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Furaltadone suppresses IgE-mediated allergic response through the inhibition of Lyn/Syk pathway in mast cells

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ABSTRACT

Mast cells are critical cells that prompt various allergic response-inducing factors, contributing to allergic diseases. While used as an antibiotic for livestock, there is no study on the effect of furaltadone on allergic response. This study investigated the effect of furaltadone on mast cells and passive cutaneous anaphylaxis (PCA). Furaltadone inhibited the degranulation of mast cells stimulated by antigen (IC_{50}, ~ 3.9 μM), and also suppressed the production of tumor necrosis factor (TNF)-α and interleukin (IL)-4 in a concentration dependent manner. In addition, furaltadone inhibited allergic responses in an acute allergy animal model, PCA. Further investigation on the mechanism for these inhibitory effects of furaltadone found that the activities of Lyn/Syk and Syk-dependent downstream proteins such as mitogen-activated protein (MAP) kinases were inhibited by furaltadone in mast cells. Taken together, this study demonstrates that furaltadone inhibits the activation of mast cells by antigen via the suppression of the Lyn/Syk pathway and ameliorates allergic responses in vivo.

1. Introduction

Incidences of allergic diseases such as allergic rhinitis, asthma, eczema, and allergic anaphylaxis have increased in developed countries (World Allergy Organization, 2014), and approximately 25% of the population has been reported to have an allergic disease (Galli et al., 2008). Mast cells are well known as one of the critical effector cells responsible for allergic diseases (Gilfillan and Beaven, 2011). Once mast cells are activated by binding antigen to immunoglobulin (Ig) E that adheres to the IgE high affinity receptor, FcεRI, on the mast cell membrane, mast cells secrete histamine or protease at the early stage of the allergic response. They further secrete prostaglandin, leukotriene and inflammatory cytokines in the later stage, by which allergic responses are induced (Yamaguchi et al., 1999; Locksley, 2010). Thus, IgE-mediated activation of mast cells is a significant event for various allergic diseases and studies have been under way to treat allergic diseases through the suppression of mast cells (Holgate et al., 2005; Galli and Tsai, 2012).

The signaling pathway for activating IgE-mediated mast cells begins with antigen binding to the IgE bound to the FcεRI receptors of mast cells. Immunoreceptor tyrosine-based activating motifs (ITAMs) that are present in β-and γ-subunits of FcεRIs are phosphorylated by Lyn, a Src family kinase, following which Syk binds to the phosphorylated ITAMs for optimal activation (Siraganian, 2003). The activated Syk directly or indirectly induces activation of downstream signaling molecules including the Src homology 2 domain-containing leukocyte protein of 76 kD, a linker for activated T cells (LAT), phospholipase (PL) C-γ, and Gab2 (Gilfillan and Rivera, 2009). Thereafter, the phosphatidylinositol-3-kinases (PI3K)/Akt pathway, Ca^{2+} mobilization, and mitogen-activated protein kinase (MAPK) are activated, leading to degranulation and the secretion of inflammatory cytokines, which in the end results in allergic responses (Kawakami and Galli, 2002). There are several reports that a deficiency of Syk in mast cells leads to the reduction of the activation of mast cells (Zhang et al., 1996, 2010; Siraganian et al., 2010). As such, the Src family kinases and Syk, which are pivotal signaling proteins for the activation of mast cells, were recently proposed as targets in the treatment of allergic diseases (Holgate and Polosa, 2008).

Furaltadone, 5-morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidinone, is a nitrofuran family antibiotic having a 5-nitrofuran ring (Vass et al., 2008). Nitrofuran family antibiotics have been broadly used as veterinary drugs (Draisci et al., 1997) and also for scalded skin infection (Vasheghani et al., 2008), cholera (Roychowdhury et al., 2008), bacterial diarrhea (Petri, 2005), and urinary tract infection

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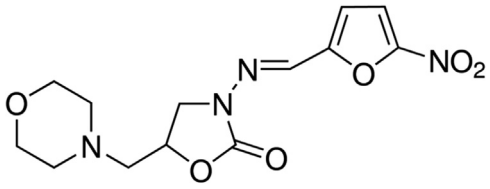

Fig. 1. The chemical structure of furaltadone.

(Guay, 2008) in humans. Although furaltadone is well known for its use as an antibiotic, there has been no study on its effect on allergic diseases. Thus, this study conducted a series of experiments at the cellular level and with animal models in order to investigate the effect of furaltadone on allergic responses. The results found that furaltadone suppressed the activation of mast cells through the inhibition of the Lyn/Syk pathway and significantly ameliorated antigen-induced allergic responses in mice.

