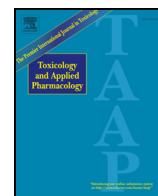




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Q1 An indoxyl compound 5-bromo-4-chloro-3-indolyl 1,3-diacetate, 2 CAC-0982, suppresses activation of Fyn kinase in mast cells and 3 IgE-mediated allergic responses in mice

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ABSTRACT

Mast cells, constituents of virtually all organs and tissues, are critical cells in IgE-mediated allergic responses. The aim of this study was to investigate the effect and mechanism of an indoxyl chromogenic compound, 5-bromo-4-chloro-3-indolyl 1,3-diacetate, CAC-0982, on IgE-mediated mast cell activation and allergic responses in mice. CAC-0982 reversibly suppressed antigen-stimulated degranulation in murine mast cells (IC₅₀, ~3.8 μM) and human mast cells (IC₅₀, ~3.0 μM). CAC-0982 also inhibited the expression and secretion of IL-4 and TNF-α in mast cells. Furthermore, CAC-0982 suppressed the mast cell-mediated allergic responses in mice in a dose-dependent manner (ED₅₀ 27.9 mg/kg). As for the mechanism, CAC-0982 largely suppressed the phosphorylation of Syk and its downstream signaling molecules, including LAT, Akt, Erk1/2, p38, and JNK. Notably, the tyrosine kinase assay of antigen-stimulated mast cells showed that CAC-0982 inhibited Fyn kinase, one of the upstream tyrosine kinases for Syk activation in mast cells. Taken together, these results suggest that CAC-0982 may be used as a new treatment for regulating IgE-mediated allergic diseases through the inhibition of the Fyn/Syk pathway in mast cells.

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Introduction

Type 1 hypersensitivity response is an allergic reaction induced by re-exposure to a specific type of antigen referred to as an allergen (Broide, 2001). The reaction could be either local or systemic, and its symptoms from mild irritation makes to sudden death by anaphylactic shock. In recent years, the prevalence of asthma and allergic diseases such as atopic dermatitis, food allergies, and allergic rhinitis has

significantly increased all over the world (Begin and Nadeau, 2014; El-Qutob et al., 2014; Holgate, 2014). According to recent studies, asthma affects more than 7% of the world's population (Akdis, 2013). In addition, allergic rhinitis, respiratory allergies, and other allergies affect around 20% of people in the United States and other industrialized countries (Castillo and Dimov, 2014).

It has been well established that these allergic responses are generally associated with the expansion of the Th2 cell subset, and mast cells are culprit immune cells to the symptoms. The aggregation of high-affinity IgE receptors (FcεRI) on mast cells via antigen triggers the release of various allergic mediators including histamine, inflammatory cytokines, and eicosanoids in humans (Metzger, 2004; Rivera and Gilfillan, 2006; Beaven, 2009). Many signaling molecules have previously been shown to be associated with the activation of mast cells, including Lyn, Fyn and Syk, and other adaptor proteins such as LAT, SLP-76, Grb2 and PLCγ (Gilfillan and Rivera, 2009; Mocsai et al., 2010; Lee et al., 2011). Three typical MAP kinases, Erk1/2, p38, and JNK, are also critical for the production of pro-inflammatory cytokines (Ishizuka et al., 1997; Duan and Wong, 2006). Collectively, these

Abbreviations: DNP-BSA, dinitrophenol-conjugated bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PIPES, 1,4-piperazinediethanesulfonic acid; BMMC, bone marrow-derived mast cell; RBL, rat basophilic leukemia; PCA, passive cutaneous anaphylaxis; ITAM, immunoreceptor tyrosine-based activation motif; Syk, spleen tyrosine kinase; LAT, linker for activation of T cells; Gab2, Grb2-associated binder 2; MAP kinase, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

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signaling pathways are essential for the degranulation and secretion of inflammatory cytokines and eicosanoids in IgE-mediated mast cells (Rivera and Gilfillan, 2006; Lee et al., 2008).

Avoidance of allergy-causing allergens before or after sensitization should be beneficial as primary or secondary prophylaxis. Various clinical approaches are also suggested for patients with allergic diseases, such as treatment with corticosteroids, β 2-adrenoceptor agonists, mediator antagonists (H1-antihistamines), allergen-specific immunotherapy, IgE blockers, inhibitors of mast cells, and cytokine-based immunotherapies (Holgate and Polosa, 2008). Given that mast cells play a critical role in IgE-mediated allergic responses, the development of inhibitors of signaling proteins involved in mast cell activation might be a good treatment approach.

In the course of the ongoing efforts to find a new compound for anti-allergic effect in vitro and in vivo, a potent inhibitor of mast cells, 5-bromo-4-chloro-3-indolyl 1,3-diacetate, CAC-0982, was selected on the basis of cell-based assays from among over 7000 compounds. However, the physiological effects and mechanism of action of the compounds in mast cells are totally unknown. This study shows for the first time that an indoxyl chromogenic compound CAC-0982 effectively suppresses mast cell activation and allergic responses in vitro and in vivo through the inhibition of a Fyn/Syk signaling pathway and its downstream signaling molecules in mast cells.

