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Inhibitory effect of taxifolin on mast cell activation and mast cell-mediated allergic inflammatory response

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ABSTRACT

The aim of the present study is to investigate the anti-inflammatory and anti-allergic effects of taxifolin on mast cells and mast cell-mediated allergic reaction. We assessed the effect of taxifolin on the activation of bone marrow-derived mast cells (BMMCs) and rat basophilic leukemia (RBL)-2H3 cells induced by immunoglobulin E (IgE)/antigen (Ag), and the activation of human mast cell line (HMC-1) induced by PMA plus A23187. Taxifolin inhibited degranulation, generation of leukotriene C_4 (LTC_4), production of interlukin-6 (IL-6), and expression of cyclooxygenase-2 (COX-2) through blocking intracellular Ca^{2+} mobilization, phosphorylation of phospholipase Cγ (PLCγ) and mitogen-activated protein kinases (MAPKs), translocation of cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase (5-LO), and Akt/IKK/NF-κB pathway, in BMMC cells. Furthermore, taxifolin suppressed phosphorylation of Syk, but without effect on Fyn and Lyn. Taxifolin also inhibited activation of RBL-2H3 and HMC-1 cells via Akt/IKK/NF-κB and MAPKs/$cPLA_2$ signal pathway. Treatment with taxifolin attenuated the mast cell-mediated passive cutaneous anaphylaxis (PCA) reaction. Our results suggest that taxifolin might become a potential drug candidate for the treatment of allergic and inflammatory diseases.

1. Introduction

Mast cells play an important role in immune response when antigen infiltrates human body. The activated mast cells release preformed granule-associated mediators such as histamine and proteases, or newly generated mediators such as eicosanoids, chemokines and inflammatory cytokines, which cause allergic reactions [1]. Mast cells secrete eicosanoids including prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4), and generate cytokine including interleukins (ILs) and tumor necrosis factor-α (TNF-α) etc. [2]. Mast cells are necessary for development of allergic reactions, through the interaction of antigen (Ag) with the immunoglobulin E (IgE) bound to high affinity IgE receptor (FcεRI) on the cell surface. Aggregation of FcεRI results in phosphorylation of the Lyn, Fyn and Syk. These kinases phosphorylate adaptor proteins linkers for activation of T cells 1 (LAT1) and 2 (LAT2), and activate the phosphoinositide 3 kinase (PI3K) pathway. Phosphorylated LAT functions as a scaffold for multimolecular signaling complexes that include cytosolic adaptors such as Gads, Grb2, SLP76, and SHC, guanosine triphosphate (GTP) exchangers including Sos and Vav1 and the signaling enzymes phospholipase Cγ1 (PLCγ1) and PLCγ2 [3,4]. PLCγ catalyzes the hydrolysis of membrane-localized phosphatidylinositol-4,5-bisphosphate (PIP_2) to form soluble inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Binding of IP_3 to IP_3 receptors (IP_3R) in the endoplasmic reticulum (ER) membrane leads to the release of Ca^{2+} from intracellular Ca^{2+} stores [5]. These signals result in mast cell degranulation, synthesis of eicosanoids (LTC_4, PGD_2) as well as gene transcription for cytokine and chemokine production (IL-6, TNFα).

Taxifolin (dihydroquercetin) (Fig. 1A) is a flavonoid isolated from *Inula japonica* Thunb. Taxifolin is known to protect against cardiac hypertrophy and fibrosis [6], enhance osteoblast differentiation and inhibit osteoclast formation [7], and inhibit the growth, migration and invasion of human osteosarcoma cells [8]. We previously reported that taxifolin suppressed generation of LTC_4 and degranulation in c-Kit ligand-induced mast cells [9]. However, the anti-inflammatory mechanism of taxifolin on mast cell activation and mast cell-mediated anaphylaxis remained unknown yet. Therefore, we recently

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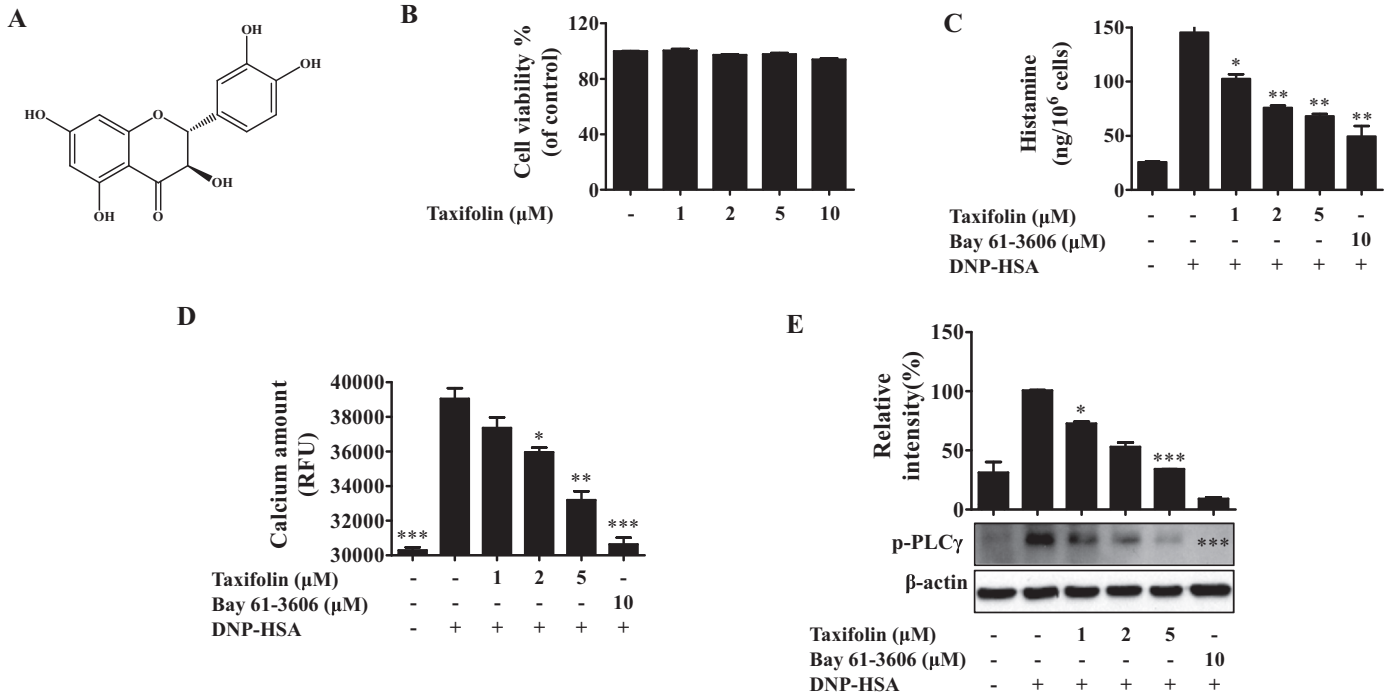

Fig. 1. Taxifolin inhibits IgE/Ag-induced mast cell degranulation. (A) Chemical structure of taxifolin. (B) Effect of taxifolin on BMMCs viability. Cytotoxicity was detected by MTT assay. (C) IgE-sensitized BMMCs were pretreated with taxifolin or bay 61-3606 for 1 h, and then stimulated by DNP-HSA for 15 min. The release of histamine was measured by ELISA. (D) IgE-sensitized BMMCs were pre-incubated with FluoForte TM dye-loading solution for 1 h. The cells were treated with taxifolin or bay 61-3606, stimulated by the DNP-HSA for 5 min and then the fluorescence was measured. (E) IgE-sensitized BMMCs were stimulated with DNP-HSA with or without taxifolin for 15 min. Cell lysates were collected for immunoblot with PLCγ antibody. Bay 61-3606 was used as positive control. The data were obtained from three independent experiments and presented as mean ± SEM. *P < .05, **P < .01, and ***P < .001, compared with the cells with IgE/Ag stimulation but without taxifolin treatment.

investigated the anti-inflammatory effect of taxifolin on IgE/Ag-stimulated mast cells including bone marrow derived mast cells (BMMCs) and rat mast cell line RBL-2H3, phorbol 12-myristate 13-acetate (PMA) plus A23187-stimulated human mast cell line (HMC-1), as well as on mast cell-mediated passive cutaneous anaphylaxis (PCA) mouse models.