2. Materials and methods

2.1. Reagents

Furaltadone (Fig. 1) was purchased from Selleckchem (Houston, TX). Cell culture medium was bought from GIBCO/Life Technologies, Inc. (Rockville, MD). Monoclonal dinitrophenol (DNP)-specific IgE, DNP-human serum albumin (DNP-HSA, antigen), Evans blue, cetirizine, and toluidine blue were obtained from Sigma (St. Louis, MO). 4-Amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine (PP2) was from Calbiochem (Lajolla, CA). Antibodies for phosphorylated proteins including Syk, LAT, Akt, Erk1/2, p38, JNK, and actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Syk and LAT antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Animals

Five to six week-old male BALB/c mice were obtained from Orient Bio, Inc. (Gyeonggi-do, Korea) and used for the preparation of bone marrow-derived mast cells (BMMCs) and PCA experiments. After approval by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University, animal experiments were conducted following institutional guidelines.

2.3. Preparation of mast cells and cell culture

Rat basophilic leukemia (RBL)-2H3 cells were obtained from the American Type Culture Collection, and cultured in a minimal essential medium supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15% fetal bovine serum. To prepare BMMCs, bone marrow cells were collected from the femoral region of 5 week-old male BALB/c, and cultured to differentiate into BMMCs for at least 4 weeks in Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES), 10% fetal bovine serum, and 10 ng/ml IL-3.

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

RBL-2H3 cells were dispensed into 24 well-plates (1.8×10^5 cells/well), followed by sensitization with 20 ng/ml DNP-specific IgE for 12 h and 2 rounds of washing with 1,4-piperazinediethanesulfonic acid (PIPES) buffer (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from a bovine serum). Thereafter, furaltadone was diluted in PIPES buffer and PP2 was also diluted separately as a control, followed by pretreatment for 30 min. After stimulating with 25 ng/ml DNP-HSA for 15 min, the supernatant and cell lysate were subjected to reaction with 1 mM 4-nitrophenyl-N-acetyl-β-D-glucosaminide at 37 °C for 1 h, followed by quenching with 0.1 M carbonate and the measurement of optical density (OD) at 405 nm in wavelength. BMMC was suspended in Tyrode buffer (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA) for experimentation. The degranulation of mast cells was measured by the ratio of β-hexosaminidase secreted relative to total β-hexosaminidase, which is the sum of secreted β-hexosaminidase in culture medium and the remaining β-hexosaminidase inside the cells.

2.5. Measurement of cell viability

After the culture of RBL-2H3 cells (2×10^4 cells/well) in 96 well-plates for 12 h, the cells were incubated with furaltadone for 4 h, and followed by culturing with a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) for 1 h. Absorbance was measured at 450 nm.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from RBL-2H3 cells by using Easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Seongnam-si, Korea). The extracted RNA was subjected to reverse transcription using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). PCR conditions were 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s. Primer sequences were as follows: rat TNF-α forward, 5′-CACCA CGCTCTTCTGTCTACTGAAC-3′; rat TNF-α reverse, 5′-CCGGA CTCCGTGATGT CTAAGTACT-3′; rat IL-4 forward, 5′-ACCTTGCTGTCA CCCTGTTC-3′; rat IL-4 reverse, 5′-TTGTGAGCGTGGACTCATTC-3′; rat GAPDH forward, 5′-GTGGAGTC TACTGGCGTCTTC-3′; rat GAPDH reverse, 5′-CCAAGGCTGTGGGCAAGG TCA-3′.

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

IgE-primed RBL-2H3 cells (5×10^5 cells/well) were stimulated with 25 ng/ml DNP-HSA for 3 h with and without furaltadone or PP2. The amount of TNF-α and IL-4 in the cultured media were determined using rat OptEIA ELISA kits, according to the manufacturer's protocol (BD Biosciences, San Jose, CA).

2.8. Western blot analysis

RBL-2H3 cells (1×10^6 cells/well) were cultured in 6 well-plates and followed by sensitization with 20 ng/ml DNP-specific IgE for 12 h. The cells were washed twice with fresh culture medium, and pretreated with furaltadone for 30 min. After stimulation with 25 ng/ml DNP-HSA for 7 min, the reaction was terminated on ice and followed by washing with cold phosphate-buffered saline (PBS) twice. Cells were lysed by adding 100 μl 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 phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 mg/ml pepstatin, and a protease-inhibitor cocktail tablet) per well. Following centrifugation of the cell lysate at $15{,}000 \times g$ for 5 min, the supernatant was mixed with 3 × Laemmli buffer and followed by protein denaturation at 95 °C for 5 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. Following incubation with each specific antibody in a TBS-T (Tris-buffered saline containing 0.1% Tween 20) buffer containing 5% BSA, the membrane was incubated with horseradish peroxidase-labeled secondary antibody. Protein bands were detected with an enhanced chemiluminescence detection kit (ThermoFisher Scientific, Waltham, MA).

