AT9283, 1-Cyclopropyl-3-(3-(5-(Morpholinomethyl)-1H-Benzo[d]Imidazole-2-yl)-1H-Pyrazol-4-yl) Urea, Inhibits Syk to Suppress Mast Cell-Mediated Allergic Response

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Abstract

Mast cells are an effector cell that plays a pivotal role in type I hypersensitive immune responses. Mast cells exist in connective tissues, such as skin and mucosal tissue, and contain granules which contain bioactive substances such as histamine and heparin in cells. The granules of mast cells are secreted by antigen stimulation to cause the type I allergic hypersensitivity. In addition, stimulated by antigen, mast cells synthesize and secrete various eicosanoids and cytokines. While AT9283 is known to have anticancer effects, the therapeutic effect of AT9283 on allergic disorders is completely unknown. In this study, it was found that AT9283 reversibly inhibited antigen-IgE binding-induced degranulation in mast cells (IC_{50}, approx. 0.58 μM) and suppressed the secretion of the inflammatory cytokines IL-4 (IC_{50}, approx. 0.09 μM) and TNF-α (IC_{50}, approx. 0.19 μM). For a mechanism of mast cell inhibition, while not inhibiting Syk phosphorylation, AT9283 suppressed the activation of LAT, a downstream substrate protein of Syk, in a dose-dependent manner. As expected, AT9283 also inhibited the activation of PLCγ1 and Akt, downstream signaling molecules of Syk/LAT, and MAP kinases such as JNK, Erk1/2, and P38. In an *in vitro* protein tyrosine kinase assay, AT9283 directly inhibited Syk activity. Next, AT9283 dose-dependently inhibited passive cutaneous anaphylaxis (PCA), an IgE-mediated allergic acute response, in mice (ED50, approx. 34 mg/kg, p.o.). These findings suggest that AT9283 has potential to use as a new drug for alleviating the symptoms of IgE-mediated allergic disorders.

Key Words: AT9283, Syk, Mast cell, Allergy, Type I hypersensitivity

INTRODUCTION

It is widely acknowledged that the number of patients suffering from allergic disease has surged worldwide in recent years (Pawankar *et al*., 2013). In the United States and Europe, particularly, about 20% of the population suffer from various allergic diseases (Galli *et al*., 2008). Type I hypersensitive allergic diseases include hay fever, atopic dermatitis, food allergy, allergic asthma, allergic rhinitis, and anaphylaxis (Akin, 2017). In these allergic diseases, mast cells play a critical role as one of the causative cells. Mature mast cells, originating from bone marrow cells, are distributed in mucosal and epithelial tissues throughout the body and are located below the epithelium of connective tissues surrounding blood cells, smooth muscle,

mucous, and hair follicles (Krystel-Whittemore *et al*., 2016).

Mast cells are activated when antigen binds to Immunoglobulin E (IgE), which is attached to FcϵRI, a high-affinity receptor for IgE, found on the extracellular surface of cell membrane. The activated mast cells secrete various inflammatory mediators and cytokines that cause allergic symptoms (Galli and Tsai, 2012). These mediators include histamine and heparin in the cytoplasmic granule of mast cells and lipid mediators such as prostaglandin, leukotrienes, and platelet-activating factor. In addition, mast cell-secreted cytokines include IL-3, IL-4, IL-5, IL-13, and TNF-α. The degranulation and cytokine secretion are critical for the induction of acute and late type I hypersensitive immune responses.

The signaling cascades of mast cells are initiated by the

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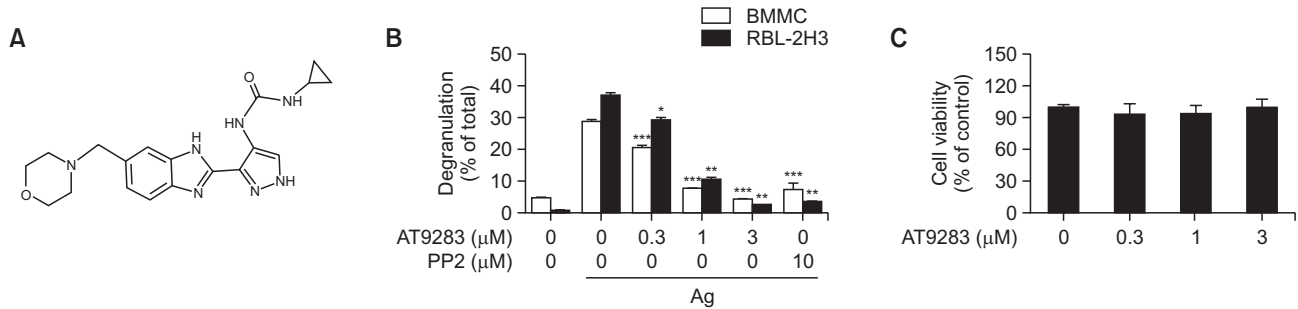
A