Materials and methods

Antibodies and reagents. The chemical library, over 7000 chemicals including 5-bromo-4-chloro-3-indolyl 1,3-diacetate (CAS Registry Number: 3030-06-6, Fig. 1), was obtained from Korea Chemical Bank (<http://www.chembank.org>, Daejeon, Korea). The code number of the compound is CAC-0982 (Fig. 1). The stock solution of CAC-0982 was made in DMSO and the solution was diluted with PBS to keep 0.1% or less DMSO during its treatment. Antibodies for the phosphorylated forms of Syk (Y346), LAT (Y191), Erk1/2, p38, c-Jun N-terminal kinase (JNK), and Akt (S473) were purchased from Cell Signaling Technology Inc. (Danvers, MA). Antibodies for Syk, LAT, and Actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody for phosphotyrosine (4G10) was obtained from Merck Millipore (Billerica, MA, USA). A prestained protein standard for the molecular weight range of 6000 to 170,000 Da was obtained from GenDEPOT (Barker, TX, USA). 4-Amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo [3,4-d] pyrimidine (PP2) and diphenylhydramine (DPH) were from Calbiochem (La Jolla, CA, USA). Evans blue, dinitrophenol-conjugated

bovine serum albumin (DNP-BSA) as antigen, and DNP-specific monoclonal IgE were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were from Invitrogen-Biosource Cytokine & Signaling (Camarillo, CA, USA). Other culture reagents were obtained from GIBCO/Life Technologies, Inc. (Rockville, MD, USA).

Preparation of rat basophilic leukemia (RBL)-2H3 cell lines and bone marrow-derived mast cells (BMMCs), human leukemic mast cell line (LAD2), and primary human mast cells (HuMCs). RBL-2H3 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in minimal essential medium (MEM) containing Earle's salts and supplemented with glutamine, antibiotics, and 15% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin-streptomycin in a humidified incubator at 37 °C, 5% CO₂ (Invitrogen, Carlsbad, CA, USA). To prepare BMMCs, bone marrow cells were isolated from femurs and tibias from Balb/c mice. The cells were cultured in an RPMI-1640 medium with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin and streptomycin, 50 mM 2-mercaptoethanol, and 10 ng/ml of mouse recombinant IL-3 (PeproTech, Rocky Hill, NJ, USA) as previously described (Jensen et al., 2006). After 4 weeks, more than 95% of the cells were characterized as BMMCs for the following experiments. LAD2 was cultured in serum-free media (StemPro-34 SFM, Life Technologies, Gaithersburg, MD, USA), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 g/ml streptomycin, and 100 ng/ml SCF. The cell suspensions were plated at a density of 10⁵ cells/ml and maintained at 37 °C and 5% CO₂. Cells were fed by hemi-depletion of media once a week. Human peripheral blood-derived CD34⁺ cells were cultured in StemPro-34 SFM, supplemented with 2 mM L-glutamine, 50 g/ml streptomycin, 100 IU/ml penicillin, 100 ng/ml SCF, and 100 ng/ml recombinant human IL-6 (PeproTech, Inc., Rocky Hill, NJ, USA). Recombinant human IL-3 (30 ng/ml) was added only for the first week. Half of the culture medium was replaced every 7 days. Cultures at 8–10 weeks consisted of ~99% HuMC.

Assessment of degranulation in mast cells. Degranulation was determined by measuring the release of a granule marker, β -hexosaminidase, as previously reported (Ozawa et al., 1993). Briefly, cells were transferred into a 24-well plate (for RBL-2H3 cells) or tube (for BMMCs) (2 × 10⁵ cells/0.5 ml) and sensitized with 20 ng/ml DNP-specific IgE in media overnight. After washing twice, the cells were incubated for 30 min with or without CAC-0982 in piperazine-N-N'-bis (1-ethanesulfonic acid) (PIPES) buffer (25 mM PIPES [pH 7.2], 159 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from a bovine serum) for RBL-2H3 cells or in a Tyrode buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, and 0.05% fatty acid-free fraction V from a bovine serum) for BMMCs. Cells were subsequently stimulated with 25 ng/ml of antigen, DNP-BSA, for 10 min. For experiments with human mast cells, biotinylated human myeloma IgE (100 ng/ml, Calbiochem, EMD Biosciences, Germany)-primed LAD2 and HuMCs were stimulated with streptavidin (SA, 100 ng/ml, Sigma, St. Louis, MO, USA) for 30 min in Tyrode buffer. Release of β -hexosaminidase was determined by use of a colorimetric assay in which the release of p-nitrophenol from p-nitrophenyl-N-acetyl- β -D-glucosaminide (p-NAG) was measured. 30 μ l of the supernatant of each group and 30 μ l of p-NAG were added into 96-well plate and then incubated at 37 °C for 1 h. Reactions were terminated by adding 250 μ l of 0.1 M carbonate buffer. The optical density of p-nitrophenol in solution was analyzed at 405 nm. Values were expressed as the percent of intracellular β -hexosaminidase that was released into the medium. All values were obtained from at least three independent experiments and every experiment was performed in triplicates.

Immunoprecipitation and western blot analysis. Mast cells (5 × 10⁶ cells/plate), which were sensitized overnight by 20 ng/ml DNP-specific IgE, 170

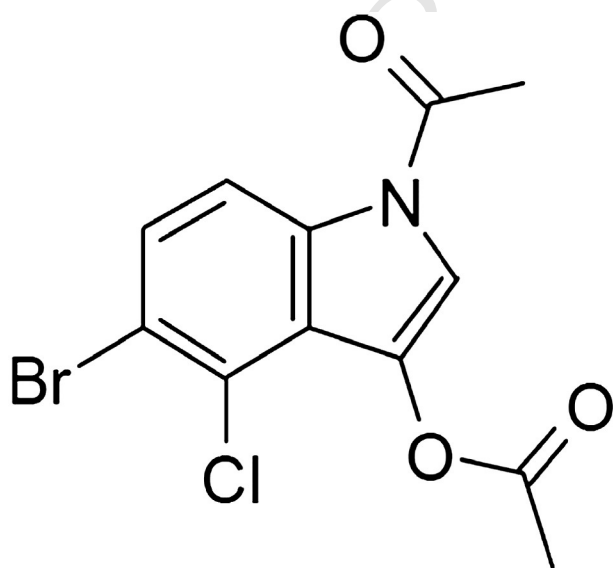


Fig. 1. Molecular structure of 5-bromo-4-chloro-3-indolyl 1,3-diacetate, CAC-0982.