2.9. In vitro protein tyrosine kinase assay

The Lyn and Fyn obtained by immunoprecipitation from a whole cell lysate of mast cells were used for in vitro tyrosine kinase assay. IgE-primed mast cells were stimulated by 25 ng/ml DNP-HSA for 7 min. After washing with cold PBS twice, cells were lysed with a 0.5 ml lysis buffer. Following centrifugation of the cell lysate at 15,000 × *g* at 4 °C for 15 min, the supernatant was incubated with 5 μg antibody for a minimum of 3 h. Fifty μl of protein A/G-agarose was added and incubated at 4 °C overnight. After washing 3 times with a washing solution (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% 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 nitrophenylphosphate, 0.7 mg/ml pepstatin, and a protease-inhibitor cocktail tablet), the activity of tyrosine kinase was analyzed with and without furaltadone by using ELISA-based Universal Tyrosine Kinase Assay Kit according to the manufacturer's protocol (Genway, San Diego, CA).

2.10. Passive cutaneous anaphylaxis (PCA)

BALB/c mice were intradermally injected with 0.5 μg DNP-IgE on back skin and, after 12 h, followed by oral administration of either furaltadone (3, 10, 30 mg/kg) or cetirizine (20 mg/kg) dissolved in 5% Arabic gum. One hour after administration, 250 μl of 5 mg/ml Evans blue solution containing 1 mg/ml DNP-HSA was intravenously injected at the tail. After 1 h, mice were euthanized, and skin on the back was removed, from which Evans blue dye was extracted in 2 ml formamide at 63 °C for 12 h. The absorbance was measured at a wavelength of 620 nm.

2.11. Histological analysis

The skin from the back of PCA mice was removed, fixed by 4% paraformaldehyde, and embedded in paraffin. The paraffin tissue was sliced by 6 μm in thickness, and stained with toluidine blue. The number of mast cells in back skin tissue was calculated from three sections per tissue ($n = 5$, total 15 sections). The percent of degranulated mast cells was presented as the ratio of degranulated mast cells to total mast cells in the tissue. Mast cells in which 10% or more of intracellular granules were released from the cells were evaluated as degranulated mast cells as previously described (Martin et al., 1989).

2.12. Statistical analysis

Experiments were independently repeated at least 3 times, and the results were expressed as the means ± S.E.M. Statistical analysis was conducted by using ANOVA and Dunnett's test. All statistical calculations were performed by using (*P < 0.05 and **P < 0.01) Sigma Stat software (SystatSoftware, Inc., Point Richmond, CA).

3. Results

3.1. Effect of furaltadone on degranulation in antigen-stimulated RBL-2H3 cells and BMMCs

To investigate the inhibitory effect of furaltadone on the degranulation of mast cells, this study measured if the secretion of β-hexosaminidase, a typical degranulation marker, by antigen in mast cells was inhibited. It was found that degranulation stimulated by an antigen was suppressed by furaltadone in a concentration-dependent manner both in RBL-2H3 cells and in BMMC (Fig. 2A). The inhibitory effect of furaltadone at the highest concentration (10 μM) was equal to that of PP2, a typical Src-family kinase inhibitor (IC_{50}, ~ 3.9 μM). To investigate whether the inhibitory effect of furaltadone was reversible, RBL-2H3 cell were pre-treated with furaltadone for 30 min, and washed with PIPES buffer 3 times, followed by a measurement of the degranulation, which found that degranulation inhibited by furaltadone was recovered by washing, indicating that the effect of furaltadone in mast cells is reversible (Fig. 2B). In addition, a cell viability test showed that furaltadone had no cytotoxicity at the experimental concentrations (Fig. 2C).

