

WZ3146 inhibits mast cell Lyn and Fyn to reduce IgE-mediated allergic responses *in vitro* and *in vivo*

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ABSTRACT

Mast cells (MCs) play an important role as effector cells that cause allergic responses in allergic diseases. For these reasons, MC is considered an attractive therapeutic target for allergic disease treatment. In this study, we investigated the inhibitory effect of WZ3146, N-[3-[5-chloro-2-[4-(4-methylpiperazin-1-yl)anilino]pyrimidin-4-yl]oxyphenyl]prop-2-enamide, and the mechanisms of its actions on the MC activation and IgE-mediated allergic response by using three types of MCs such as rat basophilic leukemia (RBL)-2H3 cells, mouse bone marrow mast cells (BMMCs), and human Laboratory of Allergic Diseases 2 (LAD2) cells. WZ3146 inhibited antigen-stimulated degranulation in a dose-dependent manner (IC₅₀, ~ 0.35 μM for RBL-2H3 cells; ~ 0.39 μM for BMMCs; ~ 0.41 μM for LAD2 cells). WZ3146 also suppressed the production of histamine, tumor necrosis factor (TNF)-α and interleukin (IL)-6, which mediate various allergic responses, in a dose-dependent manner. As the mechanism of WZ3146 to inhibit MCs, it inhibited the activation of spleen tyrosine kinase (Syk) and the downstream signaling proteins of Syk such as linker for activation of T cell (LAT) and phospholipase (PL) Cγ1 in the signaling pathway of FcεRI. In addition, WZ3146 inhibited the activation of Akt, extracellular signal-regulated kinase (ERK)1/2, p38, and c-Jun N-terminal kinase (JNK). However, WZ3146 did not inhibit degranulation of MCs by thapsigargin or ionomycin, which increase calcium concentration in cytosol. Notably, WZ3146 inhibited the activity of Lyn and Fyn, but not Syk. In an following animal experiment, WZ3146 inhibited IgE-dependent passive cutaneous anaphylaxis (PCA) in a dose-dependent manner (ED₅₀, ~ 20 mg/kg). Taken together, in this study we show that the pyrimidine derivative, WZ3146, inhibits the IgE-mediated allergic response by inhibiting Lyn and Fyn Src-family kinases, which are initially activated by antigen stimulation in MCs. Therefore, we propose that WZ3146 could be used as a new therapeutic agent for the treatment of allergic diseases.

1. Introduction

Mast cells (MCs) are distributed throughout the body, including skin, airway, gastrointestinal tract, and peritoneal cavity and secrete various allergenic mediators such as histamine, serotonin, leukotrienes, prostaglandins, and various pro-inflammatory cytokines (Galli and Tsai, 2012). Thus, MCs are one of critical cells responsible for the induction of allergic symptoms in various allergic diseases including asthma, allergic rhinitis, allergic conjunctivitis, food allergy, and anaphylaxis

(Galli and Tsai, 2012; Galli et al., 2005; Gilfillan and Tkaczyk, 2006). For this reason, in order to alleviate the symptoms of allergic diseases, studies for suppressing the activity of MCs or suppressing the action of various allergenic substances released from MCs have received much attention.

MCs have the IgE high affinity receptor called FcεRI, on the cell membrane surface, and free IgE is attached to the α subunit that is expressed on MCs. When antigen attaches to IgE attached on FcεRI, MCs start the activation of its signaling cascade. The α subunit of FcεRI

Abbreviations: MC, Mast cells; DNP-HSA, Dinitrophenol-conjugated human serum albumin; PCA, Passive cutaneous anaphylaxis; ITAM, Immunoreceptor tyrosine-based activation motif; Syk, Spleen tyrosine kinase; Gab2, Grb2-associated binder 2; LAT, Linker for activation of T cells; MAP kinase, Mitogen-activated protein kinases; ERK, Extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PIPES, 1,4-Piperazinediethanesulfonic acid

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does not have any enzyme activity, and the tyrosine residues of the immunoreceptor tyrosine-based activation motif (ITAM) of β and γ subunits are phosphorylated by Lyn or other Src-family kinases such as Fyn or Fgr (Lee et al., 2011). Next, the cytoplasmic Syk is attached to the phosphorylated ITAMs of γ subunits and then activated by Src-family kinases or auto-phosphorylation (Siraganian et al., 2010; Kovářová et al., 2001). The activated Syk phosphorylates and activates LAT and PLC γ 1. Activated PLC γ 1 then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) in the cell membrane to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The resulting IP3 binds to the IP3 receptor of the endoplasmic reticulum (ER) membrane and induces degranulation by increasing cytoplasmic Ca^{2+} from ER and from outside MCs (Beaven and Metzger, 1993; Hoth and Penner, 1992; Ozawa et al., 1993). LAT also plays as an adaptor protein to activate various proteins such as VAV, Growth factor receptor-bound protein 2 (Grb2), and SH2 domain containing leukocyte protein of 76 kDa (SLP76) (Saitoh et al., 2000). The proteins are important to activate GTPases such as Ras, Rac, and then stimulate MAP kinases including JNK, ERK1/2, and p38 which induce the synthesis of various cytokines (Jabril-Cuenod et al., 1996; Salojin et al., 2000; Teramoto et al., 1997).

In addition to the Lyn/Syk/LAT pivotal pathway in the Fc ϵ RI signaling pathway, a Fyn/Gab2/PI3K was reported as a complementary signaling pathway for the full activation of MCs (Nishida et al., 2011). In Fyn KO BMMC, the activation Phosphoinositide 3-kinases (PI3K) and MAP kinases were suppressed in antigen-stimulated MCs and eventually the degranulation and cytokine secretion were reduced (Parravicini et al., 2002), indicating that Fyn is another important signaling protein in the activation of MCs by antigen stimulation. Yu et al. also reported that Fyn as well as Lyn contributed significantly to the activation of Syk by antigen stimulation in MCs (Yu et al., 2006).

WZ3146 is a pyrimidine derivative that is an epidermal growth factor receptor (EGFR) inhibitor and has been developed as a therapeutic agent to treat lung cancer (Zhou et al., 2011). WZ3146 inhibits the protein tyrosine kinase activity of EGFR (Zhou et al., 2009). On the other hand, Lyn and Fyn kinase activity is significant in the activation of antigen-stimulated signaling in MCs (Beaven and Metzger, 1993). Therefore, the inhibitors of protein tyrosine kinase could become a potential candidate for the development of IgE-mediated allergic diseases, but the use of WZ3146 for allergic diseases has not been reported. In this study, we demonstrate for the first time that WZ3146 inhibits degranulation and cytokine production in IgE-mediated MCs and further suppresses an IgE-mediated allergic response in mice.