were washed twice in cultured media, and changed by fresh media. After incubating for 30 min with or without CAC-0982, cells were subsequently stimulated with 25 ng/ml of antigen for 10 min, then chilled with ice to terminate stimulation, and then washed twice with ice-cold PBS. The cells were lysed with an 0.5 ml of ice-cold 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 Na_3VO_4 , 1 mM phenylmethylsulfonylfluoride, 2.5 mM nitrophenylphosphate, 0.7 $\mu\text{g}/\text{ml}$ pepstatin, and protease inhibitor cocktail tablet). Lysates were kept on ice for 30 min and then centrifuged at $15,000 \times g$ for 15 min at 4°C . To perform immunoprecipitation experiments, the supernatant was “precleared” by the addition of 50 μl protein G–Agarose before the addition of the specific antibody. A 1 mg of protein was used for immunoprecipitation. Syk and LAT proteins were precipitated by overnight incubation at 4°C with 5 μg of each specific antibody and, in turn, protein G–agarose. 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 Na_3VO_4 , 1 mM PMSF, 2.5 mM nitrophenylphosphate, 0.7 $\mu\text{g}/\text{ml}$ pepstatin, and protease inhibitor cocktail tablet) and then boiled at 95°C for 5 min in a $2 \times$ Laemmli buffer. Proteins were separated by SDS–PAGE and transferred to PVDF membranes. Following incubation with blocking solution containing 5% BSA or 5% skim milk, the membranes were incubated overnight at 4°C with the appropriate primary antibodies in 5% BSA for phosphorylated protein or in 5% skim milk for others with rocking. Immunoreactive proteins were detected by use of horse-radish peroxidase (HRP)-coupled secondary antibodies. Protein bands were detected using chemiluminescence reagents according to the manufacturer's protocol. All western blot analyses were repeated at least three times with different biological samples.

Measurement of cell viability. Mouse BMMCs (5×10^4 cells/well) were plated on 96-well plates in media. Next, cells were incubated with the

indicated concentrations of CAC-0982 (1–10 μM) for 4 h. Cell viability was determined by using a cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. Briefly, CCK-8 solution (at a 1:10 ratio of CCK-8 to media) was added to each well of the plate, and the plates were incubated for 1 h in a CO_2 incubator at 37°C . The absorbance was measured at 450 nm.

Extraction of total RNA and RT-PCR. RBL-2H3 cells were plated in 6-well clustered plate (1×10^6 cells/well) and incubated overnight in a complete growth medium with 20 ng/ml DNP-specific IgE. After washing twice, cells were stimulated with 25 ng/ml of antigen for 15 min with or without CAC-0982. Total RNA was isolated from RBL-2H3 cells by use of Trizol™ Reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed with the Superscript first strand synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR was performed at 94°C for 45 s, at 55°C for 45 s, and at 72°C for 60 s for 30 cycles. The primers used were as follows: rat TNF- α forward, 5'-CACCACGCTCTTCT GTCTACTGAAC-3'; rat TNF- α reverse, 5'-CCGGACTC CGTGATGTCTAAGTACT-3'; rat IL-4 forward, 5'-ACCTTGCTGTC ACCTG TTC-3'; rat IL-4 reverse, 5'-TTGTGAGCGTGGACTCAT TC-3'; rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GTGG AGTCTAC TGGCGTCTTC-3'; rat GAPDH reverse, 5'-CCAAGGCTGT GGGCAAGG TCA-3'.

Measurement of secreted cytokines by ELISA. RBL-2H3 cells (5×10^5 cells/well) were sensitized overnight with 20 ng/ml DNP-specific IgE as described above. The cells were washed twice with cultured media and incubated for 30 min with or without CAC-0982 or PP2. Cells were, then, stimulated with 25 ng/ml antigen for 4 h at 37°C . The supernatant (50 μl) was transferred to a 96-well ELISA plate. The amounts of TNF- α and IL-4 in the culture media were then determined using ELISA kits (Invitrogen-Biosource Cytokine & Signaling). Each experiment