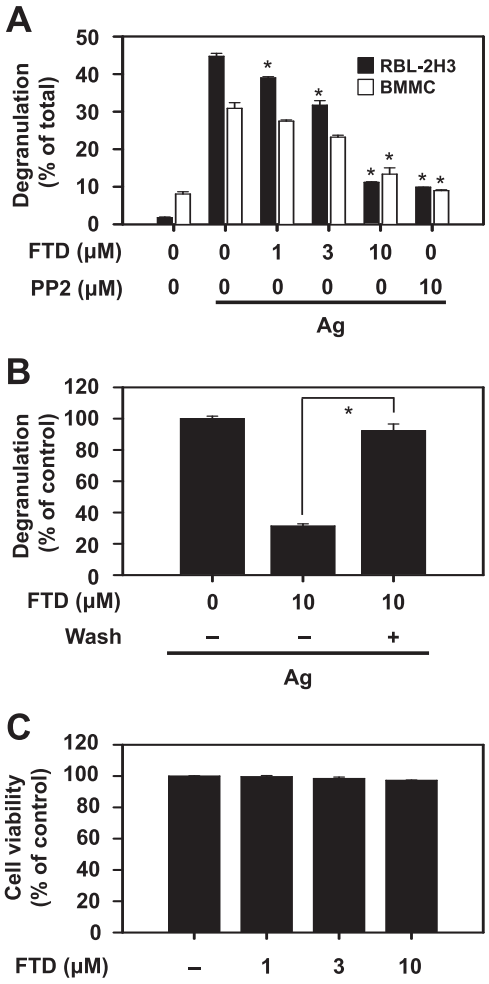

Fig. 2. Furaltadone (FTD) reversibly inhibits antigen-induced degranulation in mast cells. (A) IgE-labeled RBL-2H3 cells (2.0×10^5 cells/well) and BMMCs (2.5×10^5 cells/tube) were stimulated with 25 ng/ml DNP-HSA (antigen) for 15 min after pre-incubation with FTD or PP2 for 30 min. (B) RBL-2H3 cells were washed 5 times after incubating with FTD for 30 min and stimulated with 25 ng/ml antigen for 15 min. Degranulation rate was measured as described in the Section 2. Control refers to the mast cell degranulation rate by antigen stimulation without FTD. (C) RBL-2H3 cells (2.0×10^4 cells/well) were incubated with FTD for 4 h. CCK-8 solution (at a 1:10 ratio of CCK-8 to media) was incubated with media for 1 h. The absorbance for cell viability was measured at 450 nm. The values are expressed as the means ± S.E.M. from three independent experiments. Control refers to the survival rate without FTD. Significant differences with the values for the antigen-only groups are indicated, *P < 0.05.

3.2. Effect of furaltadone on expression and secretion of inflammatory cytokines

TNF-α and IL-4 are inflammatory cytokines secreted from mast cells, and are also critical for the induction of allergic responses (Tete et al., 2011). RT-PCR was performed to test if furaltadone also inhibited the production of TNF-α and IL-4 by antigen in mast cells. As a result, it was found that the expressions and secretion of TNF-α and IL-4 by antigen in mast cells were inhibited furaltadone in a concentration-dependent manner (Fig. 3A and B).

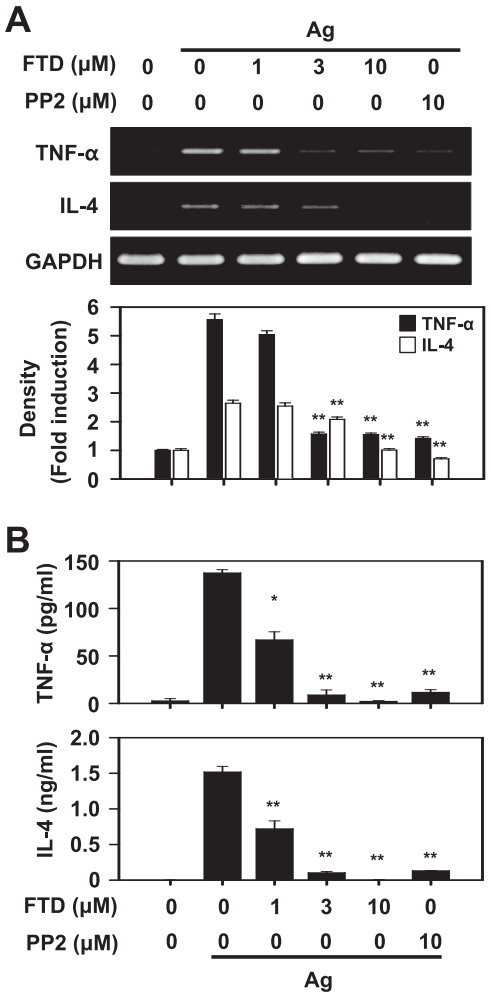

Fig. 3. Furaltadone (FTD) suppresses the gene expression and secretion of TNF-α and IL-4 in mast cells. (A) IgE-primed RBL-2H3 cells (1×10^6 cells/well) were stimulated with 25 ng/ml DNP-HSA (antigen) for 15 min after pre-incubation with or without FTD or PP2 for 30 min. The expression of TNF-α and IL-4 mRNA was measured by RT-PCR. The mean ± S.E.M. of band density for the upper panel from three independent experiments are shown. Significant differences with the values for the antigen-only groups are indicated, *P < 0.05 and **P < 0.01. (B) RBL-2H3 cells (5×10^5 cells per well) were stimulated with DNP-BSA (25 ng/ml) for 3 h with or without FTD or PP2. The secretion of TNF-α and IL-4 was measured by ELISA kits. The mean ± S.E.M. of values from three independent experiments are shown. Significant differences with the values for the antigen-only groups are indicated, *P < 0.05 and **P < 0.01.

