



The suppressive effects of YM-58483/BTP-2, a store-operated Ca^{2+} entry blocker, on inflammatory mediator release in vitro and airway responses in vivo

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Abstract

YM-58483/BTP-2, 4-methyl-4'-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide, blocks the store-operated Ca^{2+} entry (SOCE) that mediates the activation of non-excitable cells. This study investigated the pharmacological profile and therapeutic potential of YM-58483 as anti-asthma drug. YM-58483 inhibited DNP antigen-induced histamine release from and leukotrienes (LTs) production in IgE-primed RBL-2H3 cells, a rat basophilic leukemia cell line, with IC_{50} values of 460 and 310 nM, respectively. Prednisolone did not inhibit either of these responses. YM-58483 also inhibited phytohemagglutinin-P (PHA)-stimulated IL-5 and IL-13 production in human peripheral blood cells with IC_{50} values of 125 and 148 nM, respectively, which is approximately 5 times less potent than prednisolone. YM-58483 (30 mg/kg, p.o.) significantly suppressed ovalbumin (OVA)-induced bronchoconstriction in OVA-sensitized guinea pigs, whereas prednisolone did not. YM-58483 (3–30 mg/kg, p.o.) and prednisolone (100 mg/kg, p.o.) both significantly and completely suppressed airway hyperresponsiveness (AHR) caused by OVA exposure. Since YM-58483 inhibits two major characteristic symptoms of bronchial asthma, namely bronchoconstriction and AHR via the suppression of inflammatory mediator and cytokine production, SOCE inhibition is a potential approach for treatment.

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1. Introduction

Asthma is characterized by episodic airflow obstruction, airway hyperresponsiveness (AHR) to non-specific stimuli, and inflammation of respiratory tract. It has been suggested that acute inflammation is responsible for episodic airflow obstruction, while chronic inflammation contributes to AHR and fixed airflow obstruction by remodeling the airway [1].

The infiltration of inflammatory cells, such as Th2-type CD4^{+} T-lymphocytes, eosinophils, and mast cells, into the bronchial mucosa is known to occur with asthma [2–4].

Th2-type CD4^{+} T-lymphocytes produce Th2-type cytokines such as IL-13, IL-4, IL-5, and granulocyte macrophage-colony stimulation factor. Of these cytokines, IL-4 and IL-13 are thought to be the most closely associated with allergic asthma. IL-4 and IL-13 contribute to B cell class-switching, which leads to IgE production, pulmonary eosinophilia, and AHR [5]. Eosinophils release cytotoxic proteins (major basic protein, eosinophil cationic protein, and eosinophil peroxidase) that damage the airway epithelium and cause structural changes [6]. Mast cells secrete chemical mediators (histamine, leukotrienes (LTs), prostaglandin, and thromboxanes) and cytokines (IL-4 and IL-13) that contribute to bronchoconstriction and AHR [7,8].

In electrically non-excitable cells, such as immune cells, an increase in the cytosolic Ca^{2+} concentration via

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sustained Ca^{2+} influx across the plasma membrane is crucial for their activation. It is mainly mediated by the store-operated Ca^{2+} entry (SOCE) mechanism, in which emptying of intracellular Ca^{2+} stores activates the Ca^{2+} influx [9]. For example, when T-lymphocytes are stimulated by anti-CD3 antibody, Ca^{2+} is released transiently from the endoplasmic reticulum (ER), where intracellular Ca^{2+} is stored, into the cytosol. Depletion of Ca^{2+} in ER triggers a sustained influx of extracellular Ca^{2+} , which results in the activation of the Ca^{2+} -regulated transcription factor (NF-AT) that controls IL-2 gene expression. SOCE is an essential step in T-lymphocyte activation and proliferation [10]. SOCE has been observed in T-lymphocytes, basophils, macrophages, dendritic cells, and mast cells [11,12], as well as in airway smooth muscle cells [13]. At least two types of channels are reported as SOCE-related channels. One is the calcium release-activated calcium (CRAC) channel, which is a highly Ca^{2+} -selective channel, and the others are the non-selective, Ca^{2+} -permeable canonical transient receptor potential channels (TRPC). Recently, Orai1, which is a plasma membrane protein, was identified as a CRAC channel subunit in *Drosophila* cells, human T cells, and human embryonic kidney cells (HEK293) [14–16]. Orai1 and stromal interacting molecule (STIM) 1, which senses the ER Ca^{2+} level, have been reported to mediate the CRAC current as well as SOCE in *Drosophila* cells, Jurkat T cells, and HEK293 [16–18]. However, the molecular mechanisms of SOCE have yet to be determined completely.

The role of SOCE in the pathogenesis of asthma has not been established yet. However, it has been reported that SOCE plays an important role in the activation of inflammatory cells, the degranulation of mast cells [19], and the production of inflammatory cytokines in T-lymphocytes [10]. In addition, sustained contraction of rat trachea requires sustained extracellular Ca^{2+} influx [13]. Therefore, SOCE is expected to be involved in the inflammation and bronchoconstriction seen with asthma. So far, several compounds, such as econazole, SKF-96365, and methyl- β -cyclodextrin, are known to inhibit SOCE [20–22]. However, none of these compounds is very potent or selective.

