maintaining enough LTC_4 at the target organ. In our experiment, we focused on S-hexyl glutathione (S-hexyl GSH), a known synthetic substrate of γ-GTP (Bäck et al., 2001) and assumed that this substrate can be metabolized in place of LTC_4 since, like LTC_4, it has an L-γ-glutamyl-L-cysteinyl-glycine moiety (Bäck, 2002). Using S-hexyl GSH, we investigated in this study conditions under which $CysLT_2$ receptor-mediated bronchial response might be brought about in guinea pigs. Through the experiments conducted in this study, we succeeded in developing a guinea pig model of asthma mediated by $CysLT_2$ receptors alone or by both $CysLT_2$ and $CysLT_1$ receptors, depending on the dose of S-hexyl GSH. Our results clearly elucidate the contribution of $CysLT_2$ receptors in asthma-associated bronchoconstriction and airway vascular hyperpermeability *in vivo*.

2.2 Materials and methods

2.2.1 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.

2.2.2 Drugs and materials

Montelukast was obtained from Sequoia Research Products, Ltd. (Pangbourne, United Kingdom). Compound A; 1-(5-carboxy-2-{3-[4-(3-cyclohexylpropoxy)phenyl] propoxy}benzoyl)-4-piperidinecarboxylic acid was synthesized in our laboratories and used as $CysLT_2$ receptor antagonist. Others materials used in this study were purchased commercially: LTC_4, LTD_4, LTE_4, LTC_4-d5, LTD_4-d5, and LTE_4-d5 (Cayman Chemical Company, MI, USA), S-hexylglutathione and fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) (Sigma-Aldrich, MO, USA). Montelukast was suspended in 0.5 w/v% methylcellulose solution and orally administered 24 h before LTC_4 challenge. Compound A was dissolved in physiological saline containing 1 vol% WellSolve (Celeste Corporation, Tokyo, Japan) and intravenously administered approximately 1 min before, or immediately before, LTC_4 challenge.

2.2.3 Preparation of cells stably expressing guinea pig $CysLT_1$ and $CysLT_2$ receptors

Full-length DNA encoding guinea pig $CysLT_1$ receptor was chemically synthesized. Kozak consensus sequence and N-terminal hemagglutinin epitope tag sequence were artificially introduced to the vector. These fragments were subcloned into mammalian expression vector, pEF1 (Life Technologies). Full-length DNA encoding guinea pig $CysLT_2$ receptor was amplified from guinea pig heart mRNA by RT-PCR. Kozak sequence and N-terminal myc epitope tag sequence were artificially introduced to the vector. These fragments were subcloned into mammalian expression vector, pIRES (Clontech Laboratories). The receptor sequences were verified by sequencing and confirmed identity with GenBank accession number AY236968 or AY236969. Both

constructs were transfected with lipofectamine (Life Technologies) into CHO-K1 cells. After selection with geneticin, positive clones obtained by the limited dilution technique were identified by stimulation with LTD_4 or LTC_4.

2.2.4 Calcium response assay

Intracellular calcium response was measured using Fura 2-AM (Dojindo) according to the manufacture's protocol. Briefly, the CHO-K1 cells stably expressing guinea pig $CysLT_1$ and $CysLT_2$ receptors were cultured in 96 well plates (Corning Incorporated) for at least 24 h (3×10^4 cells/well). The culture medium (Ham's F-12 supplemented with 10% fetal bovine serum and 500 μg/ml geneticin) was removed, and the loading medium containing 5 μM Fura 2-AM was added to each well. After 1 h incubation, the loading medium was removed, and the cells were rinsed with the assay buffer (Hanks' Balanced Salt Solution containing 20 mM HEPES). The assay buffer was then added to each well, and the cells were further incubated at room temperature for 1 h. The cells were next 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. Receptors natural ligands, i.e. LTC_4, LTD_4 and LTE_4 (0.001 nM to 1 μM), were each added 30 s after commencement of the measurement of fluorescence intensity. In an experiment to evaluate Compound A antagonistic activity for $CysLT_1$ and $CysLT_2$, montelukast or Compound A was added 30 min before addition of the ligand (10 nM LTD_4 for the $CysLT_1$ receptor or 3 nM LTC_4 for the $CysLT_2$ receptor).

2.2.5 Preparation of lung tissue samples and quantification of CysLTs

Guinea pigs were anesthetized with pentobarbital sodium (75 mg/kg, i.p., Somnopentyl®), and a polyethylene cannula was inserted into the trachea. The other end

of the cannula was connected to a volume-limited ventilator (Model SN-480-7, Shinano Manufacturing Co., Ltd., Tokyo, Japan) providing artificial ventilation at a supply rate of 4 ml/stroke and 70 strokes/min. Another catheter was inserted into the jugular vein to secure the route for drug administration. After confirming a stable ventilation pressure, 1 vol% WellSolve (0.5 ml/kg) was injected into each animal via the catheter inserted into the jugular vein. Approximately 1 min and 50 s later, bolus doses of physiological saline or S-hexyl GSH (15 mg/kg) and LTC_4 (15 μg/kg) were intravenously injected to each guinea pig. Five min after administration of LTC_4, the chest was opened, and a sonde was inserted into the pulmonary artery from the right ventricle. The lung was then perfused with 50 ml of ice-cold perfusate (physiological saline containing 0.01 w/v% sodium citrate, 45 mM L-serine, 45 mM boric acid, 5 mM L-cysteine, 10 μM indomethacin, and 10 μM zileuton) and simultaneously a small incision was made on the left atrium. The lung subjected to perfusion was isolated together with the trachea and the bronchial cannula, and the lung lobes were separated to measure their wet weights. The isolated lung lobes were next homogenized in 4 ml of ice-cold 99.5 vol% ethanol using a polytron homogenizer (lung homogenate) and stored at -80°C until quantification of CysLTs.

To create calibration curves for quantification of CysLTs, an aliquot (0.18 ml) of the lung homogenate was allowed to stand overnight at 60°C in the presence of 1 M sodium hydroxide. Protein concentration in the lung homogenate was spectrophotometrically (Spectromax190, Molecular Devices, Tokyo, Japan) determined using the BCA™ Protein Assay Kit (Thermo Fisher Scientific Inc, MA, USA) in accordance with the manufacturer's instructions. Bovine serum albumin was used as standard for the calibration curve.

2.2.6 Measurement of CysLTs concentrations in lung tissue

The lung homogenates were centrifuged at 1870*g* for 10 min at 4°C (Hitachi Himac centrifuge, Rotor RT3S3, Tokyo, Japan), and the supernatants were collected (lung samples). A 50 μl portion of a standard solution (mixture of LTC_4, LTD_4, and LTE_4) for the calibration curve (0.6 to 600 ng/ml) was then added to 1 ml of each lung sample. A 50 μl portion of an internal standard (IS mixture; a mixture of LTC_4-d5, LTD_4-d5, and LTE_4-d5) was added to 1 ml of each lung sample for measurement and for the calibration curve. The lung samples were next evaporated to dryness by vacuum centrifuge (non-heated). The resultant residues were dissolved in 2 ml of distilled water and loaded on a solid phase extraction column (Oasis MAX cartridge 3 cc/60 mg 30 μm: Waters Corporation, MA, USA) pre-conditioned with 2 ml of methanol and 2 ml of distilled water. After washing the column with 2 ml of distilled water followed by 2 ml of 1 vol% aqueous ammonia and 2 ml of methanol, CysLTs were eluted with 2 ml of methanol containing 2 vol% formic acid. The eluate was evaporated to dryness by vacuum centrifuge and the residues were re-dissolved in 150 μl of a mixture of mobile phase A (0.2 vol% formic acid) and B (acetonitrile containing 0.2 vol% formic acid) (75/25). Individual levels of CysLTs in the samples were determined by LC/MS/MS (HPLC system: Prominence UFLC, Shimadzu, Kyoto, Japan; MS/MS: API 5000, AB Sciex, MA, USA). Concentrations of CysLTs in lung tissues were expressed in ng/g protein.

2.2.7 Measurement of bronchoconstriction

Bronchoconstriction was measured in accordance with the method described by Konzett & Rössler using ventilation pressure as an indicator (Konzett et al., 1940). Briefly, guinea pigs were anesthetized with pentobarbital sodium (75 mg/kg, i.p.), and the trachea was cannulated with a polyethylene cannula that provided artificial ventilation as described in section 2.5. Ventilation pressure was measured via a pneumotachometer

(M·I·P·S Co., Ltd., Osaka, Japan) connected to a side port of the tracheal cannula, using a Win-PULMOS-III system (Version 3.6, M·I·P·S Co., Ltd., Osaka, Japan). Compound A (1 mg/kg) and S-hexyl GSH (0, 15, 30, 60 mg/kg) were injected 1 min and 10 s prior to LTC_4 (15 μg/kg) injection, respectively, via a catheter secured in the jugular vein. Ventilation pressure was measured for at least 30 min after LTC_4 injection. The trachea was then completely blocked to obtain maximum ventilation pressure. After measurement of maximum ventilation pressure, the mechanical ventilation was stopped, and the animal was euthanized. The area under the percentage bronchoconstriction curve from 0 to 30 min after LTC_4 injection (AUC) was calculated by the trapezoidal method, and the mean percentage bronchoconstriction (%) was calculated by dividing AUC by measurement time.

2.2.8 Measurement of airway vascular permeability

Immediately after intravenous injection of FITC-BSA (2 mg/kg), the guinea pigs intravenously received S-hexyl GSH (60 mg/kg) and LTC_4 (2 μg/kg). Fifteen min after administration of LTC_4, pentobarbital sodium (Somnopentyl®: 19.4 mg/animal) was intravenously administered, and the animals were exsanguinated by cutting the femoral artery and vein followed by the abdominal aorta and vena cava. Thoracotomy was then performed, 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 the bronchoalveolar lavage fluid (BALF), which was centrifuged at 1870*g* for 5 min at 4°C (Hitachi Himac Centrifuge, Rotor RPRS3, Tokyo, Japan). Two-hundred micro-liter of the resultant supernatant was then transferred to a 96-well microplate (Corning Incorporated) under protection from light. Fluorescence intensity (Ex: 485 nm/Em: 538 nm) was measured for each supernatant 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).

2.2.9 Statistical analysis

Results are expressed as the mean ± S.E.M., except for EC_{50} and IC_{50} values, and the maximum response in calcium signal. EC_{50} and IC_{50} values, and the maximum response for CysLT-stimulated intracellular calcium responses were estimated by a 4-parameter logistic model using GraphPad Prism 5.01 (GraphPad Software. Inc, CA, USA). Data were statistically analyzed using *t*-test for comparison between 2 groups with 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.

2.3 Results

2.3.1 Calcium response assay

LTC_4, LTD_4 and LTE_4 did not induce significant intracellular calcium response in CHO-K1 cells (data not shown). The order of potency at the $CysLT_1$ receptor was LTD_4>>LTE_4≒LTC_4 with EC_{50} values of 2.27, 260 and 355 nM, respectively. On the other hand, the order of potency at the $CysLT_2$ receptor was LTC_4>>LTD_4>LTE_4 with EC_{50} values of 0.649, 25.0 and 384 nM, respectively. Unlike at the $CysLT_1$ receptor, maximum calcium signal in response to LTC_4 was significantly higher than that in response to LTD_4 or LTE_4, suggesting that LTC_4 preferentially transduce receptor-associated signal at the $CysLT_2$ receptor (Fig.1 and Table 1).

Montelukast concentration-dependently inhibited LTD_4-stimulated calcium response in $CysLT_1$ receptor-expressing cells with an IC_{50} value of 1.6 nM. This inhibition was 3

orders of magnitude more potent than that of the $CysLT_2$ receptor (IC_{50} value of 1800 nM). In sharp contrast to montelukast, Compound A preferentially inhibited $CysLT_2$ receptor-stimulated calcium signal with an IC_{50} value of 0.64 nM compared to 2600 nM at the $CysLT_1$ receptor (Table 2).

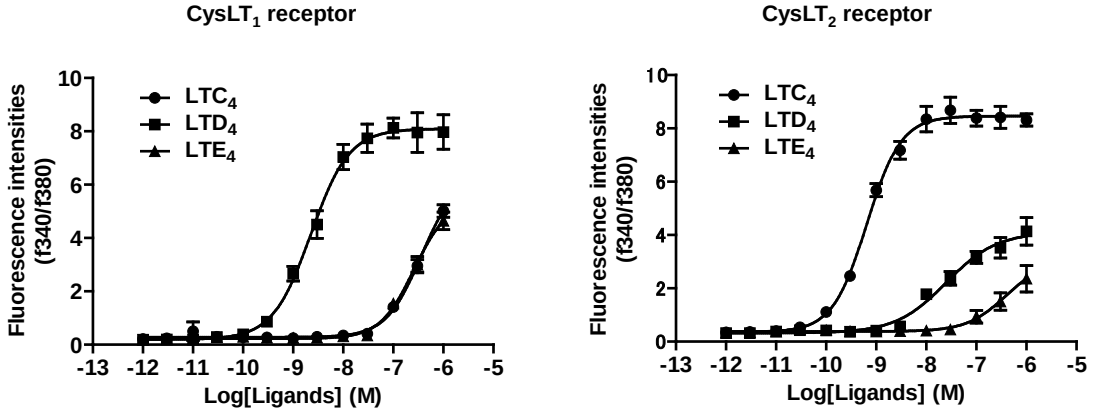

Fig.1 Intracellular calcium response stimulated by LTC_4, LTD_4 and LTE_4 in CHO-K1 cells stably expressing guinea pig $CysLT_1$ and $CysLT_2$ receptors.
Fura-2-AM-loaded CHO-K1 cells stably expressing guinea pig $CysLT_1$ or $CysLT_2$ receptor were stimulated with LTC_4, LTD_4 or LTE_4 and subsequently irradiated at 2 excitation wavelengths (340 and 380 nm) to monitor changes in fluorescence intensity at 500 nm. Maximum change in the ratio of fluorescence intensity (f340/f380) following the addition of each CysLT was determined. Each plot shows the mean ± S.E.M. for 3 independent experiments.

Table 1 Potency and efficacy of LTC_4, LTD_4 and LTE_4 for CysLT-induced intracellular calcium response in CHO-K1 cells stably expressing guinea pig $CysLT_1$ receptors and $CysLT_2$ receptors.

Guinea pig receptor	EC_{50} (nM)			Maximal response (f340/f380)		
	LTC_4	LTD_4	LTE_4	LTC_4	LTD_4	LTE_4
$CysLT_1$ receptor	355 (216-582)	2.27 (1.72-2.98)	260 (179-375)	6.31 (4.87-7.75)	8.10 (7.68-8.51)	5.41 (4.52-6.29)
$CysLT_2$ receptor	0.649 (0.541-0.779)	25.0 (14.2-44.2)	384 (56.8-2600)	8.46 (8.21-8.71)	4.11 (3.55-4.66)	3.03 (0.727-5.34)

CHO-K1 cells expressing guinea pig $CysLT_1$ or $CysLT_2$ receptor were stimulated with LTC_4, LTD_4 or LTE_4 to evaluate each receptor response using increase in intracellular calcium as an indicator. EC_{50} (nM) value and maximum calcium response, and their 95% confidence intervals (in parentheses) were estimated by the 4-parameter logistic model using data from 3 separate experiments.