3.3. Effect of furaltadone on antigen-stimulated mast cell signaling pathways

To determine the mechanism of the inhibitory effect of furaltadone in mast cells, the signaling pathways of mast cells were investigated. The activations of early signaling proteins including Syk and LAT were suppressed by furaltadone in a concentration-dependent manner (Fig. 4A). In addition, the activations of downstream signaling proteins including Akt and MAP kinases such as Erk1/2, p38, and JNK were also inhibited by furaltadone (Fig. 4A). Consistently, furaltadone inhibited the activation of Syk and LAT in BMMCs (Fig. 4B). These results suggest that furaltadone suppresses the activation of Syk and Syk-dependent signaling pathways.

3.4. Effect of furaltadone on the activity of Lyn and Fyn in vitro

The activity of the early protein kinases including Lyn and Fyn is essential for the activation of Syk in antigen-stimulated mast cells. The activity of Lyn and Fyn in vitro was measured to identify which tyrosine kinase is the direct target of furaltadone in mast cells. It was found that the activity of Lyn, but not Fyn, was inhibited by furaltadone in a concentration dependent manner in vitro (Fig. 5), suggesting that the target of furaltadone is Lyn in mast cells.

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

To test if furaltadone has an inhibitory effect on allergic response in mice as in the mast cells, a PCA experiment, a mast cell-mediated allergic animal model, was performed. IgE was subcutaneously injected to BALB/c mice on the back skin, followed by oral administration of either cetirizine (20 mg/kg), a reference drug used to treat allergies, or furaltadone (3, 10, 30 mg/kg), and an antigen was then injected to induce PCA. As a result, the skin from the backs of mice in the vehicle group showed an evident allergic response, whereas furaltadone inhibited the allergic response in a dose-dependent manner (Fig. 6A and B). The mast cell degranulation, which was increased in the skin tissue by antigen stimulation, was significantly inhibited by furaltadone (Fig. 6C). Thus, the inhibitory effect of furaltadone on allergic response was shown not only in mast cells in vitro but also in allergic mice in vivo.

3.6. Effect of nitrofuran derivatives on degranulation of mast cells and PCA

Among nitrofuran-derived antibiotics, nitrofurantoin and furazolidone have been used as medicine in various diseases and are known to be less toxic than furaltadone (Krasavin et al., 2017; Ran et al., 2016; Zhu et al., 2016). Therefore, the inhibitory effect of nitrofurantoin and furazolidone on mast cell degranulation and PCA was evaluated. As shown in Fig. 7A, both nitrofuran derivatives suppressed mast cell degranulation by antigens in a concentration-dependent manner and the inhibitory effects on mast cell degranulation were equivalent to that of furaltadone. However, unlike furaltadone, nitrofurantoin and furazolidone did not inhibit PCA in mice at a dose of 30 mg/kg (Fig. 7B). These results suggest that the unidentified specific molecular structure of furaltadone is critical for its anti-allergic activity in vivo.

4. Discussion

Mast cells are important in various allergic diseases including allergic rhinitis, food allergy, asthma, and atopic dermatitis (Gilfillan and Beaven, 2011). The IgE-mediated activation of mast cells is involved both in the early and later allergic responses, in which they induce allergic responses in the early stage through the release of histamine or protease, and in the later stage through the secretion of prostaglandin, leukotriene, and inflammatory cytokines (Yamaguchi et al., 1999; Locksley, 2010). Since the activation of mast cells is critical for an allergic response to occur, many studies have been conducted to develop compounds targeting intracellular signaling proteins, which are an important factor in mast cell activation.

