The suppressive effect of tamarixetin, isolated from *Inula japonica*, on degranulation and eicosanoid production in bone marrow-derived mast cells

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Abstract

\textbf{Background:} The study aimed to evaluate the inhibitory effect of tamarixetin on the production of inflammatory mediators in immunoglobulin E/antigen-induced mouse bone marrow-derived mast cells (BMMCs).

\textbf{Material and methods:} Tamarixetin isolated from *Inula japonica* was infected into BMMCs. The inhibitory effect of tamarixetin were analyzed by quantifying β-hexosaminidase, eicosanoid generation, intracellular calcium measurement, and Western blot analysis.

\textbf{Results:} Tamarixetin effectively decreased degranulation and the eicosanoid generation such as leukotriene C\textsubscript{4} (LTC\textsubscript{4}) and prostaglandin D\textsubscript{2} (PGD\textsubscript{2}) in BMMCs. To elucidate the mechanism involved, we investigated the effect of tamarixetin on the phosphorylation of signal molecules. Tamarixetin inhibited the phosphorylation of protein kinase B (Akt) and its downstream signal molecules including I\textkappa B kinase and nuclear factor-κB. Besides, tamarixetin downregulated the phosphorylation of cytosolic phospholipase A\textsubscript{2} and p38 mitogen-activated protein kinase.

\textbf{Conclusion:} In summary, tamarixetin inhibits degranulation and eicosanoid generation through the phospholipase C\gamma as well as Akt pathways. It could be potential for the prevention of allergic inflammatory diseases in BMMCs.

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KEYWORDS
degranulation; leukotriene C\textsubscript{4} (LTC\textsubscript{4}); prostaglandin D\textsubscript{2} (PGD\textsubscript{2}); mast cells

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**Introduction**

Host response against inflammation results in the release of various inflammatory mediators produced by innate immune cells. If left uncontrolled, the inflammatory mediators become involved in the pathogenesis of many inflammatory disorders. Among immune cells, mast cells play a vital role in allergic diseases. The binding of antigen (Ag) to the high-affinity receptor for immunoglobulin E (IgE) also known as FcεRI, is followed by crosslinking of IgE releases preformed inflammatory mediators (histamine, serotonin, and serine proteases) that generate eicosanoids prostaglandin D2 (PGD2) and leukotriene C4 (LTC4) from arachidonic acid (AA), and synthesize chemokines as well as cytokines.1,2

Activated mast cells initiate the activation of spleen tyrosine kinase (Syk) followed by phosphorylation of phospholipase C-1 (PLC-1), which triggers calcium (Ca++) release from internal stores.1-4 FcεRI signaling triggers the activation of three families of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB signaling pathways. These induce the expression of cyclooxygenase (COX)-2 and proinflammatory cytokines. Besides, the activated MAPKs also contribute to the phosphorylation of cytosolic phospholipase A2. These eventually lead to the AA release, a common precursor of eicosanoids.5

Previously, studies have shown anti-inflammatory and anti-allergic responses of britanin and tomentin.6-9 As a continuity in this study we decided to check the immune response of tamarixetin. Tamarixetin isolated from *Inulae flos* is a natural flavonoid derivative of quer cetin, and shows inhibitory effects of acetylcholinesterase and xanthine oxidase.10,11 Recently, Park et al.12 showed that tamarixetin reduced the secretion inflammatory cytokines by dendritic cells. However, no research is performed to see the immune response of the effect of tamarixetin on degranulation and AA generation in activated mast cells.

Hence, in this study we investigated the anti-inflammatory effect of tamarixetin and observed that it suppressed the degranulation through the attenuation of PLC-1 phosphorylation in IgE/Ag-induced mast cells. Tamarixetin also effectively inhibited the generation of 5-lipoxygenase (LO)-dependent LTC4 and COX-2-dependent PGD2 via the regulation of p38 as well as NF-κB pathways.

**Materials and methods**

**Plant material**

Tamarixetin (Figure 1) was isolated from the ethyl acetate extracts of the flowers of *Inula japonica* as previously described.7 Tamarixetin was dissolved in 0.1% dimethyl sulfoxide (DMSO) for all studies.