2. Materials and methods

2.1. Reagents

RPMI1640, fetal bovine serum (FBS) and the enhanced chemiluminescence (ECL) reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mouse anti-dinitrophenyl (DNP) IgE was purchased from Sigma Chemicals (St. Louis, MO, USA). DNP-human serum albumin (HSA) was from Biosearch Technologies (Petaluma, CA, USA). The LTC_4 enzyme linked immunoassay (EIA) kit, and the antibody for cyclooxygenase-2 (COX-2) were from Cayman Chemical (Ann Arbor, MI, USA). Histamine enzyme-linked immuno sorbent assay (ELISA) kit was purchased from Demeditec Diagnostics GmbH (Kiel, Germany). IL-6 and TNF-α ELISA Kit were obtained from MultiSciences Biotech Co., Ltd. (Hangzhou, Zhejiang, China). The antibodies specific for phospho-PLCγ, phospho-IKKα/β, phospho-IκBα, IκBα, phospho-p38, p38, phospho-JNK, JNK, β-actin and the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies specific for phospho-cytosolic phospholipase A_2 ($cPLA_2$), 5-lipoxygenase (5-LO) , NF-κB p65 and lamin B, Lyn, Fyn and Syk, as well as Bay 61-3606 reagent were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-Phosphotyrosine antibody was obtained from Merck (Darmstadt, Germany).

2.2. Cell Culture

BMMCs were isolated from bone marrow of Balb/c mice and differentiated as described previously [10]. Briefly, bone marrow cells from Balb/c mice were cultured in RPMI1640 medium, supplemented with 10% FBS, 0.1 μM MEM non-essential amino acid solution, 1% penicillin-streptomycin solution, and 20% pokeweed mitogen-stimulated spleen conditioned medium as a source of IL-3. After 3 weeks, > 99% of the cells were found to be BMMCs which were checked with the previously described method [11]. HMC-1 was kindly provided by Dr. J. H. Butterfield (Mayo Clinic, MN, USA), and RBL-2H3 cells were from Cell Bank of Chinese Academy of Sciences (Shanghai, China). HMC-1 and RBL-2H3 cell lines were grown in IMDM and DMEM supplemented with 10% FBS without IL-3 at 37 °C in a 5% CO_2 humidified incubator, respectively.

2.3. Determination of cell viability

Cell viability was determined using MTT assay. The cells were treated with taxifolin for 8 h or 24 h. After addition of MTT to each well, the cells were further incubated. Four hours later, the cell culture supernatant was removed and DMSO was added to dissolve the produced formazan blue. The absorbance at 490 nm was measured with microplate absorbance reader (BIO-RAD iMark, Hercules, CA, USA).

2.4. Measurement of the release of histamine, and the generation of LTC_4, IL-6 and TNF-α

BMMCs were sensitized with 500 ng/ml of anti-DNP IgE overnight, treated with taxifolin for 1 h, followed by stimulation with 100 ng/ml of DNP-HSA (Ag). After incubation for 15 min or 8 h, the levels of histamine, LTC_4 and IL-6 were determined using an ELISA kit according to

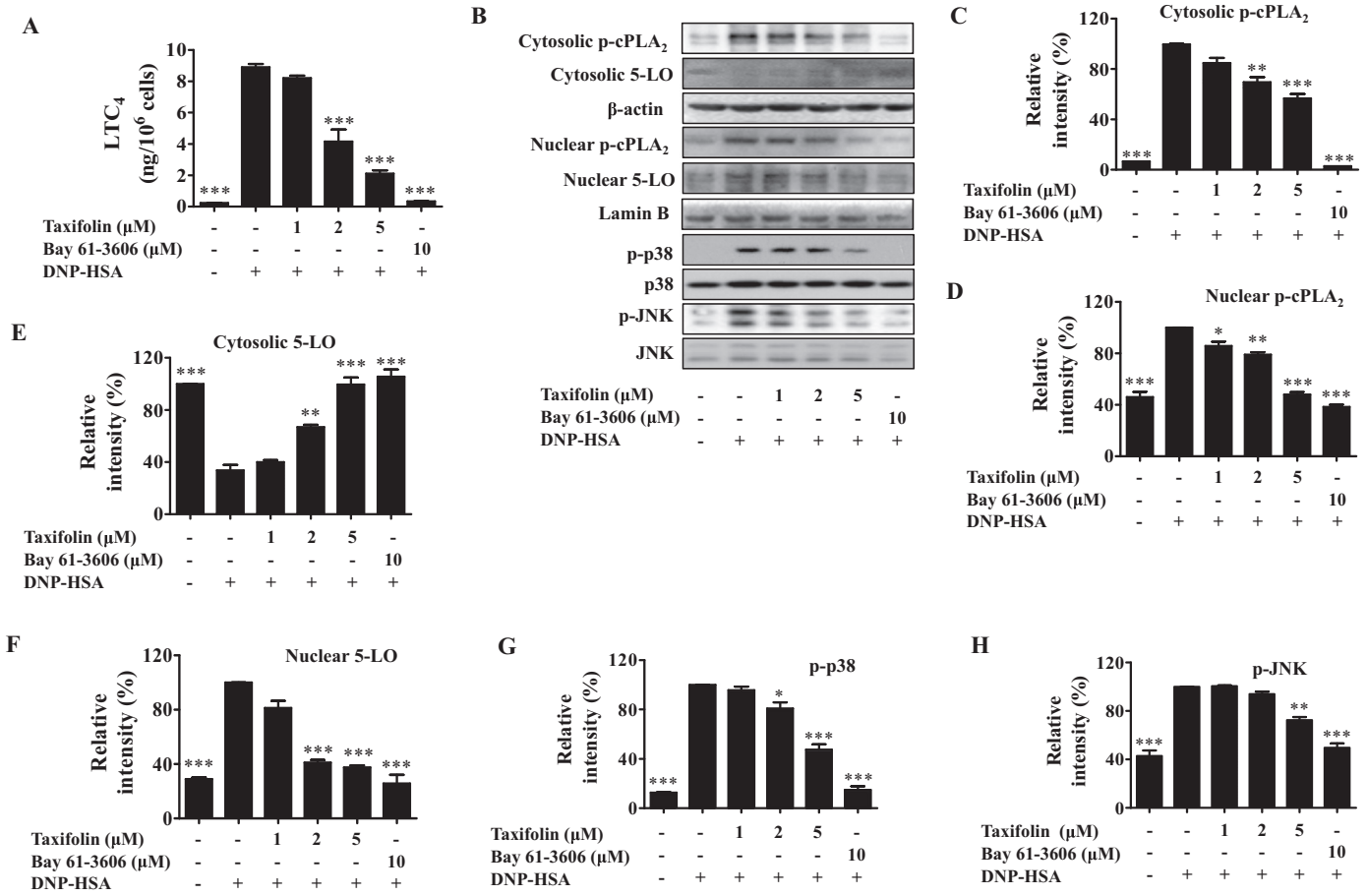

Fig. 2. Inhibition of LTC_4 generation by taxifolin in BMMCs. IgE-sensitized BMMCs were pretreated with taxifolin and then stimulated with the DNP-HSA for 15 min. Cell supernatant and cells were collected. The generation of LTC_4 (A), translocation of p-cPLA$_2$ and 5-LO, and phosphorylation of p38 and JNK (B) were analyzed by ELISA and Western blot, respectively. Densities of bands were measured by densitometry (C-H). The data were obtained from three independent experiments and presented as mean ± SEM. *P < .05, **P < .01, and ***P < .001, compared with the cells with IgE/Ag stimulation but without taxifolin treatment.