2. Materials and methods

2.1. Antibodies and reagent

WZ3146, N-[3-[5-chloro-2-[4-(4-methylpiperazin-1-yl)anilino] pyrimidin-4-yl]oxyphenyl] prop-2-enamide (Fig. 1A) was purchased from Selleckchem (Houston, TX, USA). 4-Amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2), thapsigargin, and ionomycin were obtained from Calbiochem (La Jolla, CA, USA). Monoclonal dinitrophenol (DNP)-specific IgE, DNP-human serum albumin (HSA), Evans blue, and toluidine blue O were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phosphorylated forms of Syk (Tyr352; dilution fold, 1:1000), LAT (Tyr191; dilution fold, 1:1000), PLC γ 1 (Tyr783; dilution fold, 1:1000), Akt (ser473; dilution fold, 1:1000), ERK1/2 (Thr202/Tyr204; dilution fold, 1:1000), p38 (Thr180/Tyr182; dilution fold, 1:1000), and JNK (Thr183/Tyr185; dilution fold, 1:1000) were purchased from cell signaling Technology, Inc. (Danvers, MA, USA). Antibodies against PLC γ 1 (dilution fold, 1:100), Lyn (dilution fold, 1:100), Fyn (dilution fold, 1:100), Syk (dilution fold, 1:100), Erk1/2 (dilution fold, 1:100), p38 (dilution fold, 1:100), and JNK (dilution fold, 1:100) were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against LAT (dilution fold, 1:1000) and Actin (dilution fold, 1:5000) were from EMD Millipore (Billerica, MA, USA).

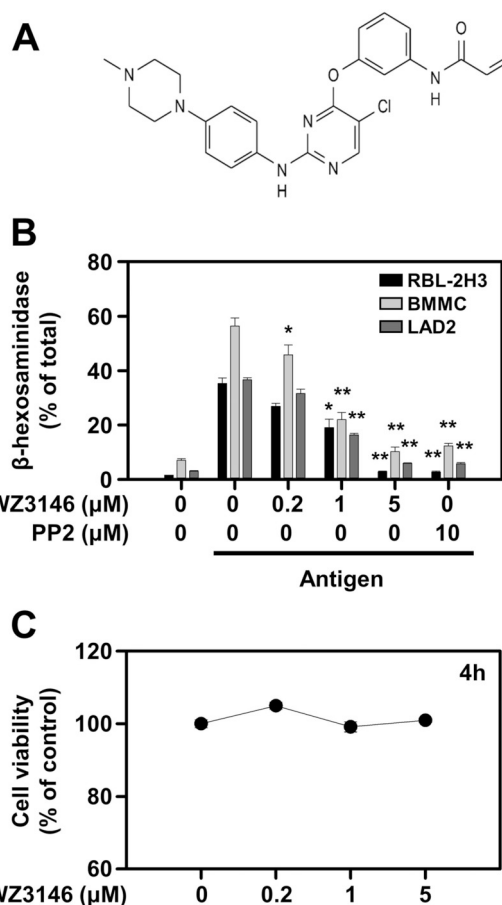


Fig. 1. WZ3146 inhibits degranulation in antigen-stimulated mast cells (MCs). (A) The molecule structure of WZ3146, N-[3-[5-chloro-2-[4-(4-methylpiperazin-1-yl)anilino] pyrimidin-4-yl]oxyphenyl] prop-2-enamide. (B) IgE-primed BMMCs (3.0×10^5 cells/well), RBL-2H3 cells (2×10^5 cells/well), or LAD2 cells (2×10^5 cells/well) were pre-treated with and without WZ3146 (0.2, 1, 5 μM) or PP2 (10 μM) for 30 min and subsequently stimulated with 50 ng/ml antigen (for BMMC and RBL 2H3 cells) or 100 ng/ml streptavidin (for LAD2 cells) for 15 min. (C) BMMCs (5.0×10^4 cells/well) incubated with/without WZ3146 (0.2, 1, 5 μM) for 4 h, and the cell viability was calculated according to the manufacturer's protocol. The control shows the value without WZ3146. (B–C) The values indicate the mean $\pm$ S.E.M. from three independent experiments (each in triplicate). The asterisks indicate significant differences from antigen-stimulated groups (B) without inhibitors, * $p < .05$, ** $p < .01$. PP2 is a general Src-family kinase inhibitor.

The media used for cell culture were from GIBCO/Life Technology, Inc. (Rockville, MD, USA).

2.2. Animal

Mice (Balb/c, 5–6 weeks of age, male) were purchased from Orient Bio. (Gyeonggi-do, Korea). The animals were used for following experiments after at least 1 week of adaptation at Konkuk University specific pathogen free animal facility. Animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

2.3. Culture of murine bone marrow-derived mast cells (BMMCs), RBL-2H3 cells, and LAD2 cells

Bone marrow cells were collected from the femur and tibia of mice and cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium containing 4 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium

pyruvate, 25 mM HEPES, 10% FBS, and 10 ng/ml interleukin (IL)-3 for differentiation of MCs. We sub-cultured twice a week with fresh culture medium. After 5 weeks, we assessed the purity of BMMCs as previously reported (Lee et al., 2015). Briefly, we analyzed the expressions of c-Kit and the α subunit of Fc ϵ RI on mature BMMCs by flow cytometer. It was observed that > 95% of all cells were MCs, which were used for the following experiments. LAD2 cells were maintained in complete StemPro-34 SFM Complete Medium supplemented with 100 ng/ml human recombinant SCF, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 mg/ml). The cells were maintained at 37 °C and 5% CO₂. Cells were fed by hemi-depletion of media once a week. RBL-2H3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and were cultured in complete MEM medium that contained 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 15% fetal bovine serum (FBS) in a humidified incubator at 37 °C, 5% CO₂.