**Reagents**

Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS), and penicillin/streptomycin were acquired from Hyclone (Logan, UT, USA). Pokeweed mitogen, mouse anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), and specific inhibitors for MAPKs (SB203580, PD98059, and SP600125) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The enzyme immunoassay kits (EIA; LTC4 and PGD2) and COX-2 antibodies were received from Cayman Chemicals (Ann Arbor, MI, USA). The primary rabbit polyclonal antibodies specific for phospho-serine/threonine-protein kinase (ERK)1/2, ERK1/2, phospho-p38, p38, phospho- c-Jun N-terminal kinase (JNK), JNK, phospho-PLCγ1, phosphoprotein kinase B (Akt), Akt, phospho-IkB kinase (IKK)α/β, phospho-IκBα, IκBα, and β-actin were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibody against phospho-cPLA2, lamin B, and Bay 61-3606 (a Syk inhibitor) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The goat anti-rabbit secondary antibody was purchased from Cell Signaling.

**Cell culture and viability**

Mouse bone marrow-derived mast cells (BMMCs) of Balb/c mice (Koatek, Seoul, Korea) were isolated and cultured for up to 10 weeks in RPMI-1640 containing 10% FBS, penicillin/streptomycin (100 U/mL/0.1%), and 20% pokeweed mitogen-spleen cell-conditioned medium as a source of interleukin-3 as described previously.13 The study protocol was approved by the animal care committee of the National Development Institute of Korean Medicine, Korea. A colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTT) assay (Promega, Madison, WI, USA) was performed to assess the cytotoxicity of tamarixetin on BMMCs as described previously.14

**Quantification of β-hexosaminidase (hex) and eicosanoid generation**

After pretreatment with or without tamarixetin for 1 h and stimulation with DNP-HSA (Ag, 100 ng/mL) for 15 min, the release of β-hex was quantified as described previously.13 To measure LTC4 generation, BMMCs (1×106 cells/mL) were sensitized with anti-DNP IgE (500 ng/mL) and pretreated with different tamarixetin concentrations for 1 h. Later, they were stimulated with DNP-HSA for 15 min and centrifuged at 120 g for 5 min at 4°C. The culture supernatants were collected and the LTC4 concentration was determined using the EIA kit (Cayman Chemical) following the manufacturer’s protocols.

To quantify COX-2-dependent PGD2 production, BMMCs (1×106 cells/mL) were preincubated with aspirin (0.1 µg/mL) for 2 h and washed to denature the preexisting COX-1. Later they were pretreated with tamarixetin for 1 h and were incubated with Ag for 7 h at 37°C. Finally, the supernatants were quantified using the PGD2 EIA kit (Cayman Chemical).
The suppressive effect of tamarixetin

Measurement of intracellular Ca$^{2+}$ level

BMMCs sensitized with anti-DNP IgE were preincubated with FluoForte™ dye-loading solution for 1 h at room temperature and were washed with Hank’s balanced salt solution (HBSS). These sensitized cells (5×10$^4$ cells) were seeded and pretreated with tamarixetin for 1 h before stimulation with DNP-HSA for 5 min. The supernatant was used to conduct the Ca$^{2+}$ assay according to manufacturer’s instructions (FluoForte Calcium Assay Kit, Enzo Life Sciences, Ann Arbor, MI, USA).

Western blot analysis

To whole-cell protein lysates were obtained using the radioimmunoprecipitation assay buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktail. The nuclear and cytoplasmatic fractions were prepared using the NE-PER protein extraction kit (Pierce) according to the manufacturer’s instructions. of the nuclear and cytoplasmatic proteins (20-30 μg) were run on 10% sodium dodecyl sulfate- polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine (Schleicher and Schull, Dassel, Germany). The membranes were initially blocked by adding TTBS buffer (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% non-fat milk for 1 h and were incubated with a variety of primary antibodies overnight. Later they were re-washed with TTBS buffer and were re-incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h. Specific protein bands were visualized using an enhanced chemiluminescence system (Pierce). The band densities were measured with the ImageQuant LAS 4000 luminescent image analyzer and quantified using the ImageQuant software system (GE Healthcare, Little Chalfont, UK).

