

The anti-anaphylactoid effects of hydroxysafflor yellow A on the suppression of mast cell Ca^{2+} influx and degranulation

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ABSTRACT

Background: Anaphylaxis is a type of potentially fatal hypersensitivity reaction resulting from the activation of mast cell mediators, especially histamine and lipid mediators. Non-IgE-mediated anaphylaxis can occur because of the direct activation of mast cells. Hydroxysafflor yellow A (HSYA) is the main chemical component of safflower (*Carthamus tinctorius*) and has been reported to have pharmacological activities. However, the anti-anaphylactoid effect of HSYA has not yet been investigated.

Purpose: The aims of this study were to evaluate the anti-anaphylactoid activity of HSYA *in vivo* and to investigate the underlying mechanism *in vitro*.

Methods: The anti-anaphylactoid activity of HSYA was evaluated in a mouse model of hindpaw extravasation. Calcium imaging was used to assess intracellular Ca^{2+} mobilization. The levels of cytokines and chemokines released by stimulated mast cells were measured using enzyme immunoassay kits. Western blotting was used to explore the related molecular signaling pathways.

Results: HSYA markedly inhibited mast cell degranulation by suppressing the activation of intracellular Ca^{2+} mobilization and preventing the release of cytokines and chemokines from mast cells in a dose-dependent manner via the PKC-PLC γ -IP3R signaling pathway.

Conclusion: In summary, HSYA has anti-anaphylactoid pharmacological activity, which makes it a potential candidate for the development of a novel agent to suppress drug-induced anaphylactoid reactions.

Introduction

Anaphylaxis is an acute, generalized, potentially life-threatening, systemic hypersensitivity reaction resulting from the sudden degranulation of mast cells and basophils (Sampson et al., 2006; Simons, 2008), which can occur via immunologic or non-immunologic mechanisms (Greenberger and Ditto, 2012; Simons, 2010). Immunologic anaphylaxis can be divided into IgE-dependent and IgE-independent pathways (Williams and Sharma, 2015). Non-immunologic anaphylaxis usually occurs due to direct stimulation of mast cells, leading to degranulation (Lieberman and Garvey, 2016; McNeil et al., 2015; Subramanian et al., 2016), by some drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) (Aun et al., 2014; Jares et al., 2015),

beta-lactam antibiotics (Jares et al., 2015), and others (Montanez et al., 2017). Medications such as quinolones, vancomycin, and opioids have been found to directly stimulate mast cells (Atkinson and Kaliner, 1992; Subramanian et al., 2016). MAS-related G protein-coupled receptor-X2 (MRGPRX2) has been found to mediate this process (Lieberman and Garvey, 2016; McNeil et al., 2015; Subramanian et al., 2016). The activation of mast cell receptors by certain drugs can promote Ca^{2+} mobilization (Holowka et al., 2016) and induce the release of cytokines and chemokines, such as β -hexosaminidase, histamine (HA), tumor necrosis factor (TNF)- α , monocyte chemotactic protein (MCP)-1, and PGD2. All of these mediators play important roles in allergic inflammation and can lead to non-allergic anaphylactic reactions (Galli and Tsai, 2012). Mast cells are well recognized as both effector

Abbreviations: HSYA, Hydroxysafflor yellow A; C48/80, Compound 48/80; HA, Histamine; TNF, Tumor necrosis factor; MCP, Monocyte chemotactic protein; IL, Interleukin; MPMCs, Mouse peritoneal mast cells; DMSO, Dimethyl sulfoxide; LAD2, Laboratory of allergic disease 2; MCDM, Mast cell dissociation media

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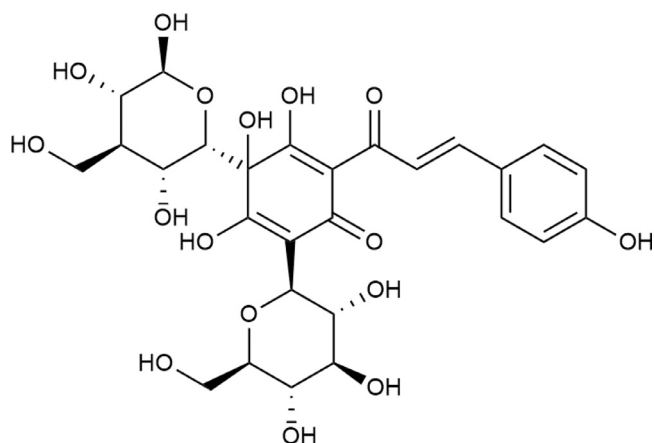


Fig. 1. Chemical structure of hydroxysafflor yellow A (HSYA).

and immunoregulatory cells that play a significant role in allergy-related disease (Metcalfe et al., 1997). It is important to inhibit the activity of mast cells to prevent anaphylaxis.