YM-58483/BTP-2 is a selective SOCE blocker that does not cross-react with voltage-operated Ca^{2+} entry, K^{+} channels, or Cl^{-} channels [22–25]. Although the precise molecular mechanism by which YM-58483 inhibits Ca^{2+} influx is not known, it suppresses CRAC channel, TRPC3 and TRPC5, and also facilitates the transient receptor potential for melastatin (TRPM) 4 channel [23–25]. YM-58483 suppresses cytokine production (IL-2, IL-4, IL-5, IL-13, etc.) in vitro [22,26–28] and T-lymphocyte-mediated immune responses in the in vivo trinitrochlorobenzene-induced delayed-type hypersensitivity model [22]. In this study, the pharmacological profiles of YM-58483 were investigated in vitro and in vivo in order to evaluate its potential as a therapeutic anti-asthma drug.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs were purchased from SLC Japan Co. (Hamamatsu, Japan). Animals (6–9 weeks old), weighing from 350 to 650 g, were used in spasmogen-induced bronchoconstriction models. Animals (4–5 weeks old), weighing from 250 to 350 g, were used in OVA-induced bronchoconstriction and AHR models. The animals were given food and water ad libitum until the day before the experiments. All experiments were carried out in accordance with regulations of the corporate Animal Ethics Committee.

2.2. Materials

YM-58483, 4-methyl-4'-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide was synthesized by Astellas Pharma Inc. (Ibaraki, Japan). Ovalbumin (OVA, Grade V), albumin bovine (BSA), urethane, mepyramine, penicillin–streptomycin and phytohemagglutinin-P (PHA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and acetylcholine was purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan), LT D₄ was purchased from Cayman Chemical Co. (Arbor, MI, USA). A 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (PAF) was purchased from Bachem AG (Bubendorf, Switzerland), gallamine triethiodide (Flaxedil[®]) was purchased from Rhone-Poulenc Rorer (Paris, France), mouse anti-DNP monoclonal IgE was purchased from Seikagaku Co., Ltd. (Tokyo, Japan), DNP-BSA was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan), rat anti-human/mouse IL-5 antibody, and biotinylated rat anti-human IL-5 antibody were purchased from Pharmingen (San Diego, CA, USA), and recombinant human IL-5 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Prednisolone was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Since prednisolone administered orally exhibited anti-asthmatic effect in rats [29], it was used as a reference compound for in vivo studies. YM-58483 and prednisolone were dissolved in and diluted with 100% DMSO, after which they were added to each assay buffer in volumes of 0.5 μl . The final in vitro concentration of DMSO was less than 0.25%. For oral administration, YM-58483 and prednisolone were suspended in 0.5% methylcellulose solution (Shin-Etsu Chemical, Tokyo, Japan) at a volume of 1 ml/kg and administered 1 and 2 h before spasmogen or OVA challenge.

2.3. DNP-induced histamine release and LTs production in RBL-2H3 cells

RBL-2H3 cells, a rat basophilic leukemia cell line with a phenotype similar to that of mucosal mast cells [30], were purchased from the American Type Culture Collection

(Manassas, VA, USA), and maintained as monolayer cultures in RPMI 1640 medium (GIBCO BRL, Rockville, MD, USA) with 10% FBS (JRH Biosciences, Lenexa, KS, USA), 25 mM HEPES, 2 g/l NaHCO₃, and penicillin (100 U/ml)/streptomycin (100 µg/ml) in a 5% CO₂-humidified atmosphere. Mediator release from RBL-2H3 cells was induced using DNP and a method described previously [31], with minor modifications. RBL-2H3 cells were cultured with monoclonal anti-DNP IgE (100 ng/ml) in a 1000-ml spinner culture bottle at a density of 3×10^5 cells/ml for 24 h. After washing, the cells that had been primed with IgE (8×10^5 cells) in an assay buffer (5 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 5 mM D(+)-glucose, pH 7.4), were stimulated with the antigen, DNP-BSA (0.1–100 ng/ml), in 96-well plates (total volume: 200 µl) at 37 °C for 20 min. After stimulation, the cells were centrifuged (250g, 10 min at 4 °C), and then the supernatants were removed and frozen at –0 °C until use. The levels of histamine and LTC₄/D₄/E₄ (LTs) in the supernatant were determined using histamine ELISA kits (ICN Pharmaceutical Inc., Costa Mesa, CA, USA) and LTC₄/D₄/E₄ EIA kits (Amersham Life Science, Piscataway, NJ, USA), respectively. To measure the effect of the test compounds, IgE-primed cells were pretreated with various concentrations of YM-58483 (30–3000 nM), or prednisolone (10 µM), and then stimulated with DNP-BSA (30 ng/ml).

2.4. PHA-induced IL-5 and IL-13 production from whole human peripheral blood

PHA was used as a stimulator to mimic the initial responses the antigens evoke in T-lymphocytes. Human peripheral blood was obtained from healthy volunteers. Heparinized human peripheral whole blood was diluted in RPMI1640 medium with penicillin-streptomycin at a rate of 1:3.5. Diluted blood (450 µl) was pretreated with various concentrations of YM-58483 (10–1000 nM) or prednisolone (1–100 nM), then stimulated with PHA (10 µg/ml) in 24-well plates (total volume: 500 µl) for 48 h at 37 °C. The plates were centrifuged (250g, for 10 min at 4 °C), and then the supernatants were harvested and frozen at –20 °C until use. The levels of IL-5 and IL-13 in the supernatant were measured ELISA using an anti-IL-5 monoclonal antibody and a human IL-13 ELISA Kit DuoSet (R&D Systems, Minneapolis, MN, USA), respectively.