B

C

Fig. 1. AT9283 inhibits antigen-induced degranulation in mast cells. (A) Molecular structure of AT9283, 1-cyclopropyl-3-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazole-2-yl)-1H-pyrazol-4-yl) urea. (B) DNP-specific IgE-labeled RBL-2H3 cells (2.0×10^5 cells/well) or BMMCs (2.5×10^5 cells/well) were pre-treated with AT9283 for 30 min and then stimulated with DNP-HSA (Ag, 25 ng/mL for RBL-2H3 cells or 50 ng/mL for BMMCs) for 15 min. The degree of degranulation was measured as the ratio of the activity of β-hexosaminidase, a granule secretion marker protein, secreted into the extracellular culture medium to the total activity. Used as a control compound was PP2, an Src-family kinase inhibitor. (C) RBL-2H3 cells (2.0×10^5 cells/well) were treated with AT9283 and cultured for 4 h, and cell viability was measured with CCK-8. The CCK-8 solution was incubated for 1 h at a 1:10 ratio (CCK-8: medium). The absorbance of cell viability was measured at 450 nm. (B, C) The values indicate the mean ± SEM. from three independent experiments (each in triplicate). The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

crosslinking of antigen to the IgE that is attached to FcεRI α chain (Oettgen, 2016). Lyn, a Src family kinase adjacent to receptors on the cell membrane of mast cells, phosphorylates immunoreceptor tyrosine-based activating motifs (ITAMs) of the cytoplasmic domains of FcεRI β and γ chains. Subsequently, cytosolic Syk tyrosine kinase is recruited to phosphorylated ITAMs of the FcεRI γ chain for activation, and Syk phosphorylates various downstream signaling proteins (Galli et al., 2005). For example, activated Syk phosphorylates Linker for activation of T cells (LAT), an adapter protein essential for mast cell activation, followed by the binding of phospholipase C γ (PLCγ) to the phosphorylated LAT (Siraganian et al., 2010). PLCγ hydrolyzes membrane phosphatidylinositol bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 increases the cytoplasmic Ca^{2+} level, while DAG stimulates the activation of protein kinase (PK) C to induce degranulation of mast cells. Activated signals also stimulate the phosphorylation of mitogen-activated protein (MAP) kinases, such as JNK, Erk1/2 and P38, as well as phosphatidylinositol-3-kinases and Akt (Kawakami and Galli, 2002). The activation of MAP kinases leads to the formation and secretion of several inflammatory cytokines such as IL-4, IL-5, and IL-13 (Rivera and Gilfillan, 2006). Particularly, extracellular receptor-activated kinase (Erk1/2) induces the synthesis of lipid mediators, such as prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4), by activating phospholipase A_2 (PLA_2) (Beaven, 2009). Collectively, the activation of the series of IgE-mediated signals eventually causes an acute response, such as anaphylaxis, or a chronic response such as atopic dermatitis.

AT9283, 1-cyclopropyl-3-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-yl) urea, is a pyrazole-benzimidazole derivative. This pyrazole compound is being used as a core structure in agrochemical and pharmaceutical fields. The AT9283 compound is a multi-targeted kinase inhibitor and has been reported to inhibit the activity of not only Aurora A and Aurora B but also several kinases such as JAKs, Abl, and Flt3 (Howard et al., 2009). With this mechanism in effect, AT9283 has been clinically applied as an antitumor substance, while it was reported that the compound had inhibition effect

on the growth or survival of multiple solid tumors in multiple myeloma (Tanaka et al., 2010). However, there has been no report, to date, of the therapeutic effect of AT9283 in allergic diseases. This study demonstrated for the first time that AT9283 not only inhibits the degranulation of mast cells by antigen, via inhibiting Syk activity, but also suppresses type I allergic responses in mice.

MATERIALS AND METHODS

Antibodies and reagents

1-Cyclopropyl-3-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-yl) urea (AT9283 catalog no. S1134) (Fig. 1) was obtained from Selleckchem (Houston, TX, USA), and the stock solution was made with DMSO. Monoclonal dinitrophenol (DNP)-specific IgE (catalog no. D8406), DNP-human serum albumin (HSA, catalog no. A6661), Evans blue (catalog no. E2129), and toluidine blue O (catalog no. 198161), which were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phosphorylated forms of ZAP-70: Tyr319/Syk Tyr352 (catalog no. 2701), LAT: Tyr191 (catalog no. 3584), PLCγ: Tyr783 (catalog no. 2821), Akt: ser473 (catalog no. 9271), Erk1/2: Thr202/Tyr204 (catalog no. 9106), p38: Thr180/Tyr182 (catalog no. 9211), SAPK/JNK: Thr183/Tyr185 (catalog no. 9251), and beta-Actin: HRP-conjugated (catalog no. 5125) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against Syk (catalog no. sc-51703) were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies of LAT (catalog no. 06-807) were bought from EMD Millipore (Billerica, MA, USA). The RPMI media for cell culture were obtained from GIBCO/Life Technology, Inc. (Rockville, MD, USA).

Animals

Five-week-old BALB/c male mice were obtained from Orient Bio, Inc. (Seongnam, Korea) and used to prepare bone marrow-derived mast cells (BMMCs) and PCA experiments. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk Univer-

sity (Seoul, Korea) and conducted in accordance with the institutional guidelines.

Culture of rat basophilic leukemia (RBL)-2H3 cells and bone marrow-derived mast cells (BMMCs)

Rat basophilic leukemia (RBL)-2H3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in complete MEM (minimal essential medium) that was supplemented with L-glutamine, 100 Units/mL penicillin, 100 μg/mL streptomycin, and 15% heat-inactivated fetal bovine serum in 37°C, CO_2. To prepare BMMCs, bone marrow cells were isolated from tibias and femurs of 5-week-old mice and then cultured in complete Roswell Park Memorial Institute (RPMI) 1640 culture medium. The RPMI complete media contains 10% FBS, 4 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 mM 2-mercaptoethanol, and 10 ng/mL mouse recombinant interleukin (IL)-3. After 4 weeks, cells were differentiated into BMMCs with the purity of mast cells reaching above 95%.