the manufacturer's protocol. The HMC-1 cells were pretreated with taxifolin for 1 h, then stimulated with PMA and calcium ionophore A23187 for 8 h. Levels of TNF-α and IL-6 in the culture media were measured using ELISA kit.

2.5. Western blot

Western blot analysis was carried out as we previously reported [12]. Cells were collected and the protein concentration of each sample was determined by the BCA protein assay kit. Equal amount of protein was separated by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the PVDF membrane. After being blocked with 5% skim milk, the membranes were incubated with each primary antibody, and then the horseradish peroxidase-conjugated secondary antibody. The signals were detected with ChemiDoc™ XRS + System (BIO-RAD, Hercules, CA, USA) after exposure to ECL reagent.

2.6. Immunoprecipitation (IP)

Total cell lysates containing equal amounts of protein were immunoprecipitated with anti-Syk, anti-Fyn or anti-Lyn antibody overnight, and the immune-complexes were precipitated with protein A/G plus agarose. Precipitates were subjected to SDS-PAGE and immunoblotted with the respective antibodies.

2.7. Extraction of nuclear protein

The nuclear proteins were prepared using Nuclear Extraction Kit according to the manufacturer's protocol (Panomics, Fremont, CA, USA).

2.8. Measurement of intracellular Ca^{2+} level

Intracellular Ca^{2+} level was determined with the FluoForte Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, MI, USA) as described previously [10]. Briefly, IgE-sensitized BMMCs were pre-incubated with FluoForte TM dye-loading solution for 1 h, treated with taxifolin for 1 h. After stimulation with DNP-HSA for 5 min, the fluorescence was monitored with VICTOR™*X*5 multilabel plate reader at Ex = 485 nm/Em 535 nm (Perkin Elmer, Waltham, MA, USA).

2.9. Passive cutaneous anaphylaxis (PCA)

An 80 ng of mouse anti-DNP IgE (Sigma) was injected subcutaneously into one ear of ICR mouse (7 weeks old male). After 24 h, taxifolin or dexamethasone (Dexa) was orally administered. One hour later, the mice were intravenously injected with 60 μg of DNP-HSA in 200 μl of PBS containing 1% (w/v) Evans blue. The mice were euthanized, and one ear of each mouse was excised and placed in 400 μl of formamide. The Evans blue dye in the ear was extracted at 63 °C overnight, and quantified by determining the absorbance at 630 nm with microplate reader (BIO-RAD iMark, Hercules, CA, USA). On the

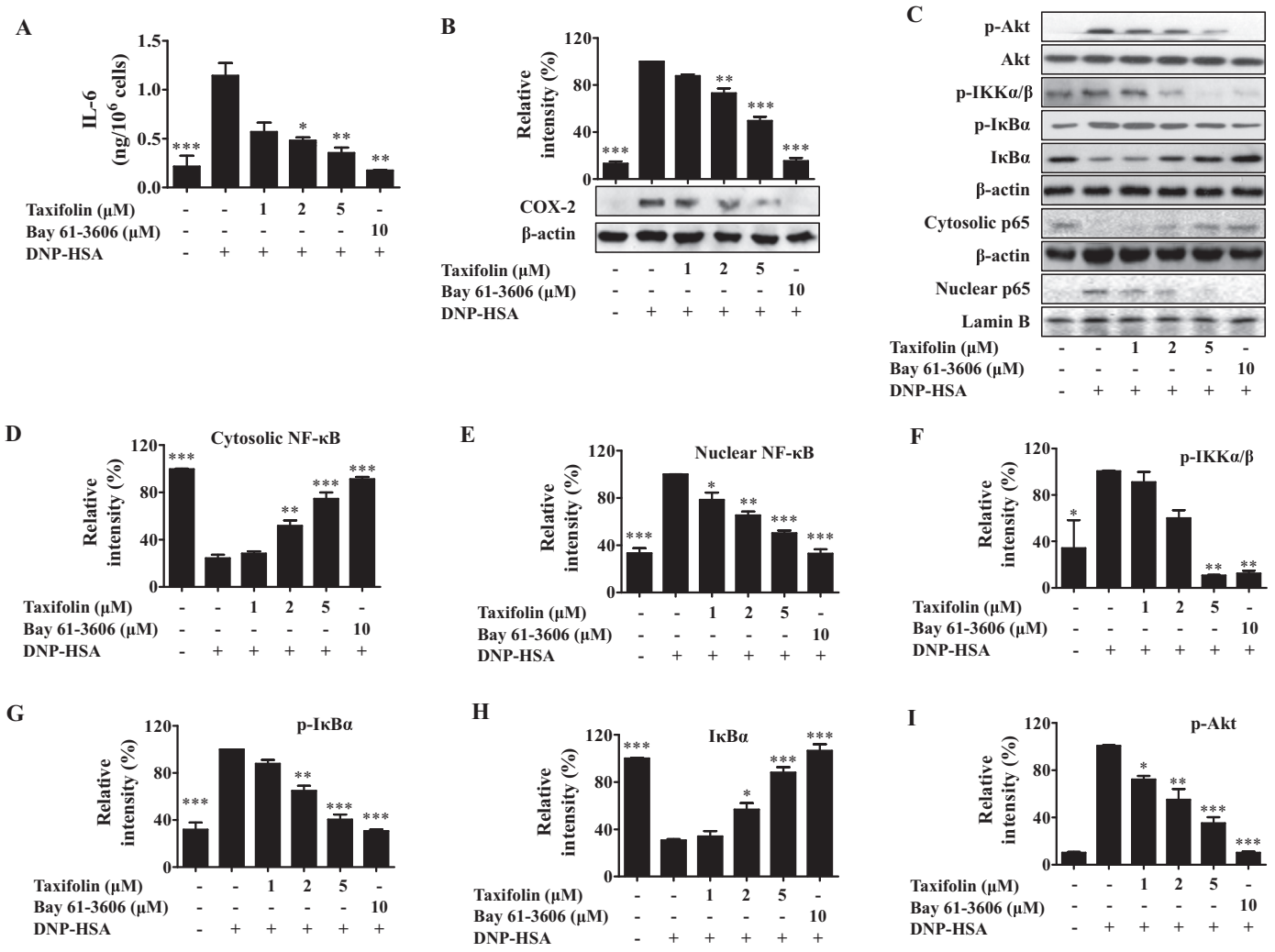

Fig. 3. Taxifolin reduces IL-6 level and COX-2 expression. IgE-sensitized BMMCs were stimulated by DNP-HSA with or without taxifolin for 8 h or 7 h. The secretion of IL-6 (A) and expression of COX-2 (B) were then measured by ELISA and Western blot, respectively. (C) IgE-sensitized BMMCs were stimulated with DNP-HSA for 15 min after pre-incubation with or without taxifolin for 1 h, and the cell lysates were subjected to immunoblot analysis with specific antibodies including p-Akt, p-IKK, p-IκBα, IκBα, and cytosolic or nuclear p65. Densities of bands were measured by densitometry (D-I). The data were obtained from three independent experiments and presented as mean ± SEM. *P < .05, **P < .01, and ***P < .001, compared with the cells with IgE/Ag stimulation but without taxifolin treatment.

other hand, the other ear from the same mouse was fixed with 4% formaldehyde and embedded in paraffin. Four μm sections of the tissues were prepared and stained with toluidine blue to count the number of mast cells. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University.