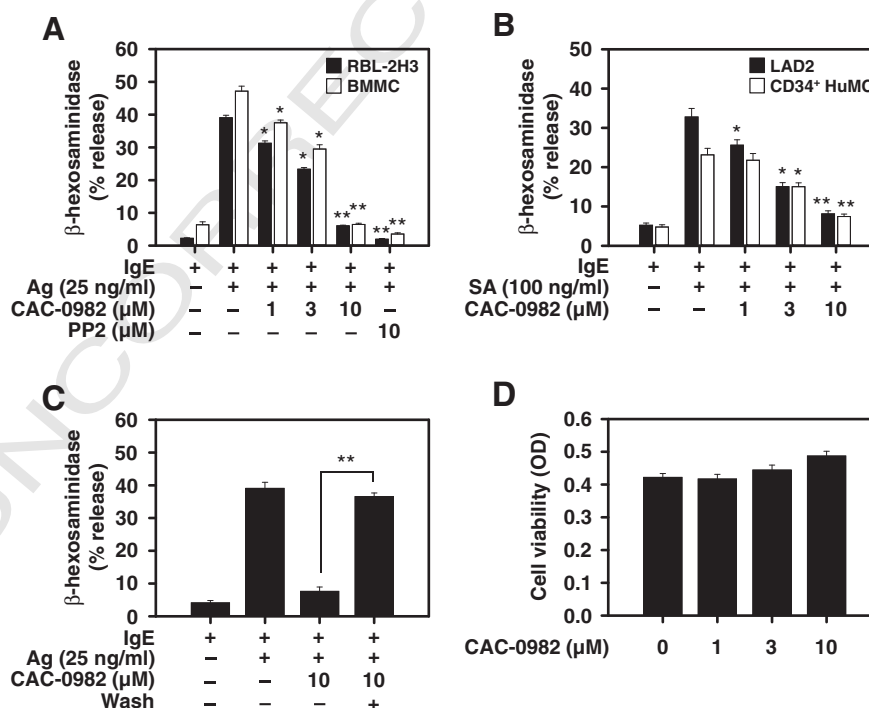


Fig. 2. Effect of CAC-0982 on antigen-stimulated degranulation and its reversibility in mast cells. (A) After pulsing mast cells with 20 ng/ml of DNP-specific IgE overnight, RBL-2H3 cells and BMMCs were stimulated by 25 ng/ml antigen (10 min) with or without CAC-0982 or PP2. (B) Biotinylated human myeloma IgE (100 ng/ml)-primed LAD2 and CD34⁺ human mast cells (HuMCs) were stimulated by streptavidin (100 ng/ml, 30 min) with or without CAC-0982. (C) RBL-2H3 cells were stimulated with or without CAC-0982 for 1 h, washed five times with PIPES buffer, and then stimulated for 10 min with 25 ng/ml antigen. Mast cell degranulation was determined by measuring the activity of β -hexosaminidase in the culture medium. (D) BMMCs were incubated with CAC-0982 for 4 h and the cell viability, then, was determined by using a cell counting kit-8 as described in the “Materials and methods”. The values indicate mean $\pm$ SEM from three independent experiments. Significant differences with the Ag-stimulated controls without CAC-0982 are indicated as follows: ** $p < 0.05$; * $p < 0.01$. Ag, antigen; SA, streptavidin; PP2, a Src-family kinase inhibitor.

included two replicates and all values were obtained from three different experiments.

Induction of IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. Balb/c mice (male; aged 4 weeks) were purchased from the Dae Han Experimental Animal Center (Eumseong, Korea) and the mice were housed in a laminar air flow room maintained at $22 \pm 1^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$. PCA was induced as previously described (Lee et al., 2008). Mice were injected intradermally in the left ear with $0.5\ \mu\text{g}$ of anti-DNP IgE, and in the right ear with the same volume of phosphate-buffered saline (PBS) as a control. After 24 h, $250\ \mu\text{g}$ of antigen, DNP-BSA, in PBS containing 4% Evans blue was injected into the tail vein. To assess the effect of CAC-0982, this compound was orally administered 1 h prior to the antigen challenge. The mice were euthanized 1 h after the challenge, followed by the removal of an ear for measurement of the amount of dye extravasated by the antigen. The dye was eluted overnight from the ear tissue in $700\ \mu\text{l}$ of formamide at 63°C . The absorbance of the eluted dye solution was measured at 620 nm. The animal study was performed using a protocol pre-approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University (Approval No. KU-14111).

Measurement of tyrosine kinase activity. The key protein tyrosine kinases for mast cell activation, Lyn, Fyn, Fgr, or Syk were immunoprecipitated by using each specific antibody from whole cell lysates (1 mg of total protein) of BMMCs stimulated with or without CAC-0982 as described above. The kinase activity of precipitates was assayed with an ELISA-based Universal Tyrosine Kinase Assay Kit by the manufacturer's instructions (Genway, San Diego, CA, USA).

Statistical analysis. The data were presented as the mean $\pm$ SEM from at least three independent experiments. Statistical analysis was performed by using one-way ANOVA and the student's t-test for unpaired values unless stated otherwise. All statistical calculations (*, $p < 0.05$ and **, $p < 0.01$) were performed with the SigmaStat software (Systat Software Inc., San Jose, CA, USA).

Results

Effect of CAC-0982 on degranulation in antigen-stimulated mast cells

To examine the effect of CAC-0982 (Fig. 1) on antigen-stimulated degranulation of mast cells, the release of β -hexosaminidase from RBL-2H3 cells or mouse BMMCs was measured with or without antigen. The degranulation was dose-dependently inhibited by CAC-0982 in murine (Fig. 2A, $\text{IC}_{50} = \sim 3.8\ \mu\text{M}$) and human (Fig. 2B, $\text{IC}_{50} = \sim 3.0\ \mu\text{M}$) mast cells. The effect of CAC-0982 was comparable to that by PP2, a typical Src-family kinase inhibitor (Fig. 2A). However, this suppression disappeared when the mast cells were washed with PIPES buffer after a 1 h pre-treatment with $10\ \mu\text{M}$ CAC-0982 (Fig. 2C), indicating that the effect of CAC-0982 on mast cells was reversible. No cytotoxic effect was observed in the experimental dose range (Fig. 2D).