Measurement of β-hexosaminidase release in RBL-2H3 cells and BMMCs

Briefly, RBL-2H3 cells (2.0×10⁵ cells/well) and BMMCs (2.5×10⁵ cells/well) were sensitized with 20 ng/mL and 50 ng/mL DNP-specific IgE, respectively, in media overnight. The cells were transferred into Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 4 mM MgCl 2, 1 mM CaCl 2, and 0.5% BSA) for RBL-2H3 cells or, for BMMCs, Tyrode buffer (pH 7.4, 135 mM NaCl, 5 mM KCl, 20 mM HEPES, 5.6 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% BSA). And AT9283 was prepared in Siraganian buffer and Tyrode buffer. The cells were incubated with or without AT9283 for 30 min and then stimulated with 25 ng/mL for RBL-2H3 cells and 50 ng/mL for BMMCs of DNP-HSA for 15 min. 30 μL of supernatant and 30 μL of p-nitrophenyl N-acetyl-beta-D-glucosamine (p-NAG) were added into a 96-well plate and incubated at 37°C for 1 h. The reaction was terminated by adding 250 μL of 0.1 M carbonate buffer. The absorbance was measured at 405 nm. The degree of degranulation of mast cells was expressed as % of released β-hexosaminidase secreted out of total β-hexosaminidase.

Measurement of cell viability

After culturing RBL-2H3 cells (2×10⁴ cells/well) in 96 well plates for 12 h, the cells were treated with AT9283 for 4 h. Cell viability was measured with Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) for 1 h in accordance with the manufacturer's protocol. Absorbance was measured at 450 nm.

Measurement of cytokines by enzyme-linked immunosorbent assay (ELISA)

RBL-2H3 cells (8×10⁵ cells/well) were sensitized overnight with 20 ng/mL DNP-IgE. IgE-sensitized RBL-2H3 cells were stimulated with 25 ng/mL DNP-HSA for 3 h at 37°C with and without AT9283. The amounts of tumor necrosis factor (TNF)-α and IL-4 in the cultured media were measured using rat OptEIA ELISA kits in accordance with the manufacturer's protocol (BD Biosciences, San Jose, CA, USA).

Western blot analysis

RBL-2H3 cells (8×10⁵ cells/well) and BMMCs (2×10⁶ cells/well) were cultured in 6 well-plates and sensitized with 20 ng/mL and 50 ng/mL DNP-specific IgE, respectively, for 12 h. The cells were washed twice with fresh culture media and treated with AT9283 for 30 min. They were subsequently stimulated with 25 ng/mL and 50 ng/mL of DNP-HSA for RBL-2H3 cells and BMMCs, respectively, for 15 min and the reaction was terminated on the ice. They were lysed using 70 μL lysis buffer (pH 7.5, 20 mM HEPES, 150 mM NaCl, 1% Nonidet P–40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenyl phosphate, 0.7 mg/mL pepstatin, and a protease-inhibitor cocktail tablet) and 70 μL 0.2% SDS containing lysis buffer per well. Following the centrifugation of the cell lysate at 15,000×g for 5 min, the supernatant was mixed with 4× Nu-PAGE™ sample buffer (Thermo Fisher Scientific, Waltham, MA, USA) and denatured at 100°C heating block for 5 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. After incubating with each specific primary antibody in a TBS-T (Tris-buffered saline containing 0.1% Tween 20) buffer containing 5% BSA or skim milk, the membrane was incubated with horseradish peroxidase-labeled secondary antibody. Protein bands were detected with an enhanced Luminata Crescendo Western HRP substrate (EMD Millipore).

In vitro protein tyrosine kinase assay

RBL-2H3 cells were sensitized using DNP-IgE prior to stimulating with 25 ng/mL of DNP-HSA for 15 min. After washing with cold PBS, the cells were lysed in a NP-40 based lysis buffer (1% Nonidet p-40, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 60 mM octyl-β-glucoside, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenyl phosphate, 0.7 μg/mL pepstatin, and a protease inhibitor cocktail tablet). Lysates were centrifuged at 13,000 rpm at 4°C, and then the supernatant were bound with Syk antibody. After 2 h, the supernatant and Syk antibody were added into 50 μL of protein G-agarose and incubated at 4°C overnight. The agarose was then washed 7 times with washing buffer (0.1% Nonidet p-40, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2.5 mM nitrophenyl phosphate, 0.7 μg/mL pepstatin, and a protease inhibitor cocktail tablet). The activity of tyrosine kinase was analyzed, with or without AT9283, using a Universal Tyrosine Kinase Assay kit (GenWay Biotech Inc., San Diego, CA, USA) in accordance with the manufacturer's recommendation.