2.5. OVA-induced contractions in tracheal tissue isolated from actively sensitized guinea pigs

Male Hartley guinea pigs (6–9 weeks, 350–650 g) were actively sensitized by intraperitoneal injection of OVA (5 µg) containing Alum [Al(OH)₃, 10 mg]. This was repeated a total of three times at 1 week intervals. One week after the last sensitization, the guinea pigs were sacrificed by exsanguination under anesthesia, and the tracheas were removed immediately. Each trachea was cut

into 3-mm-wide ring segments, suspended in a 10-ml organ bath containing Krebs-Henseleit solution (118 mM NaCl, 4.6 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, and 10 mM glucose), and then equilibrated under a resting tension of 1g for 30 min at 37 °C. Each tracheal ring was treated with carbachol (1 µM) to define the maximum contraction level for each trachea. After washing and stabilizing for 30 min, the tracheal rings were pretreated with vehicle or YM-58483 (0.1–10 µM) for 15 min, then stimulated by cumulative addition of OVA (0.1–1000 µM). After the response at each concentration reached a plateau, the next concentration of OVA was added. Tracheal responses were recorded isometrically using a force transducer (TB-611T, Nihon Kohden, Tokyo, Japan) connected to a force amplifier (AP-621G, Nihon Kohden, Tokyo, Japan) and a pen recorder (Model 3056, Yokogawa Electric Co., Tokyo, Japan). Results were expressed as the percentage of maximum contraction induced by carbachol.

2.6. OVA-induced bronchoconstriction in actively sensitized guinea pigs

Male Hartley guinea pigs (4–5 weeks, 350–650 g) were actively sensitized by intraperitoneal injection of OVA (5 µg) containing Alum (10 mg). This was repeated a total of three times at 2 week intervals. OVA-induced bronchoconstriction was assessed 1 week after the last sensitization. Animals were anesthetized with urethane (1.2 g/kg, i.p.), and then the jugular vein and trachea were cannulated. The animals were placed individually in a whole body plethysmograph (Buxco Electronics, Inc., Sharon, CT, USA), and airway resistance was calculated from airflow and transpulmonary pressure using a pulmonary mechanics analyzer (Model 6; Buxco Electronics, Inc.). To suppress spontaneous breathing, gallamine (1 mg/kg, i.v.) was injected through the jugular vein cannula, and animals were mechanically ventilated at a rate of 60 strokes/min and a volume of 10 ml/kg body weight per cycle using a constant-volume respirator (Model 683; Harvard Bioscience, South Natick, MA, USA). Before OVA challenge, a steady baseline was measured over 8 min. To prevent anaphylactic shock, the animals were pretreated with mepyramine (2 mg/kg, i.v.), an H1-histamine receptor antagonist, 2 min prior to a 5-min challenge with aerosolized OVA solution (10 mg/ml) via an ultrasonic nebulizer (NE-U12, Omuron, Kyoto, Japan) attached to the trachea cannula. Airway resistance was measured continuously throughout the experiment until 15 min after OVA challenge. Data were collected for each breath and averaged at 1-min intervals. The results were expressed as the time course of increased airway resistance and the area under the time-response curve (AUC) for the first 15 min after OVA challenge. Negative control animals were actively sensitized by OVA and exposed to aerosolized 0.9% saline. It was confirmed that mepyramine did not affect basal airway resistance.

2.7. Spasmogen-induced bronchoconstriction in normal guinea pigs

Acetylcholine, LTD₄, and PAF-induced bronchoconstriction were performed according to a previously described method [32], with minor modifications. Male Hartley guinea pigs were anesthetized with urethane (1.2 g/kg, i.p.) and artificial respiration and measurement of airway resistance were carried out as described above. The animals were injected with acetylcholine (100 µg/kg, i.v.), LTD₄ (300 ng/kg, i.v.), or PAF (300 ng/kg, i.v.), and then airway resistance was measured for 2 or 5 min after challenge. Data were calculated for each breath and averaged at 10-s intervals.

2.8. OVA-induced AHR in actively sensitized guinea pigs

Male Hartley guinea pigs were sensitized by intraperitoneal injection of OVA (10 µg) containing Alum (10 mg). One week later, sensitized animals were exposed to an aerosolized solution of OVA (5 mg/ml) for 5 min via whole body exposure using an ultrasonic nebulizer. This was repeated a total of three times at 1 week intervals as a booster. To avoid anaphylactic shock, the animals were pretreated with mepyramine (10 mg/kg, i.p.) 30 min before each OVA booster. One week after the final booster, the animals that had been pretreated with mepyramine (10 mg/kg, i.p.) were challenged with an aerosolized OVA solution (5 mg/ml) for 30 min. Negative control animals were actively sensitized to OVA, and exposed and challenged with aerosolized 0.9% saline. AHR was assessed as the change in airway reactivity to aerosolized acetylcholine 24 h after OVA challenge. The animals were anesthetized with urethane (1.2 g/kg, i.p.) and artificial respiration was carried out as described above. Airway pressure was continuously measured using a low-pressure transducer (TP-101T; Nihon Kohden) connected to a carrier-amplitude amplifier (AG620G or AG621G, Nihon Kohden), and recorded on a polygraph (RM-6100, Nihon Kohden) configured with a Macintosh computer using the MacLab 8 software system (AD Instruments, Castle Hill, NSW, Australia). The animals were exposed to aerosolized saline (baseline) followed by increasing concentrations of acetylcholine solution (1, 3, 10, 30, and 100 mg/ml) using an ultrasonic nebulizer via the trachea cannula. Each concentration was delivered over six strokes of the respirator, with 3-min intervals between the different concentration strengths. The maximum airway pressure after each exposure to acetylcholine was taken and expressed as the increase in airway pressure relative to the baseline. The log of the acetylcholine concentration required to produce an airway pressure increase equal to 200% of the baseline value was calculated using log-linear interpolation and was represented as PC₂₀₀. Lower log PC₂₀₀ values indicate greater airway reactivity.