Table 2 IC_{50} values of montelukast and Compound A for inhibition of CysLT-induced intracellular calcium response.

Guinea pig receptor	IC_{50} (nM)	
	Montelukast	Compound A
$CysLT_1$ receptor	1.6 (1.1-2.2)	2600 (2100-3100)
$CysLT_2$ receptor	1800 (1600-2000)	0.64 (0.53-0.76)

Fura 2-AM-loaded CHO-K1 cells expressing guinea pig $CysLT_1$ or $CysLT_2$ receptor were stimulated with 10 nM LTD_4, for the $CysLT_1$ receptor, or 3 nM LTC_4, for the $CysLT_2$ receptor. IC_{50} values were estimated by the 4-parameter logistic model using data obtained from 5 separate experiments. Parameters top and bottom were constrained by 100 and 0, respectively. Values indicated in parentheses represent 95% confidence intervals of the IC_{50} value.

2.3.2 Concentrations of CysLTs in lung tissues following LTC_4 administration

The amounts of CysLTs in lung tissues 5 min after intravenous injection of LTC_4 were determined in guinea pigs treated or non-treated with S-hexyl GSH. In the absence of S-hexyl GSH, the predominant CysLT was LTD_4 (724 ng/g protein) compared to LTE_4 (73 ng/g protein) and LTC_4 (9 ng/g protein), suggesting rapid metabolism of LTC_4 in *in vivo*. In contrast, LTC_4 (669 ng/g protein) was the most abundant CysLT in the lung tissues of S-hexyl GSH-treated animals, with LTD_4 (285 ng/g protein) being approximately at a concentration half that of LTC_4. Absolute concentrations of LTC_4 in the lungs of S-hexyl GSH-treated animals were significantly higher than those in the non-treated animals. Contrary to LTC_4, the concentrations of LTD_4 and LTE_4 in the S-hexyl GSH-treated animals were significantly lower than those in the non-treated animals (Fig.2). $LTC_4/(LTD_4+LTE_4)$ ratio, an indicator of LTC_4 metabolism to LTD_4, in the lungs of S-hexyl GSH-treated animals was much higher than that in the non-treated animals (1.64 vs. 0.01).

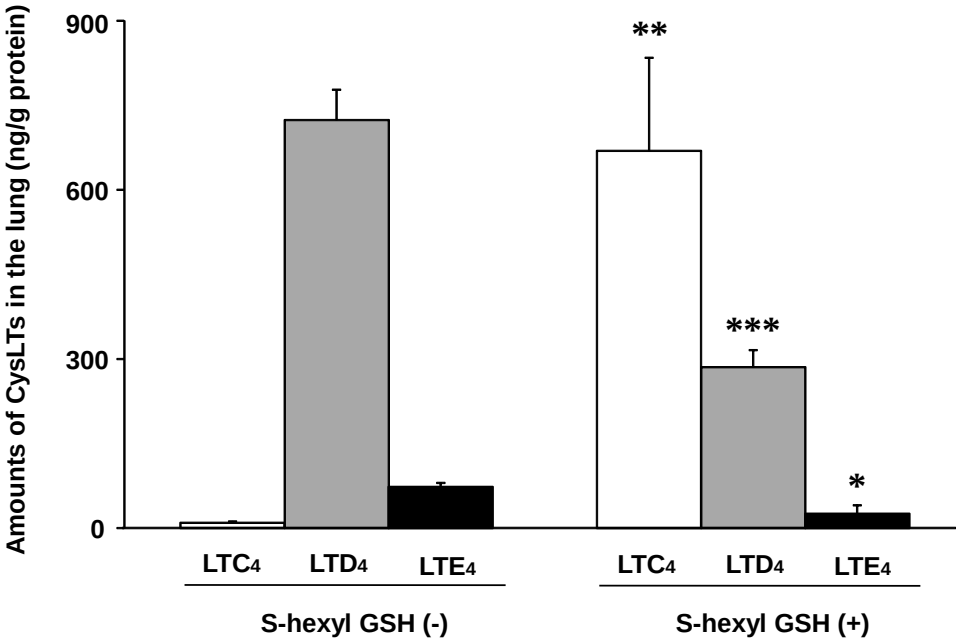

Fig.2 Concentrations of LTC_4, LTD_4 and LTE_4 in guinea pigs lung tissues treated or non-treated with S-hexyl GSH.

Animals were intravenously injected with LTC_4 (15 μg/kg), and lung tissues were isolated 5 min later. S-hexyl GSH (15 mg/kg) or physiological saline was intravenously administered approximately 10 s before LTC_4 injection. Each column represents the mean ± S.E.M. for 6 animals.

*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$: *t*-test vs. corresponding CysLT in S-hexyl GSH(-) group.

2.3.3 LTC_4-induced bronchoconstriction

In the absence of S-hexyl GSH, LTC_4 (15 μg/kg) elicited bronchoconstriction that was abrogated by montelukast, but not by Compound A. However, LTC_4 at the same dose caused a more pronounced bronchoconstriction when the animals were treated with S-hexyl GSH at 15 mg/kg or more. This LTC_4-induced bronchoconstriction was partially inhibited by either montelukast or Compound A. At the highest dose of S-hexyl GSH (60 mg/kg), the inhibitory effect of montelukast on LTC_4-induced bronchoconstriction was completely abolished (Fig.3). On the other hand, Compound A inhibition of LTC_4-induced bronchoconstriction increased with increasing dose of S-hexyl GSH. Combination of montelukast and Compound A completely blocked this response at every

evaluation point in S-hexyl GSH (15 mg/kg)-treated animals (Fig.4).

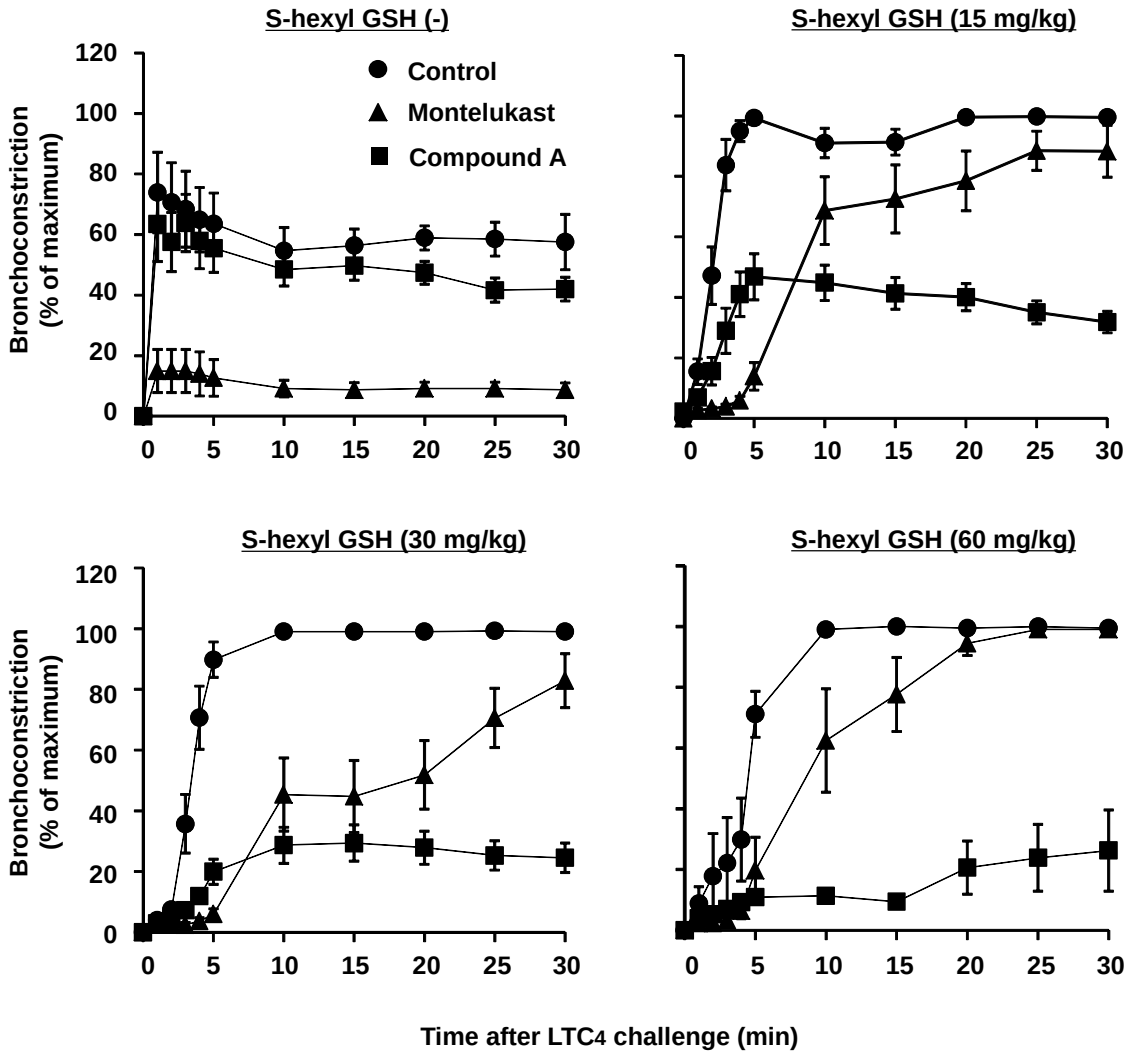

Fig.3 Effects of montelukast and Compound A on LTC_4-induced bronchoconstriction in guinea pigs treated with varying doses of S-hexyl GSH.

Animals treated with S-hexyl GSH (0, 15, 30, 60 mg/kg) were challenged with LTC_4 (15 μg/kg) under anesthesia. Montelukast was orally administered 24 h before LTC_4. Compound A was intravenously administered approximately 1 min before LTC_4. Each column represents the mean ± S.E.M. for 6-11 animals.

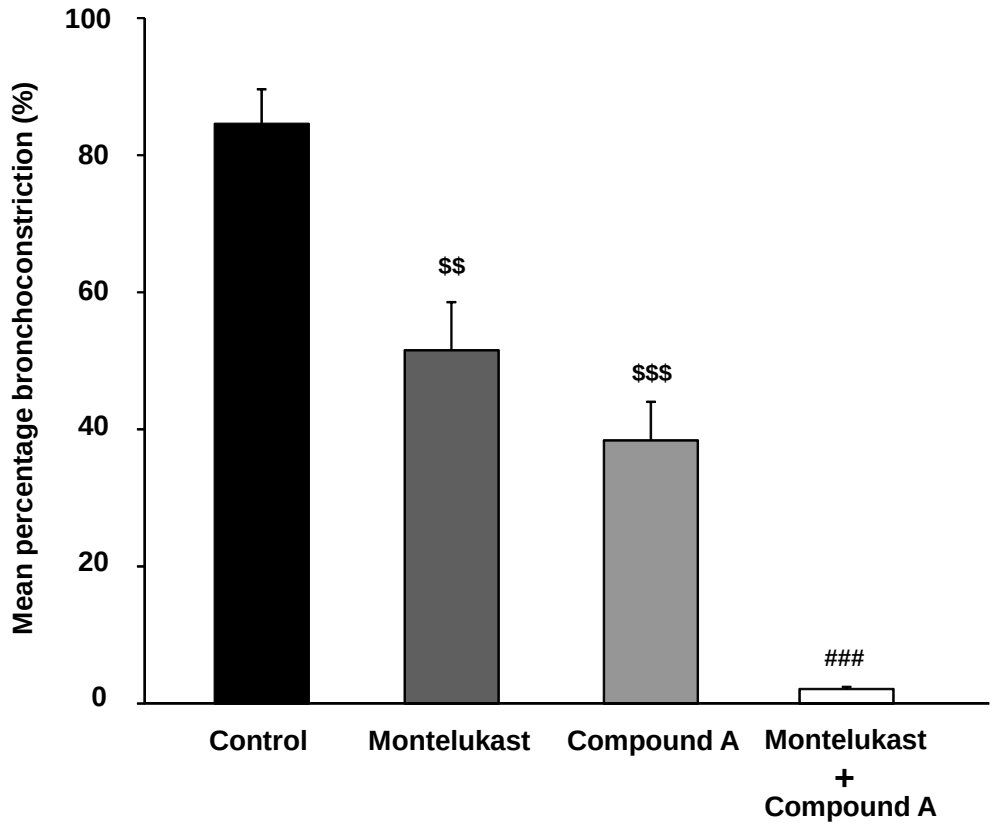

Fig.4 Effects of montelukast, Compound A, and combination of montelukast and Compound A on LTC_4-induced bronchoconstriction in S-hexyl GSH-treated guinea pigs.

Animals treated with S-hexyl GSH (15 mg/kg) were challenged intravenously with LTC_4 (15 μg/kg) under anesthesia. Montelukast was orally administered 24 h before LTC_4. Compound A was intravenously administered approximately 1 min before LTC_4. Each column represents the mean ± S.E.M. for 10 animals.

$$; $P < 0.01$: $$$; $P < 0.001$: *t*-test vs. control group. ###; $P < 0.001$: *t*-test vs. montelukast group.

2.3.4 LTC_4-induced airway vascular hyperpermeability

Intravenous injection of LTC_4 induced airway vascular hyperpermeability in both S-hexyl GSH-treated and non-treated guinea pigs. Montelukast completely inhibited this hyperpermeability in the non-treated animals (data not shown), but failed to do so in S-hexyl GSH-treated animals. On the other hand, Compound A inhibited LTC_4-induced hyperpermeability to a level comparable to that of normal or S-hexyl GSH (60 mg/kg)-treated guinea pigs that received a combination of montelukast and Compound A (Fig.5).