2.4. Measurement of degranulation in MCs

BMMCs were sensitized overnight with 50 ng/ml of DNP-specific IgE and the MCs were spun down the next day. The BMMCs are suspended in Tyrode buffer (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% BSA). The cells are transferred to 96-well plates (3.0 $\times$ 10⁵ cells/well), treated with or without WZ3146 or PP2 and then incubated for 30 min at 37 °C. The MCs are stimulated with 50 ng/ml antigen (DNP-HSA), 1 μ M ionomycin or 300 nM thapsigargin and allowed to react for 15 min. LAD2 cells were plated at a density of 2 $\times$ 10⁵ cells/ml per well and sensitized overnight with biotinylated IgE (0.1 μ g/ml, Calbiochem, EMD Biosciences, Germany). The cell suspensions were washed with HEPES-BSA buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄, 5.6 mM glucose, 1.8 mM CaCl₂, 1.3 mM MgSO₄, and 0.04% BSA), and then the cells were stimulated by 100 ng/ml streptavidin (SA) (Sigma, St Louis, MO, USA) for 15 min with or without WZ3146 or PP2. RBL-2H3 cells (2.0 $\times$ 10⁵ cells/well) were sensitized with 50 ng/ml of DNP-specific IgE for 4 h. The cells were transferred into Siraganian-buffer (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, and 0.1% BSA). The cells were treated with or without WZ3146 or PP2 and then incubated for 30 min at 37 °C. The cells were stimulated with 50 ng/ml antigen for 15 min. The degree of degranulation of MCs was expressed as the % of the activity of β -hexosaminidase secreted out of the cells compared to the total activity of β -hexosaminidase.

2.5. Measurement of cell viability

MCs (5.0 $\times$ 10⁴ cells/well) were cultured in a 96-well plate for 4 h with or without WZ3146. Cell viability was measured with a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol.

2.6. Assessment of histamine release from MCs

DNP-specific IgE sensitized BMMCs (2.5 $\times$ 10⁵ cells/well) were stimulated with 50 ng/ml of antigen for 15 min with or without WZ3146 or PP2 in a Tyrode buffer. Histamine EIA kits were used to measure the amount of histamine according to the manufacturer's instructions (Beckman Coulter, Prague, Czech Republic).

2.7. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

IgE-sensitized BMMCs in 6-well plates (5.0 $\times$ 10⁶ cells/well), and the cells were stimulated with 50 ng/ml of antigen for 30 min. The total RNA was extracted using the Easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Seongnam-si, Korea). Reverse transcription was using the Superscript First-Strand Synthesis System

(Invitrogen, Carlsbad, CA, USA), and PCR was performed at 94 °C for 30 s, at 55 °C for 20 s, and at 72 °C for 30 s for 30 cycles. The primer sequences used in PCR analysis were (forward) 5'-GGCAGGTCTACTT TAGA GTCATTGC-3' and (reverse) 5'-ACATTCGAGGCTCCAGTGAATT CGG-3' for TNF- α , (forward) 5'-ATGAAGTTCCTCT CTGCAAGAGACT-3' and (reverse) 5'-CACTAGGTTTGC CGAGTAGATCTC-3' for IL-6, and (forward) 5'-TTGGCCGTATTGGGCGCCTG-3' and (reverse) 5'-ATCGGC AGAAGG GCGGAGA-3' for GAPDH.

2.8. Measurement of secreted TNF- α and IL-6 by ELISA

IgE-sensitized MCs (1.0 $\times$ 10⁶ cells/well) were stimulated with 50 ng/ml DNP-HSA for 3 h, with or without WZ3146. The amount of TNF- α and IL-6 in cultured media were measured using OptEIA™ ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

2.9. Western blot analysis

The cells were lysed using RIPA buffer (Thermo Fisher scientific, Waltham, MA, USA) in 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 mM *p*-nitrophenyl phosphate (pNPP), 0.7 μ g/ml pepstatin, and protease-inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Centrifuged supernatant was mixed with a NuPAGE™ LDS sample buffer (Thermo Fisher scientific) and denatured by heating for 5 min at 100 °C. Proteins were separated by 7.5–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane using transfer cassettes (Bio-Rad, Hercules, CA, USA). After incubating with each primary antibody in TBS-T buffer (tris-buffered saline with 0.1% tween 20) contained 5% BSA or 5% skim milk at 4 °C overnight. The membrane were washed three times with TBS-T and incubated a horse-radish peroxidase-coupled secondary antibody (dilution fold, 1:2000) for 1 h. The blots were detected using chemiluminescence reagents (Thermo Fisher scientific) according to the manufacturer's protocol.

2.10. Immunoprecipitation and in vitro tyrosine kinase assay

This experiment was performed as described previously (Nam et al., 2017). Briefly, IgE primed MCs were stimulated by 50 ng/ml DNP-HSA for 7 min. The cells were lysed in a NP-40 based lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 60 mM octyl- β -glucoside, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 2.5 mM pNPP, 0.7 μ g/ml pepstatin, and a protease inhibitor cocktail tablet). One milligram of protein was incubated with 5 μ g each antibody (Lyn, Fyn, or Syk) and 50 μ l protein G agarose at 4 °C for overnight. Then the agarose was washed 5 times with a washing buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Nonidet p-40, 10% glycerol, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 2.5 mM pNPP, 0.7 μ g/ml pepstatin, and a protease inhibitor cocktail tablet). Tyrosine kinase activity was measured using the Universal Tyrosine Kinase Assay Kit (Cat. No. GWB-24C8F9, GenWay Biotech Inc., San Diego, Calif., USA). Briefly, the immunoprecipitates were diluted with Kit reacting buffer (by the ratio of 1:25) and the suspensions were transferred into a reaction plate (40 μ l per well). And, next, 10 μ l of 40 mM ATP solution was added and reacted for 30 min at 37 °C. The reaction wells were washed with the Washing buffer 4 times to terminate the reaction. Then, the tyrosine kinase activity was measured with Anti-phosphotyrosine antibody according to the manufacturer's protocol.

2.11. Passive cutaneous anaphylaxis (PCA)

Mice (Balb/c, ~ 6 weeks of age, male) were injected intradermally in back skin with 50 ng of DNP-specific IgE in 20 μ l PBS. After 12 h, mice were orally administered with or without WZ3146 (10, 20, or 50 mg/kg) in 5% Arabic gum (Sigma-Aldrich, St. Louis, MO, USA). After 1 h, mice were intravenously injected with 250 μ l of 5 mg/ml Evans

blue with or without 100 μ g DNP-HSA. The mice were euthanized 1 h after the DNP-HSA injection, followed by the removal of back skin. The dye was extracted from back skin tissues in 2 ml of formamide at 63 °C for 24 h. The intensity of the absorbance was measured at 620 nm.