2.10. Statistical analyses

Data represent the mean ± SEM. The percent inhibition values were calculated for unstimulated and control stimulated levels. The concentration and doses causing 50% inhibition were calculated using non-linear regression. The statistical significance of the difference between groups was determined using either a one-way ANOVA followed by Dunnett's multiple comparison test or the Student's *t*-test. *P* values of less than 0.05 were considered significant. All data analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Effect of YM-58483 on DNP-induced histamine release and LTs production in RBL-2H3 cells

The IgE-primed RBL-2H3 cells did not generate LTs by themselves, however, the presence of DNP-BSA (1–100 ng/ml) stimulated LTs production in a concentration-dependent manner (Fig. 1A). The histamine released from IgE-primed RBL-2H3 cells was also stimulated by DNP-BSA (3–100 ng/ml) in a concentration-dependent manner (Fig. 1B). In order to investigate the effect of YM-58483 on these reactions, 30 ng/ml DNP-BSA was added to stimulate the release of submaximal amounts of histamine as well as LTs production in RBL-2H3 cells. YM-58483 inhibited both LTs production and histamine release in a concentration-dependent manner, with IC₅₀ values of 310 ± 16 and 460 ± 160 nM, respectively (Fig. 2). In contrast, prednisolone had little effect on either response, even at 10 µM.

3.2. Effect of YM-58483 on PHA-induced IL-5 and IL-13 production in whole human peripheral blood

It has been reported that PHA activates SOCE in T-lymphocytes [33]. PHA significantly stimulated the production of IL-5 and IL-13 in human peripheral blood cells compared to unstimulated blood (IL-5; PHA (+) 171 ± 23 pg/ml vs. PHA (–) 6 ± 3 pg/ml, IL-13; PHA (+) 356 ± 36 pg/ml vs. PHA (–) 0 pg/ml). YM-58483 inhibited IL-5 and IL-13 production in a concentration-dependent manner, with IC₅₀ values of 125 ± 11 and 148 ± 18 nM, respectively (Fig. 3). Production of both cytokines was completely inhibited at 1 µM. Prednisolone inhibited IL-5 and IL-13 production with IC₅₀ values of 26 ± 6 and 30 ± 5 nM, respectively. YM-58483 is 4–6 times less potent than prednisolone in this Th2 cytokine production assay.

3.3. Effect of YM-58483 on OVA-induced contraction of trachea isolated from OVA-sensitized guinea pigs

The cumulative addition of OVA (0.1–1000 ng/ml) evoked concentration-dependent contractions in the tracheal rings prepared from OVA-sensitized guinea pigs.

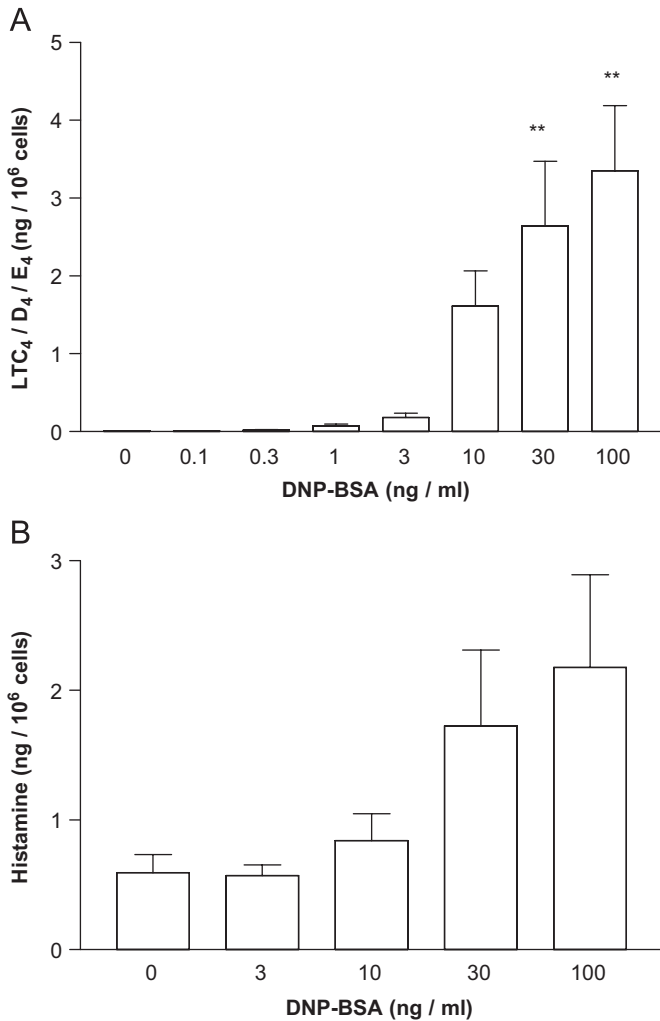


Fig. 1. DNP-induced LTC₄/D₄/E₄ production (A) and histamine release (B) in IgE-primed RBL-2H3 cells. Data are expressed as the mean $\pm$ SEM of 3–4 independent experiments. ** P < 0.01: significant difference from vehicle (without DNP-BSA) as determined using Dunnett's multiple range test.

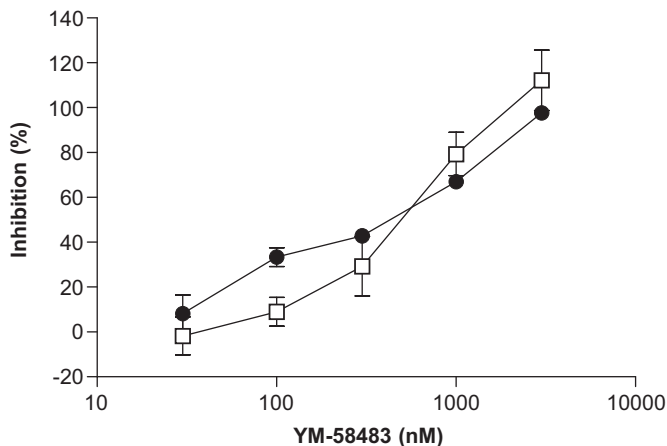


Fig. 2. Effect of YM-58483 on DNP-BSA (30 ng/ml)-induced LTC₄/D₄/E₄ (●) and histamine (□) production in IgE-primed RBL-2H3 cells. Data are expressed as the mean $\pm$ SEM of 3–4 independent experiments.