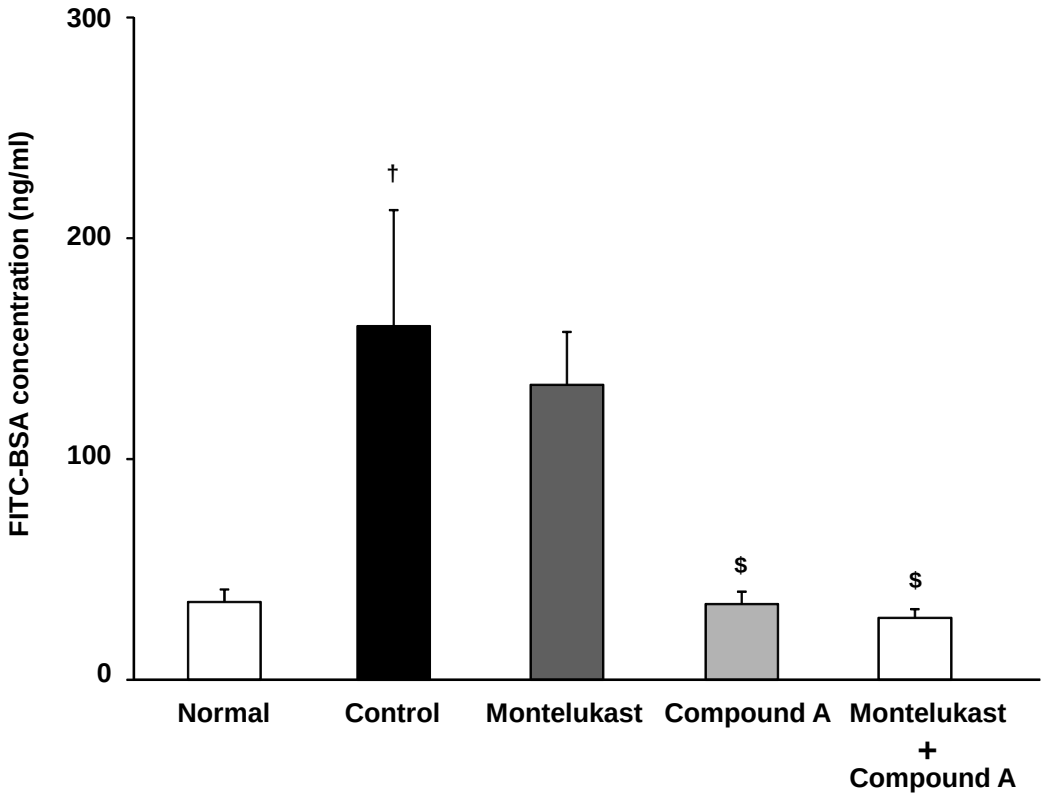

Fig.5 Effect of montelukast, Compound A and combination of montelukast and Compound A on LTC_4-induced airway vascular hyperpermeability in S-hexyl GSH-treated guinea pigs.

Immediately after FITC-BSA injection, S-hexyl GSH (60 mg/kg) and LTC_4 (2 μg/kg) were intravenously administered. Fifteen min later, BALF was collected, and FITC-BSA level in the BALF supernatant was determined. Montelukast was orally administered 24 h before LTC_4. Compound A was intravenously administered just before LTC_4. 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.

2.4 Discussion

Due to species difference in the affinity of LTD_4 for $CysLT_2$ receptors between human and the guinea pig, studying the functional role of $CysLT_2$ receptors in asthma using guinea pig models has been very difficult. By inhibiting LTC_4 metabolism using S-hexyl GSH, we succeeded in developing a guinea pig model of asthma where bronchoconstriction and airway vascular permeability are mediated not only via $CysLT_1$ receptors, but also via $CysLT_2$ receptors. Inhibition of LTC_4 metabolism resulted in an increase of this ligand concentration in guinea pig lung tissue, leading to a $CysLT_2$

receptor-dependent biological response as evidenced by Compound A (a specific $CysLT_2$ receptor antagonist) inhibition of LTC_4-induced bronchoconstriction. The developed model may therefore be useful to elucidate the functional role of $CysLT_2$ receptors in various diseases including asthma.

It has been shown that LTC_4 is more than a hundred times more potent than LTD_4 in activating calcium mobilization through $CysLT_2$ receptors in guinea pigs (Ito et al., 2008). This finding indicates that the guinea pig $CysLT_2$ receptor is preferentially activated by LTC_4. In the present study, we confirmed that LTC_4 selectively stimulates guinea pig $CysLT_2$, while LTD_4 readily binds to $CysLT_1$ receptors (Table 1). On the other hand, in humans, CysLTs affinity for $CysLT_1$ receptors is reported to be in the order of $LTD_4 > LTC_4 \fallingdotseq LTE_4$, while that for $CysLT_2$ receptors is in the order of $LTC_4 = LTD_4 >> LTE_4$ (Lynch et al., 1999; Heise et al., 2000). Therefore, in humans, LTD_4 is the most potent natural agonist for both $CysLT_1$ and $CysLT_2$ receptors. In guinea pigs, on the other hand, LTC_4 is quickly metabolized to LTD_4, making it difficult to induce $CysLT_2$ receptor-mediated biological responses.

Considering that LTC_4 is rapidly transformed to LTD_4 by metabolizing enzymes, such as γ-GTP (Orning et al., 1980; Snyder et al., 1984) and gamma-glutamyl leukotrienase (Lieberman et al., 1999; Han et al., 2002), we measured CysLTs levels in guinea pigs plasma and lung tissue after LTC_4 administration. As expected, LTC_4 was hardly detected in the plasma and lung tissue, instead high levels of LTD_4 were measured (Fig.2). In fact, we confirmed that intravenous administration of LTC_4 induces bronchoconstriction in normal guinea pigs and that this bronchoconstriction is fully abrogated by the $CysLT_1$ selective antagonist, montelukast, but not by the $CysLT_2$ receptor antagonist Compound A (Fig.3). These results indicate that exogenously administered LTC_4 is rapidly

metabolized to LTD_4 and the LTD_4 induces bronchoconstriction via a $CysLT_1$ receptor-dependent pathway in normal guinea pigs.

To suppress LTC_4 metabolism in guinea pig plasma and lung tissue, we focused on GSH derivatives, which are synthetic substrates of γ-GTP known as γ-GTP inhibitors (Bäck et al., 2001). As anticipated, the concentration of LTC_4 and the ratio of $LTC_4/(LTD_4+LTE_4)$ in the lung tissue dramatically increased in S-hexyl GSH-treated animals. In the presence of S-hexyl GSH, intravenous administration of LTC_4 induced bronchoconstriction to a degree much higher than that the untreated animals. The inhibitory effect of montelukast on LTC_4-induced bronchoconstriction subsided with increasing doses of S-hexyl GSH, although that of Compound A became more potent, suggesting that the contribution of $CysLT_1$ and $CysLT_2$ receptors to bronchoconstriction changed depending on LTC_4 concentration in the body (Fig.3). In animals treated with 15 mg of S-hexyl GSH, dual blockage of $CysLT_1$ and $CysLT_2$ by combination treatment with montelukast and Compound A abolished LTC_4-induced bronchoconstriction, indicating involvement of both receptors in this condition. Thus, inhibition of LTC_4 metabolism allowed the involvement of both $CysLT_1$ and $CysLT_2$ receptors in LTC_4-induced biological response in guinea pigs. As LTD_4 is known to activate both human $CysLT_1$ and $CysLT_2$ receptors, it would be interesting to see if both $CysLT_1$ and $CysLT_2$ receptors are involved in human bronchoconstriction. It has been shown that a $CysLT_1$ receptor antagonist does not completely inhibit tracheal contraction induced by high concentrations of LTD_4 and that residual contraction to LTD_4 is inhibited by the $CysLT_1/LT_2$ receptor antagonist BAY u9773 (Bäck et al., 2001; Tudhope et al., 1994).

In addition to bronchoconstriction, CysLTs also induce potent airway vascular hyperpermeability (Nakagawa et al., 1992). As is the case with bronchoconstriction, intravenous administration of LTC_4 to guinea pigs induced airway vascular

hyperpermeability, which was fully abrogated by the $CysLT_1$ receptor antagonist montelukast. However, our study shows that in the presence of 60 mg/kg S-hexyl GSH, LTC_4-induced airway vascular hyperpermeability was not inhibited by montelukast, but was almost completely abolished by Compound A. These results clearly demonstrate that LTC_4 induced airway vascular hyperpermeability via both $CysLT_1$ and $CysLT_2$ receptors.

Interestingly, LTC_4-induced bronchoconstriction was rather aggravated by S-hexyl GSH. We have previously found in an *in vitro* study that LTC_4 in the presence of a γ-GTP inhibitor elicits more potent contraction of guinea pig trachea than LTD_4 or LTC_4 in the absence of the γ-GTP inhibitor (Obata et al., 1992). Additionally, in the S-hexyl GSH (15 mg/kg)-treated guinea pigs, montelukast seemed to suppress the early phase of LTC_4-induced bronchoconstriction, while Compound A inhibited the later phase. It is therefore suggested that $CysLT_2$ receptor-mediated bronchoconstriction is more severe and sustained than that mediated by the $CysLT_1$ receptor. In fact, we have already confirmed that inhaled LTC_4 causes more severe and sustained bronchospasm in conscious guinea pigs treated with S-hexyl GSH than inhaled LTD_4 (data not shown). However, the difference between the effect of LTC_4 and that of LTD_4 cannot be explained by receptor density and calcium signal response due to the following reasons. First, both $CysLT_2$ and $CysLT_1$ receptors are expressed in the lung of guinea pigs. Second, the expression level of mRNA for the guinea pig $CysLT_2$ receptor is even lower than that of the $CysLT_1$ receptor in the lung tissue (data not shown). Finally, there appeared to be no significant difference in the maximum response of calcium signal between the two receptors. Further studies are needed to address the functional difference between guinea pig $CysLT_1$ and $CysLT_2$ receptors. In addition, the physiological function of the $CysLT_2$ receptor is not fully clear, although $CysLT_2$ receptors are expressed on many cell types including eosinophil (Mita et al., 2001), monocyte/macrophage (Figueroa et al., 2003), mast cell (Mellor et al., 2003),

airway smooth muscle cell (Heise et al., 2000), platelet (Hasegawa et al., 2010), cardiac Purkinje cell (Heise et al., 2000) and endothelial cell (Sjöström et al., 2003). The animal model constructed in this study using S-hexyl GSH may be useful for studying the role of the $CysLT_2$ receptor not only in bronchoconstriction and airway vascular hyperpermeability, but also in other conditions.

Currently, selective $CysLT_1$ receptor antagonists as well as inhaled corticosteroids are widely used in the treatment asthma (Montuschi et al., 2010). However, a sizable proportion of patients do not respond fully to these therapies. As demonstrated in this study, $CysLT_2$ receptors, like $CysLT_1$ receptors, can mediate asthmatic response, suggesting that dual antagonist for $CysLT_1/LT_2$ receptors antagonists can be more useful for the treatment of asthma than specific $CysLT_1$ receptor antagonists. The developed model may therefore become a powerful tool for screening and characterization of such new anti-asthmatic agents.

In conclusion, we have established a novel guinea pig model of asthma mediated via the $CysLT_2$ receptor using S-hexyl GSH. This model may be useful to study the functional role of $CysLT_2$ receptors in various diseases, including asthma.

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

3.1 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 and Shahidi, 2010). Those patients were prescribed with cysteinyl leukotriene 1 receptor antagonists (LTRA) or long-acting β2-agonists in addition to inhaled corticosteroids.

Cysteinyl leukotrienes (CysLTs: i.e.; LTC_4, LTD_4 and LTE_4) 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., 1982; 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 ($CysLT_1$) and cysteinyl leukotriene 2 ($CysLT_2$) receptors, have been identified and cloned (Lynch et al., 1999; Heise et al., 2000). Like $CysLT_1$ receptor, $CysLT_2$ 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 et al., 2003, Duah et al., 2013). In addition, it has been shown that the $CysLT_1$ receptor antagonist ICI 198,615 does not completely inhibit high LTD_4 concentrations-induced contractions of guinea pig tracheal spiral preparations and that such contractions are inhibited by the $CysLT_1/LT_2$ receptor antagonist BAY u9773 *in vitro* (Bäck et al., 2001). These findings raise the possibility that CysLTs-induced asthmatic responses are mediated via both $CysLT_1$ and $CysLT_2$ receptors, and that a dual antagonist for $CysLT_1/LT_2$ receptors would be more useful for the treatment of asthma than current $CysLT_1$ specific LTRAs. Using CHO-K1 cells expressing human and guinea pig $CysLT_2$ and $CysLT_1$ receptors, we have found a series of indole derivatives with potent dual $CysLT_1/LT_2$ receptors antagonistic activity.

However, *in vivo* evaluation of antagonists targeting the $CysLT_2$ receptors is not an easy task, since guinea pig $CysLT_2$ receptors, unlike human $CysLT_2$ receptors, are preferentially stimulated by LTC_4 (not LTD_4), which is rapidly metabolized to LTD_4 in the body. It is therefore difficult to detect $CysLT_2$ receptor-mediated biological responses in guinea pigs (Orning and Hammarström, 1980; Ito et al., 2008). By inhibiting LTC_4 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 $CysLT_1$ receptors but also via $CysLT_2$ receptors. Inhibition of LTC_4 metabolism increases tissue LTC_4 concentration in guinea pigs, which in turn results in a $CysLT_2$ receptor dependent biological response as evidenced by inhibition of LTC_4-induced bronchoconstriction by a specific antagonist for $CysLT_2$ 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 $CysLT_1$ and $CysLT_2$ 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, $CysLT_1$ selective receptor antagonist.

3.2 Materials and methods

3.2.1 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.

3.2.2 Drugs and materials

ONO-6950 (Fig. 1), 4,4'-[4-Fluoro-7-(2-{4-[4-(3-fluoro-2-methylphenyl)butoxy]phenyl}ethynyl)-2-methyl-1*H*-indole-1,3-diyl]dibutanoic acid was synthesized in our laboratories. Montelukast was purchased from Sequoia Research Product Inc. (Pangbourne, United Kingdom). $BayCysLT_2RA$ [1-(5-carboxy-2-{3-[4-(3-cyclohexylpropoxy)phenyl]propoxy}benzoyl)-4-piperidinecarboxylic acid] (Harter et al., 2006) was synthesized in our laboratories. Other materials used in this study were purchased commercially: LTC_4 and LTD_4 (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. Bay$CysLT_2$RA (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.