Effect of CAC-0982 on the expression and secretion of TNF- α and IL-4

Besides degranulation, mast cells are also involved in the production of inflammatory mediators during the allergic process (Theoharides and Kalogeromitros, 2006). Therefore, we also tested whether CAC-0982 suppressed the expression and secretion of TNF- α and IL-4 in antigen-stimulated mast cells. Consistently with the previous reports (Park et al., 2011; Wang et al., 2014), the increase of expression of IL-4 and TNF- α was evident by antigen stimulation for 15 min in mast cells (Fig. 3A). Notably, CAC-0982 inhibited the expression of TNF- α and IL-4 mRNA derived by antigen stimulation (Fig. 3A). In addition, the releases of cytokines were also significantly suppressed by CAC-0982 in

a dose-dependent manner (Figs. 3B and C). These results showed that CAC-0982 has an inhibitory effect on the production of pro-inflammatory cytokines from mast cells.

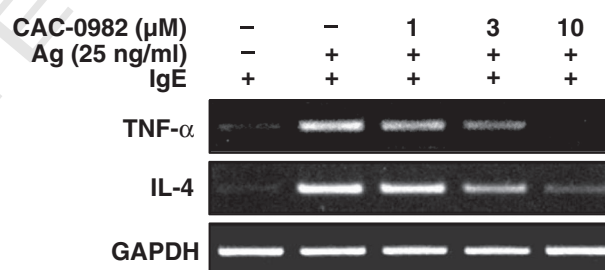
Effect of CAC-0982 on Fc ϵ RI-associated mast cell signaling pathways

The initial signaling events that follow Fc ϵ RI aggregation are regulated by a series of protein phosphorylation events. Upon Fc ϵ RI aggregation, Lyn becomes activated, with a resulting activation of the tyrosine kinase Syk and downstream signaling molecules such as PLD2, LAT, SLP-76 and PLC γ (Kawakami and Galli, 2002; Lee et al., 2006). The next step was to investigate whether or not CAC-0982 inhibited Fc ϵ RI-associated proximal signaling molecules such as Syk and LAT in antigen-stimulated mast cells. CAC-0982 significantly suppressed the phosphorylation of Syk and LAT in a dose-dependent manner (Fig. 4A, upper panel) as well as the downstream proteins Akt and three typical MAP kinases (Fig. 4A, lower panel). Consistently, CAC-0982 also inhibited the activating phosphorylation of Syk and LAT in mouse primary BMMCs (Fig. 4B), suggesting that CAC-0982 might be involved in the suppression of the early signaling molecules associated with the Fc ϵ RI receptors.

Effect of CAC-0982 on activation of protein tyrosine kinases

The IgE high affinity receptor-associated Lyn, most possibly, is the first tyrosine kinase activated by antigen stimulation in mast cells while Fyn and Fgr are also activated in this event (Gilfillan and Rivera, 2011

A



B

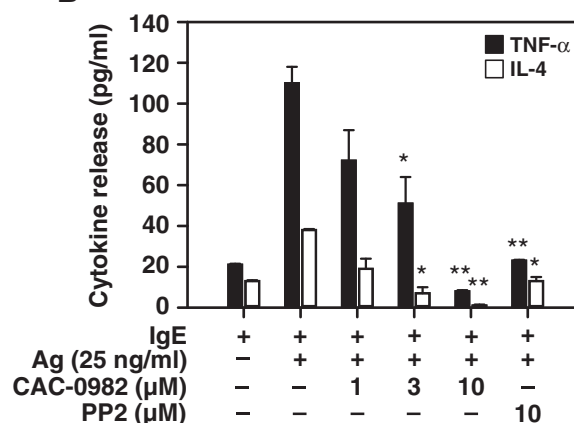


Fig. 3. Effect of CAC-0982 on the expression and secretion of TNF- α and IL-4 in RBL-2H3. (A) IgE-pulsed cells were stimulated with 25 ng/ml antigen for 15 min in the presence of the indicated concentrations of CAC-0982. Total RNA was isolated using Trizol, and polymerase chain reaction was performed using TNF- α - or IL-4-specific primers as described in the Materials and methods. (B) RBL-2H3 cells were stimulated with 25 ng/ml antigen for 8 h with or without CAC-0982. The concentration of TNF- α and IL-4 in the conditioned medium were determined using ELISA assay kits. The values indicate mean $\pm$ SEM from three independent experiments. Significant differences with the antigen-stimulated controls without CAC-0982 are indicated as follows: ** $p < 0.05$; * $p < 0.01$. Ag, antigen; PP2, a Src-family kinase inhibitor.

2009; Lee et al., 2011). Following those activations, an essential protein tyrosine kinase Syk is activated during this stimulation and is followed by many downstream signaling molecules including LAT and MAP kinases (Beaven, 2009). Next, this study investigated which protein tyrosine kinases were targeted by CAC-0982 in mast cells. Notably, the activity of Fyn, but not Lyn and Fgr, was inhibited by CAC-0982 in antigen-stimulated mast cells in a dose-dependent manner. In addition, the activity of Syk was also suppressed by CAC-0982 (Fig. 5). These findings suggest that the inhibition of the IgE-mediated mast cell activation by CAC-0982 was caused by the inhibition of Fyn/Syk-dependent signals.

Effect of CAC-0982 on passive cutaneous anaphylaxis (PCA) in mice

To determine the effect of CAC-0982 on allergic response in vivo, the experiments were performed using the IgE-dependent type I hypersensitive mouse model, PCA. It is well established that PCA is an IgE/mast cell-mediated allergic response in mice (Okudaira et al., 1980; Ujike

et al., 1999). The antigen-induced PCA reaction was significantly inhibited by CAC-0982 in a dose-dependent manner. The effect of CAC-0982 at a dose of 100 mg/kg (ED₅₀, 27.9 mg/kg) was comparable to that by diphenhydramine, DPH (50 mg/kg) (Figs. 6A and B). These observations strongly suggested that CAC-0982 effectively suppresses IgE-mediated allergic responses in mice.