2.12. Histological analysis

After the PCA reaction, the back skin of the mice was fixed with 4% paraformaldehyde in PBS. The fixed skin is then paraffinized and then cut to 4- μ m thick slices and then stained with toluidine blue. The degree of degranulation of MCs was calculated as the ratio of the number of degranulated MCs to the total number of MCs in the same skin tissue.

2.13. Statistical analysis

The data were expressed as the mean $\pm$ SEM from three independent experiments. In each animal experiment, at least five mice were used per an experimental group. *In vitro* experiments were carried out in triplicate for each experiment. Statistical analysis was performed by using one-way ANOVA and the student's *t*-test for unpaired values. All statistical calculations were performed with the SigmaStat software (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Effect of WZ3146 on degranulation by antigen in human and murine MCs

We first assessed whether WZ3146 inhibits the degranulation in IgE-mediated BMMCs, RBL-2H3 cells, and LAD2 cells. We preliminarily measured the experimental concentration range of WZ3146 in each MC. WZ3146 had no inhibitory effect below 200 nM and showed the maximal effect at 5 μ M or more in all MCs. We next performed further experiments in the range of 0.2 to 5 μ M of WZ3146 for the following experiments with MCs. WZ3146 inhibited the degranulation by antigen in RBL-2H3 cells (IC_{50} , $\sim$ 0.35 μ M), BMMCs (IC_{50} , $\sim$ 0.39 μ M), and LAD2 cells (IC_{50} , $\sim$ 0.41 μ M) in a dose-dependent manner (Fig. 1B). Notably, the degranulation inhibition rate of WZ3146 at a concentration of 5 μ M was $>$ 90% and WZ3146 exhibited similar potency to that of PP2 at 10 μ M (Fig. 1B) in all three MCs. However, WZ3146 did not affect cell viability of MCs at the experimental concentrations (Fig. 1C). Based on these results, we found for the first time that WZ3146 may be effective in suppressing IgE-mediated acute allergic responses *in vivo*.

3.2. Effect of WZ3146 on histamine secretion and pro-inflammatory cytokine production by antigen in MCs

Histamine is a substance released from MCs by antigen stimulation, and it plays a pivotal role in initially inducing various allergic responses (Thangam et al., 2018). We measured whether WZ3146 inhibits histamine secretion from BMMCs by antigen. As a result, WZ3146 inhibited histamine secretion in a dose-dependent manner, and the inhibition rate of WZ3146 was $\sim$ 75% at the highest concentration (5 μ M) compared to the control (Fig. 2A).

Pro-inflammatory cytokines secreted by MCs are also critical for the induction of allergic responses, especially for late phase allergic responses, in IgE-mediated allergic diseases (Theoharides and Kalogeromitros, 2006). Thus, we next examined whether the expression and secretion of TNF- α and IL-6, the typical cytokines secreted by MCs, is inhibited by WZ3146. According to the results of the RT-PCR and ELISA analysis, WZ3146 suppressed the expressions and secretions of TNF- α and IL-6 from MCs by antigen in a dose-dependent manner (Fig. 2B and C). Notably, WZ3146 inhibited the expression and secretion of the cytokines almost entirely at 5 μ M compared to the control (Fig. 2B and C), which suggests that not only did WZ3146 suppress the secretion of preformed cytokines, it also inhibited the *de novo* synthesis of the cytokines by antigen stimulation.

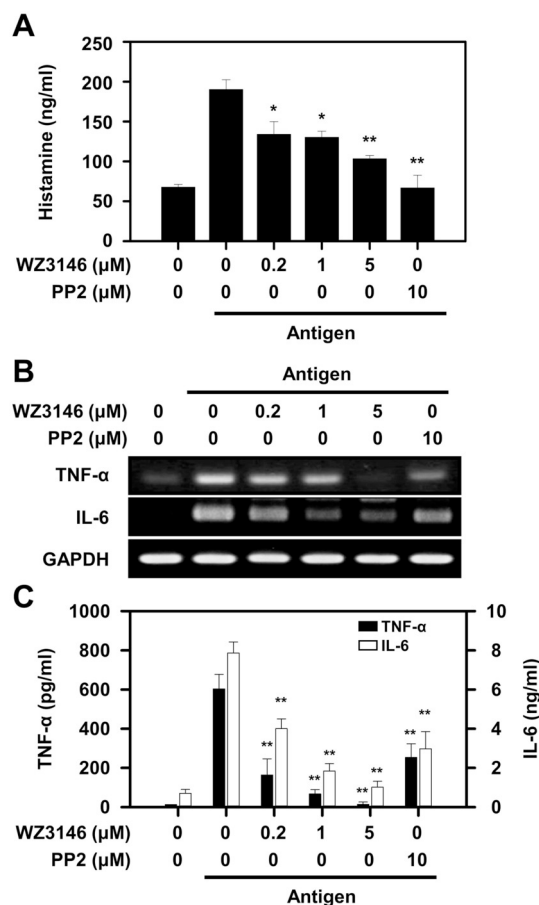


Fig. 2. WZ3146 reduces histamine release, expression, and secretion of pro-inflammatory cytokines from antigen-stimulated MCs. (A) IgE-primed BMMCs (2.5×10^5 cells/well) were stimulated with 50 ng/ml antigen for 15 min with or without WZ3146 or PP2. The measurement of histamine secretion was performed as described in "Materials and Methods". (B–C) IgE-primed BMMCs (1×10^6 cells/well) were stimulated with 50 ng/ml of antigen for 30 min (for RT-PCR) or 3 h (for ELISA) after pre-treated with or without WZ3146 or PP2 for 30 min. The expressions of TNF- α and IL-6 were determined by electrophoresis after RT-PCR (B), and the amount of TNF- α and IL-6 were assessed by ELISA (C) as described in "Materials and Methods". The representative images (B) are shown and the values (A and C) indicate the mean $\pm$ S.E.M. from three independent experiments. The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, **p* < .05, ***p* < .01. PP2 is a general Src-family kinase inhibitor.