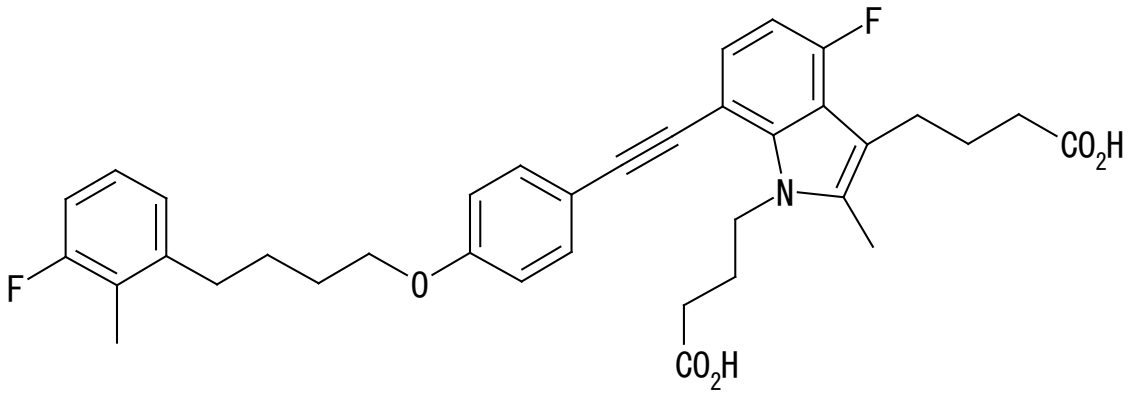

Fig.1 Chemical structure of ONO-6950

3.2.3 Preparation of cells stably expressing human and guinea pig $CysLT_1$ and $CysLT_2$ receptors

Full-length cDNAs encoding human $CysLT_1$ or $CysLT_2$ receptor were amplified from human spleen poly (A) + RNA by RT-PCR. A Kozak consensus sequence was artificially introduced, and PCR fragments encoding $CysLT_1$ receptor were subcloned into the mammalian expression vector pCI-neo (Promega Corporation). PCR fragments encoding $CysLT_2$ 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 $CysLT_1$ receptor or AB038269 for $CysLT_2$ receptor. Both constructs were transfected by lipofection into CHO-K1 cells. After selection with geneticin, positive clones were identified by LTD_4-stimulated intracellular calcium response. Cell-lines expressing guinea pig $CysLT_1$ and $CysLT_2$ receptors were prepared as described previously (Yonetomi et al., 2015).

3.2.4 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 $CysLT_1$ and $CysLT_2$ 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 h 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 $CysLT_1$/$CysLT_2$ receptors, ONO-6950 or montelukast was added 30 min before addition of the ligand, i.e. 100 nM LTD_4 for human $CysLT_1$ receptor, 0.3 nM LTD_4 for human $CysLT_2$ receptor, 10 nM LTD_4 for guinea pig $CysLT_1$ receptor and 3 nM LTC_4 for guinea pig $CysLT_2$ receptor.

3.2.5 Measurement of LTD_4 and LTC_4-induced bronchoconstriction

Ventilation pressure was measured using the method of Konzett & 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, LTD_4 (0.3 μg/kg) or LTC_4 (15 μg/kg) was intravenously administered via the catheter secured in the jugular vein. For assessment of LTC_4-induced bronchoconstriction, S-hexyl GSH (15 mg/kg) was intravenously administered 10 s prior to LTC_4 injection. Ventilation pressure was measured for at least 10 min after LTD_4 or LTC_4 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 LTD_4 or LTC_4 injection was calculated by the trapezoidal method, and mean percentage bronchoconstriction (%) was calculated by dividing AUC by measurement time.

3.2.6 Measurement of airway vascular hyperpermeability

Immediately after intravenous injection of FITC-BSA (2 mg/kg), the guinea pigs were intravenously given LTD_4 (1 μg/kg) or S-hexyl GSH (60 mg/kg) followed by LTC_4 (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 1870*g* 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).

3.2.7 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 μl 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 Days 15, 20 and 21, the sensitized animals were anesthetized, and ventilation pressure was measured according to the procedure described in Section 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 A_2. 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 Section 2.5.

3.2.8 Statistical analysis

Mean percentages of bronchoconstriction and FITC-BSA levels in the BALF are expressed as mean ± S.E.M. Differences in mean values between 2 groups (normal vs. control, control vs. montelukast, control vs. BayCysLT$_2$RA and montelukast vs. montelukast + BayCysLT$_2$RA) 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. IC_{50} values are shown as estimated values with the corresponding 95% confidence intervals. Nonlinear regression analysis for estimation of the IC_{50} values was performed by a 4-parameter logistic model using GraphPad Prism Ver. 5.01 (GraphPad Software. Inc., CA, USA).

3.3 Results

3.3.1 Calcium response assay

LTC_4, LTD_4 and LTE_4 elicited a concentration-dependent increase in intracellular calcium in CHO-K1 cells expressing $CysLT_1$ or $CysLT_2$ receptors with different potency. In addition, LTD_4-induced activation of $CysLT_2$ receptors was species-dependent. LTD_4 was rather more potent than other CysLTs in stimulating both human and guinea pig $CysLT_1$ receptors. On the other hands, LTD_4 stimulation of human $CysLT_2$ receptors was equivalent to that of LTC_4. LTC_4 was the only potent stimulant of guinea pig $CysLT_2$ receptors. Based on these results, a sub-maximum concentration of LTD_4 was selected to stimulate human $CysLT_1$, human $CysLT_2$, and guinea pig $CysLT_1$ receptors for evaluation of test-compounds antagonistic activity. LTC_4, on the other hand, was used to activate guinea pig $CysLT_2$ receptors (data not shown).

Both ONO-6950 and montelukast inhibited human $CysLT_1$ receptor-mediated calcium response with IC_{50} values of 1.7 and 0.46 nM, respectively (Table 1). ONO-6950 also inhibited human $CysLT_2$ receptor-mediated calcium response with an IC_{50} value of 25 nM, whereas montelukast inhibition of this receptor was 3 orders less potent than that of human $CysLT_1$ receptors with an IC_{50} value of 1800 nM. As observed with human $CysLT_1$ and $CysLT_2$ receptors, ONO-6950 potently inhibited both guinea pig $CysLT_1$ and $CysLT_2$ receptors with IC_{50} values of 6.3 and 8.2 nM, respectively. Montelukast, on the other hand, preferentially inhibited guinea pig $CysLT_1$ receptors with an IC_{50} value of 1.6 nM compared to 1800 nM for inhibition of guinea pig $CysLT_2$ receptors. The IC_{50} values of ONO-6950 and montelukast for inhibition of human $CysLT_1$ and $CysLT_2$ receptor-mediated intracellular calcium response shown in Table 1 are already published by Sekioka et.al. (2015).

Table 1 IC_{50} values of ONO-6950 and montelukast for inhibition of $CysLT_1$ and $CysLT_2$ 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 $CysLT_1$ or $CysLT_2$ receptor were stimulated with 100 nM LTD_4, for the $CysLT_1$ receptor, or 0.3 nM LTD_4, for the $CysLT_2$ receptor. Fura 2-AM-loaded CHO-K1 cells expressing guinea pig $CysLT_1$ or $CysLT_2$ receptor were stimulated with 10 nM LTD_4, for the $CysLT_1$ receptor, or 3 nM LTC_4 for the $CysLT_2$ receptor. IC_{50} 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 IC_{50} values.

3.3.2 CysLT-induced bronchoconstriction

In the absence of S-hexyl GSH, LTD_4 (0.3 μg/kg) elicited bronchoconstriction in anesthetized guinea pigs. Montelukast (0.01-1 mg/kg, p.o.) dose-dependently attenuated LTD_4-induced bronchoconstriction with almost complete inhibition at 0.3 mg/kg or more. In contrast, the selective $CysLT_2$ receptor antagonist Bay$CysLT_2$RA at 1 mg/kg, i.v. did not inhibit LTD_4-induced bronchoconstriction (data not shown), indicating that LTD_4-induced bronchoconstriction in normal guinea pigs depends on a $CysLT_1$-mediated pathway. ONO-6950 (0.03-3 mg/kg, p.o.) dose-dependently inhibited LTD_4-induced bronchoconstriction with maximum effect at 1 mg/kg (Fig. 2).

In the presence of S-hexyl GSH (15 mg/kg), LTC_4 (15 μg/kg) elicited

bronchoconstriction in anesthetized guinea pigs. Treatment with montelukast or BayCysLT$_2$RA partially inhibited this bronchoconstriction, while combination of montelukast and BayCysLT$_2$RA fully abrogated animals' spasmogenic response (Fig.3). These findings indicate that LTC$_4$-induced bronchoconstriction in S-hexyl GSH-treated guinea pigs involves both CysLT$_1$ and CysLT$_2$ pathways. ONO-6950 at 0.03-10 mg/kg, p.o. dose-dependently attenuated LTC$_4$-induced bronchoconstriction with almost complete inhibition at 3 mg/kg. The inhibitory effect of ONO-6950 on LTC$_4$-induced bronchoconstriction was significantly stronger than that of montelukast at the dose of 1 mg/kg or more (Fig. 3).

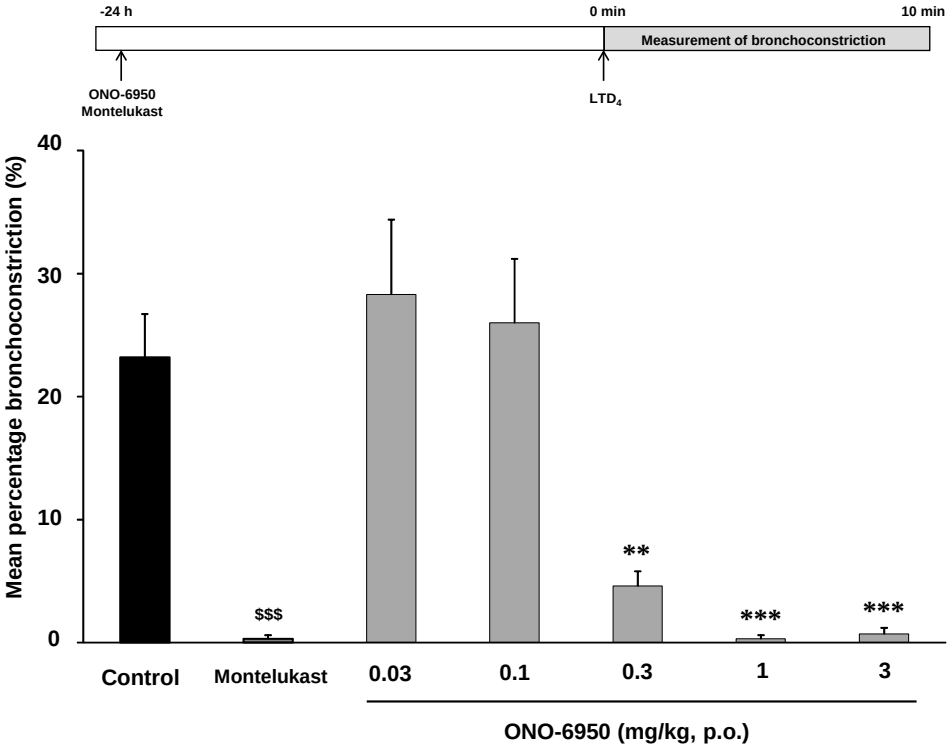

Fig.2 Effects of ONO-6950 and montelukast on LTD$_4$-induced bronchoconstriction

Anesthetized guinea pigs were challenged intravenously with LTD$_4$ (0.3 μg/kg). ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before LTD$_4$ challenge. Vertical axis represents mean percentage bronchoconstriction for 10 min following LTD$_4$ 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.

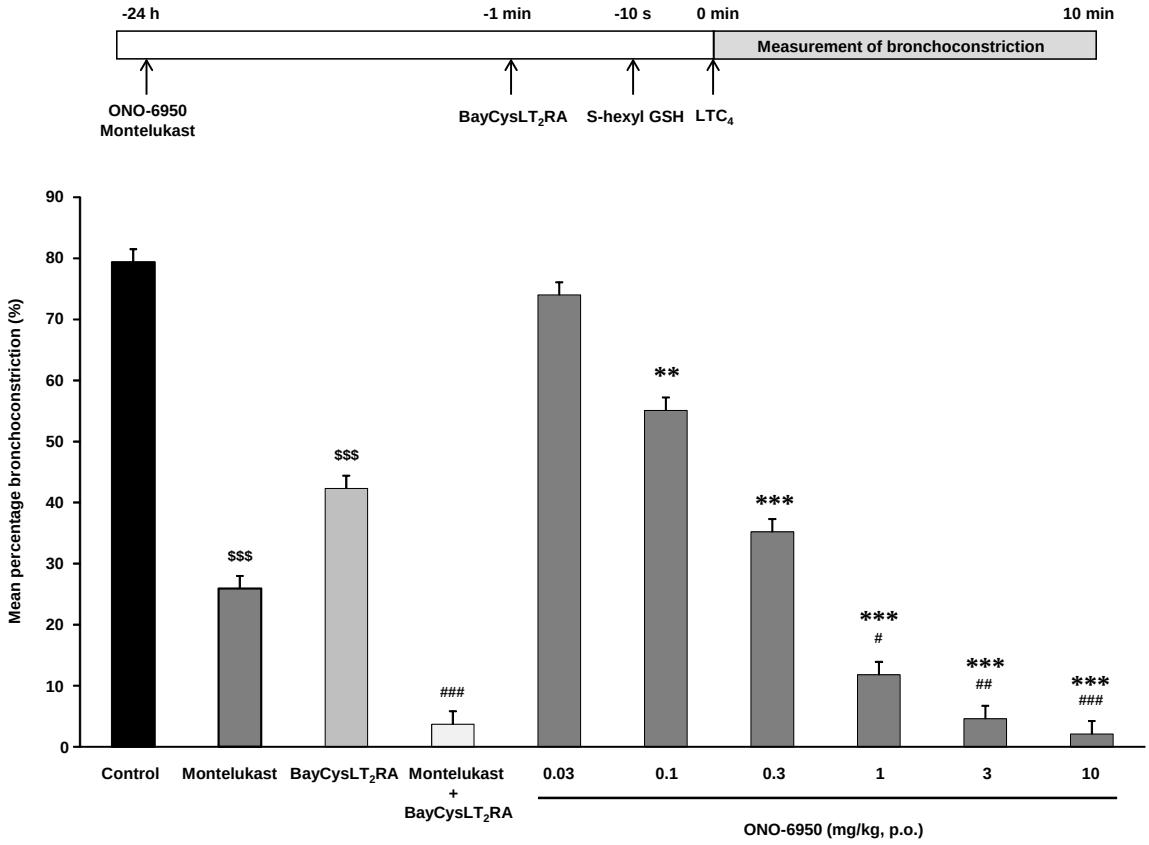

Fig.3 Effects of ONO-6950, montelukast, BayCysLT$_2$RA and combination of montelukast and BayCysLT$_2$RA on LTC$_4$-induced bronchoconstriction

Anesthetized guinea pigs treated with S-hexyl GSH (15 mg/kg) were challenged intravenously with LTC$_4$ (15 μg/kg). ONO-6950 or montelukast were orally administered to the guinea pigs 24 h before LTC$_4$ challenge. BayCysLT$_2$RA, a cysLT$_2$ receptor antagonist, was intravenously administered 1 min before LTC$_4$ challenge. Vertical axis represents mean percentage bronchoconstriction for 10 min following LTC$_4$ 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.