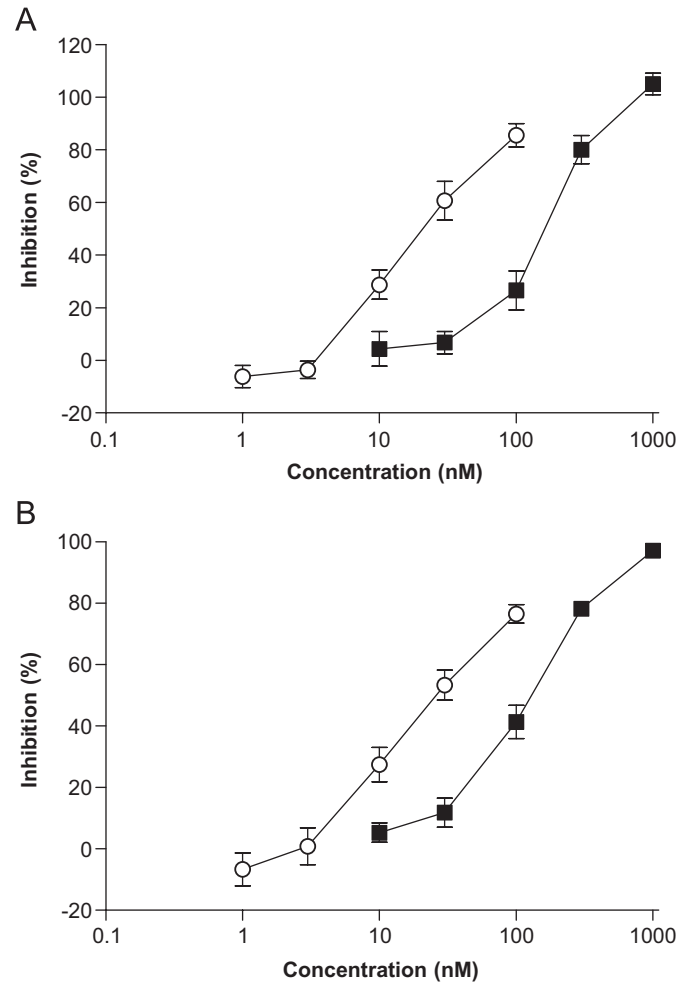


Fig. 3. Effect of YM-58483 (■) and prednisolone (○) on IL-5 (A) and IL-13 (B) production in the supernatant from PHA-stimulated human peripheral blood. Data are expressed as the mean $\pm$ SEM of 6–7 independent experiments.

At 1000-ng/ml doses of OVA, contraction was approximately 80% of that evoked by 1 μ M carbachol (Fig. 4). YM-58483 (0.1–10 μ M) did not affect basal tone (data not shown), but concentration-dependently inhibited OVA-induced contractile responses (Fig. 4).

3.4. Effect of YM-58483 on OVA-induced bronchoconstriction in OVA-sensitized guinea pigs

When OVA-sensitized guinea pigs were challenged with aerosolized OVA solution, an increase in airway resistance occurred that continued for more than 15 min. YM-58483 (0.3–30 mg/kg, p.o.), which did not affect basal airway resistance, inhibited the increase in airway resistance dose-dependently (Fig. 5A). This inhibition was statistically significant at 30 mg/kg, which was a AUC reduction of approximately 35% (AUC was 3.50 ± 0.41 and 2.14 ± 0.34 for vehicle and YM-58483, respectively) (Fig. 5B). In contrast, prednisolone did not significantly inhibit the increase in airway resistance, even at 100 mg/kg (AUC was

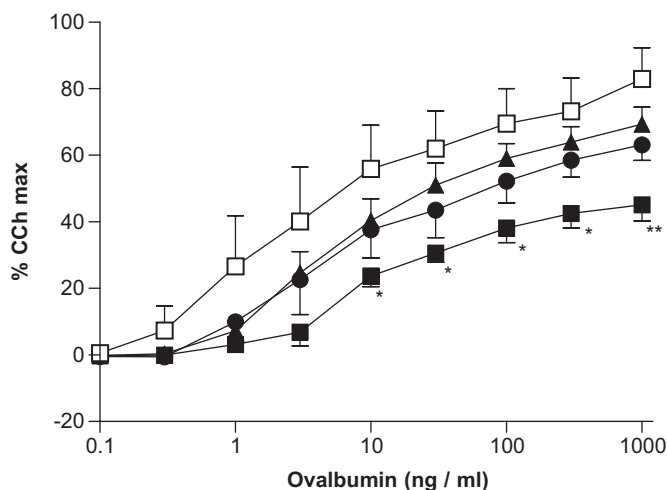


Fig. 4. Effect of YM-58483 (vehicle: □, 0.1 μM: ▲, 1 μM: ●, 10 μM: ■) on the OVA-induced contractile response of isolated trachea from OVA-sensitized guinea-pigs. Results are expressed as a percentage of the strongest contraction induced by 1 μM carbachol (CCh max). Data are shown as the mean ± SEM of the results from five animals. * $P < 0.05$, ** $P < 0.01$: significant difference from the control as determined using Dunnett's multiple range test.

2.45 ± 0.29 and 2.37 ± 0.36 for vehicle and prednisolone, respectively).