Discussion

IgE-mediated mast cell activation is involved in the process of allergic responses and associated with allergic disorders such as allergic rhinitis, atopic dermatitis, allergic asthma, and some food allergies (Theoharides and Kalogeromitros, 2006; Galli et al., 2008). Mast cells are activated by antigen through the high affinity receptors for IgE, FcεRI, on the membrane, which then release granules that contain preformed inflammatory mediators such as histamine as well as de novo synthesis of eicosanoids

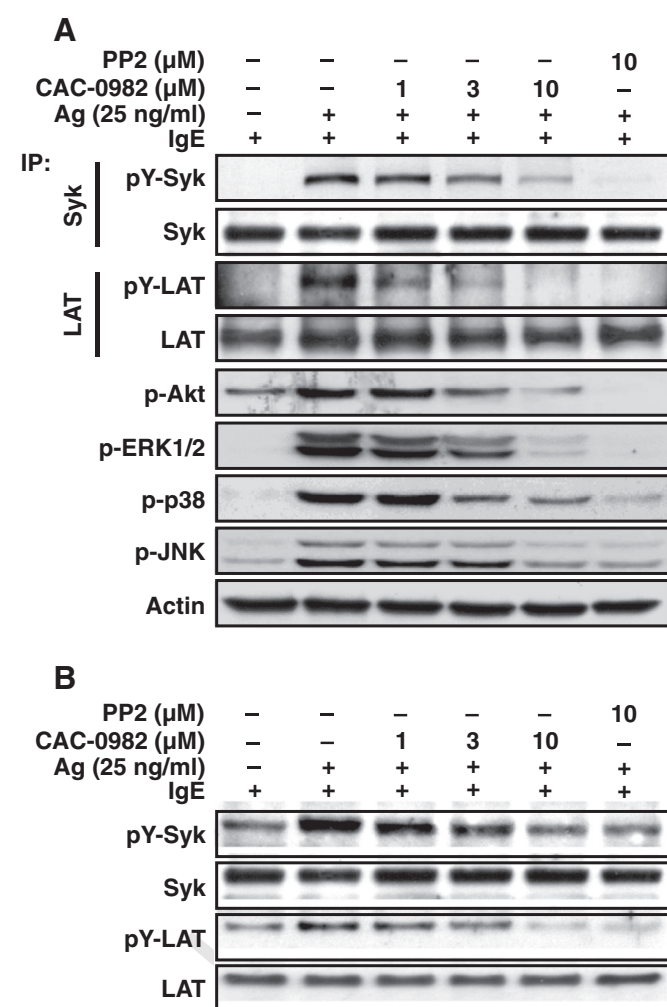


Fig. 4. Effect of CAC-0982 on the phosphorylation of IgE-mediated signaling pathway in mast cells. (A) RBL-2H3 cells were primed overnight with 20 ng/ml DNP-specific IgE, and then stimulated with 25 ng/ml of antigen for 10 min with or without CAC-0982 and PP2. The proteins derived from the cell lysates were immunoprecipitated with specific antibodies against Syk or LAT. The immunoprecipitates for Syk and LAT or whole cell lysate for Akt and three MAP kinases were then measured by western blot analysis using specific antibodies for phosphorylated tyrosine residues (clone 4G10), Akt, and three typical MAP kinases. (B) BMMCs were incubated overnight with 20 ng/ml DNP-specific IgE, and then stimulated with 25 ng/ml of antigen for 10 min with or without CAC-0982 and PP2. The western blot analysis was done for phosphorylated forms (pY) or total of Syk and LAT using specific antibodies. Representative images from three independent experiments are shown. Ag, antigen; PP2, a Src-family kinase inhibitor.