3.3.3 CysLT-induced airway vascular hyperpermeability

In the absence of S-hexyl GSH, LTD$_4$ (1 μg/kg) induced airway vascular hyperpermeability in conscious guinea pigs. Montelukast (0.1 mg/kg, p.o.) completely inhibited this hyperpermeability. ONO-6950 (0.03-1 mg/kg, p.o.) dose-dependently attenuated LTD$_4$-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), LTC_4 (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 LTD_4-induced airway vascular hyperpermeability in S-hexyl GSH untreated guinea pigs. Bay$CysLT_2RA$ at 1 mg/kg, i.v. inhibited LTC_4-induced hyperpermeability to a level comparable to that of normal guinea pigs (no LTC_4 treatment) (Fig.5). ONO-6950 at 0.1-3 mg/kg, p.o. dose-dependently inhibited LTC_4-induced airway vascular hyperpermeability with statistical significance at 3 mg/kg.

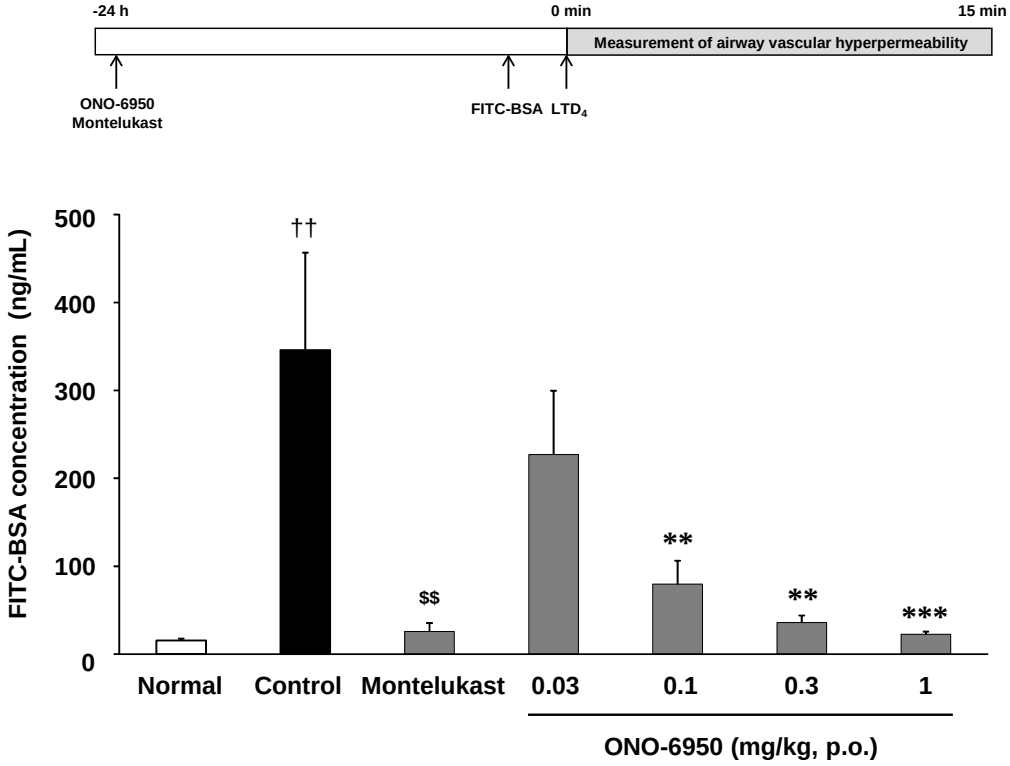

Fig.4 Effects of ONO-6950 and montelukast on LTD_4-induced airway vascular hyperpermeability

LTD_4 (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 LTD_4 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.

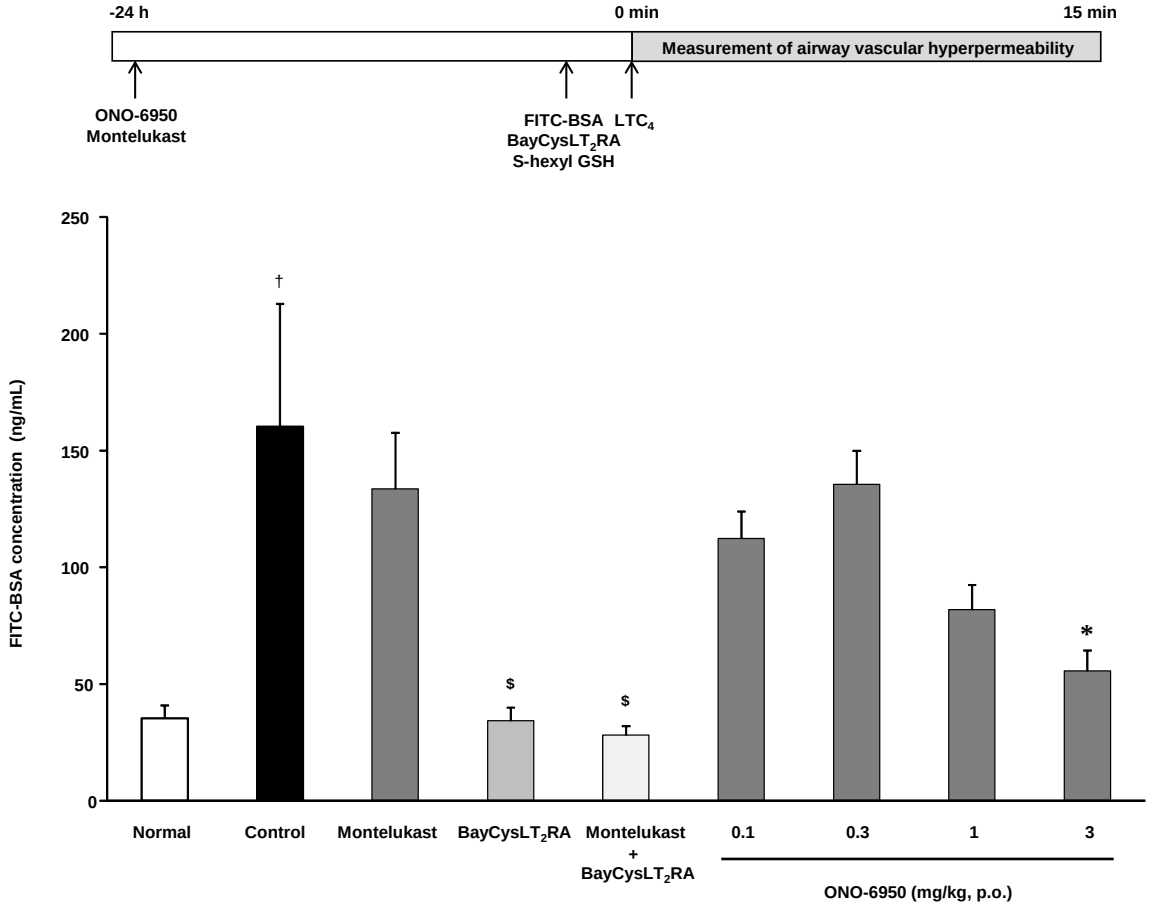

Fig.5 Effects of ONO-6950, montelukast, BayCysLT$_2$RA and combination of montelukast and BayCysLT$_2$RA on LTC$_4$-induced airway vascular hyperpermeability

LTC$_4$ (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 LTC$_4$ injection. BayCysLT$_2$RA was intravenously administered just before LTC$_4$ 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.

3.3.4 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 LTC$_4$ to LTD$_4$ 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 BayCysLT$_2$RA 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 0.1-3 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 BayCysLT$_2$RA (Fig. 6).

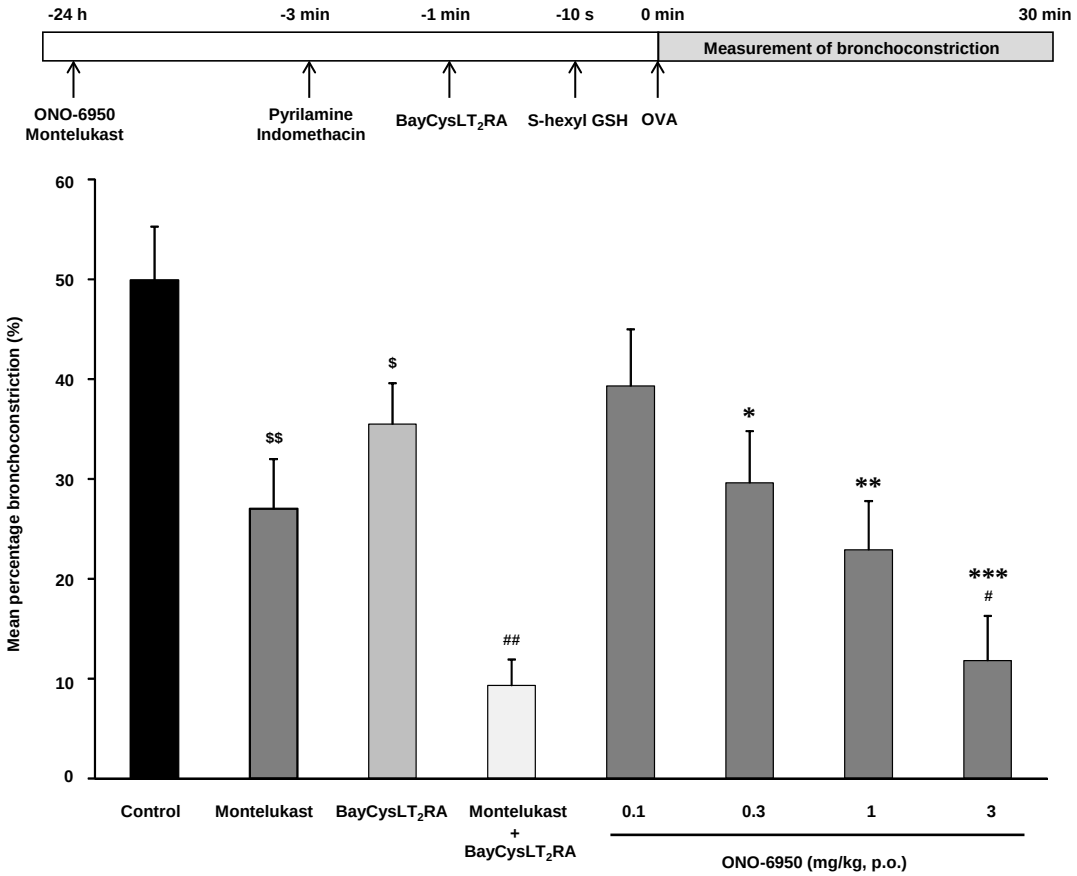

Fig.6 Effects of ONO-6950, montelukast, BayCysLT$_2$RA and combination of montelukast and BayCysLT$_2$RA on OVA-induced bronchoconstriction

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. BayCysLT$_2$RA, a cysLT$_2$ 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 ± 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.

3.4 Discussion

In the present study, we demonstrated that ONO-6950 fully suppresses not only LTD_4-induced bronchoconstriction (through the $CysLT_1$ receptor) in normal guinea pigs, but also LTC_4-induced bronchoconstriction and airway vascular hyperpermeability (mainly through the $CysLT_2$ 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 $CysLT_1$ receptor antagonist montelukast in S-hexyl GSH-treated guinea pigs. These findings suggest that ONO-6950 is an orally active $CysLT_1$ and $CysLT_2$ 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). $CysLT_1$ receptor antagonists are now widely prescribed for the treatment of asthma (Montuschi and Peters-Golden, 2010; Matsuse and Kohno, 2014). However, implication of $CysLT_2$ receptor in the pathophysiology of asthma is still unclear despite the fact that $CysLT_2$ receptors, like $CysLT_1$ receptors, are 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 et al., 2003; Duah et al., 2013). Studies on the involvement of $CysLT_2$ receptors in the pathophysiology of asthma have been hampered by lack of appropriate animal models. However, we have recently reported that exogenously administered LTC_4 can elicit $CysLT_2$-mediated bronchoconstriction and airway vascular hyperpermeability in S-hexyl GSH-treated guinea pigs (Yonetomi et al., 2015). In this study, antigen challenge induced $CysLT_2$ 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 LTC_4 concentration high in target organs, allowing investigation of $CysLT_2$ receptors involvement in asthma pathophysiology.

ONO-6950 antagonized intracellular calcium signaling via human and guinea pig $CysLT_1$ and $CysLT_2$ receptors with IC_{50} 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 LTD_4-induced bronchoconstriction and airway vascular

hyperpermeability, respectively. These LTD_4-induced airway responses were mediated via $CysLT_1$ receptors, because montelukast fully attenuated both responses. ONO-6950 at 3 mg/kg, p.o. or more almost completely inhibited LTC_4-induced bronchoconstriction and airway vascular hyperpermeability in S-hexyl GSH-treated guinea pigs. These LTC_4-induced airway responses were largely mediated via $CysLT_2$ receptors, because montelukast showed only partial or negligible inhibition of these responses, while the $CysLT_2$ receptor antagonist Bay$CysLT_2$RA strongly inhibited both events. Although higher doses were needed to inhibit $CysLT_2$-mediated response compared to $CysLT_1$-mediated response, ONO-6950 inhibited both $CysLT_1$ and $CysLT_2$ receptor-mediated airway responses at a similar dose range. These results clearly demonstrate that ONO-6950 antagonizes $CysLT_1$ and $CysLT_2$ 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 Bay$CysLT_2$RA. These results indicate that ONO-6950 inhibits asthmatic responses mediated via both $CysLT_1$ and $CysLT_2$ receptors. As previously reported, we found a species difference between human and guinea pig in the reactivity of $CysLT_2$ receptor to natural ligands. LTC_4 is the only potent stimulator of $CysLT_2$ receptors in guinea pigs, whereas LTC_4 and LTD_4 are equally potent stimulators of $CysLT_2$ receptors in human (Ito et al., 2008; Heise et al., 2000). Therefore, it would be reasonable to speculate that both $CysLT_1$ and $CysLT_2$ 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, $CysLT_2$ receptors, like the $CysLT_1$ receptors, can mediate bronchoconstriction and airway vascular hyperpermeability in guinea pigs. However,

$CysLT_2$-mediated airway vascular hyperpermeability seems to be minor compared to the $CysLT_1$-mediated one, because the maximal response induced by LTC_4 in the presence of S-hexyl GSH was smaller than that induced by LTD_4 or LTC_4 in the absence of S-hexyl GSH (data not shown). On the contrary, $CysLT_2$-mediated bronchoconstriction seems to be major compared to the $CysLT_1$ receptor-induced one, because bronchoconstriction induced by LTC_4 in the presence of S-hexyl GSH was more severe and sustained than that induced by LTC_4 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 remains unclear, our observations suggest that $CysLT_1$ and $CysLT_2$ receptors contribution to asthmatic responses is triggered via different pathways. With regards to bronchoconstriction, we are currently investigating the possibility that $CysLT_2$ receptors contribute to peripheral airway obstruction, because activation of $CysLT_2$ pathway induces more prominent lung collapse in anesthetized guinea pigs (unpublished data). Further studies are needed to elucidate the different action of $CysLT_1$ and $CysLT_2$ receptors in various airway responses.