Statistical analysis

All data are presented as mean ± S.E.M. One-way analysis of variance by Dunc’s multiple range tests was utilized to determine statistical differences and a $p < 0.05$ was considered statistically significant.

Results

Tamarixetin decreases degranulation and intracellular Ca$^{2+}$ through inhibition of PLCγ1 phosphorylation

We performed an MTS assay to determine the cytotoxicity of tamarixetin in BMMCs and found that tamarixetin was not toxic at a concentration of up to 6.3 μM (Figure 2A). Thus, concentrations lesser than 6.3 μM were used for subsequent experiments. To examine the inhibitory effect of tamarixetin on degranulation, IgE-sensitized BMMCs were

**Figure 2** Effect of tamarixetin on (A) Degranulation. IgE-sensitized BMMCs were pretreated with tamarixetin for 1 h and stimulated with DNP-HSA (Ag) for 15 min. The amount of released β-hex into the supernatant was quantified, and cell lysates were used to assess for PLCγ1 phosphorylation. (B) Ca$^{2+}$ mobilization. IgE-sensitized BMMCs were preincubated with Fluoro\textsuperscript{Tm} Dye-Loading for 1 h and washed with HBSS. BMMCs were pretreated with tamarixetin for 1 h before stimulation with DNP-HSA. Intracellular Ca$^{2+}$ levels were measured with a multilabel plate reader at an excitation of 485 nm and emission of 535 nm, and (C) PLCγ1 phosphorylation. Data are expressed as the means ± S.E.M. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were compared to DNP-HSA stimulated-BMMCs.
pretreated with various concentrations of tamarixetin for 1 h followed by 15 min of Ag stimulation, and the supernatants were quantified to determine β-hex release. An increase in the β-hex release was recorded, but pretreatment with tamarixetin dose-dependently suppressed β-hex release (Figure 2B).

Next, the effect of tamarixetin on the intracellular Ca²⁺ levels were analyzed. The activation of BMMCs induced an increase of intracellular Ca²⁺ levels, a reduction with tamarixetin treatment was noted (Figure 2C). Finally, the effect of tamarixetin on PLCγ reduction of PLCγ-1 phosphorylation was noted when IgE-sensitized BMMCs underwent Ag treatment (Figure 2D). The outcomes showed an increase when IgE-sensitized BMMCs only were treated with tamarixetin. A statistically significant increase in the β-hex release was recorded, but pretreatment with tamarixetin for 1 h and stimulated with DNP-HSA for 30 min. Total lysates were subjected to Western blot analysis to evaluate the phosphorylation of cPLA2, p-p38, p-JNK, and p-ERK MAPKs. Data are expressed as the means ± S.E.M. * p < 0.05, ** p < 0.01, and *** p < 0.001 were compared to DNP-HSA stimulated-BMMCs.

**Tamarixetin inhibits LTC₄ generation by decreasing cPLA₂ phosphorylation**

cPLA₂ is a critical enzyme for AA release and LT synthesis. To determine the effect of tamarixetin on the IgE-induced LTC₄ generation, the LTC₄ concentration was measured with or without tamarixetin pretreatment. The Ag stimulation significantly increased the LTC₄ levels, but tamarixetin consistently reduced LTC₄ concentrations in a dose-dependent manner (Figure 3A). Besides, we examined the effect of tamarixetin on cPLA₂ phosphorylation and observed similar outcomes to that of LTC₄ (Figure 3B), suggesting that tamarixetin decreased LTC₄ generation by inhibiting cPLA₂ phosphorylation.

![Figure 3](image-url)

(A) Effect of tamarixetin on LTC₄ and the phosphorylation of cPLA₂ and MAPKs. IgE-sensitized BMMCs were pretreated with the tamarixetin for 1 h followed by stimulation with DNP-HSA for 15 min. (B) LTC₄ generation in the supernatant was determined using an EIA kit. Cell lysates were used for Western blot analysis to evaluate the phosphorylation of cPLA₂. (C) BMMCs were pretreated with tamarixetin for 1 h and stimulated with DNP-HSA for 30 min. Total lysates were subjected to Western blot analysis for phosphorylation of p38, JNK, and ERK MAPKs. Data are expressed as the means ± S.E.M. * p < 0.05, ** p < 0.01, and *** p < 0.001 were compared to DNP-HSA stimulated-BMMCs.
To study the involvement of MAPKs in cPLA$_2$ phosphorylation, we further investigated the effect of tamarixetin on phosphorylation of MAPKs in IgE-sensitized BMMCs. Ag stimulation in IgE-sensitized BMMCs induced the phosphorylation of all three MAPKs without disrupting the total protein and tamarixetin attenuated p38 phosphorylation (Figure 3C). However, tamarixetin did not affect the phosphorylation of JNK and ERK.