Passive cutaneous anaphylaxis (PCA)

After BALB/c mice (n=5) were intradermally injected in the ear with 50 ng of DNP-IgE for 24 h, AT9283 (10, 30, 100 mg/kg) and cetirizine (50 mg/kg) dissolved in 5% Arabic gum were orally administered. After 30 min, 100 μg of DNP-HSA in PBS containing 5 mg/mL Evans blue was intravenously injected into the tail vein. Mice were euthanized 30 min after antigen injection and the ears were removed with Evans blue dye extracted in 1 mL of formamide at 63°C for 12 h and absorbance measured at 620 nm.

Histological analysis

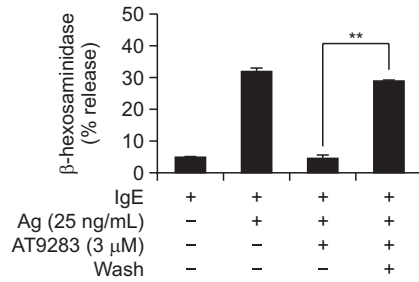The mice ears were removed and fixed using 4% parafor-

Fig. 2. AT9283 reversibly inhibits antigen-induced mast cell degranulation. RBL-2H3 cells were pre-treated for 30 min with or without AT9283, washed 3 times, and then stimulated with antigen for 15 min to induce degranulation. The degree of degranulation was measured as the ratio of the activity of β-hexosaminidase, a granule secretion marker protein, secreted into the extracellular culture medium to the total activity. The values indicate the mean ± SEM. from three independent experiments (each in triplicate). The asterisks indicate significant differences as indicated, **p<0.01.

Fig. 3. AT9283 inhibits IL-4 and TNF-α secretion from antigen-stimulated RBL-2H3 cells. IgE-primed RBL-2H3 cells (8.0×10^5 cells/well) were pretreated with or without AT9283 for 30 min and then stimulated with 25 ng/mL antigen for 3 h. Then, only the cell culture supernatant was taken and used to measure the amount of cytokines. The concentrations of secreted TNF-α and IL-4 were measured using the BDTM ELISA kit in accordance with the manufacturer's protocol. The values indicate the mean ± SEM from three independent experiments (each in triplicate). The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, *p<0.05, **p<0.01.

maldehyde. After dehydrated with ethanol, the ear tissues were embedded in paraffin. The paraffinized tissues were cut into 4 μm-sized slices and stained with toluidine blue for 1 min and 40 s. To determine the percentage of degranulated mast cells in ear tissues, the number of degranulated mast cells was measured in three sections per ear tissue (n=5 mice, 3 sections per ear). The percentage of degranulated mast cells was presented as the ratio of degranulated mast cells to total mast cells in the tissue.

Statistical analysis

Data were presented as mean ± SEM from at least three independent experiments. Statistical analysis was performed using one-way ANOVA, Dunnett's test, and the student's t-test for unpaired values unless stated otherwise. All statistical calculations (*p<0.05 and **p<0.01) were carried out with the SigmaStat software (Systat Software Inc., San Jose, CA, USA). IC$_{50}$ values were calculated using the AAT bioquest IC$_{50}$ calculator (https://www.aatbio.com/ tools/ic50-calculator).

RESULTS

Effect of AT9283 on degranulation of mast cells by antigen

To determine whether AT9283 has an inhibitory effect on degranulation in antigen-stimulated RBL-2H3 cells, we measured the extracellular secretion of β-hexosaminidase, a granule secretion marker of mast cells. Antigen-stimulated degranulation of RBL-2H3 cells and BMMCs was dose-dependently inhibited by AT9283 (Fig. 1B, IC$_{50}$, approx. 0.58 μM for RBL-2H3 cells; approx. 0.50 μM for BMMCs). Next, to determine whether AT9283 has cytotoxicity against RBL-2H3 cells, the cells were cultured with AT9283 (0.3, 1, 3 μM) for 4 h. This cell viability test revealed that AT9283 had no cytotoxicity in the experimental concentration range of RBL-2H3 cells (Fig. 1C). To confirm the reversibility of the inhibitory effect of AT9283 on mast cell degranulation, RBL-2H3 cells were treated with 3 μM AT9283 and washed 3 times with PIPES buffer after 1 h, which resulted in an observation that mast cell granules were released in equivalent level for both the group treated with
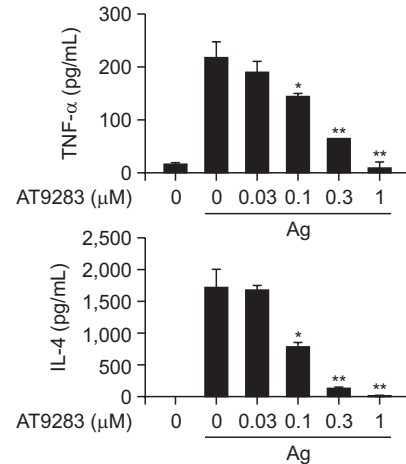
AT9283 and subsequently washed and the group not treated with AT9283 (Fig. 2). This finding indicates that the inhibitory effect of AT9283 on mast cell was reversible.