In conclusion, ONO-6950 is a novel orally active dual $CysLT_1/LT_2$ receptor antagonist that reduces asthmatic responses mediated by both $CysLT_1$ receptor and $CysLT_2$ 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.

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4 CHAPTER IV：感作モルモットの抗原誘発遅発型気道抵抗における $CysLT_2$受容体の関与：Involvement of $CysLT_2$ receptor in antigen-induced late phase airway resistance in actively sensitized guinea pigs.

4.1 はじめに

喘息は世界で 3 億人以上が罹患する代表的なアレルギー性疾患である [1]．喘息患者が抗原に曝露されると，しばしばアレルギー反応特有の二相性の反応が誘発される．抗原誘発直後に認められる即時型喘息反応（IAR）は一過性であるのに対して，抗原誘発数時間後に再度出現する好酸球浸潤を伴う遅発型喘息反応（LAR）は持続的である [2) 3)]．気管支拡張剤である β_2 刺激薬は IAR を改善するが LAR には十分な効果を示さないのに対して，抗炎症薬であるステロイドは IAR には十分な効果を示さず LAR を改善すること [4) 5)]から，IAR は気道収縮が主たる原因で引き起こされる気道反応，LAR は気道炎症によりもたらされる気道反応であると考えられている．喘息反応に関与しているメディエーターはこれまでに多くの報告がなされているが，これらの中でも，システイニルロイコトリエン（CysLTs：LTC_4，LTD_4，LTE_4）は肥満細胞や好酸球などの種々の炎症細胞より遊離され，気道収縮や気道血管透過性亢進及び粘液分泌の増加など多様な作用を有している [6)~8)]．更に，CysLTs は抗原誘発後の肺組織において持続的な上昇を認めることから，IAR 及び LAR への関与が考えられている [3) 9)]．

CysLTs の受容体は，$CysLT_1$ と $CysLT_2$ 受容体の存在が報告されている [10) 11)]．これまで，CysLTs による気道収縮や気道血管透過性亢進などの作用は $CysLT_1$ 受容体を介すると考えられてきたが，我々の最近の研究から，LTC_4 により $CysLT_2$ 受容体を介した気道収縮や気道血管透過性亢進を誘発できることが明らかになり，$CysLT_2$ 受容体も $CysLT_1$ 受容体と同様に喘息反応に関与している可能性が示唆された [12)]．そこで我々は感作モルモットに抗原を連続吸入して誘発される喘息反応における $CysLT_2$ 受容体の関与について検証を重ねてきた．

モルモットは喘息のモデル動物として汎用されているものの，CysLT 分子種の $CysLT_1$ と $CysLT_2$ 受容体に対する親和性のパターンはヒトとモルモットで異なっており，ヒトでは LTC_4 及び LTD_4 いずれも両受容体に作用するのに対して，モルモットにおいては LTC_4 が $CysLT_2$ 受容体に，LTD_4 が $CysLT_1$ 受容体に選択的に作用する [11) 13)]．ただし，体内において LTC_4 は速やかに LTD_4 に代謝される [12) 14)]ため，モルモットにおいて $CysLT_2$ 受容体を介した作用を見出すことは困難であった．我々は最近，LTC_4 代謝阻害剤である S-ヘキシル GSH[15)] を処置することで，LTC_4 誘発による気道収縮や気道血管透過性亢進並びに抗原誘発による気道収縮が $CysLT_2$ 受容体を介することを見出した [12)]．しかしながら，これまで検討してき

た抗原誘発による気道収縮は即時型の喘息反応であり，遅発型の喘息反応における $CysLT_2$ 受容体の関与は未だ不明である．本研究では遅発型気道抵抗における $CysLT_2$ 受容体の役割を明らかにするため，S-ヘキシル GSH を処置した条件下で抗原誘発モルモット喘息モデルにおける $CysLT_1$ 受容体拮抗薬であるモンテルカストと $CysLT_1/LT_2$ 受容体拮抗薬である ONO-6950 の効果について比較検討した．

4.2 実験材料及び方法

4.2.1 動物

実験には 6 週齢の雄性ハートレー系モルモット（九動株式会社）を使用した．動物は，照明照射周期 12 時間，温度：許容範囲；20～26℃，湿度：許容範囲；35～75％の空調設備を設けた部屋にて，自由摂餌節水下にて飼育した．本試験は，「動物実験に関する指針」を遵守して実施した．

4.2.2 薬物及び試薬

ONO-6950 （ 4,4’-[4-Fluoro-7-(2-{4-[4-(3-fluoro-2-methylphenyl)butoxy]phenyl}ethynyl)-2-methyl-1*H*-indole-1,3-diyl]dibutanoic acid）の合成は小野薬品工業株式会社にて行った．モンテルカストは，Sequoia Research Product Inc.より購入した．卵白アルブミン（OVA, grade V, Sigma-Aldrich）は生理食塩液にて溶解した．S-ヘキシル GSH（Sigma-Aldrich）に等モルの 1 mol/L 水酸化ナトリウム溶液を添加し，溶解するまで超音波処理を施した．生理食塩液を加え，更に適宜超音波処理を施して溶解を確認した．ギムザ染色液は，原液を希釈したリン酸緩衝液（濃度 1/150 mol/L）で約 20 倍希釈して使用した．ONO-6950 及びモンテルカストは 0.5％メチルセルロース溶液に懸濁し，経口投与した．

4.2.3 能動感作及び誘発

モルモットをポリプロピレン製 BOX（W 300×H 390×D 570 mm）に収容した．1 w/v％ OVA 含有生理食塩液を超音波ネブライザー（NE-U17，オムロン株式会社）を用いて霧化し（風量 10，霧化量 10），動物に 1 日に約 10 分間，連続 8 日間吸入させ感作した．最終感作の 1 週間後，動物の首にカラーを装着し，Pulmos チャンバー（W 115×H 140×D 410 mm）内に収容，保定した．非誘発群には生理食塩液を，それ以外の群には 2 w/v％OVA 含有生理食塩液を超音波ネブライザー（NE-U17）を用いて霧化し（風量 2，霧化量 2），Pulmos チャンバーに導いた．同時に吸引ポンプ（SPP-3EBS，株式会社テクノ高槻）にて 3 L/min の流速で吸引する条件下で，生理食塩液又は 2 w/v％OVA 含有生理食塩液を 5 分間動物に吸入させ，抗原抗体反応を誘発した．吸入は 1 匹ずつ行い，吸入開始 24 及び 1 時間前にメチラポン

（10 mg/kg）を後肢末梢静脈内に，吸入開始 30 分前にピリラミンマレイン酸塩（10 mg/kg）を腹腔内にそれぞれ投与した．更に，S-ヘキシル GSH を使用する場合，吸入開始直前（1 分前）及び吸入開始 4 時間後の気道抵抗測定 1 分前に S-ヘキシル GSH 溶液を後肢末梢静脈内に投与した．実験の概略図を図 1 に示した．

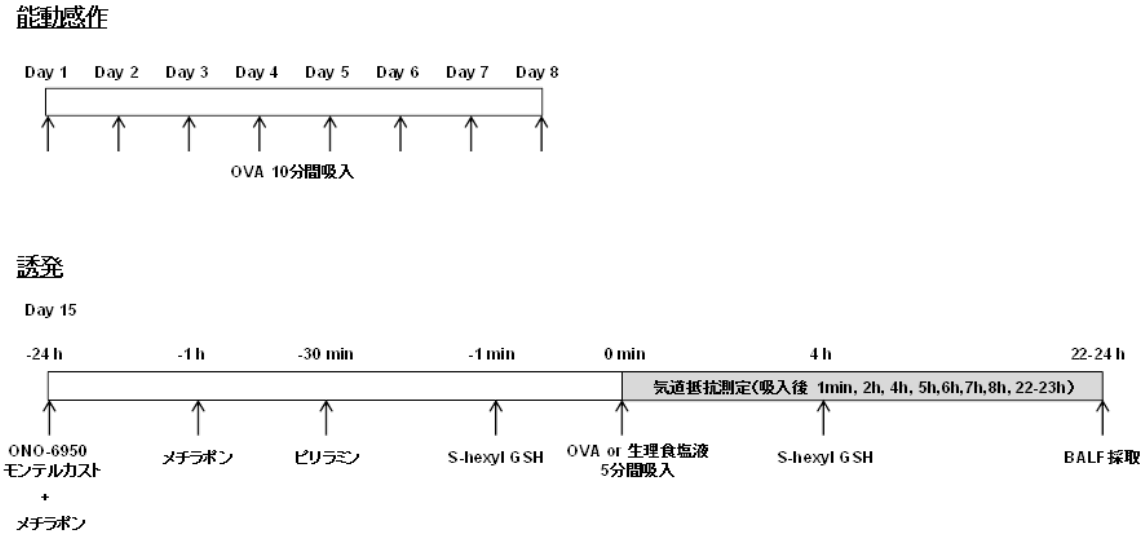

図 1　実験の概略図

4.2.4　喘息モデル

1）　気道抵抗の測定

動物の首にカラーを装着後，Pulmos チャンバー内に収容，保定した．総合呼吸機能解析システム（Pulmos-Ⅰ，株式会社エム・アイ・ピー・エス）を用い，誘発日のメチラポン投与前，生理食塩液又は OVA 吸入終了 1 分後，吸入終了約 2，4，5，6，7，8 及び 22～23 時間後に 1 回，それぞれ気道抵抗（specific airway resistance，sRaw）を 1 匹ずつ測定した．各測定時間における sRaw の値は，100 呼吸分の平均値とした．なお，メチラポン投与前の sRaw 測定前に，動物の首にカラーを装着後，Pulmos チャンバー内に収容，保定する操作を 1 回行い，チャンバーに対する馴化を実施した．各測定時間の sRaw の測定値より，総合呼吸機能解析システムの解析ソフト（WinPUL16 ver. 1.23，株式会社エム・アイ・ピー・エス）上で，次の計算式を用いて sRaw 増加率を算出した．

各測定時間の sRaw 増加率（%）＝

$$\frac{\text{各測定時間の sRaw}-\text{誘発前の sRaw}}{\text{誘発前の sRaw}} \times 100$$

被験物質の評価指標は，各測定時間における sRaw 増加率，生理食塩液又は OVA 吸入終了 1 分後の sRaw 増加率：即時型気道抵抗及び吸入終了 4～8 時間後の sRaw 増加率から算出した

曲線下面積（AUC4-8h：遅発型気道抵抗）とした．AUC4-8h は以下の計算式に従い台形法にて算出した．

AUC4-8h＝%inc.4h/2＋%inc.5h＋%inc.6h＋%inc.7h＋%inc.8h/2

%inc.*h：*時間後の sRaw 増加率

2） 炎症細胞浸潤

生理食塩液又は OVA 吸入終了 22～23 時間後の sRaw 測定後に気管支肺胞洗浄及び洗浄液中細胞数の計数を行った．動物をペントバルビタールナトリウム（50 mg/kg，i.p.）麻酔下で腹部大静脈を切断して脱血致死させ，開胸した．気管を切開して気管内にカニューレを挿入後に結紮固定し，これを介して日本薬局方 生理食塩液 5 mL を肺内に注入後吸引した．この注入・吸引操作を 2 回繰り返し（全 10 mL），その回収液を肺胞洗浄液（BALF）とした．回収した BALF は氷中で取り扱った．なお，BALF の採取は，生理食塩液又は OVA 吸入終了 22～24 時間後に実施した．

BALF を 230*g*，4℃，10 分間遠心分離し，沈殿物（ペレット）を得た．ペレットに 0.5 mL の 0.2 w/v%塩化ナトリウム液を加えることにより溶血させ，その約 1 分後に 1.6 w/v%塩化ナトリウム液を 0.5 mL 加えることで等張化した．再度上記条件で遠心分離し，上清を除いたペレットを日本薬局方 生理食塩液 1 mL に懸濁し，その一部（20 μL）を自動希釈装置（AD-270，シスメックス株式会社）を用いセルパックで 500 倍希釈した．クイックライザⅡを 3 滴加え，軽く振り混ぜて溶血させた後，自動血球計数装置（Sysmex F-820，シスメックス株式会社）にて，生理食塩液に懸濁した調製液 1 μL 当たりの細胞数を計数した．引き続き，残りの懸濁液を再度上記条件で遠心分離した．

得られたペレットに日本薬局方 生理食塩液を加え，2×10^6 cells/mL 以下の細胞懸濁液となるように希釈した．セトリングチャンバー（DC8/DC8AP，家田貿易株式会社）にセットしたスライドガラスに希釈された細胞懸濁液をマイクロピペットで 20 μL ずつのせ，48.6*g*，4℃で約 30 秒間遠心した．乾燥後，メイグリンワルド・ギムザ染色を行った．顕微鏡（OLYMPUS CX31 10×40，オリンパス株式会社）にて約 500 個の白血球（全観察白血球数）を観察し，好中球，好酸球，マクロファージ及びリンパ球（分画後の各細胞数）を計数した．全観察白血球数に対する分画後の各細胞数の比率を算出し，この比率に BALF 中の総細胞数（1 μL 当たりの細胞数×1000（μL））を乗じて BALF 中の各細胞数を算出した．評価は，BALF 中の総細胞数及び各細胞数について行った．

4.2.5 薬物投与

ONO-6950 及びモンテルカストは，OVA 誘発の 24 時間前に経口投与した．

4.2.6　統計解析

結果は平均±標準誤差で表示した．検定は t 検定若しくは Dunnett 検定を行った．ONO-6950 の用量依存性は，直線回帰分析の傾きの検定により行った．用量依存性が認められた場合は，ONO-6950 の高用量群からの下降閉手順による t 検定によりモンテルカスト群との比較を行った．t 検定，Dunnett 検定及び直線回帰分析の傾きの検定はいずれも両側検定とし，有意水準は 5%とした．有意差検定には，SAS 9.1.3 Service Pack 4［EXSUS Version 7.7.1, SAS Institute Japan 株式会社（株式会社シーエーシー）］を用いた．

4.3 結果

モルモットにおける抗原誘発即時型及び遅発型気道抵抗上昇は $CysLT_1$ 受容体に依存した反応であることが報告されている．本研究においても初めに，S-ヘキシル GSH 非処置下での即時型及び遅発型気道抵抗上昇に対する ONO-6950 とモンテルカストの効果を比較した．反復吸入感作したモルモットに抗原を吸入すると，吸入終了 1 分後をピークとする即時型気道抵抗並びに誘発 7 時間後をピークとする遅発型気道抵抗が二相性に上昇した．図 2 に示すように，モンテルカストは即時型及び遅発型の気道抵抗上昇を有意に抑制し，その効果は 1 mg/kg で頭打ちした．また，ONO-6950 は即時型及び遅発型気道抵抗上昇をいずれも 3 mg/kg で有意に抑制し，その効果は $CysLT_1$ 受容体拮抗薬であるモンテルカスト（1 mg/kg）と同程度であった．