2.10. Statistical analysis

All values are expressed as means ± SEM of triplicate values. One-way ANOVA followed by Tukey's Multiple Comparison Test was utilized to determine the statistical significance with GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Differences were considered statistically significant when P < .05.

3. Results

3.1. Taxifolin potently suppressed IgE/Ag-induced histamine release in BMMCs

At first, the effect of taxifolin on cell viability of BMMCs was evaluated. As shown in Fig. 1B, taxifolin did not reveal significant cell cytotoxicity at the concentrations < 10 μM (Fig. 1B). Thus, 1, 2 and 5 μM concentrations of taxifolin were used in all subsequent experiments.

Mast cell has been recognized as an important mediator-secreting cell involved in allergic diseases by degranulating histamine, serotonin, etc. [13]. Histamine participates in both early and late-phase allergic responses with multiple effects such as vasodilation and constriction of smooth muscle, which are mediated by specific surface receptors in target cells [14]. Therefore, we investigated the effect of taxifolin on histamine release in IgE/Ag-stimulated BMMCs. IgE-senstized BMMCs were treated with different concentrations of taxifolin for 1 h, and then stimulated with DNP-HSA for 15 min. As shown in Fig. 1C, IgE/Ag treatment potently induced histamine release, and this induction was decreased by taxifolin in a dose-dependent manner. Bay 61–3606, inhibitor of Syk, was used as positive control.

Phosphorylated PLCγ catalyzed the hydrolysis of PIP_2 to yield DAG and IP_3. The generated IP_3 causes liberation of intracellular Ca^{2+} from ER, leading to mast cell degranulation and cytokine production [15]. Therefore, next we investigated the effect of taxifolin on Ca^{2+} mobilization in IgE/Ag-stimulated BMMCs. As shown in Fig. 1D, taxifolin

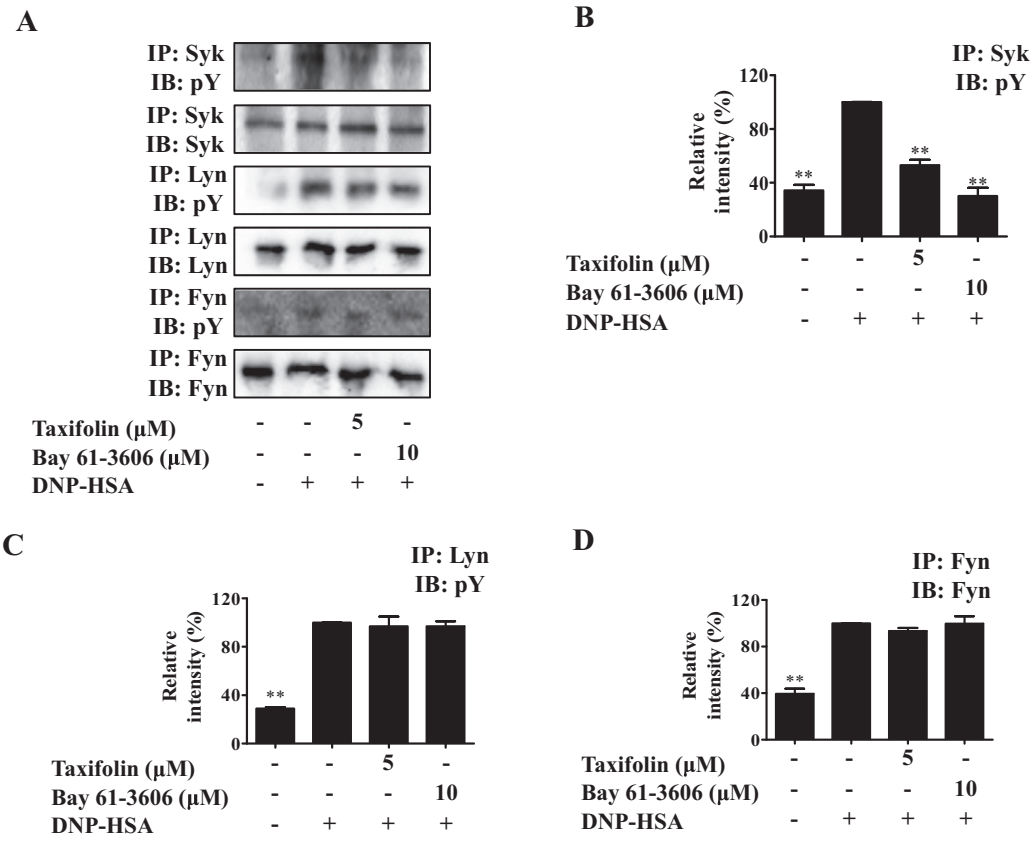

Fig. 4. Taxifolin inhibits the activation of Syk, but not Lyn and Fyn. IgE-sensitized BMMCs were stimulated with DNP-HSA with or without taxifolin for 5 min. The cell lysates were immune-precipitated and immunoblotted for the phosphorylated forms of Syk, Lyn and Fyn (A). Densities of bands were measured by densitometry (B-D). The data were obtained from three independent experiments and presented as mean ± SEM. **P < .01 compared with the cells with IgE/Ag stimulation but without taxifolin treatment.

inhibited IgE/Ag-induced Ca^{2+} mobilization. Consistent with this, we found that PLCγ phosphorylation was inhibited after treatment with taxifolin (Fig. 1E). These results indicate that taxifolin reduced the release of histamine through inhibiting phosphorylation of PLCγ and mobilization of Ca^{2+}.

3.2. Taxifolin down-regulated LTC_4 generation in BMMCs

LTs are known to play pivotal roles in a variety of acute and chronic inflammatory or allergic diseases including asthma, rhinitis and atopic dermatitis [16]. To examine whether taxifolin affects the synthesis of LTC_4, IgE-sensitized BMMCs were treated with various concentrations of taxifolin for 1 h before DNP-HSA stimulation. As shown in Fig. 2A, IgE/Ag stimulation induced LTC_4 generation, which was decreased dose-dependently by taxifolin treatment.