Furaltadone inhibited the degranulation of mast cells by antigen in a concentration-dependent manner (Fig. 2A). In general, most drugs acting on proteins are reversible, so if the concentration of the drug falls below a certain concentration, the protein and drug separate and the protein recovers its original activity. In the case of irreversible drugs, however, the drug is permanently bound to the protein to exert its activity. The protein cannot regain its normal activity regardless of the concentration or half-life of the drug in the body and therefore, the irreversibility of these drugs is likely to be a cause of serious side effects due to various mechanisms in the body (Lee et al., 2015). For this

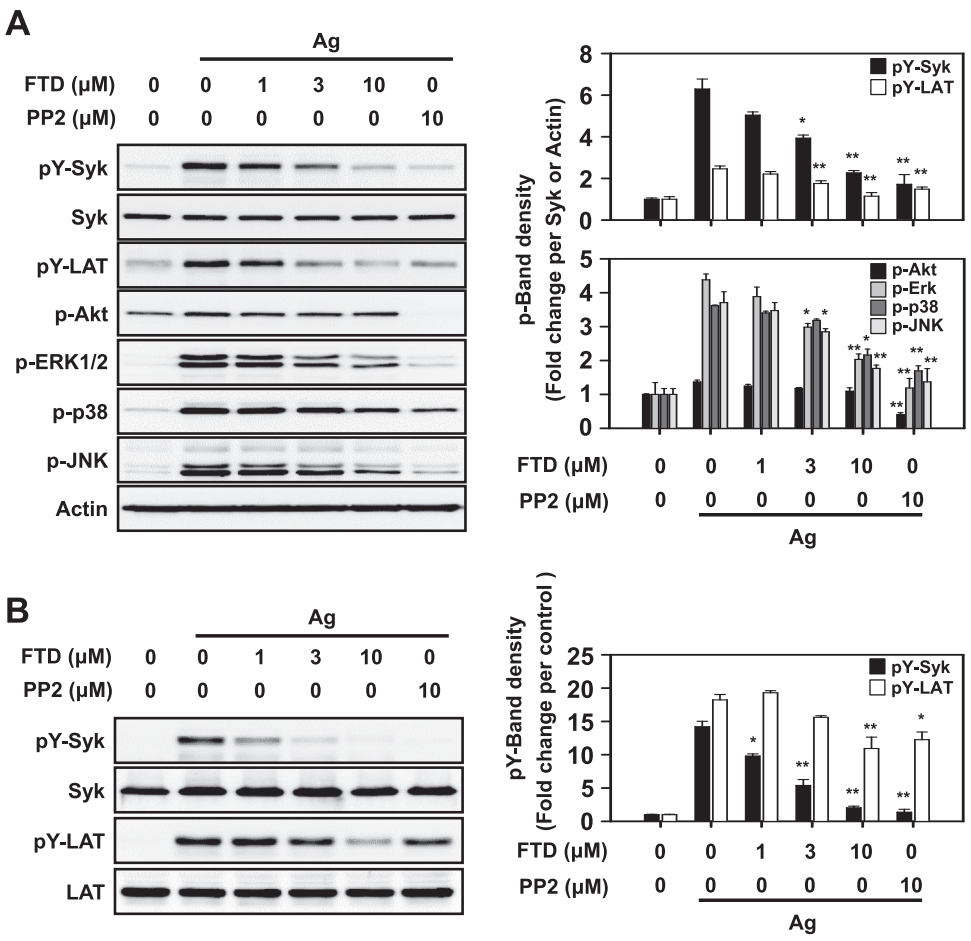

Fig. 4. Furaltadone (FTD) inhibits phosphorylation of antigen-stimulated signaling pathway in mast cells. IgE-primed (A) RBL-2H3 cells (1.0×10^6 cells/well) and (B) BMMCs (5.0×10^6 cells/tube) were stimulated with 25 ng/ml DNP-HSA for 10 min after pre-incubation with FTD or PP2 for 30 min. The cell lysates were subjected to Western blot analysis. Representative images are shown from three independent experiments. The mean ± S.E.M. of band density for each upper panel from three independent experiments are shown. Significant differences with the values for the antigen-only groups are indicated, *P < 0.05 and **P < 0.01.

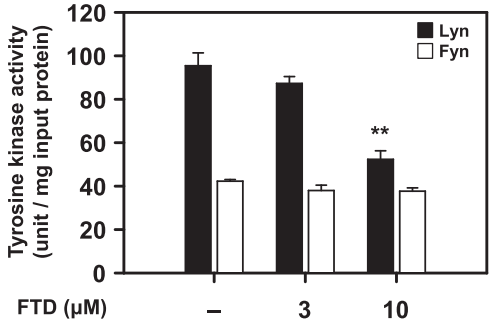

Fig. 5. Furaltadone (FTD) inhibits Lyn in vitro. Lyn and Fyn were immunoprecipitated as described in the Section 2 and Lyn and Fyn were then subjected to in vitro kinase assay with or without FTD at 3 and 10 μM. The mean ± S.E.M. from three independent experiments are shown. Significant differences with the value without FTD are indicated, **P < 0.01.

reason, the reversibility of drugs in the development of new drugs is a main concern. To test the reversibility of furaltadone, the degranulation of mast cells was measured after the treatment of cells with furaltadone for 30 min followed by washing with the buffer. Washing caused the action of furaltadone to disappear, indicating that the effect of furaltadone is reversible (Fig. 2B). In addition, it was also demonstrated that furaltadone has no cytotoxicity to mast cells at the experimental concentrations (Fig. 2C). These results were sufficient to lead to an experiment investigating the inhibitory effect of furaltadone on allergic responses in mice.