LTC_4 から LTD_4 への代謝を抑制する S-ヘキシル GSH を処置することで $CysLT_2$ 受容体を介した即時型喘息反応を誘発できることを既に報告してきたが，遅発型喘息反応の形成においても同様の反応を誘発できるか検討した．S-ヘキシル GSH（0，5，10 及び 15 mg/kg，i.v.）を抗原曝露直前及び 4 時間後に処置すると S-ヘキシル GSH 非処置下と同様に 2 相性の気道抵抗の上昇は認められるものの，S-ヘキシル GSH 存在下でのモンテルカスト（3 mg/kg）の効果は即時型気道抵抗において 67.9%から 9.1%まで，遅発型気道抵抗において 67.7%から 27.8%まで減弱した．また，遅発型気道抵抗における S-ヘキシル GSH の効果は 10 mg/kg で頭打ちした（図 3）．

そこで，S-ヘキシル GSH 10 mg/kg 処置下における即時型及び遅発型気道抵抗上昇に対する ONO-6950 とモンテルカストの効果を同時比較した．図 4 に示すように，即時型の気道抵抗上昇は 3 mg/kg 以上の ONO-6950 とモンテルカスト（3 mg/kg）のいずれにおいても有意に抑制され，それぞれの最大抑制率は 55.4 及び 45.9%であった．一方，遅発型の気道抵抗上昇は 1 mg/kg 以上の ONO-6950 により有意に抑制されたが，モンテルカスト（3 mg/kg）では

有意に抑制されなかった．遅発型の気道抵抗上昇に対する ONO-6950 の最大抑制率は 85.4％であり，モンテルカストの抑制率は 33.9％であった．

更に，抗原吸入終了 22～24 時間後に採取した BALF 中の総細胞数及び分画後の細胞数を調べ，両薬剤の効果を比較した（表 1）．抗原を吸入した対照群では生理食塩液を吸入した正常群と比較して，総細胞，マクロファージ，好酸球及び好中球が有意に上昇した．ONO-6950 は 0.3 mg/kg 以上で総細胞並びに好酸球を，10 mg/kg でマクロファージを有意に抑制した．また，モンテルカストは，総細胞，好酸球及びマクロファージを有意に抑制し，その効果は ONO-6950 と同程度であった．

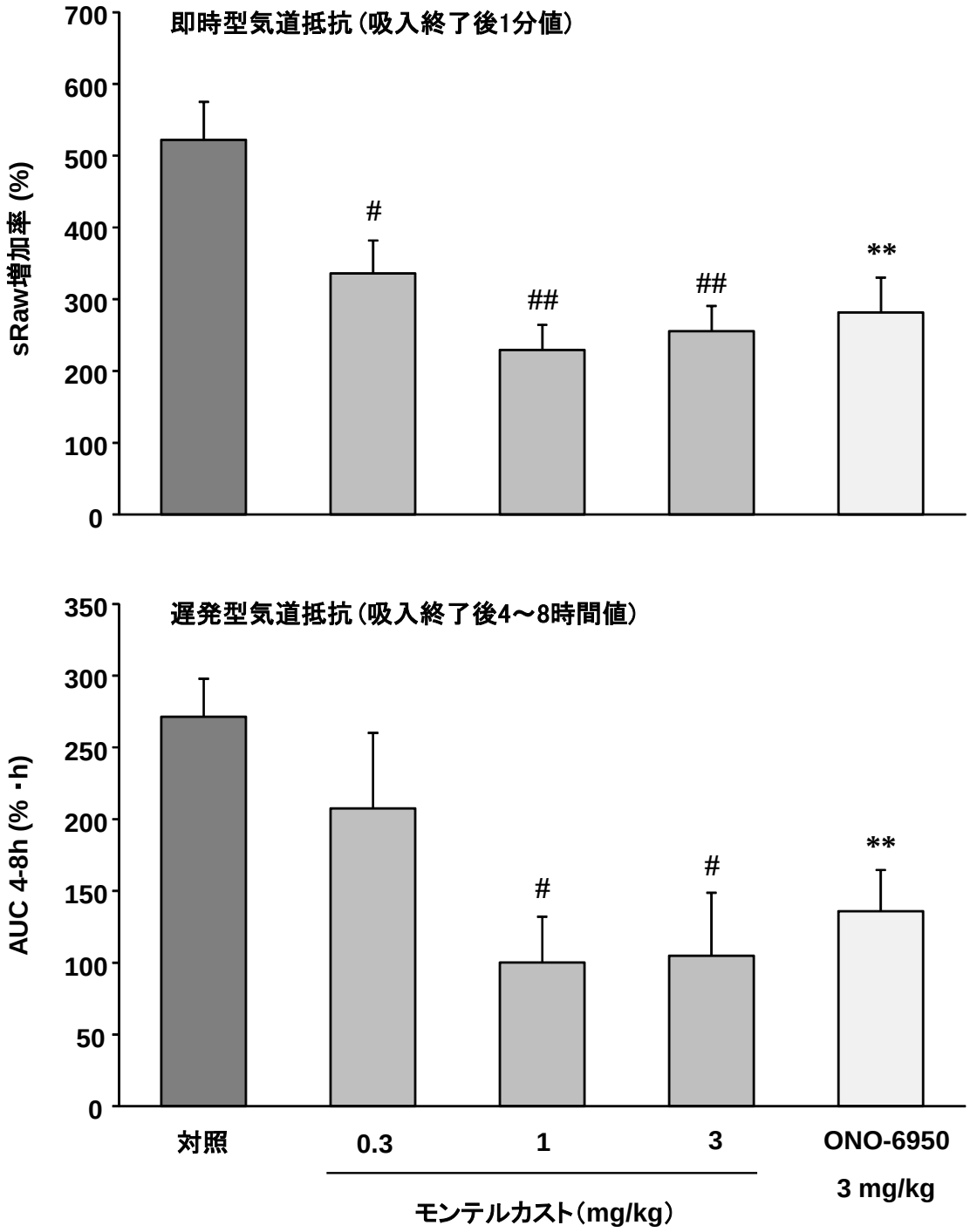

図 2　能動感作モルモットにおける即時型及び遅発型気道抵抗上昇に対する ONO-6950 及びモンテルカストの効果

感作モルモットに OVA を吸入し，抗原吸入終了 1 分後の気道抵抗値並びに抗原吸入終了 4～8 時間後の気道抵抗率の曲線下面積を示した．ONO-6950 及びモンテルカストは抗原誘発 24 時間前に経口投与した．結果は各群 8 匹の動物の平均±標準誤差を示す．#: $p<0.05$, ##: $p< 0.01$; 対照群と比較して有意差有り（Dunnett 検定）．**: $p< 0.01$; 対照群と比較して有意差有り（t 検定）．

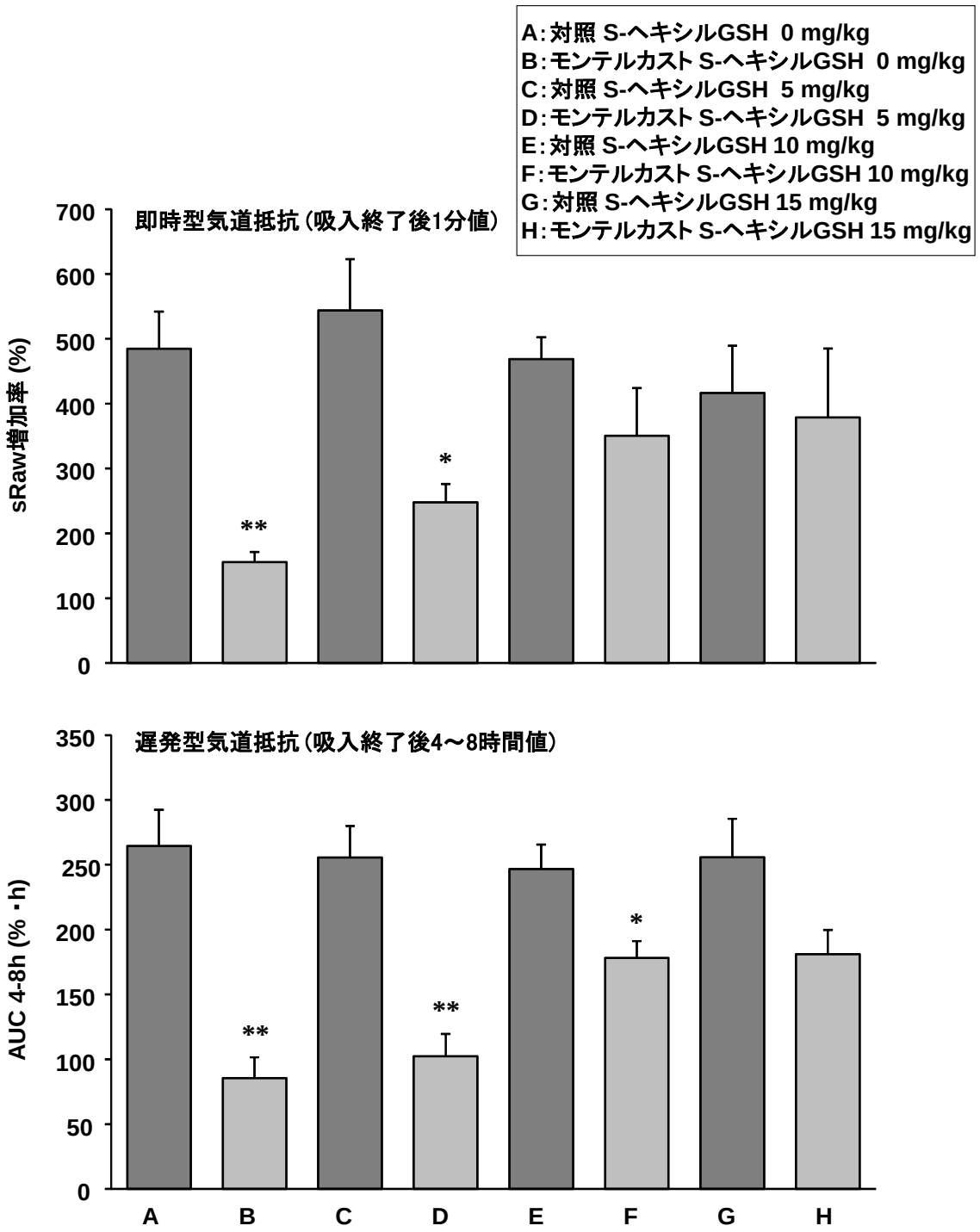

図 3　抗原誘発即時型及び遅発型気道抵抗上昇に及ぼす S-ヘキシル GSH の作用

感作モルモットに OVA を吸入し，抗原吸入終了 1 分後の気道抵抗値並びに抗原吸入終了 4～8 時間後の気道抵抗率の曲線下面積を示した．モンテルカストは抗原誘発 24 時間前に経口投与した．S-ヘキシル GSH は抗原誘発直前及び抗原誘発 4 時間後の測定直前に静脈内投与した．結果は各群 6 匹の動物の平均±標準誤差を示す．*: $p<0.05$, **: $p< 0.01$; 対照群と比較して有意差有り（t 検定）．

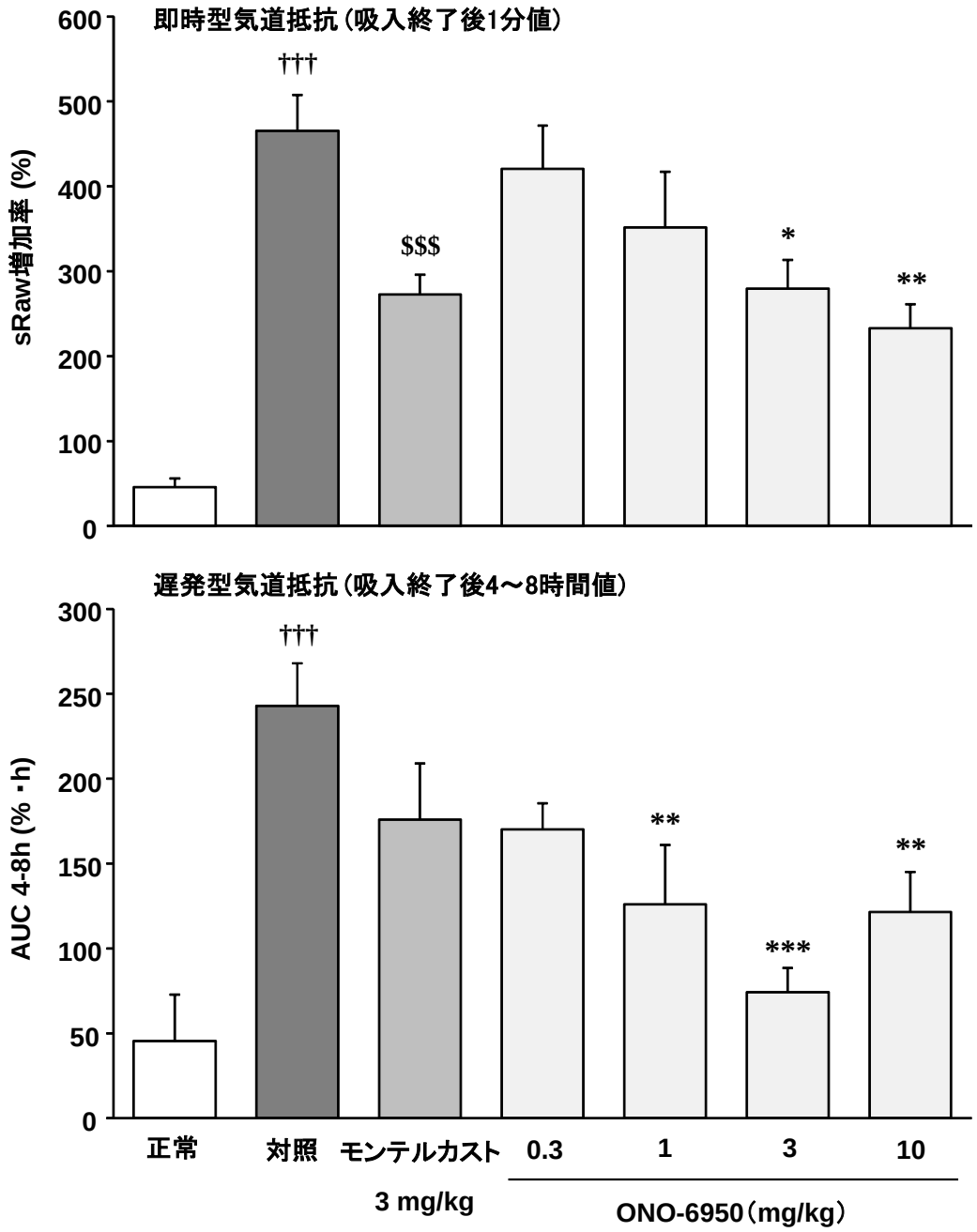

図 4　S-ヘキシル GSH を処置した能動感作モルモットにおける即時型及び遅発型気道抵抗上昇に対する ONO-6950 及びモンテルカストの効果

感作モルモットに OVA を吸入し，抗原吸入終了 1 分後の気道抵抗値並びに抗原吸入終了 4～8 時間後の気道抵抗率の曲線下面積を示した．ONO-6950 及びモンテルカストは抗原誘発 24 時間前に経口投与した．S-ヘキシル GSH は抗原誘発直前及び抗原誘発 4 時間後の測定直前に静脈内投与した．結果は各群 10 匹の動物の平均±標準誤差を示す．†††: p< 0.001; 正常群と比較して有意差有り(t 検定)．$$$: p< 0.001; 対照群と比較して有意差有り(t 検定)．*: p< 0.05, **: p< 0.01, ***:p< 0.001; 対照群と比較して有意差有り(Dunnett 検定)．ONO-6950 の即時型気道抵抗のみ用量依存性有り．