LTs are metabolites of arachidonic acid (AA) which is hydrolyzed by $cPLA_2$ [16]. The $cPLA_2$ is regulated by phosphorylation and calcium-induced translocation to the nuclear envelope [17]. As the key enzyme in the biosynthesis of the LTs, upon cell activation, 5-LO translocates to the nuclear envelope and co-localizes with $cPLA_2$ and 5-lipoxygenase activating protein (FLAP) [18]. Finally, AA is converted to LTs by 5-LO in the presence of FLAP [16]. In order to clarify whether $cPLA_2$ and 5-LO are involved in the mechanism of taxifolin's action, the cytosolic and nuclear level of p-$cPLA_2$ and 5-LO was analyzed by Western blot. As shown in Fig. 2B–F, upon stimulation with IgE and DNP-HSA, phosphorylated $cPLA_2$ in both cytoplasm and nucleus was increased, and 5-LO in cytoplasm decreased while that in nucleus increased, suggesting that both $cPLA_2$ and 5-LO translocated from cytosol to nuclear envelope. After treatment by taxifolin, the translocation of $cPLA_2$ and 5-LO was suppressed. The $cPLA_2$ is known to be phosphorylated following phosphorylation of JNK and p38 MAPKs [17,19]. Therefore, next we evaluated the effect of taxifolin on activation of these MAPKs. As a result, taxifolin inhibited IgE/Ag-induced phosphorylation of p38 and JNK (Fig. 2B, G–H).

These data suggest that taxifolin suppressed LTC_4 production in BMMCs, the mechanism of which might be related to the inactivation of MAPK, $cPLA_2$ and 5-LO.

3.3. Taxifolin inhibited IL-6 generation and COX-2 expression in BMMCs

IL-6 is produced at the site of inflammation and plays an important role in the acute phase immune response as defined by a variety of clinical and biological features such as the synthesis of acute phase proteins [20]. The effect of taxifolin on the IL-6 production was determined by ELISA. Taxifolin showed inhibitory effect on production of IL-6 in concentration-dependent manner in IgE/Ag-stimulated BMMCs (Fig. 3A).

PGs are potent biologically active lipid molecules derived from AA that are generated after upregulation of COX-2 in response to innate stimuli. Therefore COX-2 plays a key role in inflammatory processes [21]. In order to measure the inhibitory activity of taxifolin on COX-2, the IgE-sensitized BMMCs were pre-incubated with aspirin (1 μg/ml) for 2 h to irreversibly inactivate any preexisting COX-1. And then BMMCs were stimulated with DNP-HSA for 7 h in the presence or absence of taxifolin. As shown in Fig. 3B, COX-2 expression in BMMCs was significantly increased by IgE/Ag, but the stimulated expression was blocked dose-dependently by taxifolin.

NF-κB is an important transcription factor mainly involved in inflammatory and immune responses. NF-κB is located in the cell cytoplasm bound to its inhibitory protein IκB, IκB is phosphorylated by IκB kinase (IKK), resulting in its ubiquitination and degradation. Liberated NF-κB enters the nucleus, and then binds the B site of DNA to promote the transcription of the target genes [22]. The NF-κB signal is implicated in the expression of IL-6 and COX-2 [23]. Therefore, next we investigated the effect of taxifolin on IKK/NF-κB signal pathway. As shown in Fig. 3C–E, stimulation with IgE and DNP-HSA caused nuclear

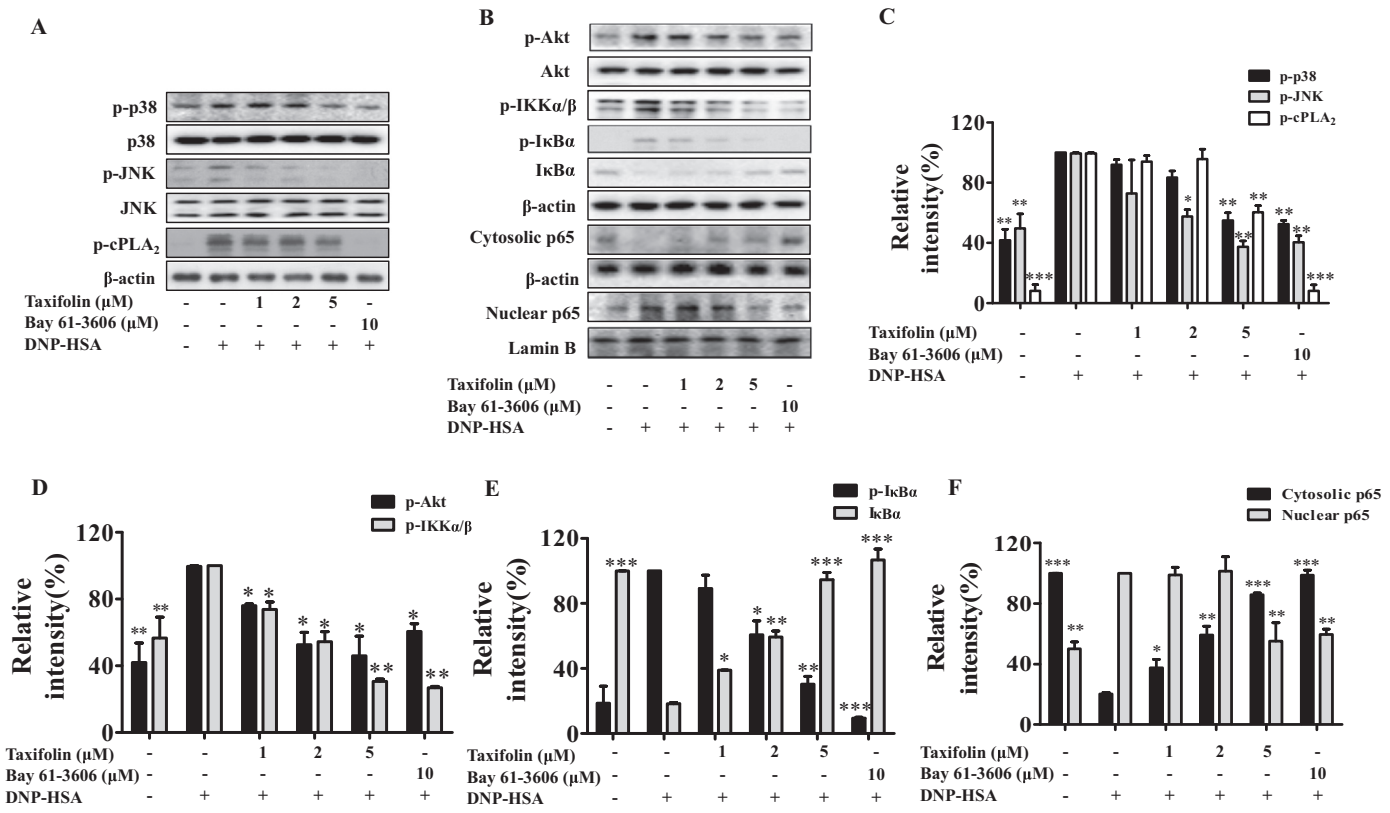

Fig. 5. Taxifolin down-regulated MAPK/cPLA$_2$ and Akt/NFκB pathway in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were pretreated with or without taxifolin, then incubated with DNP-HSA for 15 min. The cell lysates were collected and the p-p38, p-JNK, p-ERK, cPLA$_2$ (A), and p-Akt, p-IKKα/β, p-IκBα, IκBα, and cytosolic or nuclear p65 (B) were assayed by Western blot. (C–F) Relative amounts of the respective proteins were determined by analyzing immunoblot band intensities. The data were obtained from three independent experiments and presented as mean ± SEM. *P < .05, **P < .01, and ***P < .001, compared with the cells with IgE/Ag stimulation but without taxifolin treatment.

translocation of the p65 subunit of NF-κB, which was weakened after treatment with taxifolin. Taxifolin also inhibited the phosphorylation of IKKα/β, phosphorylation and degradation of IκBα (Fig. 3C, F–H). Since Akt pathway is known to activate IKK complex, we investigated the effect of taxifolin on Akt. As indicated in Fig. 3C and I, Taxifolin treatment markedly decreased the phosphorylation of Akt. These data suggest that taxifolin inhibits IL-6 production and COX-2 expression, the mechanism of which might be related to the downregulation of Akt, IKKα/β, IκBα and NF-κB.