Hydroxysafflor yellow A (HSYA) (Fig. 1) is a highly water-soluble monomer of *Flos Carthami*, which is a type of traditional Chinese herbal medicine used in the treatment of cardiocerebrovascular diseases (Kelly et al., 1997; Sun et al., 2009). HSYA has been demonstrated to have neuroprotective effects, and it has the potential to be used as a neuroprotective agent for the treatment of focal cerebral ischemia (Zhu et al., 2003). Xinbing et al. demonstrated that HSYA can exert a neuroprotective effect against cerebral ischemia-reperfusion injury in rats (Wei et al., 2005). The inhibitory effect of HSYA on pulmonary inflammation in mice (Sun et al., 2010) and its ability to attenuate lipopolysaccharide-induced inflammatory signal transduction in human alveolar epithelial A549 cells (Song et al., 2013) have also been recently reported. HSYA also exerts beneficial effects on oxygen free radical-scavenging, anti-inflammatory actions and anti-apoptotic mechanisms (Ji et al., 2009; Tian et al., 2008; Wu et al., 2012). In addition, studies have also shown that HSYA can significantly decrease rat mast cell degranulation and reduce the release of inflammatory mediators *in vitro* (Han et al., 2015). However, the anti-anaphylactoid effect and the underlying therapeutic mechanisms of HSYA remain poorly understood.

In recent years, there has been important progress in the discovery of several mast cell-regulated inhibitory mechanisms. It is important to find more effective drugs to inhibit mast cell responses during anaphylactoid reactions. Basic secretagogues can induce mast cell degranulation, of which compound 48/80 (C48/80) is the most potent [15]. C48/80 acts directly on G-proteins rather than on high-affinity IgE receptors to promote mast cell degranulation (McNeil et al., 2015). The anti-anaphylactoid effects of any molecule can be validated by its ability to prevent C48/80-induced degranulation in mast cells. In this study, C48/80 was used to promote degranulation in mast cells and thereby to initiate anaphylactoid reactions. Mouse peritoneal mast cells (MPMCs) and human LAD2 mast cells were used to study the inhibitory effect of HSYA on mast cells via the suppression of intracellular Ca^{2+} mobilization and degranulation. HSYA also inhibited the release of β -hexosaminidase and HA triggered by ciprofloxacin and LL-37, which further confirmed its inhibitory effect on mast cells. Results from Western blotting demonstrated that HSYA inhibits the activation of mast cells via the PKC-PLC γ -IP3R signaling pathway in LAD2 cells. HSYA can suppress a C48/80-induced cutaneous flare in a dose-dependent manner. These findings demonstrate the anti-allergic activity of HSYA and its underlying mechanism, which indicates the potential of HSYA to be used as a novel therapeutic agent for the treatment of drug-induced anaphylactoid reactions.

Materials and methods

Drugs and reagents

C48/80 (a classic mast cell activator and canonical basic secretagogue), dimethyl sulfoxide (DMSO), MTT, Evans blue and p-nitrophenyl N-acetyl- β -D-glucosamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). HSYA was obtained from Chengdu Pufei De Biotech Co., LTD (Chengdu, Sichuan, China) and the purity is $\geq 98\%$ based on HPLC analysis. Saline was purchased from Shandong Qidu Pharmaceutical Co., Ltd. A Human IL-8 ELISA Kit, Human MCP-1 ELISA Kit and Human TNF- α ELISA Kit were purchased from ExCell Biology, Inc. (Shanghai, China). Fluo-3 AM was procured from Thermo Fisher Scientific (Waltham, MA, USA). Pluronic F-127 gel was procured from Biotium (Fremont, CA, USA). The anti-PLC γ 1 (#5690), anti-phosphorylated-PLC γ 1 (P-PLC γ 1, Ser1248) (#8713), anti-IP3R (#3763), anti-phosphorylated-IP3R (P-IP3R, Ser1756) (#8548), anti-phosphorylated PKC (P-PKC, Ser1756) (#9371), anti-P38 (#8690), anti-phosphorylated-P38 (P-P38, Thr180/Tyr182) (#4511), anti-Akt (#4691), anti-phosphorylated-Akt (P-Akt, Ser473) (#4060), anti-Erk1/2 (p44/42 MAPK (Erk1/2), #9102), anti-phosphorylated-Erk1/2 (P-Erk1/2, P-p44/42 MAPK (Erk1/2) [Thr202/Tyr204], #9101) and anti-GAPDH (#2118) were procured from Cell Signaling Technology (Danvers, MA, USA). The anti-PKC (#GR257247-4) were procured from Abcam (Cambridge, MA, USA).