表 1　S-ヘキシル GSH を処置した能動感作モルモットにおける気管支肺胞洗浄液中の炎症細胞浸潤に対する ONO-6950 及びモンテルカストの効果

群名	投与用量 (mg/kg)	細胞数（×10^5）				
		総細胞	マクロファージ	好酸球	好中球	リンパ球
正常	0	28.7 ± 4.1	12.6 ± 2.3	7.2 ± 1.5	8.4 ± 3.0	0.5 ± 0.1
対照	0	105.3 ± 16.0 †††	27.7 ± 5.2 †	39.2 ± 6.1 †††	37.7 ± 10.8 †	0.8 ± 0.2
モンテルカスト	3	48.5 ± 6.0 $$	14.2 ± 2.7 $	12.9 ± 2.5 $$$	21.1 ± 4.5	0.3 ± 0.1
ONO-6950	0.3	59.4 ± 13.1 *	16.2 ± 3.0	19.1 ± 2.2 **	23.5 ± 8.7	0.6 ± 0.1
ONO-6950	1	65.0 ± 13.0	17.9 ± 3.8	18.7 ± 3.8 **	28.2 ± 8.4	0.3 ± 0.1
ONO-6950	3	49.6 ± 11.8 *	15.5 ± 3.1	13.2 ± 2.7 ***	20.4 ± 6.9	0.5 ± 0.2
ONO-6950	10	42.7 ± 8.0 **	13.9 ± 2.9 *	13.5 ± 3.0 ***	14.9 ± 3.3	0.4 ± 0.1

感作モルモットに OVA を吸入し，抗原吸入終了 22～24 時間後の気管支肺胞洗浄液中の炎症細胞浸潤数を示した．ONO-6950 及びモンテルカストは抗原誘発 24 時間前に経口投与した．S-ヘキシル GSH は抗原誘発直前及び抗原誘発 4 時間後の測定直前に静脈内投与した．結果は各群 10 匹の動物の平均±標準誤差を示す．†: $p< 0.05$, †††: $p< 0.001$; 正常群と比較して有意差有り(t 検定)．$: $p< 0.05$, $$: $p< 0.01$, $$$: $p< 0.001$; 対照群と比較して有意差有り(t 検定)．*: $p< 0.05$, **: $p< 0.01$, ***:$p< 0.001$; 対照群と比較して有意差有り(Dunnett 検定).

4.4 考察

本研究より，モルモット喘息モデルでは，遅発型気道抵抗上昇に $CysLT_2$ 受容体が関与すること，並びに $CysLT_1$ 及び $CysLT_2$ 両受容体を阻害することにより効果的な喘息反応の制御が可能であることが示唆された.

LTC_4 代謝阻害剤である S-ヘキシル GSH 処置下の感作モルモットに抗原を連続吸入することにより，S-ヘキシル GSH 非処置下と同様に二相性の喘息反応が誘発された．S-ヘキシル GSH 非処置下での遅発相の気道抵抗上昇に対してモンテルカスト及び ONO-6950 はほぼ同等の強い抑制効果を示した．これに対して，S-ヘキシル GSH 処置下の遅発型の気道抵抗上昇に対して ONO-6950（3 mg/kg）は有意な抑制効果を示したものの，$CysLT_1$ 受容体を十分に拮抗する用量のモンテルカストは有意な抑制効果を示さなかった．我々は，ONO-6950 が 3 mg/kg の用量で $CysLT_1$ 及び $CysLT_2$ いずれの受容体を介した気道反応もほぼ最大に抑制することを報告しており [16]，今回の結果は，モルモットにおいて S-ヘキシル GSH を処置することにより $CysLT_2$ 受容体を介して遅発型の気道抵抗上昇が誘発されることを示唆している.

我々が知る限り，今回の研究が遅発型喘息反応における $CysLT_2$ 受容体の関与を示唆した初めての報告である．

遅発相の気道抵抗上昇は気管支拡張薬である β_2 刺激薬により抑制されないため，遅発型喘息反応の本体は気道収縮でなく気道炎症であると考えられている．つまり，持続的な抗原吸入により気道への各種炎症細胞浸潤や炎症細胞から遊離されるメディエーターによる気道粘膜浮腫が遅発相の気道抵抗上昇をもたらす．気道粘膜浮腫の原因となる気道の血管透過性亢進は CysLTs により誘発されることが知られており，我々は外因性に投与した LTC_4 が $CysLT_1$ 及び $CysLT_2$ 受容体のいずれを介しても気道の血管透過性亢進を誘発できることを報告してきた [12]．したがって，今回の S-ヘキシル GSH 処置下における $CysLT_2$ 受容体を介した遅発型の気道抵抗上昇に気道の血管透過性亢進が関与していることが推察された．

気道炎症に係る炎症細胞の中でも特に好酸球は喘息の病態形成に重要な役割を果たしている [17]．これまでに CysLTs の吸入は気道に好酸球を浸潤させること [18]，また $CysLT_1$ 受容体拮抗薬は喘息患者の末梢血好酸球数を低下させること [19]が報告されている．これらの事実から，好酸球浸潤には $CysLT_1$ 受容体が関与していると考えられている．一方，好酸球機能における $CysLT_2$ 受容体の役割に関しては報告されていないが，好酸球は $CysLT_1$ 受容体に加え $CysLT_2$ 受容体も発現していること [20]から，$CysLT_2$ 受容体は $CysLT_1$ 受容体と同様に好酸球浸潤に関与している可能性が考えられた．今回，遅発型の気道抵抗上昇モデルを用いて好酸球浸潤に対する ONO-6950 とモンテルカストの効果を比較した結果，モンテルカストは S-ヘキシル GSH を処置しているにも関わらず好酸球浸潤を 80％以上抑制した．また，ONO-6950 も 0.3 mg/kg で好酸球浸潤を有意に抑制した．これまでの検討で，$CysLT_1$ 受容体拮抗作用を発揮する ONO-6950 の用量は 0.3 mg/kg であることが明らかとなっていることから，本モデルにおいて ONO-6950 は $CysLT_1$ 受容体拮抗作用を介して好酸球浸潤を抑制したと推察される．これらの結果から，今回の試験条件では好酸球浸潤に $CysLT_2$ 受容体は関与しておらず，主に $CysLT_1$ 受容体を介した反応であることが示唆された．しかしながら，本試験の S-ヘキシル GSH は抗原曝露 4 時間後以降には投与されていないため，BALF 液を回収した 22～24 時間後までの間に $CysLT_2$ 受容体を介した炎症細胞浸潤が消失した可能性も否定はできない．したがって，好酸球浸潤における $CysLT_2$ 受容体の関与については，今後の更なる検討が必要と思われる．

我々は以前に，S-ヘキシル GSH 処置下での即時型の気道抵抗上昇に対して ONO-6950 がモンテルカストよりも強く抑制すること [16]を報告した．しかし，今回の試験において即時型気道抵抗上昇に対して両薬物間の差異は認められなかった．我々がこれまでに実施した数多くの試験において S-ヘキシル GSH 処置下で即時型の気道抵抗上昇が $CysLT_2$ 受容体を介した反応であることを確認してきたため，今回この矛盾が生じた原因としては気道抵抗測定の

評価ポイントの違いが考えられる．$CysLT_1$ 受容体を介する反応は誘発直後から強く認められるが，$CysLT_2$ 受容体を介する気道収縮は誘発 10 分後以降に強く認められる．今回の試験では抗原誘発 6 分後に気道抵抗を評価したため，S-ヘキシル GSH 処置下においてではあるが，今回の試験においては偶々$CysLT_1$ 優位な収縮反応を捉え $CysLT_2$ 受容体を介した収縮反応を捉えられなかったものと考えられる．

モルモットにおいては LTC_4 が $CysLT_2$ 受容体に選択的に作用する [13]ため，生体において $CysLT_2$ 受容体を介した反応を捉えるためには LTC_4 代謝を抑制する必要があるが，ヒトにおいては LTC_4 及び LTD_4 はいずれも $CysLT_2$ 受容体に作用する [11]ため，喘息等の CysLTs 産生が亢進した状況において $CysLT_2$ 受容体を介した反応が誘発されると考えられる．本試験において，S-ヘキシル GSH 処置下で ONO-6950 がモンテルカストよりも強く遅発型の気道抵抗上昇を抑制した事実は，喘息患者においても $CysLT_1$ 及び $CysLT_2$ 受容体を抑制することにより，効果的に気道炎症に基づく気道反応を抑制する可能性を示唆している．気道炎症に基づく気道反応として，遅発型の気道抵抗上昇に加え気道過敏性の亢進や気道リモデリングが報告 [21) 22)]されており，気道炎症における $CysLT_2$ 受容体の関与を明確にするためには，これらに関する検討も考慮すべきである．

4.5 結論

モルモット喘息モデルにおいて LTC_4 代謝阻害剤 S-ヘキシル GSH を処置することにより抗原誘発後の遅発型気道抵抗上昇に $CysLT_2$ 受容体が重要な役割を演じることを示した．$CysLT_1/LT_2$ 受容体拮抗薬である ONO-6950 は $CysLT_1$ 受容体拮抗薬であるモンテルカストよりも強く気道炎症に基づく気道反応を抑制することが期待される．

4.6 文献

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5 CHAPTER V：SUMMARY AND CONCLUSION

Two CysLTs receptors, i.e. $CysLT_1$ and $CysLT_2$ receptors have identified and cloned. Although $CysLT_1$ receptor antagonists are currently available for asthma treatment, little is known about the pathophysiological role of $CysLT_2$ receptors in asthma. In this study, to elucidate the possible involvement of $CysLT_2$ receptors in bronchoconstriction and airway vascular hyperpermeability, we have established a novel guinea pig model of asthma. *In vitro* study confirmed that CHO-K1 cells, expressing guinea pig $CysLT_2$ and $CysLT_1$ receptors are selectively stimulated by LTC_4 and LTD_4, respectively. However, when LTC_4 was intravenously injected to guinea pigs, it caused $CysLT_1$-mediated airway responses as evidenced by the fact that the bronchoconstriction was fully abrogated by montelukast, indicating the possibility of rapid metabolism of LTC_4 to LTD_4 in the body. We found that treatment with S-hexyl GSH, an inhibitor of γ-GTP, significantly increased LTC_4 content and LTC_4/(LTD_4 plus LTE_4) ratio in the lung. Under these circumstances, LTC_4-induced bronchoconstriction became resistant to montelukast, but sensitive to a $CysLT_2$ receptor antagonist, depending on the dose of S-hexyl GSH. Combination with montelukast and $CysLT_2$ receptor antagonist completely abrogated this spasmogenic response. Additionally, we confirmed that LTC_4 elicits airway vascular hyperpermeability via $CysLT_2$ receptors in the presence of high dose of S-hexyl GSH as evidenced by complete inhibition of LTC_4-induced hyperpermeability by $CysLT_2$ receptor antagonist, but not montelukast. These results suggest that $CysLT_2$ receptors, like $CysLT_1$ receptors, mediate bronchoconstriction and airway vascular hyperpermeability in guinea pigs. Therefore, the developed model became a powerful tool for screening and characterization of $CysLT_2$ receptor antagonist.

After screening of many compounds, we have successfully produced a novel and potent $CysLT_1/LT_2$ receptor antagonist. In second research, we showed that ONO-6950 antagonized $CysLT_1$ and $CysLT_2$ receptor-mediated intracellular calcium responses with IC_{50} values of 1.7 and 25 nM in human, and IC_{50} values of 6.3 and 8.2 nM in guinea pigs, respectively. Also, we investigated effects of ONO-6950 on airway responses mediated by both $CysLT_1$ and $CysLT_2$ receptors in S-hexyl GSH-untreated or -treated guinea pigs, and compared them to those of montelukast. In S-hexyl GSH-untreated guinea pigs, both ONO-6950 and montelukast fully attenuated $CysLT_1$-mediated bronchoconstriction and airway vascular hyperpermeability induced by LTD_4. On the other hand, in S-hexyl GSH-treated guinea pigs ONO-6950 almost completely inhibited bronchoconstriction and airway vascular hyperpermeability elicited by LTC_4, while montelukast showed only partial or negligible inhibition of these airway responses. More importantly, ONO-6950 suppressed antigen-induced bronchoconstriction in sensitized guinea pigs although montelukast did not suppress it sufficiently. These findings suggest that ONO-6950 is an orally active $CysLT_1$ and $CysLT_2$ dual antagonist that may alleviate asthmatic response more potently than montelukast.

Asthma is a chronic inflammatory disorder. Therefore, it is important to evaluate the effect of chronic asthma model, characterized by late asthmatic response. In final research, we examined the effect of ONO-6950 and montelukast on $CysLT_1$ and $CysLT_2$ receptor mediated late phase airway resistance. In non-S-hexyl GSH treated guinea pigs, ONO-6950 and montelukast equally attenuated antigen-induced increase in late phase airway resistance. On the other hand, in S-hexyl GSH-treated guinea pigs only ONO-6950, but not montelukast, significantly inhibited antigen-induced increase in late phase airway resistance. This result suggests that $CysLT_2$ receptors play an important role in the development of late phase airway resistance.

In conclusion, we clearly demonstrate that (a) $CysLT_2$ receptors mediate bronchoconstriction and airway vascular hyperpermeability in guinea pigs, and (b) $CysLT_2$ receptor as well as $CysLT_1$ receptor is involved in both immediate and late asthmatic response in sensitized guinea pigs. From these results, development of $CysLT_1/LT_2$ receptor antagonist may provide a novel therapeutic option for patients with asthma than current $CysLT_1$ specific LTRAs.

6. CHAPTER VI：ACKNOWLEDGMENT

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