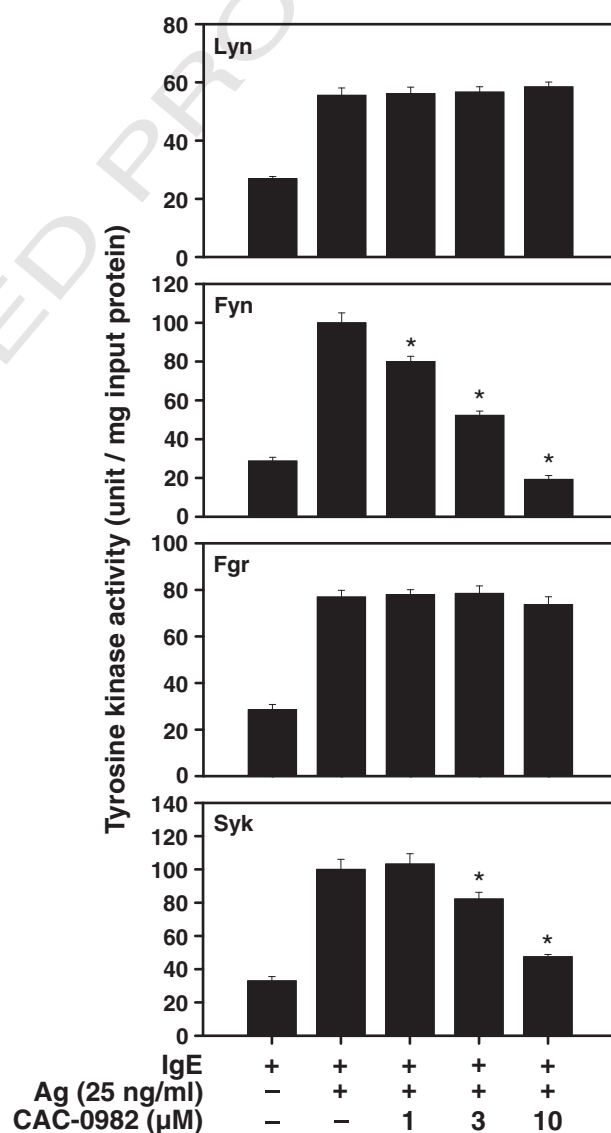


Fig. 5. Effect of CAC-0982 on tyrosine kinase activity. RBL-2H3 cells were stimulated with 25 ng/ml of antigen in the presence of the indicated concentrations of CAC-0982 after IgE pulsing. Protein lysates were prepared, and whole cell lysates were immunoprecipitated with antibodies against Lyn, Fyn, Fgr, and Syk coupled to protein G beads. The isolated proteins were used to perform the tyrosine kinase activity assay. Each section of data represents the mean $\pm$ SEM of three independent experiments. Statistical significance is indicated as follows: * $p < 0.05$. Ag, antigen; PP2, a Src-family kinase inhibitor.

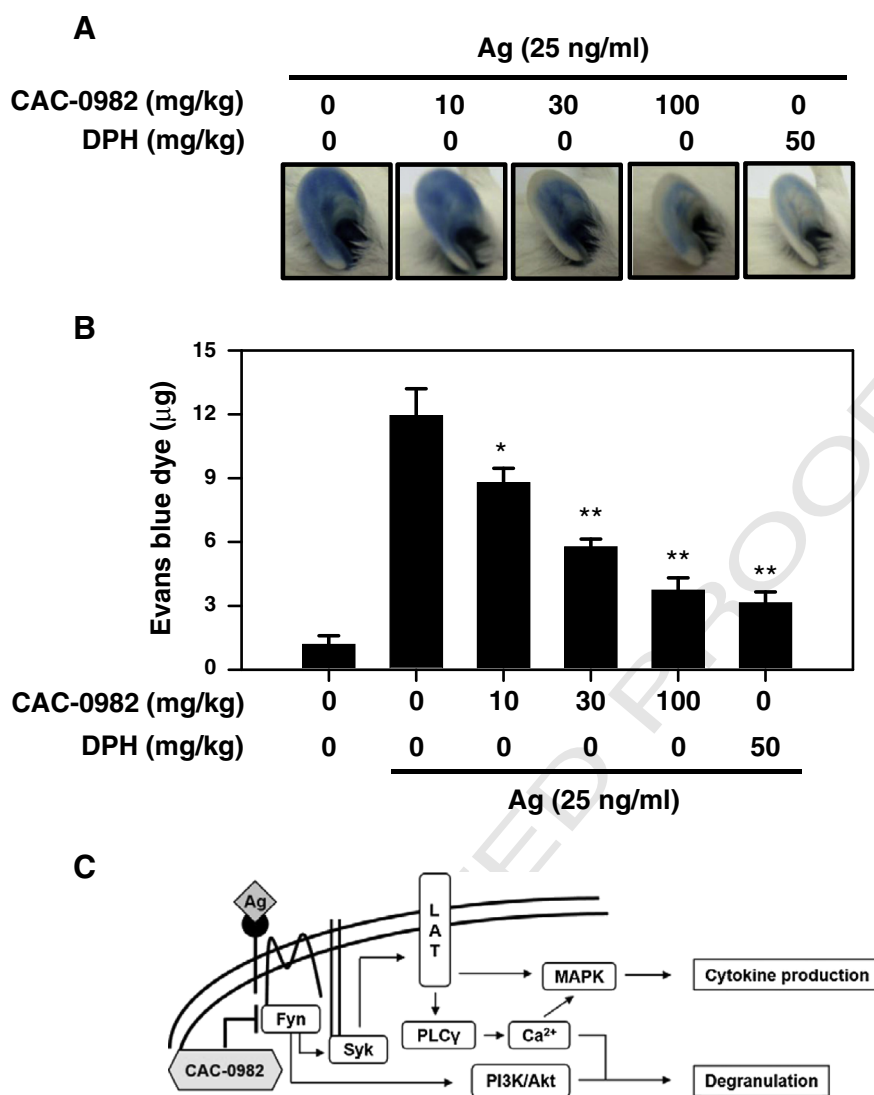


Fig. 6. Effect of CAC-0982 on the IgE-mediated PCA reaction. Left ears of mice ($n = 5$) were intradermally injected with DNP-specific IgE. Right ears of the same mice were injected with the same volume of $1 \times$ PBS as controls. CAC-0982 was administered orally 1 h prior to the challenge, 250 μ g of antigen, DNP-BSA, in PBS containing 4% Evans blue was injected intravenously into the tail vein. (A) Representative images from three independent experiments are shown. (B) Values of extravasated dye into ear tissue by antigen are expressed as the means $\pm$ SEM from three independent experiments. Significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$. Ag, antigen; PP2, a Src-family kinase inhibitor. (C) A proposed scheme for the mechanism of CAC-0982 action in mast cells.

and various cytokines (TNF- α , IL-1 β , IL-4, IL-6, and TGF- β) in humans (Beaven, 2009; Stone et al., 2010).

Although various treatments have been applied to allergic disorders (Holgate, 2014; Akdis, 2013; Burks et al., 2013), the treatments sometimes cause unwanted side effects including: corticotherapy, alterations in fat distribution, gastric irritation, and peptic ulcer (Holgate and Polosa, 2008); clinical therapy with Omalizumab, high cost and partial efficacy (Poole et al., 2005; Kopp, 2011); allergen-specific immunotherapy, long duration of treatment and occasional hazards (Sheehan et al., 1993; Hayen et al., 2014). Consequently, novel approaches are being explored to develop new therapies for allergic disorders.