Cell lines

Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). Cells were maintained in StemPro-34 medium supplemented with StemPro nutrient supplement, 100 U penicillin, 100 U streptomycin, 2 mM L-glutamine and 100 ng/ml human stem cell factor in an incubator with 5% CO_2 at 37 °C. The culture medium was replaced every 7 days, and the cells were maintained at a density of 2×10^6 cells/ml.

Purification of mouse peritoneal mast cells (MPMCs)

Two- to five-month-old adult mice were sacrificed through CO_2 inhalation. A total of 12 ml ice-cold mast cell dissociation media (MCDM; HBSS with 3% fetal bovine serum and 10 mM HEPES, pH 7.2) was used to obtain two to three sequential peritoneal lavages, which were then centrifuged at 200 g and 4 °C for 5 min. The cells from each mouse were resuspended in 2 ml MCDM, layered over 4 ml of an isotonic 70% Percoll suspension (2.8 ml Percoll, 320 μ l 10 $\times$ HBSS, 40 μ l 1 M HEPES, and 830 μ l MCDM) and centrifuged at 500 g and 4 °C for 20 min. Mast cells were collected from the pellet, and their purity was confirmed at $>95\%$ by morphological analysis and toluidine blue staining. The mast cells were resuspended at 5×10^5 to 1×10^6 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) with 25 ng/ml recombinant mouse stem cell factor (SCF) and seeded in 96-well plates.

Intracellular Ca^{2+} mobilization assay

LAD2 cells or MPMC were incubated with 0, 50 μ M, 100 μ M or 200 μ M HSYA diluted in calcium imaging buffer (CIB; 125 mM NaCl, 3 mM KCl, 2.5 mM CaCl_2 , 0.6 mM MgCl_2 , 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO_3 , and 20 mM sucrose, adjusted to pH 7.4 with NaOH) for 30 min. The incubation buffer consisted of 4 μ M Fluo-3 AM with 0.1% Pluronic F-127. For imaging, cells were washed twice in CIB. Fluo-3,AM-loaded cells were imaged with excitation at 488 nm. Unless otherwise specified, 30 μ g/ml of C48/80 was slowly added to each well beginning from 10 s, and responses were monitored at 3 s intervals for an additional 120 s.

Cell viability assay

The effects of HSYA on the viability of LAD2 cells were assessed using the MTT assay. Briefly, exponentially growing LAD2 cells were plated at a density of 5×10^4 cells/well in 96-well plates and then cultured for 24 h. The cells were subsequently treated with HSYA at 1 μ M, 10 μ M, 50 μ M, 100 μ M, 200 μ M or 400 μ M for 24 h. Then, the cell proliferation reagent MTT was added to each well, and the cells were incubated for another 4 h at 37 °C in an atmosphere with 5% CO₂. The resulting formazan crystals were dissolved in DMSO (150 μ l/well) with constant agitation for 15 min. Absorbance of the plates was read using a model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm.

β -hexosaminidase release assay

LAD2 cells were seeded at 2×10^4 cells/well in a 96-well plate and incubated overnight. Then, the culture medium was removed, added 100 μ l of 30 μ g/ml C48/80 and 0, 50 μ M, 100 μ M or 200 μ M HSYA diluted in modified Tyrode's solution (120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 5.5 mM glucose, and 5 mM BSA) were added, followed by incubation for 40 min. For IgE-mediated mast cell degranulation, LAD2 cells were incubated with 1 μ g/ml IgE for 12 h, and then remove the medium, added 100 μ l of 100 ng/ml DNP-BSA and 0, 50 μ M, 100 μ M or 200 μ M HSYA incubated for 40 min. The 96-well plate was centrifuged at 200 g for 5 min. To analyze the total β -hexosaminidase content, the cells were lysed with 0.1% Triton X-100 in modified Tyrode's solution. β -Hexosaminidase released into the supernatant and in cell lysates was quantified by the hydrolysis of p-nitrophenyl N-acetyl- β -D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was stopped by the addition of stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11.0), and the absorbance was measured at 405 nm using a microplate spectrophotometer. C48/80 was used as the positive control.