3.3. Mechanism of the action of WZ3146 on MCs

From the above results, we found that WZ3146 inhibits the secretion of antigen stimulation-induced allergic mediators from MCs. Next, we investigated the inhibitory effect of WZ3146 on the activation of the signaling pathway of MC by antigen in order to find the target protein of WZ3146 in the cells. The intracellular mobilization of Ca^{2+} is very critical for degranulation and cytokine secretion in MCs (Nishida et al., 2005). Antigen stimulation in mast cells stimulates the calcium signal after the activation of Lyn/Fyn-Syk-LAT pathway (Wernersson and Pejler, 2014; Beaven and Metzger, 1993). To find a direct target protein of WZ3146 that inhibits mast cells, we first investigated whether the direct target is upstream or downstream of the calcium signal in MCs. We tested whether WZ3146 inhibits MC degranulation by the increase of intracellular Ca^{2+} . We used ionomycin and thapsigargin to increase cytosolic concentration of Ca^{2+} . Ionomycin is a Ca^{2+} ionophore that allows extracellular Ca^{2+} to enter cells (Liu and Hermann, 1978). On the other hand, thapsigargin inhibits the uptake of cytosol Ca^{2+} into endoplasmic reticulum in the cells (Thastrup et al., 1990). Notably,

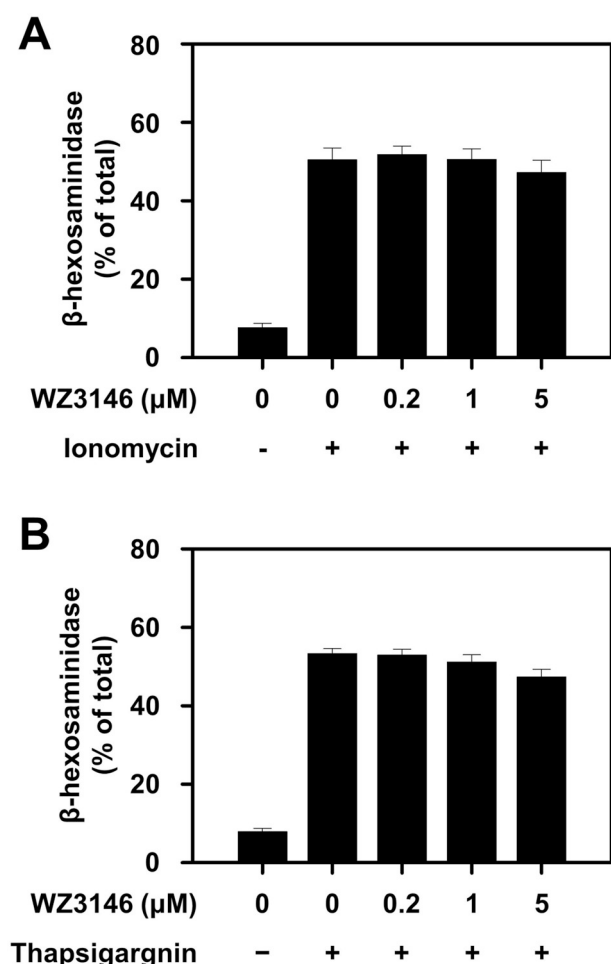


Fig. 3. WZ3146 does not affect degranulation by Ca^{2+} influx. The BMMCs (3.0×10^5 cells/well) were pre-incubated with or without WZ3146 (0.2, 1, 5 μM) for 30 min and then stimulated with 1 μM ionomycin (A) or 300 nM thapsigargin (B) for 15 min. The values indicate the mean $\pm$ S.E.M. from three independent experiments (each in triplicate).

WZ3146 did not inhibit degranulation by ionomycin (Fig. 3A) or thapsigargin (Fig. 3B). These results suggested that WZ3146 inhibits some upstream signaling proteins that are activated earlier than calcium signal in $\text{Fc}\epsilon\text{RI}$ signaling pathway of MCs. Based on these result, we checked the inhibitory effect of WZ3146 on signaling proteins above the calcium signal activated by antigen stimulation. As the results, the phosphorylations of Syk, LAT, and $\text{PLC}\gamma 1$ were reduced by WZ3146 in a dose-dependent manner (Fig. 4A). Notably, WZ3146 almost completely inhibited the activation of the Syk-LAT- $\text{PLC}\gamma$ pathway at the concentration of 5 μM compared to the control (Fig. 4A). In addition, the phosphorylations of Akt, ERK1/2, p38, and JNK were also remarkably suppressed in a dose-dependent manner (Fig. 4B). These results indicated that WZ3146 suppresses MC activation through the inhibition of Syk or its upstream signaling proteins.

3.4. Effect of WZ3146 on protein kinase activity in vitro

The initial signaling proteins of IgE-mediated mast cell activation by antigen are Src-family kinases such as Lyn and Fyn (Parravicini et al., 2002; Wernersson and Pejler, 2014). We measured *in vitro* protein kinase activity whether WZ3146 directly inhibits Lyn, Fyn, or Syk in order to find the direct target protein of WZ3146. As the results, WZ3146 significantly inhibited Lyn and Fyn kinase activity (Fig. 5). However, it was of interest that WZ3146 did not inhibit the activity of Syk kinase which is a downstream protein of Lyn and Fyn (Fig. 5). Our

results suggest that WZ3146 suppresses degranulation and cytokine secretion from IgE-mediated MCs via the direct inhibition of Lyn and Fyn in MCs.

3.5. Effect of WZ3146 on passive cutaneous anaphylaxis (PCA)

PCA is well-known as a typical animal model for human IgE-mediated Type I allergic response that causes local allergic symptoms, especially in the skin (Finkelman, 2007). We performed this experiment after setting a dosage range of 10 to 50 mg/kg in the preliminary animal experiments. WZ3146 showed minimal anti-allergic effect at concentrations below 10 mg/kg and a maximum effect at 50 mg/kg or more. In further studies, WZ3146 suppressed the PCA response in a dose-dependent manner (Fig. 6A and B; ED_{50} , ~20 mg/kg). WZ3146 exerted ~44% inhibition at 10 mg/kg, ~60% at 20 mg/kg, and ~86% at 50 mg/kg compared to the control (Fig. 6B). WZ3146 stayed consistent with the results and significantly inhibited degranulation of MCs in the skin (Fig. 6C). Taken together, these results indicated that WZ3146 inhibits IgE-mediated allergic responses through the inhibition of MC in mice.