Effects of AT9283 on inflammatory cytokine secretions in mast cells

In addition to degranulation, mast cells secrete various pro-inflammatory cytokines during allergic reactions. Typical cytokines, secreted by antigen-stimulated mast cells in allergic conditions, are IL-4 and TNF-α (Galli and Tsai, 2012). Consequently, a test was conducted to find out if AT9283 inhibits the antigen-stimulated cytokine secretion of RBL-2H3 cells, which resulted in the finding that the secretion of IL-4 and TNF-α was inhibited by AT9283 in a dose-dependent manner (Fig. 3). This finding indicates that AT9283 has an inhibitory effect on the secretion of pro-inflammatory cytokines in RBL-2H3 cells (IC$_{50}$, approx. 0.017 μM for TNF-α, IC$_{50}$, approx. 0.09 μM for IL-4). Based on these findings, it is speculated that AT9283 plays a role in not only alleviating the early symptoms of allergy induced by mast cell degranulation, but also suppressing the chronic allergic symptoms induced by cytokines.

Effects of AT9283 on signaling proteins in mast cells stimulated by antigen

Next, an experiment was conducted to identify the inhibitory mechanism of AT9283 on degranulation and cytokine secretion in antigen-stimulated mast cells. In RBL-2H3 cells, AT9283 did not inhibit the phosphorylation of Syk, the most crucial early signaling protein, stimulated by antigen (Fig. 4A). However, AT9283 dose-dependently inhibited the phosphorylation of LAT and PLCγ1, which are widely known as downstream signaling proteins of Syk (Fig. 4A). In addition, also dose-dependently inhibited by AT9283 was the phosphoryla-
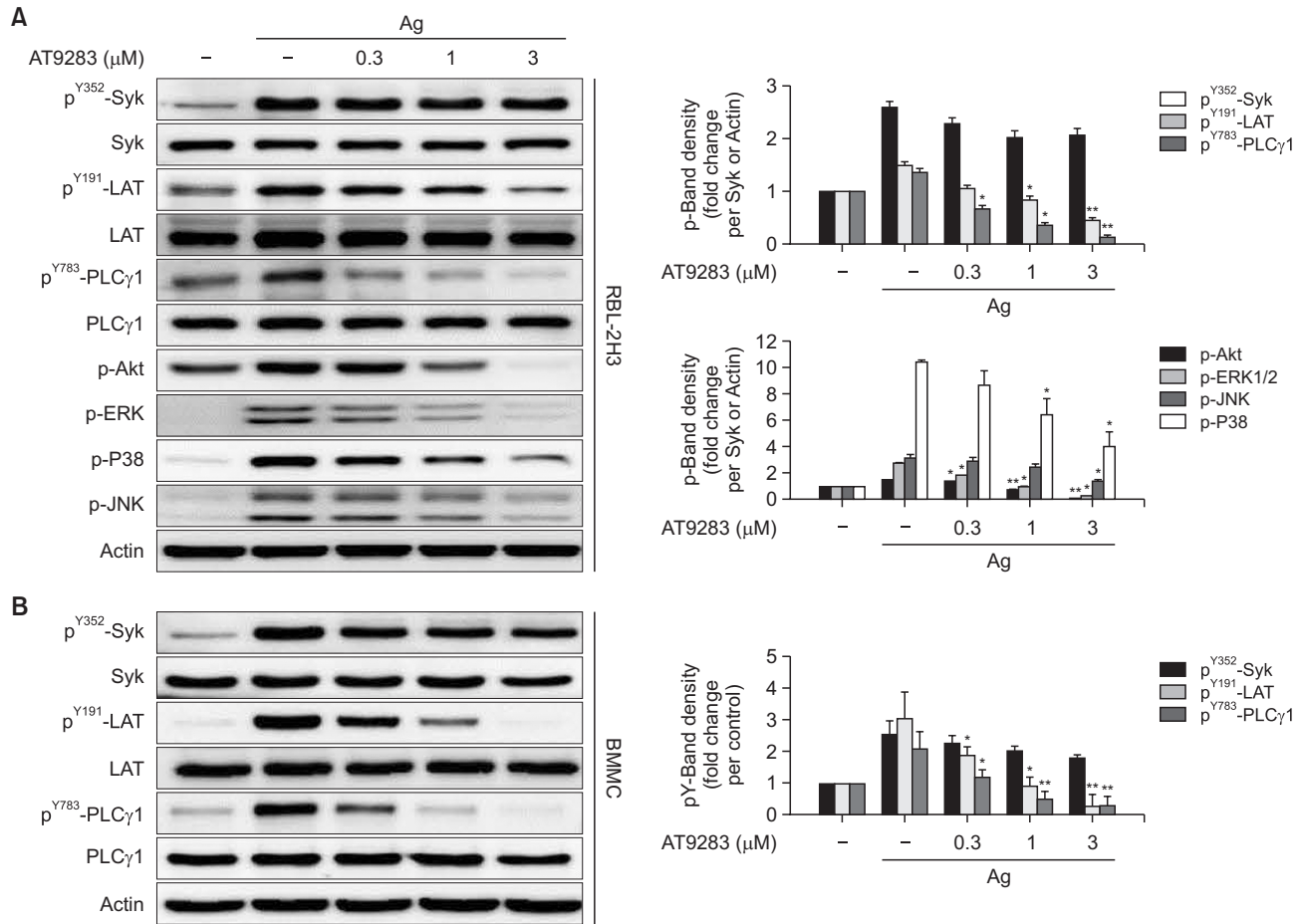
Fig. 4. AT9283 inhibits IgE-mediated signaling cascade in antigen-stimulated RBL-2H3 cells and bone marrow-derived mast cells. (A) RBL-2H3 cells (8.0×10^{5} cells/well) and (B) BMMCs (2.0×10^{6} cells/well) were sensitized with 20 ng/mL (A) or 50 ng/mL (B) of DNP-IgE overnight. RBL-2H3 cells and BMMC were pretreated on the following day for 30 min with AT9283 as indicated, followed by stimulation with 25 ng/mL (A) and 50 ng/mL (B) of antigen for 15 min, respectively. Next, the cell culture medium was removed, and Western blotting was performed using the cells remaining after washing with ice-cold PBS buffer. The detailed method of Western blot analysis is described in the "MATERIALS AND METHODS" section. The representative images and band densities of phosphorylated proteins were obtained from three independent experiments. Densitometric analysis of phosphorylated proteins was performed using Multi Gauge Ver 3.0 (FUJIFILM, Valhalla, NY, USA). (B) The values indicate the mean ± SEM from three independent experiments (each in triplicate). The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, *p<0.05, **p<0.01.