**Tamarixetin suppresses COX-2 dependent PGD$_2$ production by decreasing Akt phosphorylation or Akt-mediated signal pathway**

PGD$_2$ is a key prostanoid synthesized from AA via the catalytic activities of COX and PGD$_2$ in mast cells. To assess COX-2-dependent PGD$_2$ generation, BMMCs were pretreated with aspirin to remove the preexisting COX-1 activity. Tamarixetin dose-dependently suppressed the PGD$_2$ generation with a reduced COX-2 expression at the concentration of 6.3 μM (Figure 4A,B). Since NF-κB, an essential transcription factor for COX-2 expression lies downstream of the Akt pathway. We examined the effect of tamarixetin on the NF-κB signal pathway in IgE/Ag-stimulated BMMCs. As shown in Figure 4C, tamarixetin inhibited the phosphorylation of Akt, IKK, and IκBα, the degradation of IκBα, and the nuclear translocation of NF-κB, implying that the Akt-mediated signal pathway regulates the decreased generation of COX-2-dependent PGD$_2$.

**Discussion**

Mast cells are effector cells of innate immunity and can serve to amplify adaptive immunity. Activated mast cells degranulate and release preformed mediators such as histamine and AA metabolites that are responsible for various inflammatory diseases. This study demonstrated that
tamarixetin decreased β-hex release dose-dependently in BMMCs (Figure 2B). Since PLCγ-mediated Ca²⁺ mobilization is a prerequisite for mast cell degranulation in activated mast cells, we examined intracellular Ca²⁺ influx and PLCγ phosphorylation with tamarixetin treatment. The results showed that tamarixetin decreased intracellular Ca²⁺ levels and PLCγ phosphorylation activation (Figure 2C,D), suggesting that the inhibitory effect of tamarixetin on mast cell degranulation resulted from the suppression of PLCγ phosphorylation and intracellular Ca²⁺ levels.

LTC₄ is a potent lipid mediator involved in asthma and inflammation. LTC₄ synthesis is orchestrated by translocation to the nuclear envelope with cPLA₂, 5-lipooxygenase, and FLAP endonuclease in response to the concentration of increased intracellular Ca²⁺. cPLA₂ plays a role in mediating AA and eicosanoid production. The present study showed that tamarixetin suppressed LTC₄ generation by inhibiting cPLA₂ phosphorylation (Figure 3A,B). Moreover, since MAPK can synthesize LTC₄ through cPLA₂ phosphorylation, we examined the involvement of MAPK and demonstrated that tamarixetin suppressed the increased phosphorylation of p38 (Figure 3C). Thus, these data indicate that tamarixetin inhibits LTC₄ synthesis by suppressing cPLA₂, p38 activation, and intracellular Ca²⁺ release in BMMCs.

In mast cells, AA is metabolized to PGD₂ through the COX pathway that occurs in a biphasic manner. COX-1 dependent PGD₂ production occurs within a few minutes followed by the delayed phase of the production of PGD₂ that is dependent on de novo-induced COX-2. NF-κB, a transcription factor, regulates several genes, including COX-2 and Akt activates the NF-κB pathway through direct activation of IKK. Our study showed that tamarixetin inhibited the PGD₂ generation and COX-2 expression along with the reduced phosphorylation of Akt, IKK, and IκBα, the degradation of IκBα, and the nuclear translocation of NF-κB (Figure 4), implying that Akt-mediated signal pathway regulates the decreased generation of COX-2-dependent PGD₂. Moreover, since several reports demonstrated that the MAPKs and NF-κB /Akt pathways are critical for COX-2

**Conflicts of interest**

The authors declare that they have no conflict of interest.

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References