4. Discussion

Allergic diseases such as atopic dermatitis, allergic rhinitis, allergic asthma, and food allergy are very common diseases in about one out of every five people in developed countries, and these diseases are continuously increasing (Pawankar et al., 2013). However, the cause of allergic diseases is still unclear. Therefore, the best way to prevent allergic diseases at present is to identify and avoid allergenic substances. However, it is impossible to completely avoid allergens because the substances that cause allergic symptoms vary from person to person and mostly exist in various forms in our living environments. Immunologically, a variety of immune cells such as Th2 cells, eosinophils, and MCs play a critical role in the induction of allergic diseases (Gould et al., 2003). In particular, MCs are well known as critical cells responsible for allergic inflammation. Therefore, it is generally accepted that inhibiting the activity of MCs is a viable treatment option. In this study, WZ3146 inhibited degranulation (Fig. 1B), histamine release (Fig. 2A), and pro-inflammatory cytokine production (Fig. 2B and C) from MCs by inhibiting the activity of Lyn and Fyn tyrosine kinase (Fig. 5). Notably, WZ3146 inhibited degranulation by antigen in human LAD2 cells as well as mouse BMMCs and rat RBL-2H3 cells (Fig. 1B), which indicates that WZ3146 inhibits the activation process by antigen in MCs originating from various species. WZ3146 also inhibited allergic reactions by antigen in the IgE-mediated allergic animal model (Fig. 6). These results suggest that we can use WZ3146 as a therapeutic compound for type I hypersensitive allergic diseases.

Drug repositioning (or repurposing) refers to the reuse of commercially available drugs and drugs that have been discontinued at clinical test phases 1, 2, and 3 for the treatment of other diseases. The entire process of developing new drugs is usually accompanied by a long time and too much cost of at least 10 years (Ashburn and Thor, 2004). According to Thomas et al.' report, the success rate for FDA-approved new drugs from the first phase of the clinical study is 9.6% (Thomas et al., 2016). For this reason, many multinational pharmaceutical companies have begun to be interested in drug repositioning in order to reduce the cost and to shorten the development time for new drug development. For example, thalidomide, which was developed as a morning sickness treatment but caused critical problems with birth defects, has been marketed as a drug for multiple myeloma (Singhal et al., 1999). In addition, sildenafil (Viagra®) was developed as a treatment for angina, but has also been reborn as an erectile dysfunction treatment (Goldstein et al., 1998). Pharmaceutical companies are now investing in drug development through drug repositioning based on successful results which account for 10–50% of the R & D investment (Novac, 2013). We have focused on compounds capable of inhibiting protein tyrosine

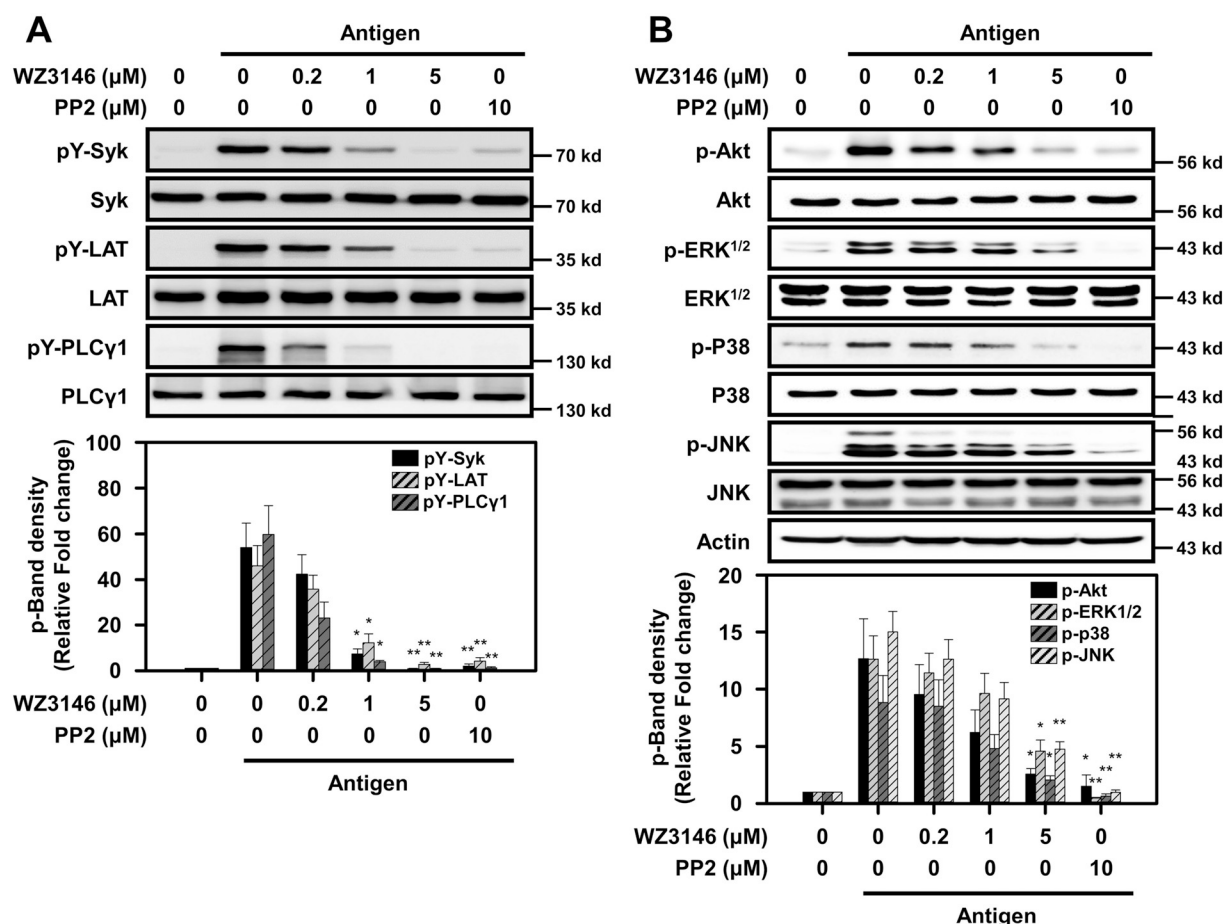


Fig. 4. WZ3146 inhibits the activations of signaling proteins in antigen-stimulated MCs. IgE-primed BMMCs (3.0×10^6 cells/well in 6-well plate) were pre-treated with or without WZ3146 (0.2, 1, 5 μ M) or PP2 (10 μ M) for 30 min and stimulated with 50 ng/ml antigen for 10 min. The Western blotting analysis was performed as described in the “Materials and Methods”. (Upper panels) Representative images are shown from three independent experiments (each in triplicate). (Lower panels) The band densities were expressed as the mean $\pm$ S.E.M. from three independent experiments. The asterisks indicate significant differences from antigen-stimulated groups without inhibitors, * $p < .05$, ** $p < .01$. PP2 is a general Src-family kinase inhibitor.