tion of Akt and MAP kinases, such as Erk1/2, p38, and JNK, which are signaling proteins that play a role in mast cell survival and cytokine secretion (Fig. 4A). Next, a test was conducted to find out if the effect of AT9283 on RBL-2H3 cells resembles that on bone marrow-derived mast cells (BMMCs). In BMMCs, while not inhibiting Syk phosphorylation as in RBL-2H3 cells, AT9283 effectively inhibited LAT and PLCγ1 phosphorylation in a dose-dependent manner (Fig. 4B). This finding suggests that AT9283 has mast cell inhibitory effects via inhibiting the Syk-dependent downstream signaling pathway among FcεRI-associated signaling pathways.

Effects of AT9283 on the activity of Syk *in vitro*

In the earlier experiment, it was found that while not inhibiting Syk phosphorylation in antigen-stimulated mast cells, AT9283 effectively suppressed phosphorylation of several signaling proteins in the Syk-dependent signaling proteins (Fig. 4), which led to the speculation that AT9283 could directly in-

hibit the activity of Syk. Subsequently, a tyrosine kinase assay was conducted to determine whether AT9283 inhibits Syk activity *in vitro*, which revealed that AT9283 inhibited the tyrosine kinase activity of Syk in a dose-dependent manner (Fig. 5). This finding suggests that AT9283 inhibits the mast cell degranulation and cytokine secretion through an inhibition of the Syk activity in IgE-mediated mast cell activation.

Effects of AT9283 on passive cutaneous anaphylaxis (PCA) in mice

The earlier finding indicated that AT9283 had the inhibitory effect on degranulation and cytokine secretion, which were responsible for allergic responses. A PCA experiment followed to find out if AT9283 suppresses allergic symptoms in an animal model of allergy. The PCA mouse model used in this experiment is widely known as a typical mast cell-mediated allergic reaction in mice (Lee *et al*., 2008; Lee *et al*., 2020). 50 ng of IgE was intradermally injected into both ears of mice, and
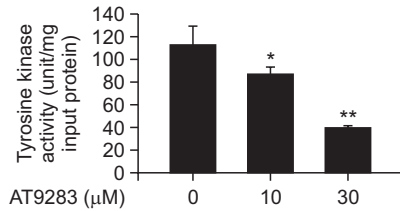
Fig. 5. AT9283 inhibits Syk kinase activity *in vitro*. IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL antigen for 15 min. The cells were lysed with NP40-based lysis buffer, and using the lysate, Syk was immunoprecipitated as described in the "MATERIALS AND METHODS" section. Then, using the immunoprecipitated Syk, an *in vitro* kinase assay was performed with the Universal Tyrosine Kinase Assay Kit to measure the direct inhibitory effect of AT9283 on Syk. The values indicate the mean ± SEM from three independent experiments (each in triplicate). The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, *$p<0.05$, **$p<0.01$.

24 h later, AT9283 (10, 30, 100 mg/kg) and cetirizine (reference drug, 50 mg/kg) were orally administered to mice. Thirty min after oral administration of AT9283 and cetirizine, antigen was injected into mice via tail vein, which led to the finding that the efflux amount of Evans blue into the ear tissue was suppressed in a dose-dependent manner in the group treated with AT9283 compared to the control group (Fig. 6A). Particularly, when compared with the group treated with cetirizine, the group treated with AT9283 had a sharp decrease in efflux at the highest AT9283 concentration of 100 mg/kg (Fig. 6A, 6B, ED_{50}, approx. 34 mg/kg, p.o.). In addition, measuring the ratio of degranulated mast cells to total mast cells in ear tissue indicated that the ratio was significantly lower in the group treated with AT9283 (100 mg/kg) in comparison to the control group (Fig. 6C). Consequently, these experiment findings show that AT9283 not only suppresses the mast cell degranulation and cytokine secretion but also inhibits the antigen-stimulated allergic response in mice.

Fig. 6. AT9283 inhibits IgE-mediated passive cutaneous anaphylaxis (PCA) reaction in mice. DNP-specific IgE (50 ng) was injected intradermally into the ears of mice. AT9283 (10, 30, 100 mg/kg) and Cetirizine (CZ, 50 mg/kg) were orally administered on the following day 30 min prior to injecting the mixture of Evans blue and Antigen into the tail vein. (A) Representative ear images are shown 1 h after PCA in mice. (B) Ear tissues were extracted with formamide and then the amount of Evans blue was measured at 620 nm. (C) Histological analysis was performed on the ear tissues that had undergone the same experiment as (A), the ear sections were stained with toluidine blue, and the numbers of total mast cells and degranulated mast cells were measured with the ratio indicated. (A, B) The values indicate the mean ± SEM from three independent experiments (each in triplicate). The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, *$p<0.05$, **$p<0.01$.