Many research groups are exploring novel candidates that could target early signaling molecules like Src-family kinases and Syk for suppressing mast cell activation (Bradding, 2008). R112, a Syk inhibitor, ameliorated acute allergic rhinitis disorder through the inhibition of the allergen-induced release of mast cell mediators and the production of most eicosanoids and cytokines (Masuda and Schmitz, 2008). Saucerneol F (Lu et al., 2013) and morin (Kim et al., 2009) were reported to inhibit mast cells and allergic responses in vitro and in vivo through suppressing Fyn kinase and its downstream signaling cascades in mast

cells. In this study, the indoxyl compound CAC-0982 suppressed the activation of Fyn, but not Lyn and Fgr, and its downstream signaling protein Syk in antigen-stimulated mast cells.

The indoxyl compound is a class of chromogenic substrates, especially in microbial detection, with a wide range of applications (Pohanka and Drtinova, 2013; Juergensmeyer, 2006). To our best knowledge, no pharmacological effect of indoxyl chromogenic compounds including CAC-0982 has yet been reported. In this study, we demonstrated for the first time that CAC-0982 inhibited significantly degranulation in antigen-stimulated mast cells for suppression of IgE-mediated allergic responses (Figs. 2A and B).

It has been usually reported that most actions of a drug on a receptor or an enzyme are reversible. After a period of time, the drug releases from the receptor or enzyme and resumes its normal function. Sometimes an interaction is largely irreversible, and the drug's effect persists until the body makes more enzymes anew. Therefore, an irreversible drug should be utilized carefully in patient treatment because of the more plausible side effect. To check the reversibility of the effect of CAC-0982 on mast cells, we washed away the compound from the reaction media 1 h after its incubation with mast cells and, then,

degranulation of the cells was assessed. The degranulation of mast cells was restored by washing away the compound from the medium (Fig. 2C), suggesting that the action of CAC-0982 on mast cells was reversible. However, further study should be performed to investigate its possible side effects before application to patients.

Mast cells can secrete an array of cytokines including IL-4, IL-5, and TNF- α by antigen stimulation (Bischoff, 2007). These cytokines are known to induce pathogenic inflammatory symptoms, especially in the later stages of an allergic reaction (Carter et al., 1999). CAC-0982 significantly suppressed expression and secretion of TNF- α and IL-4 in antigen-stimulated mast cells in a dose-dependent manner (Fig. 3), suggesting that the compound could suppress chronic allergic symptoms. It is also well established that MAP kinases are essential for the production of various cytokines from mast cells; ERK1/2 for IL-5, TNF- α , IL-3, and IL-13 (Lorentz et al., 2003); p38 for IL-4 production (Frossi et al., 2007). In the study, CAC-0982 inhibited the expression and secretion of TNF- α and IL-4 (Figs. 3A and B) as well as the activation of three typical MAP kinases (Fig. 4A), indicating that these effects could be associated with the inhibition of MAP kinases.

The release of allergic mediators from antigen-stimulated mast cells is regulated through intracellular signaling events (Lee et al., 2011). The cross-linking of antigen-stimulated receptors activates the Src-family kinase, such as Lyn, Fyn, Fgr, and downstream signaling molecules such as Syk, LAT, SLP-76, PLD2, and PLC γ (Rivera and Gilfillan, 2006; Lee et al., 2011). Although the Lyn-dependent pathway leads to the initial activation of Fc ϵ RI proximal signals in mast cells, it involves also negative signaling processes in the cells to maintain its homeostasis (Roth et al., 2008; Ingley, 2012). On the other hand, Fyn kinase shows largely a positive regulatory role for the induction of degranulation, synthesis and release of leukotrienes, and the production of cytokines via the Gab2/PI3K pathway (Gomez et al., 2005; Parravicini et al., 2002) and/or Syk (Yu et al., 2006). As the mechanism of CAC-0982, it inhibited the activation of Syk and the downstream substrate protein LAT in antigen-stimulated mast cells (Fig. 4). Of particular interest, CAC-0982 inhibited the activation of Fyn, but not Lyn and Fgr, in the cells (Fig. 5). These findings strongly suggest that CAC-0982 significantly suppressed mast cell degranulation by reversibly inhibiting the Fyn/Syk pathway.

Basophils are Th2 type reaction-associated cells that share functional and developmental similarities as mast cells (Voehringer, 2013). They produce a similar allergic mediators, including proteases, histamine, various proinflammatory cytokines, and lipid mediators. These mediators are rapidly released by the activation of the IgE high-affinity receptor (Fc ϵ RI) that is expressed on the surface of basophils. Thus, basophils may contribute, at least partially, to IgE-mediated allergic responses (Voehringer, 2013). Therefore, it would be of interest to determine whether CAC-0982 suppresses the release of allergic mediators from basophils by antigen stimulation.

In summary, this study demonstrated for the first time that a synthetic substance CAC-0982 inhibited the activation of murine and human mast cells, and IgE/mast cell-mediated allergic responses. CAC-0982 suppressed IgE-mediated allergic responses through inhibition of the activation of Fyn/Syk pathway and its downstream signaling cascades (Fig. 6C). These results suggest, therefore, that further evaluation is warranted of its usefulness in the treatment of acute and chronic allergic diseases in patients.

Conflict of interest statement

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

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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