3.5. Effect of YM-58483 on acetylcholine-, LTD₄- and PAF-induced bronchoconstriction in normal guinea pigs

Intravenous injection of acetylcholine, LTD₄, and PAF in guinea pigs provoked transient bronchoconstriction, with peak times of 20, 30, and 30 s, respectively (data not shown). YM-58483 (0.3–30 mg/kg, p.o.) dose-dependently inhibited PAF-induced increases in airway resistance, but did not inhibit those induced by LTD₄ or acetylcholine at doses of 30 mg/kg (Table 1). In contrast, prednisolone (100 mg/kg, p.o.) did not significantly inhibit any agonist-induced increases in airway resistance (Table 1).

3.6. Effect of YM-58483 on OVA-induced AHR in OVA-sensitized guinea pigs

AHR is a typical and pivotal condition that occurs with bronchial asthma. Repeated exposure of OVA-sensitized guinea pigs to OVA solution resulted in an increase in airway reactivity to acetylcholine and a decrease in the PC₂₀₀ value compared to the negative control group (Fig. 6). YM-58483 (1–30 mg/kg, p.o.), which did not affect baseline airway pressure (data not shown), dose-dependently suppressed airway hyperreactivity to acetylcholine and increased the PC₂₀₀ value, with complete inhibition at 30 mg/kg (Fig. 6A, B). Prednisolone (3–100 mg/kg, p.o.) also dose-dependently suppressed airway hyperreactivity to acetylcholine and increased the PC₂₀₀ value, with complete inhibition at 100 mg/kg (Fig. 6C, D). The ED₅₀ values of orally administered YM-58483 and prednisolone were 1.0 and 12.2 mg/kg,

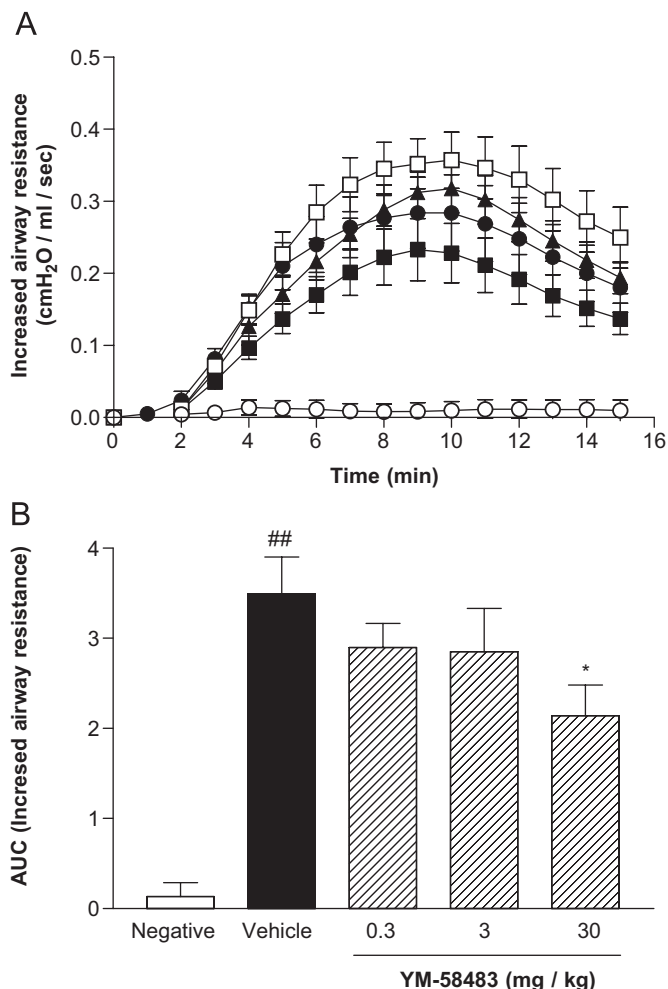


Fig. 5. Effect of YM-58483 (negative: ○, vehicle: □, 0.3 mg/kg: ▲, 3 mg/kg: ●, 30 mg/kg: ■, p.o.) on increases in airway resistance induced by OVA challenge in OVA-sensitized guinea pigs. The results are expressed as the time course of increased airway resistance for 15 min after OVA challenge (A) and as the AUC (B). Data are shown as the mean ± SEM of the results from 7 to 9 animals. ## $P < 0.01$: significant difference from negative control as determined using the Student's *t*-test. * $P < 0.05$: significant difference from the control as determined using Dunnett's multiple range test.

respectively, which indicated that YM-58483 was more potent than prednisolone.

4. Discussion

In this study, the effect of YM-58483/BTP-2, an SOCE blocker, was investigated in several in vitro and in vivo asthma related models to evaluate the pharmacological profile and therapeutic potential of this compound for bronchial asthma.

Airway mast cells are key players in the initiation of acute antigen-induced histamine release and LTs production [34,35]. The importance of SOCE for both degranulation and LTs production in mast cells was suggested by the following two findings, which resulted from experimentation with a mast cell-like cell line, RBL-2H3: (1) the rate of

Table 1

Effects of YM-58483 and prednisolone on increases in airway resistance induced by acetylcholine, LTD₄, and PAF

Compound	Dose (mg/kg, p.o.)	Maximum response (increased airway resistance [cmH ₂ O/ml/s])		
		Acetylcholine	LTD ₄	PAF
YM-58483	Vehicle	0.427 ± 0.034	0.151 ± 0.03	0.233 ± 0.040
	0.3	NA	NA	0.227 ± 0.047
	3	NA	NA	0.154 ± 0.041
	30	0.429 ± 0.083	0.141 ± 0.024	0.099 ± 0.020*
Prednisolone	Vehicle	0.379 ± 0.032	0.133 ± 0.034	0.148 ± 0.038
	100	0.404 ± 0.035	0.162 ± 0.019	0.147 ± 0.068

Results are expressed as increased airway resistance. Data are shown as the mean ± SEM from 5 to 7 animals.