3.4. Taxifolin inhibited Syk phosphorylation, but not Lyn and Fyn in BMMCs

Aggregation of the FcεRI results in the activation of the Lyn and Fyn, the recruitment of Syk to the receptor complex and its subsequent activation, as an important step for mast cell activation [24]. The effect of taxifolin on Syk, Lyn and Fyn activation was evaluated using IP assay. Treatment of IgE-sensitized BMMCs with taxifolin decreased Syk phosphorylation (Fig. 4A–B). However, no significant change of the Fyn and Lyn phosphorylation was found (Fig. 4A, C–D).

3.5. Taxifolin inhibited cPLA$_2$ and NF-κB activation in RBL-2H3 cells

We also examined the anti-inflammatory effect of taxifolin by use of RBL-2H3, a rat mast cell line often used for the study of IgE-FcεRI interactions involved in various intracellular signal pathways [25]. No significant cytotoxicity of taxifolin was observed at concentrations of 1, 2, 5 and 10 μM in RBL-2H3 cells (data not shown). Consistent with the result for BMMCs, treatment with taxifolin also inhibited the phosphorylation of p38, JNK and cPLA$_2$ (Fig. 5A, C), and the phosphorylation of Akt, IKKα/β and IκBα, degradation of IκBα, and translocation of NF-κB p65 from cytosol into the nucleus in IgE/Ag-stimulated RBL-2H3 cells (Fig. 5B, D–F).

3.6. Taxifolin decreased IL-6 and TNF-α production in HMC-1 cells

To further study whether taxifolin has inhibitory effect on mast cell activation, another human mast cell line HMC-1 was used. HMC-1 originates from a patient with mastocytosis, and does not express FcεRI [26]. Therefore, many researchers utilize phorbol esters like PMA and calcium ionophore A23187 to activate HMC-1. No significant cytotoxicity was found for taxifolin at concentrations of 1, 2, 5 and 10 μM in HMC-1 (data not shown). As shown in Fig. 6A and B, the levels of IL-6 and TNF-α were significantly elevated by PMA and A23187 stimulation, and taxifolin inhibited the production of IL-6 and TNF-α. In addition, taxifolin reduced the PMA plus A23187-induced phosphorylation of p38, JNK, cPLA$_2$, Akt, IKKα/β, PLCγ and IκBα, as well as the degradation of IκBα (Fig. 6C–G). Furthermore, taxifolin inhibited the nuclear translocation of NF-κB p65 (Fig. 6D, H).

3.7. Taxifolin attenuated PCA reaction in mast cell-mediated allergic mouse model

Mast cells degranulate allergic mediators such as histamine and various proteases after antigen and IgE aggregation, causing allergic symptoms like anaphylaxis [27]. The effect of taxifolin on allergic response was examined in vivo by use of PCA mouse model. IgE and Ag significantly induced PCA reaction evidenced by augment of dye diffusion and ear thickness, and treatment with taxifolin or Dexa attenuated the dye diffusion and ear thickness (Fig. 7A–D). In addition,

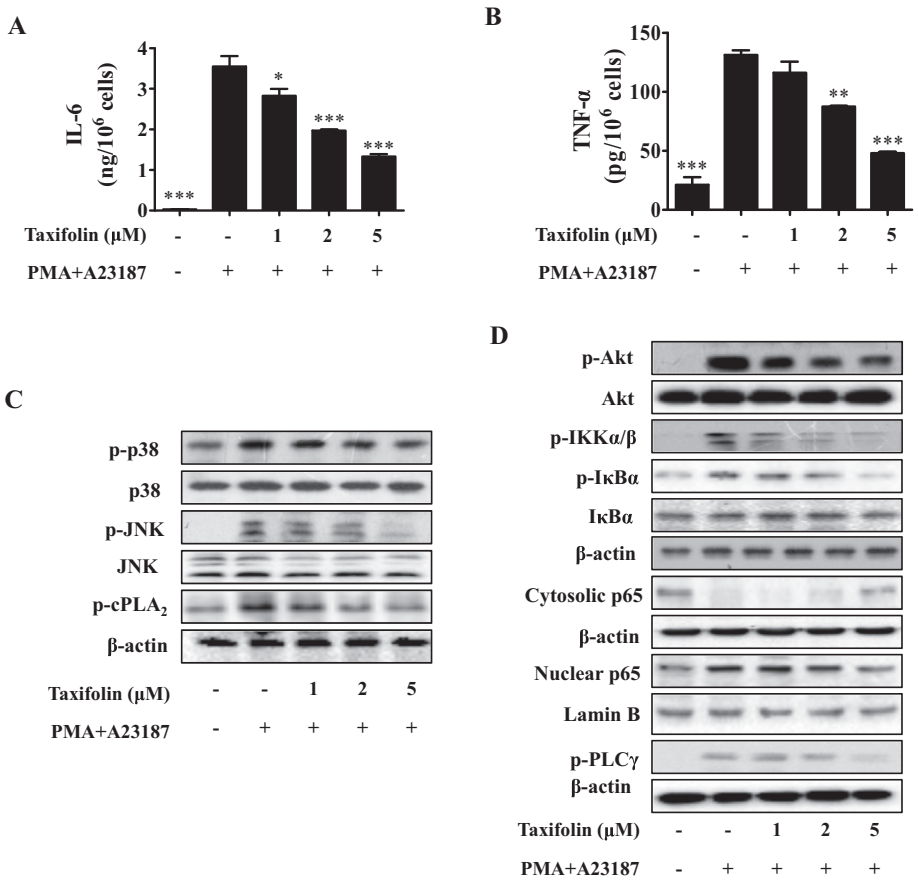

Fig. 6. Taxifolin inhibited the IL-6 and TNF-α levels in HMC-1. HMC-1 cells were treated with taxifolin for 1 h before stimulation with PMA and A23187, the cell supernatants were subjected to ELISA to detect generation of IL-6 (A) and TNF-α (B), and cell lysates were subjected to immunoblot analysis with specific antibodies to detect p-p38, p-JNK1/2, p-Akt, p-IKK, p-IκBα, IκBα, cytosolic or nuclear p65, and p-PLCγ (C–D). (E–H) Relative amounts of the respective proteins were determined by analyzing immunoblot band intensities. The data were obtained from three independent experiments and presented as mean ± SEM. *P < .05, **P < .01, and ***P < .001, compared with the cells with PMA plus A23187 stimulation but without taxifolin treatment.

taxifolin and Dexa also inhibited phosphorylation of IKKα/β (Supplementary Fig. 1). To verify whether the decreased PCA reaction was attributed to the reduction of mast cells, the number of mast cells at the site of ear was counted. As a result, no reduction of the mast cells was found (Fig. 7E). These results suggest that taxifolin attenuated the allergic response through inhibiting the activation of mast cells, without affecting the number of the cells.