DISCUSSION

The World Allergy Organization (WAO) estimates that more than 20% of the population in developed countries suffer from allergy symptoms (Pawankar *et al*., 2013). Most common cases of allergic diseases include allergic rhinitis, asthma, hay fever, and life-threatening anaphylaxis. Among them, anaphylaxis, a typical type I allergic reaction, develops in approx. 1 in every 1000 of the general population. Mast cells are an effector cell that plays a central role in those allergic diseases, in particular, type I allergic disease (Lee *et al*., 2011; Kim *et al*., 2013). When IgE-mediated mast cell activation is initiated by antigen stimulation, mast cells initiate signaling cascades through FcεRI to secrete intracellular granules, histamine, various lipid mediators, and cytokines (Kawakami and Galli, 2002; Lee *et al*., 2006). Mast cells are found in mucosal and epithelial tissues throughout the body and play an important role in not only immediate hypersensitivity that occurs within a few minutes to repeated allergen exposure, but also symptom development of chronic allergic diseases that occur chronically over a long period of time.

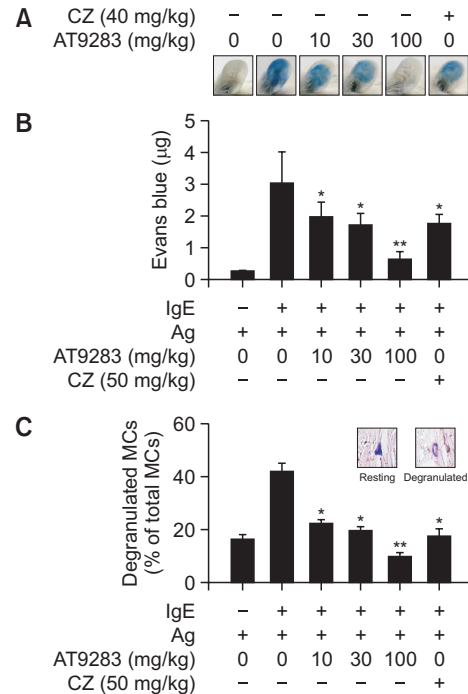Several drugs are currently used as a therapeutic agent to alleviate allergic diseases (Warrington *et al*., 2018). Examples include corticosteroids, antihistamines, and adrenaline. Nevertheless, although these treatments are effective in treating allergic diseases, they also coincide with side effects. For example, corticosteroids have a side effect of Cushing's syndrome, which is caused by an excess of cortisol. A typical side effect of antihistamines is drowsiness, and the tolerance due to repeated administration is an obstacle to treatment (Liu *et al*., 2018; Randall and Hawkins, 2018). Consequently, studies on therapeutic agents are to continue to identify substances that have anti-allergic effects with minimized side effects.

Global pharmaceutical companies are endeavoring to develop a therapeutic agent that relieves allergic diseases. However, the development of a drug that effectively treats allergy without any side effect has not yet come to fruition. Meanwhile, many multinational pharmaceutical companies are investing heavily in a drug repositioning research, that is, to develop and identify a drug with no or minor side effects from the drugs that have already entered clinical trials. AT9283, 1-cyclopropyl-3-(3-(5-(morpholinomethyl)-1H-benzo[d]imidazol-2-yl)-1H-pyrazol-4-yl) urea used in this study, is a pyr-

azole-benzimidazole compound. Several pyrazole-containing derivatives have been used for therapeutic purposes, and the structural modification of pyrazole broadens the spectrum of biological activity (Da Costa *et al.*, 2018). Some studies have already shown that pyrazole derivatives have the efficacy of being anti-microbial, anti-convulsant, anti-inflammatory, anti-viral, and anti-cancer (Karrouchi *et al.*, 2018). Consequently, it could be inferred that AT9283 is most likely to have such diverse therapeutic effects as well. Known as a small-molecule multi-targeted kinase inhibitor, in particular, AT9283 inhibits the activity of Aurora A and Aurora B as well as the activity of several kinases such as JAKs, Abl, and Flt3 (Howard *et al.*, 2009). AT9283 also affects the growth and survival of multiple solid tumors. In multiple myeloma, it was reported that AT9283 inhibits cell-growth to induce apoptosis (Santo *et al.*, 2011). As mentioned earlier, AT9283 is known to inhibit the activity of Janus kinase (JAK), and one study also suggested that other JAK inhibitors may have an immunosuppressive effect on immune diseases (O'Shea *et al.*, 2013). In addition, AT9283 was progressed to a clinical trial phase II, as a drug for anti-multiple myeloma by the NCIC Clinical Trials Group (Collaborate with Canadian Cancer Trial Groups) (Hay *et al.*, 2016), and as a drug for Leukemia by Cancer Research UK under clinical trial phase 1 (Vormoor *et al.*, 2017). AT9283 is also known to inhibit other kinases such as Abl, Tyk2, and Yes at relatively high concentrations (Howard *et al.*, 2009). However, inhibitory effects against Lyn, Fyn, and Syk, which are important tyrosine kinases for degranulation of mast cells by antigen, have not been reported. As shown in our results, AT9283 suppressed the degranulation of mast cells by antigen through inhibiting the activity of Syk (Fig. 5), although it did not affect the activities of Lyn and Fyn in the IgE signaling pathway based on the fact that AT9283 did not inhibit Syk phosphorylation (Fig. 4). Consequently, in this study, experiments were performed to determine whether AT9283 has an inhibitory role on allergic disorders, which found that AT9283 inhibited antigen-stimulat-

ed mast cell activation (Fig. 1) and IgE-mediated PCA in mice in a dose-dependent manner (Fig. 6). This finding suggests that AT9283 has the potential to use as an allergy treatment in clinical practice.