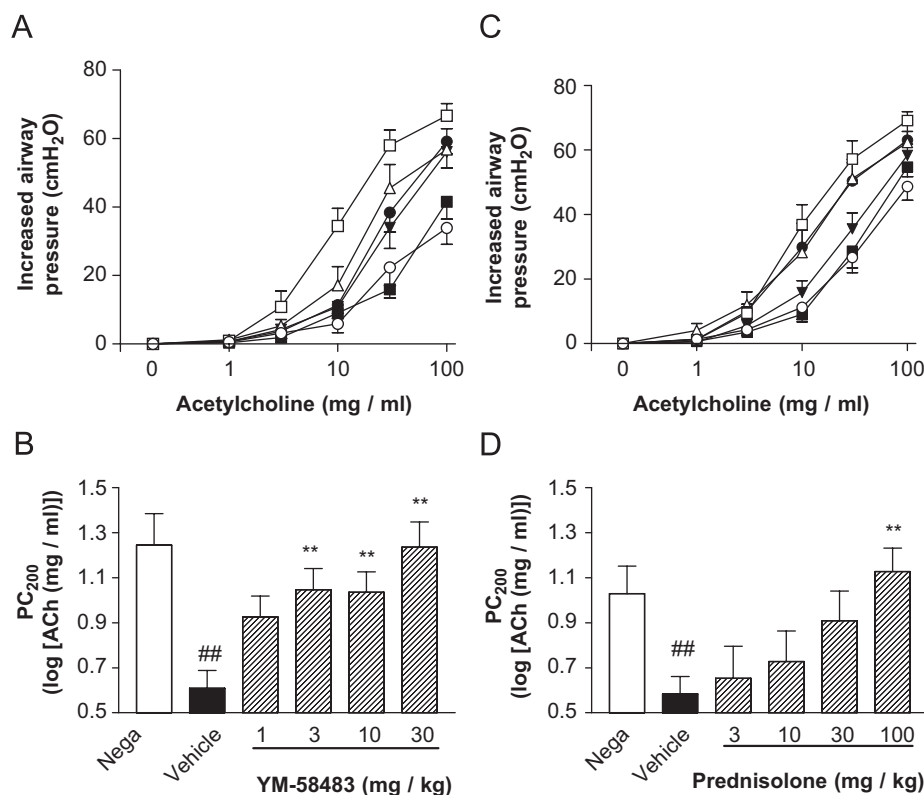
**P* < 0.05: significant difference from vehicle guinea pigs as determined using Dunnett's multiple range test. NA, not available.

Fig. 6. Effect of YM-58483 (A, B) and prednisolone (C, D) on OVA-induced airway hyperresponsiveness in OVA-sensitized guinea pigs. Results are expressed as airway reactivity to acetylcholine (A: YM-58483; vehicle: □, 1 mg/kg, ▲: 3 mg/kg, ●: 10 mg/kg, ▼: 30 mg/kg, ■: p.o., C: prednisolone; vehicle: □, 3 mg/kg, ▲: 10 mg/kg, ●: 30 mg/kg, ▼: 100 mg/kg, ■: p.o.) and the PC₂₀₀ value of acetylcholine (ACh) (B, D). Data are shown as the mean ± SEM of the results from 8 to 10 animals. ##*P* < 0.01: significant difference from negative control (Nega, ○) as determined using the Student's *t*-test. **P* < 0.05, ***P* < 0.01: significant difference from the vehicle as determined using Dunnett's multiple range test.

antigen-induced degranulation in RBL-2H3 cells decreased after treatment of methyl-β-cyclodextrin, an SOCE inhibitor [19]; and (2) the amount of antigen-induced LTC₄ production correlates with the increase in sustained intracellular Ca²⁺ concentration in RBL-2H3 cells [31]. YM-58483 potently inhibited histamine release and LTs production induced by the cross-linking of Fc ε RI on RBL-2H3 cells (IC₅₀: 460 and 310 nM, Fig. 2). These IC₅₀ values are very similar to the IC₅₀ value which YM-58483 inhibited CRAC current in RBL-2H3 cells (IC₅₀: 500 nM)

[24]. Recently, Orai1 was identified as a CRAC channel subunit [14–16]. Involvement of the CRAC channel in SOCE has been reported in Jurkat T cells and HEK293 cells based on the role of the Orai1 message knockdown cells [16]. In RBL-2H3 as well as Jurkat T cells and HEK293 cells, the co-overexpression of Orai1 and STIM1 has been reported to increase the CRAC current substantially [17,18]. SOCE and the CRAC current were induced by the depletion of Ca²⁺ from intracellular stores in RBL-2H3 [19,24]. These results suggest the involvement of the

CRAC channel in the SOCE in RBL-2H3. YM-58483 inhibited the function of overexpressed TRPC3 and TRPC5 in the range of 100–1000 nM, but activated overexpressed TRPM4 in HEK293 at a lower concentration (10 nM) [23,24]. YM-58483 also activated TRPM4 in Jurkat T cells with an EC_{50} value of 8 nM [24]. These results suggest it is unlikely that TRPM4 is responsible for the inhibition of histamine or LT release because their IC_{50} values (histamine: 460 nM, LT: 310 nM) are much larger than the EC_{50} of TRPM4 activation. It has been reported that YM-58483 does not affect voltage-dependent Ca^{2+} entry K^+ channels, such as K_v 1.3 and SK_{Ca} , or Cl^- channels [22–25]. Given this, it seems plausible that YM-58483 inhibits histamine and LT release via the suppression of the CRAC channel, TRPC3, or TRPC5. The relationship between YM-58483 and Oria1 will have to be investigated in future studies to fully explore this hypothesis.