4. Discussion

Taxifolin is a flavonoid we isolated from *Inula japonica* Thunb. While various activities of this compound were known, the anti-inflammatory effect as well as the involved mechanism remained rarely reported. In this paper, we first reported the anti-inflammatory effect on IgE/Ag-, and PMA plus A23187-stimulated mast cells, as well as the related mechanism. The in vivo efficacy on mast cell-mediated PCA mouse models was also indicated.

Mast cells are effector cells activated by the IgE binding to FcεRI and subsequent cross-linking with multivalent Ag, with a key role in IgE-mediated immediate hypersensitivity and allergic diseases [5]. Mast cells store large amounts of histamine, which is released by degranulation in response to immunologic and non-immunologic stimuli. Histamine is a potent vasoactive agent, bronchial smooth muscle constrictor, and stimulant of nociceptive itch nerves [14]. In our study, the release of histamine was dramatically enhanced in IgE/Ag-stimulated BMMCs compared with non-stimulated BMMCs, and treatment with taxifolin potently inhibited the release of histamine (Fig. 1C). PLCγ activation leads to the hydrolysis of the PIP_2 thus liberating IP_3 and DAG. These products liberate Ca^{2+} from the ER and activate PKC, respectively [5]. Ca^{2+} is an important intracellular messenger, which is involved in numerous cellular signals of physiologic or pathologic events [28]. Elevated cytosolic Ca^{2+} not only leads to the mast cell degranulation, but also plays a key role in signal for synthesis of cytokines. We found that taxifolin showed significant inhibitory effects on mobilization of Ca^{2+} and phosphorylation of PLCγ (Fig. 1D, E). These data suggest that taxifolin might inhibit PLCγ phosphorylation, Ca^{2+} mobilization, and therefore reduce histamine release in BMMCs.

LTs are important mediators of allergic diseases such as asthma and rhinitis [29]. Taxifolin treatment dose-dependently decreased synthesis of LTC_4 in BMMCs (Fig. 2A). Activated $cPLA_2$ translocates to the cell membrane and then catalyzes the hydrolysis of the esterified form of AA. The free AA is converted to LTs by 5-LO [30]. Therefore, $cPLA_2$ and 5-LO appear to be important for LT production. In this study, we found that taxifolin significantly inhibited IgE/Ag-induced translocation of $cPLA_2$ and 5-LO in BMMCs (Fig. 2B). The $cPLA_2$ is regulated by intracellular Ca^{2+} and MAPKs [17,19]. Taxifolin inhibited FcεRI-mediated p38 and JNK activation (Fig. 2B), but without effect on ERK (Data not shown). Our results revealed that taxifolin could remarkably decrease synthesis of LTC_4, which might be related to the inhibition of $cPLA_2$ and 5-LO, as well as JNK and p38 MAPK, and mobilization of Ca^{2+}. As p38, JNK and ERK all belong to MAPK family, why taxifolin does not affect ERK remains to be elucidated.

IL-6 is a pleiotropic cytokine that mediates various immunomodulatory and inflammatory pathways [31]. IL-6 is not

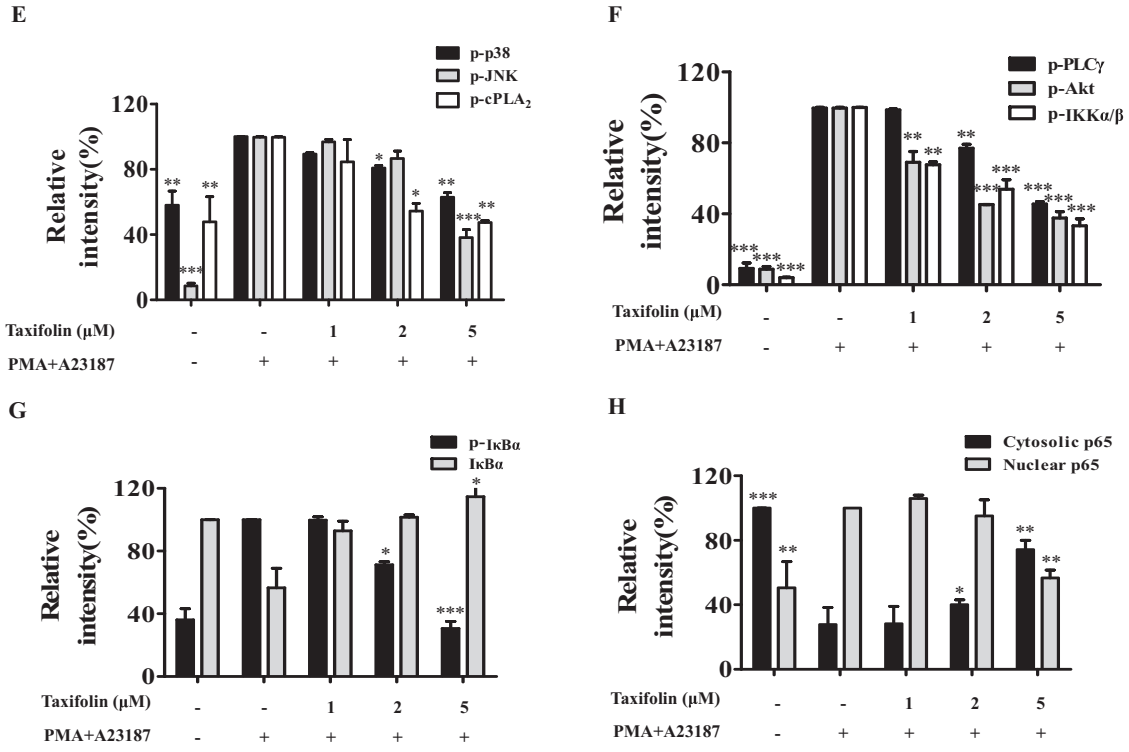

Fig. 6. (*continued*)

expressed in healthy individuals, but rapidly synthesized with infection and inflammation. Abnormal and excessive production of IL-6 has been found responsible for the pathogenesis of numerous autoimmune, chronic inflammatory diseases, even cancers [32]. COX-2, also known as PGs synthase, is an enzyme responsible for the generation of PGs from AA [33]. Our results showed that taxifolin treatment greatly decreased IL-6 and COX-2 expression (Fig. 3A, B). NF-κB is one of the best characterized transcription factors that regulate inflammation, innate and adaptive immune reactions [22]. IKK phosphorylates the inhibitory IκB proteins and therefore leads to their ubiquitination and degradation, releasing the NF-κB to nucleus [34]. The active NF-κB promotes the expression of over 150 target genes including IL-6 and COX-2 [23]. Our findings showed that translocation of p65 NF-κB was suppressed after treatment with taxifolin (Fig. 3C). Akt is known to play important roles in NF-κB activation [35]. We demonstrated that taxifolin suppressed phosphorylation of Akt, IKKα/β and IκBα, as well as the degradation of IκBα (Fig. 3C). These results suggest that taxifolin might reduce production of IL-6 and COX-2 via down-regulating Akt/IKK/IκBα/NF-κB pathway.