kinase that are also critical for the initial activation of IgE-high affinity receptor signals in MCs (Beaven and Metzger, 1993). EGFR inhibitors have limited drug efficacy for the treatment of lung cancer due to drug resistance caused by mutations in the EGFR (commonly T790 M) (Pao et al., 2005; Kobayashi et al., 2005). Therefore, many scientists have developed compounds with high activity against the EGFR T790 M mutant. With this in mind, we sought out to find compounds that inhibit MCs by using compounds that are more effective on EGFR T790 M than wild-type EGFR. Compounds that inhibit MCs among the EGFR mutant-specific compounds are less likely to cause side effects because of their low affinity with wild-type EGFR. In this study, we eventually found that WZ3146, a molecule that targets EGFR T790 M, inhibits MC activation to inhibit IgE-mediated allergic response (Fig. 1B).

EGFR-targeting inhibitors usually have severe toxicity because they inhibit wild-type EGFR (Zhou et al., 2009). For this reason, many researchers have been trying to develop drugs with more affinity to the EGFR T790 M mutant that is more frequently found in lung cancer than in normal tissues to avoid toxicity or drug resistance. Zhou et al. found pyrimidine EGFR inhibitors (WZ3146, WZ4002, and WZ8040) that are highly reactive to EGFR T790 M and are developing them as an anticancer drug (Zhou et al., 2009; Zhou et al., 2011). However, the WZ3146 study was discontinued because WZ4002 was more selective for EGFR. Interestingly, WZ4002 did not inhibit the degranulation of IgE-mediated MCs by antigen in our preliminary study, and we also found WZ8040 to be cytotoxic at low concentrations under 10 μ M. Notably, WZ3146 inhibited MC degranulation without cytotoxicity and IgE-

mediated allergic response in mice (Figs. 1 and 6). According to the previous reports (Zhou et al., 2009; Zhou et al., 2011), WZ3146 selectivity for mutant EGFR is likely to have fairly low toxicity. However, further studies on the specificity of activity and toxicity of WZ3146 in other proteins and cell systems should be interesting.

The degranulation of MCs is an important process leading to allergic reactions in allergic diseases (Galli and Tsai, 2012). MCs contain histamine, heparin, serotonin, platelet activating factor (PAF), and various other allergic mediators in cytoplasmic granules (Moon et al., 2014). Among them, histamine is a very important mediator to be involved in the induction of various allergic responses in diseases such as asthma, allergic rhinitis, and anaphylaxis (Thangam et al., 2018). In addition, antigen-stimulated MCs newly synthesize and secrete the pro-inflammatory cytokines such as IL-4, IL-6, IL-13, and TNF- α and chemokines. These secretions recruit various immune cells to allergic tissues and thus cause chronic allergic symptoms (Metcalf et al., 1997). WZ3146 inhibited the degranulation of murine and human MCs by antigen stimulation in our study (Fig. 1B). WZ3146 also inhibited the production of pro-inflammatory cytokines such as TNF- α and IL-6 (Fig. 2B and C) as well as Lyn and Fyn, the initially activated tyrosine kinases, in antigen-stimulated MCs. Thus, the results suggest that WZ3146 inhibits the expression and secretion of other cytokines in addition to TNF- α and IL-6 in MCs. Collectively, our results demonstrate that WZ3146 suppress allergic responses by inhibiting degranulation, histamine release, and cytokine secretion.

Syk plays a key role in signal transduction of Fc ϵ RI of MCs. When

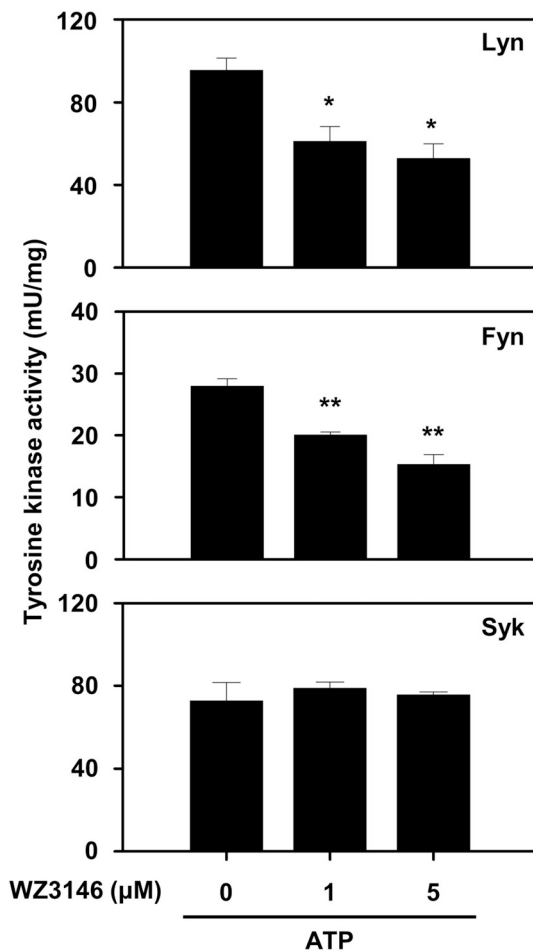


Fig. 5. WZ3146 inhibits Lyn and Fyn, but not Syk, *in vitro* kinase activity. One mg of whole protein was obtained by NP-40-based lysis buffer. Lyn, Fyn, and Syk were immunoprecipitated as described in the “Materials and Methods” using specific antibodies against Lyn, Fyn, or Syk. And then kinase activity was determined using ELISA-based *in vitro* assay kit according to the manufacturer's instructions. The values were expressed as the mean $\pm$ S.E.M. from three independent experiments (each in triplicate). The asterisks indicate significant differences from without WZ3146 group, * $p < .05$, ** $p < .01$. ATP, Adenosine triphosphate.