YM-58483 inhibited OVA-induced bronchoconstriction in vivo (Fig. 5). It is known that histamine and LTs are potent bronchoconstricting agents [36]. Since a histamine receptor antagonist was used to protect against fatal anaphylaxis in this OVA-induced bronchoconstriction model, YM-58483 appears to inhibit bronchoconstriction by suppressing LT production rather than histamine release. However, SOCE has also been identified in airway smooth muscle cells [13], and is known to play a role in the KCl^- or histamine-induced contraction of isolated trachea [13,37]. In addition, it is also known that sustained Ca^{2+} influx is induced by the cross-linking of $Fc \epsilon RI$ on airway smooth muscle cells [38]. For these reasons, the direct effect of YM-58483 on airway smooth muscle cannot be ruled out.

It is known that SOCE is essential during PAF-stimulated cell activation in various cells, such as eosinophils, macrophages, and leukemia cells. PAF induces sustained Ca^{2+} influx in eosinophils and macrophages [39–41]. In this study, PAF-induced superoxide production was inhibited by SKF96365, an SOCE inhibitor, in leukemia HL-60 cells [41]. In fact, YM-58483 dose-dependently suppressed PAF-induced bronchoconstriction in guinea pigs (Table 1), that peaked at 30 s and did not disappear within 5 min (data not shown). This result suggests that the influx of extracellular Ca^{2+} is important in PAF-induced bronchoconstriction. However, YM-58483 did not inhibit LTD_4 - or acetylcholine-induced bronchoconstriction in guinea pigs. In intact human small bronchioles, LTD_4 -induced bronchoconstriction has been reported to be dependent on Ca^{2+} influx from outside the cell. However, this influx is induced by non-selective cation channels and is apparently independent of the phosphatidylinositol 3-induced emptying of the intracellular Ca^{2+} store [42]. These results suggest that, during LTD_4 -induced bronchoconstriction, SOCE does not play an important role in Ca^{2+} influx. Acetylcholine induces both transient Ca^{2+} release from intracellular Ca^{2+} stores and sustained Ca^{2+} influx from outside the cell in isolated rat trachea

[13]. However, acetylcholine induced only transient bronchoconstriction that peaked at 20 s and disappeared within one min (data not shown), and the transient contraction was induced in the absence of extracellular Ca^{2+} [13]. This suggests that Ca^{2+} release from intracellular Ca^{2+} stores is associated with acetylcholine-induced bronchoconstriction. The effect and the mechanism of extracellular Ca^{2+} influx during bronchoconstriction is likely to differ among stimulators.

YM-58483 (3 mg/kg, p.o.) significantly suppressed OVA-induced AHR (Fig. 6A, B). The following findings suggest that IL-13 is important for the induction of AHR: (1) transfer of antigen-specific $CD4^+$ T-lymphocytes from IL-13-deficient mice to naive IL-5/eotaxin double-deficient mice fails to develop antigen-induced AHR, and, conversely, the instillation of mIL-13 in the lungs of these mice reverses AHR [43]; and (2) IL-13-deficient mice fail to develop antigen-induced AHR, despite the presence of two other Th2 cytokines, IL-4 and IL-5, which cause eosinophilic pulmonary inflammation [44]. The involvement of LTs in AHR development was also suggested by two studies: (1) LT receptor antagonist inhibited OVA-induced AHR in guinea pigs [45]; and (2) treatment with LTs receptor antagonist in addition to low dose corticosteroid had an additive effect on AHR in patients with mild to moderate asthma [46]. Recently, Kim et al. [47] reported that IL-5 and IL-13 mRNA expression in the lung and AHR were significantly reduced in LTC_4S^{null} mice compared to the wild-type. This suggests that LTs-induced Th2 cytokine production is involved in AHR development. YM-58483 inhibited IL-13 production in human peripheral blood with an IC_{50} value of 148 nM (Fig. 3), and DNP BSA-induced LTs production in IgE-primed RBL-2H3 cells with an IC_{50} of 310 nM (Fig. 2). When YM-58483 (3 mg/kg, p.o.) was orally administered to guinea pigs, the maximum plasma concentration (480 nM) exceeded both IC_{50} values, and its plasma half-life was relatively long ($t_{1/2} = 13.3$ h) (data not shown). Although the direct effect of YM-58483 on the airway smooth muscle cells cannot be ruled out, YM-58483 mainly suppressed AHR via inhibition of the production of both IL-13 and LTs.

Corticosteroids are used predominantly for the treatment of bronchial asthma to suppress airway inflammation and AHR [48]. Prednisolone inhibited IL-5 and IL-13 production in human peripheral blood (Fig. 2) and OVA-induced AHR (Figs. 6C and D) as effectively as YM-58483. However, prednisolone did not affect histamine release or LTs production in RBL-2H3 cells, nor did it affect OVA-induced bronchoconstriction, even at the 100-mg/kg level. Smith et al. [49] reported that dexamethasone had no significant effect on $Fc \epsilon RI$ -dependent histamine release or the synthesis and release of LTs from mature human mast cells. In fact, corticosteroids did not induce bronchodilation during clinical treatment. In this study, YM-58483 potently inhibited two characteristic symptoms of bronchial asthma, bronchoconstriction and AHR, via the suppression of an inflammatory mediator as well as

cytokine production. In addition, YM-58483 inhibited acute eosinophilia in OVA-induced rat asthma models [50]. Therefore, the inhibition of SOCE is believed to be a promising approach for the treatment of bronchial asthma.

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