Crosslinking of FcεRI by IgE and allergen activates Fyn, Lyn and Syk, and therefore forms multi-molecular signaling complexes with adaptor LAT which serves as a scaffold, finally leads to mast cell degranulation, eicosanoids and cytokine production [36]. Our study revealed that the Syk phosphorylation was inhibited by taxifolin (Fig. 4A). However, treatment with taxifolin did not affect the IgE/Ag-induced phosphorylation of Fyn and Lyn. These findings suggest that Fyn and Lyn might not be involved in the inactivation of BMMCs by taxifolin. Therefore, taxifolin might reduce production of pro-inflammatory mediators including histamine, LTC_4, IL-6 and COX-2 via blocking Syk-dependent pathway. While the direct target of taxifolin remains unclear yet, we postulate that the molecule to which taxifolin binds might be upstream of Syk and downstream of FcεRI in the mast cells.

We also used another cell RBL-2H3, a mast cell line that originates from rat basophilic leukemia, to demonstrate the anti-inflammatory activities of taxifolin. Taxifolin reduced the IgE plus DNP-HSA stimulated activation of p38, JNK, $cPLA_2$, Akt, IKKα/β, IκBα, and NF-κB (Fig. 5A–F).

The HMC-1 is often used in an immediate-allergy experiment because it produces inflammatory cytokines when stimulated with PMA and A23187. In our experiment, PMA plus A23187 enhanced the production of IL-6 and TNF-α in HMC-1, which were abolished by taxifolin (Fig. 6A, B). Taxifolin also attenuated PMA plus A23187-induced phosphorylation of p38, JNK, $cPLA_2$, Akt, IKKα/β, IκBα, PLCγ and nuclear translocation of NF-κB (Fig. 6C–H). These results are consistent with those from BMMCs and RBL-2H3 experiments, suggesting that the inhibition of taxifolin against TNF-α and IL-6 generation depends on attenuation of phosphorylation of PLCγ, and MAPK/$cPLA_2$ and Akt/IKK/IκBα/NF-κB pathway.

Multiple mast cell mediators including histamine, LTC_4 and PGD_2 cause an increase in vascular permeability and induce the constriction of smooth muscle cells [2]. The FcεRI/mast cell axis is potently involved in triggering several intracellular signaling molecules to induce degranulation, production of lipid mediators and various cykokines, leading to the induction of allergy and anaphylaxis [37]. In this study, we verified that taxifolin inhibited the allergic response in PCA reaction. The results showed that treatment with taxifolin decreased the IgE/Ag-elevated exudation of dye (Fig. 7A, B) and ear thickness (Fig. 7C, D), without changing the number of mast cells at ear site (Fig. 7E).

Collectively, our results indicated that taxifolin potently inhibited mast cell activation in vitro in BMMCs, RBL-2H3 and HMC-1 cells, and attenuated mast cell-mediated PCA reaction in vivo, suggesting that taxifolin might become a potential drug candidate for the treatment of allergic and inflammatory diseases.

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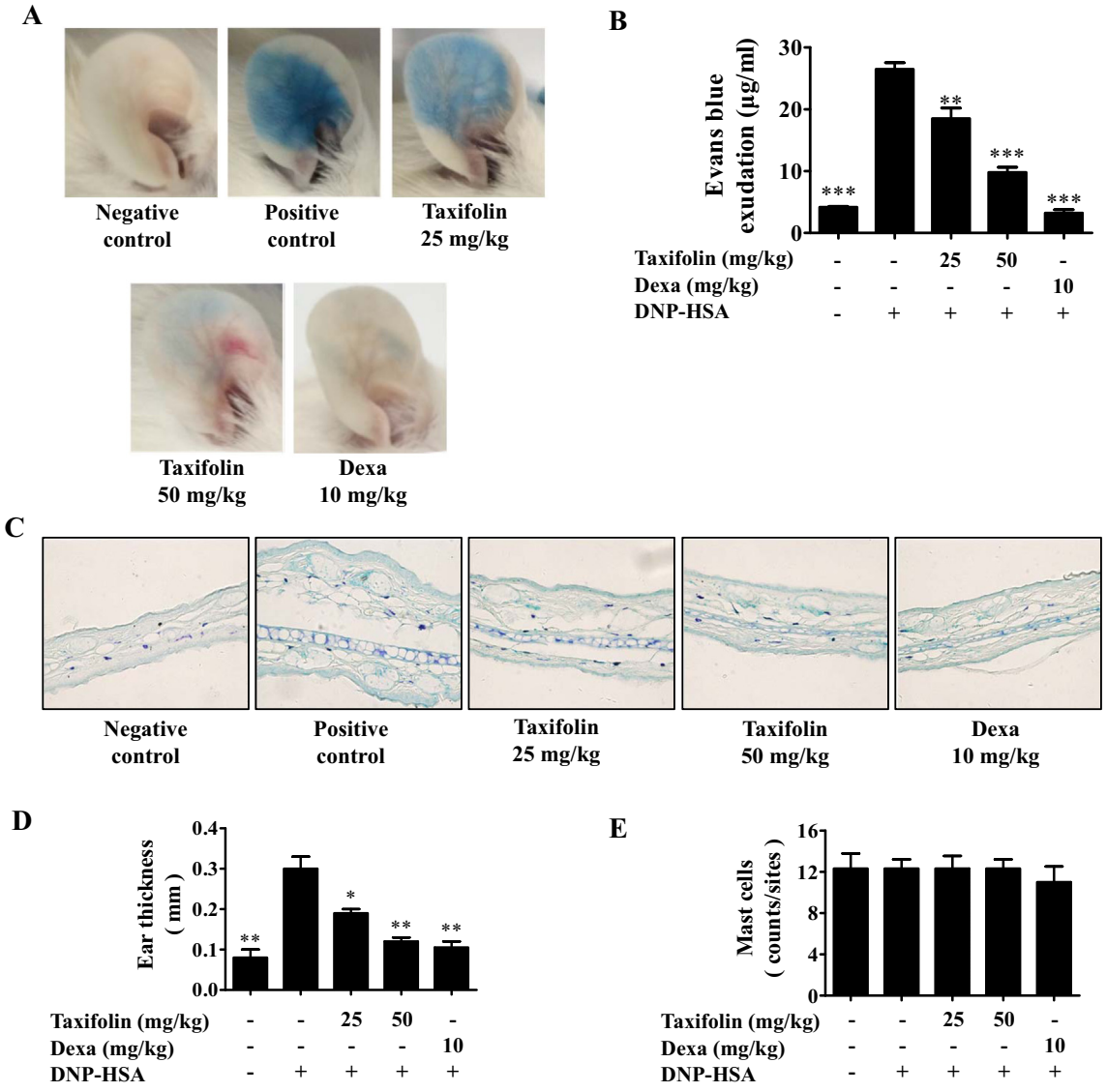

Fig. 7. Taxifolin inhibited IgE-mediated PCA reaction in vivo. The mouse ear was sensitized with an intradermal injection of IgE for 24 h, and taxifolin was orally administered 1 h before the intravenous injection of DNP-HSA. The mouse was euthanized 1 h after the antigen challenge, and the ear was then removed for measurement of amount of the dye extravasated (A–B), ear thickness (C–D) and mast cell amount (E). The data were obtained from three independent experiments and presented as mean ± SEM. *P < .05, **P < .01, and ***P < .001, compared with the mice challenged with IgE and Ag but without taxifolin treatment.

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Author disclosure statement

The authors have declared that no competing interest exists.

Author contributions

M. Jin and D. Kong designed the experiments and acquired funding for the study; S. Pan, X. Zhao, N. Ji, C. Shao and B. Fu performed the experiments; Z. Zhang, R. Wang and Y. Qiu provided technical assistances; S. Pan and M. Jin wrote the manuscript; D. Kong edited the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.03.038.

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