In mast cells, Syk, a member of the non-receptor tyrosine kinase family, plays an essential role as an initial signaling protein in the signaling cascade, which is initiated after FcεRI crosslinking via the IgE-Ag complex. As presented in our experimental findings, AT9283 inhibited mast cell degranulation (Fig. 1B) and cytokine secretion (Fig. 3) in a dose-dependent manner. Earlier studies have suggested that Syk-inhibiting inhibitors have potentials as a therapeutic drug to treat allergic diseases (Seow *et al.*, 2002; Yamamoto *et al.*, 2003; Matsubara *et al.*, 2006). Other studies reported that R112, which has been a subject of clinical studies as a Syk inhibitor, reduces clinical symptoms of allergic rhinitis (Meltzer *et al.*, 2005). More recently, furaltadone and CYC116 reduced the allergic response in PCA in mice by inhibiting the activity of Src-family kinases in mast cells (Nam *et al.*, 2018; Park *et al.*, 2019). It has been generally acknowledged that Syk is a subject of great interests for the treatment of various allergic diseases (Shao *et al.*, 2020). This study found that while not inhibiting the antigen-stimulated phosphorylation of Syk in mast cells, AT9283 inhibited the phosphorylation of Syk-dependent downstream signaling proteins (Fig. 4). Generally, in mast cells, Syk phosphorylation is increased by Src-family kinases including Lyn and Fyn (Rivera and Gilfillan, 2006). As shown in our results, AT9283 did not affect the phosphorylation of Syk and inhibited phosphorylation of LAT, a downstream substrate protein of Syk (Fig. 4A). These results mean that AT9283 inhibits the activity of phosphorylated Syk without affecting the activity of Src-family kinases. In addition to these interpretations, we obtained the result that AT9283 inhibited the activity of Syk *in vitro* (Fig. 5). Taken together, these results suggest that AT9283 does not inhibit the activity of Src-family kinase, but inhibits the activity of its downstream Syk, thereby
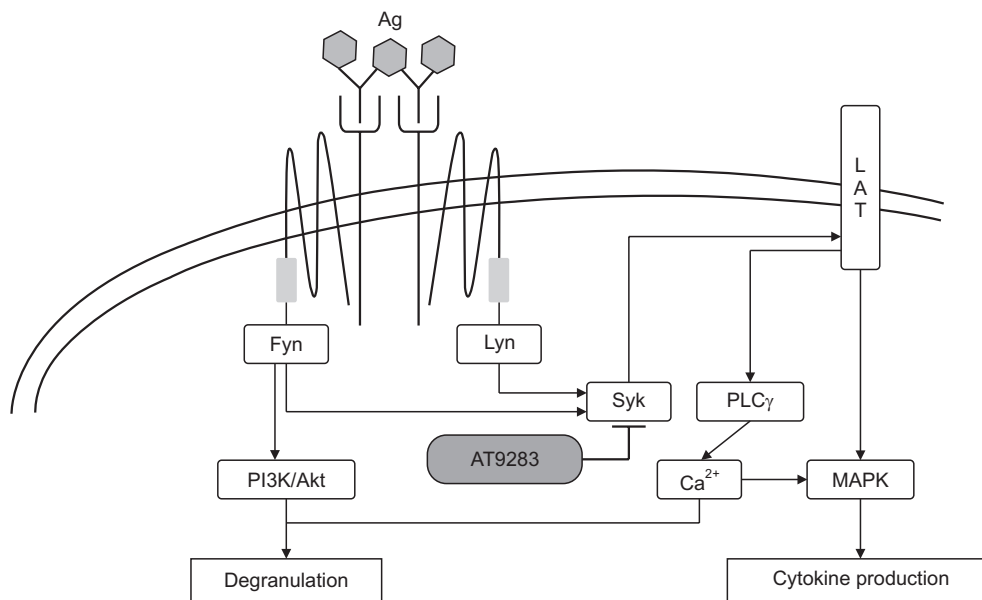

Fig. 7. Scheme showing the mechanism of AT9283 in mast cells to suppress PCA in mice.

inhibiting LAT and its downstream signaling pathway. However, we cannot exclude the possibility that AT9283 partially suppresses mast cell activation through inhibition of other protein tyrosine kinases.

To sum up the study findings, AT9283 inhibited mast cell activation by IgE/antigen cross-linking and consistently suppressed mast cell-mediated allergic responses in mice. For a mechanism of action, AT9283 was found to inhibit mast cells by inhibiting Syk activity and reducing the activation of Syk-dependent downstream signaling proteins (Fig. 7). This study revealed for the first time that AT9283 has significant potentials for the future development of a drug for allergic disease treatment.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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