IgE-mediated activation of mast cells is initiated through the aggregation of FcεRI's by antigen, and followed by phosphorylation on ITAM motifs in β and γ subunits of FcεRI by Lyn, a Src family kinase (Siraganian, 2003). Subsequently, the Syk in the cytosol binds to the phosphorylated ITAM motifs, being phosphorylated, which activates the signal cascade of Src homology 2 domain-containing leukocyte protein of 76 kD, LAT, PLC-γ, and Gab2 for the production and secretion of allergic mediators, resulting in the activation of downstream signaling proteins (Kawakami and Galli, 2002). According to previous reports, the deficiency or suppression of the Syk in mast cells brought about the inhibition of mast cell activation and allergic response (Zhang et al., 1996, 2010). In this study, it was observed that furaltadone inhibited Syk activation as well as downstream signaling proteins by antigen (Fig. 4). These results suggest that furaltadone inhibits the activation of mast cells through the suppression of Syk in mast cells by antigens.

MAP kinases are also activated by antigen stimulation in mast cells and the activities of these signals are important for the production of various inflammatory cytokines (Tete et al., 2011). The results of this study demonstrated that furaltadone inhibited the activations of MAP kinases including Erk1/2, p38, and JNK (Fig. 4A), suggesting that the decrease in TNF-α and IL-4 production is attributable to suppressed MAP kinases' activities. Inflammatory cytokines including TNF-α and IL-4 are well known to play an important role in later phase of allergic responses (Locksley, 2010; Metcalfe et al., 1997). Thus, these results suggest that furaltadone exerts its inhibitory effect in chronic allergic diseases as well as inhibiting acute allergic reactions.

PCA is well known as a local allergic reaction mediated by antigen-stimulated mast cells in vivo (Inagaki and Nagai, 2009). This study found that PCA was inhibited by the administration of furaltadone in mice (Fig. 6A and B). In addition, this study also showed that the number of degranulated mast cells in skin tissue from the back was decreased by furaltadone (Fig. 6C). These results demonstrate that the inhibition of allergic responses in mice by furaltadone is attributable to