Syk is deficient in MCs, the Fc ϵ RI signaling is not activated and degranulation and cytokine secretion do not occur (Costello et al., 1996). Therefore, many researchers are studying an anti-allergic treatment that inhibits Syk in MCs (Ulanova et al., 2005). In our study, WZ3146 inhibited the activation of Syk in MCs by antigen stimulation (Fig. 4A). In congruence, WZ3146 further inhibited the activation of LAT and PLC γ 1, the downstream signaling proteins of Syk, in mast cells by antigen stimulation (Fig. 4A). These results suggest that WZ3146 suppresses MCs by the inhibition of Syk and Syk-dependent signaling pathway. According to many reports, the activation of a Fyn/Gab2/PI3K pathway as an auxiliary signaling pathway as well as the Lyn/Syk/LAT pivotal pathway in the activation of MCs by antigen is very important for activating MC sufficiently (Nishida et al., 2011; Parravicini et al., 2002; Yu et al., 2006). For this reason, regulating the activity of Fyn as well as Syk to inhibit the activation of MCs is another important target. In this study, WZ3146 inhibited the activities of Lyn and Fyn kinase *in vitro*. Interestingly, however, it did not inhibit the activity of Syk (Fig. 5). Thus, our results indicate that WZ3146 suppresses allergic responses by inhibiting Src-family kinases such as Lyn and Fyn for the IgE-mediated MC activation by antigen.

Passive cutaneous anaphylaxis (PCA) is a typical IgE/MC-dependent local allergic response. PCA is generated by the activation of local tissue

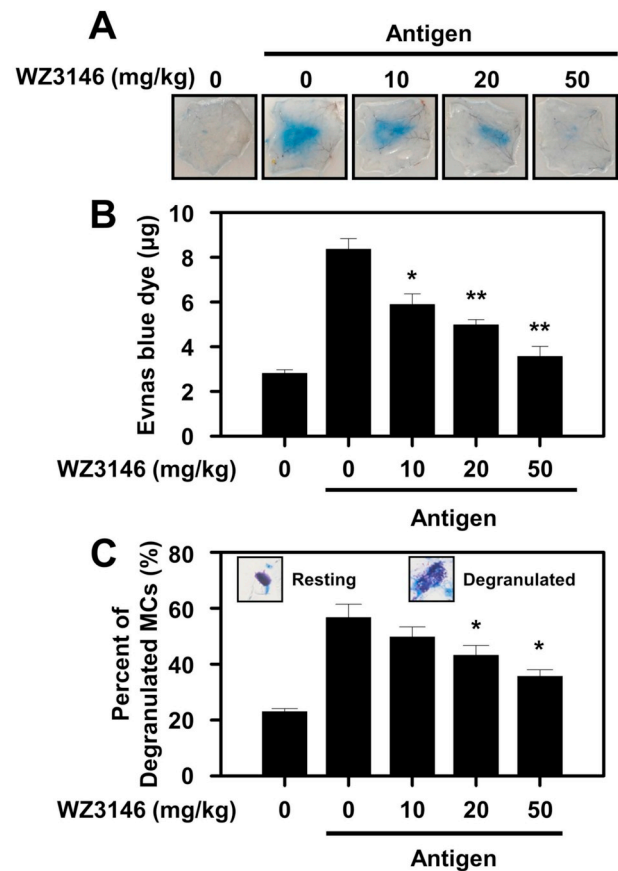


Fig. 6. WZ3146 suppresses IgE-mediated passive cutaneous anaphylaxis (PCA). The DNP-specific IgE (50 ng) was intradermally injected into mouse back skin. Next day, WZ3146 in 5% Arabic gum was orally administrated as indicated. After 1 h, 250 μl of Evans blue (5 mg/ml) with or without antigen (100 μg) was injected into mouse tail vein. The mice were euthanized 1 h after the injection of antigen and the back skins where IgE was injected were isolated for the extraction of Evans blue dye and histology. All data were obtained from three independent experiments (each experiment, $n = 5$ per group). (A) Representative images for the back skin are shown. (B) The amount of Evans blue dye extracted from the back skin are shown as the mean $\pm$ S.E.M. (C) The percent of degranulated MCs of total MCs were presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resident MCs by stimulating them with antigen (Finkelman, 2007). In this study, we used the PCA mouse model to determine whether WZ3146 inhibited the allergic response by its administration *in vivo*. WZ3146 suppressed the extravasation induced by local PCA response in a dose-dependent manner (Fig. 6A and B). At the highest dose (50 mg/kg), the extravasation was almost completely suppressed (Fig. 6B). Mast cells are widely acknowledged to cause PCA response, which does not occur in MC-deficient *Kit^{W-sh/W-sh}* mice (Finkelman, 2007; Kim et al., 2015). Therefore, we measured whether degranulation of MCs in tissues of PCA mice was inhibited by WZ3146 and found that it dose-dependently inhibited degranulation of tissue-resident MCs by antigen (Fig. 6C). These results indicate that the inhibitory effect of WZ3146 on PCA responses in mice is exerted by inhibiting the activity of MCs in tissues.

In summary, we first demonstrate in this study that WZ3146, a pyrimidine derivative N-[3-[5-chloro-2-[4-(4-methylpiperazin-1-yl)anilino]pyrimidin-4-yl]oxyphenyl]prop-2-enamide, inhibits IgE-mediated MC activation both *in vitro* and *in vivo*. WZ3146 inhibits the degranulation and cytokine production by inhibiting the activation Lyn and Fyn in antigen-stimulated MCs (Fig. 7). Furthermore, WZ3146 inhibited IgE-mediated allergic response in mice. Therefore, we propose that WZ3146 could be a new therapeutic agent for allergic diseases.

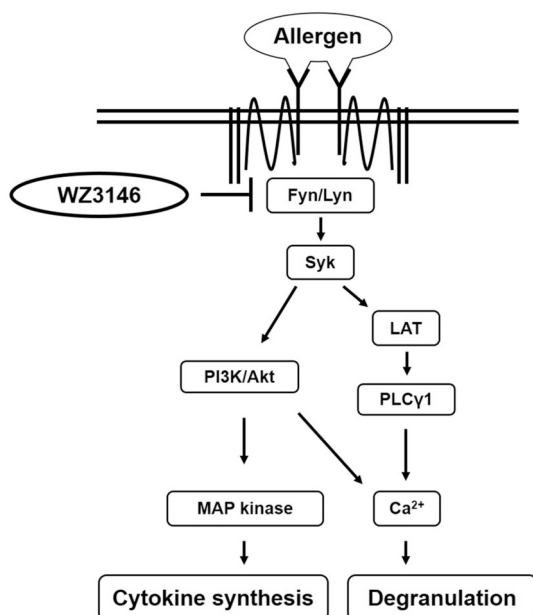


Fig. 7. Proposed scheme for the inhibitory mechanism of WZ3146 in mast cells.

Declaration of competing interest

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

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