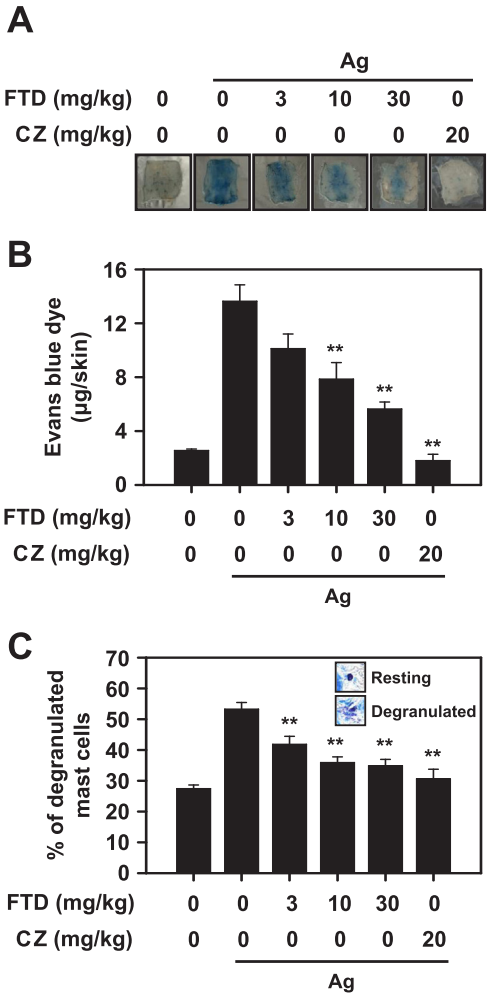

Fig. 6. Furaltadone (FTD) inhibits passive cutaneous anaphylaxis (PCA) reaction in mice. The DNP-specific IgE (0.5 μg) was intradermally injected into mouse back skin (n = 5). After 12 h, FTD or cetirizine (CZ) as a reference drug was orally treated 1 h before 250 μg DNP-HSA (antigen) containing 5 mg/ml Evans blue was intravenously injected into the mouse tail. The back skin was excised 1 h after injection of the antigen. Evans blue from the back skin was extracted in formamide for 12 h. The absorbance was measured at 620 nm. (A) The representative images of back skins are shown. (B) The amount of Evans blue dye extravasated into back skins was measured as described in the Section 2. The values indicate the means ± S.E.M. (n = 5). Significant differences with the values for the antigen-only groups are indicated, **P < 0.01. (C) The representative histological images for mast cells (inset) and percentage of degranulated mast cells from back skin sections are shown. The values indicate the means ± S.E.M. (n = 5). Significant differences with the values for the antigen-only groups are indicated, **P < 0.01.

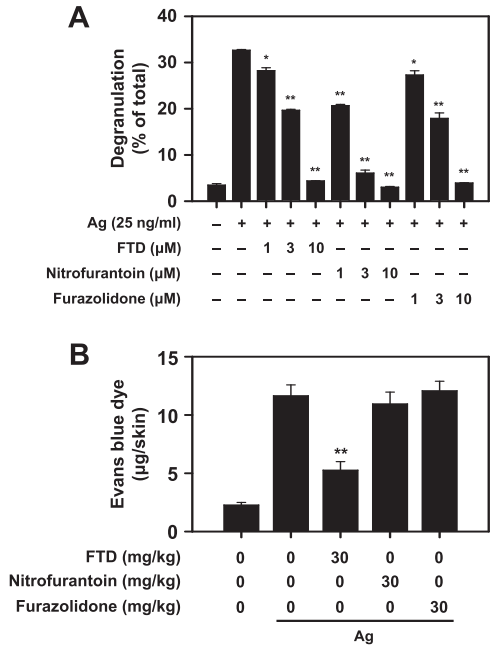

Fig. 7. Nitrofurantoin and furazolidone suppress degranulation by antigen in mast cells, but not passive cutaneous anaphylaxis in mice. (A) IgE-primed RBL-2H3 cells (1.8×10^5 cells/well) were stimulated by 25 ng/ml DNP-HSA (antigen) for 15 min after pre-incubation with nitrofurantoin, furazolidone or furaltadone for 30 min. The rate of degranulation was assessed as described in the Section 2. (B) PSA experiments were performed as described in the Section 2. The values are presented as the means ± S.E.M. from three independent experiments (A) or values from 5 mice per group (B). Significant differences with the values for the antigen-only groups are shown, *P < 0.05 and **P < 0.01.

the inhibition of mast cell activation in mice.

Nitrofuran-derived antibiotics were mostly used as feed additives for livestock to prevent and treat infection by certain bacteria or protozoa (Draisci et al., 1997; Dann and Möller, 1947). Since, however, it was reported that long exposure to the metabolites of nitrofuran could cause cancer or other side effects (McCalla, 1983; Vroomen et al., 1990), its use in livestock has been prohibited (Khong et al., 2004). It has been attempted to avoid adverse reactions through structural modification of nitrofuran compounds (Krasavin et al., 2017; Ran et al., 2016; Zhu et al., 2016). With these efforts, some other derivatives are in limited used for human or veterinary treatment. For example, nitrofurazone was locally used for scalded skin infection (Vasheghani et al., 2008), and furazolidone was used to treat cholera (Roychowdhury et al., 2008) and bacterial diarrhea (Petri, 2005) in human. Nitrofurantoin was effective for treatment of urinary tract infections in human (Guay, 2008). In this study, furazolidone and nitrofurantoin were similar to furaltadone in inhibiting mast cell degranulation in vitro (Fig. 7A). But at the highest concentration of 30 mg/kg, furazolidone and nitrofurantoin were not effective in suppressing PCA in mice (Fig. 7B). At present, the discrepancy of in vitro and in vivo effects cannot be accounted for but it is possible that the difference in absorption and distribution of each compound could affect their anti-allergic effect in vivo. Overall, considering the toxicity of furaltadone mentioned above, furaltadone is difficult to use over a long period of time as a remedy to treat allergic diseases in human. For this reason, the creation of derivatives of furaltadone that reduce its toxicity is currently underway.

In conclusion, this study demonstrated for the first time that furaltadone inhibited the IgE-mediated allergic response in vivo as well as in vitro. The inhibitory mechanism of furaltadone was found to suppress the antigen-stimulated Lyn/Syk pathway and its downstream signaling molecules through the direct inhibition of Lyn in mast cells.

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Conflict of interest

The authors declare no competing financial interests.

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