Histamine (HA) release assay

HA was purchased from Sigma-Aldrich. HA•2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific. Mass spectrometry-grade formic acid was obtained from Sigma-Aldrich. For liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used. HA content was evaluated on the system employing a HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 μ m, Agela Technologies, Tianjin, China) and an isocratic elution with mixed solution of acetonitrile-water added with 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min.

Measurements of cytokines

The levels of chemotactic factors in cell supernatants were detected using human chemokine array kits according to the manufacturers' instructions. After cells were incubated with 100 μ l of 30 μ g/ml C48/80 and 0, 50 μ M, 100 μ M or 200 μ M HSYA diluted in modified Tyrode's solution for 6 h, the levels of TNF- α , interleukin (IL)-8 and MCP-1 were measured using commercially available ELISA kits.

Western blot analysis

Protein was extracted from LAD2 cells treated with 30 μ g/ml C48/80 and 0, 50 μ M, 100 μ M and 200 μ M HSYA for 24 h using RIPA lysis buffer containing 10% protease inhibitor and phosphatase inhibitor cocktails (Roche Diagnostics) on ice for 30 min. The protein lysate was

cleared by centrifugation at 13,500 g for 10 min at 4 °C. The concentration of protein was determined using a BCA Protein Quantification kit according to the manufacturer's instructions. The cell lysates were denatured by boiling in 5 $\times$ loading sample buffer (Thermo Fisher Scientific) for 5 min and separated by 10% SDS-PAGE (Shaanxi Pioneer Biotech Co., Ltd.). Following electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Hangzhou Microna Membrane Technology Co., Ltd., Hangzhou, China) and blocked with 5% non-fat milk in Tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd.) for 2 h at room temperature with continuous agitation. The membranes were then incubated with specific primary antibodies, including anti-PLC γ 1 (1:1000), anti-phosphorylated-PLC γ 1 (P-PLC γ 1, Ser1248) (1:1000), anti-IP3R (1:1000), anti-phosphorylated-IP3R (P-IP3R, Ser1756) (1:1000), anti-PKC (1:1000), anti-phosphorylated PKC (P-PKC, Ser1756) (1:500), anti-P38 (1:1000), anti-phosphorylated-P38 (P-P38, Thr180/Tyr182) (1:1000), anti-Akt (1:1000), anti-phosphorylated-Akt (P-Akt, Ser473), (1:1000), anti-Erk1/2 (1:1000), anti-phosphorylated-Erk1/2 (P-Erk1/2, P-p44/42 MAPK (Erk1/2) [Thr202/Tyr204], 1:1000) or anti-GAPDH (1:2000) antibodies overnight at 4 °C, followed by three washes with TBST every 10 min and incubation with secondary antibodies at a dilution of 1:20,000 in TBST for 1 h at 37 °C. The membranes were then washed three times with TBST for 10 min and developed using an ECL kit. A Lane 1DTM transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to capture images of the blots. Image-Pro Plus 5.1 (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

Mouse model

C57BL/6 mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Adult mice aged 6 to 8 weeks were used in all the experiments in this study. All experiments involving equal treatments in animals were conducted by investigators blinded to the conditions.

Ethics statement

The study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for the use of mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045).

Hindpaw swelling and extravasation assay

Adult mice (6 to 8 weeks old) were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Fifteen minutes after the induction of anesthesia, each mouse was intravenously (i.v.) injected with 12.5% Evans blue and 0, 2.5 mg/kg, 5 mg/kg or 10 mg/kg HSYA in saline. Thirty minutes later, a vernier caliper was used to measure the thickness of the paw before the injection of any test substances, and then 5 μ l of 10 μ g/ml C48/80 was injected in one paw in each mouse, and saline was injected in the other paw as a negative control. Fifteen minutes later, paw thickness was measured again and recorded. Mice were then sacrificed by decapitation, and the paw tissues were collected and dried at 50 °C and individually weighed. Next, Evans blue dye was extracted from the tissues by adding 500 μ l mixture of acetone-saline (7:3) and incubating at 37 °C for 12 h. The tissues were then cut into pieces, dissociated for 10 min in an ultrasonic machine, and centrifuged at 3000 rpm for 20 min. The supernatant was equally distributed in 200- μ l aliquots into 96-well plates, and the optical density (OD) value was read at 620 nm using a spectrophotometer.

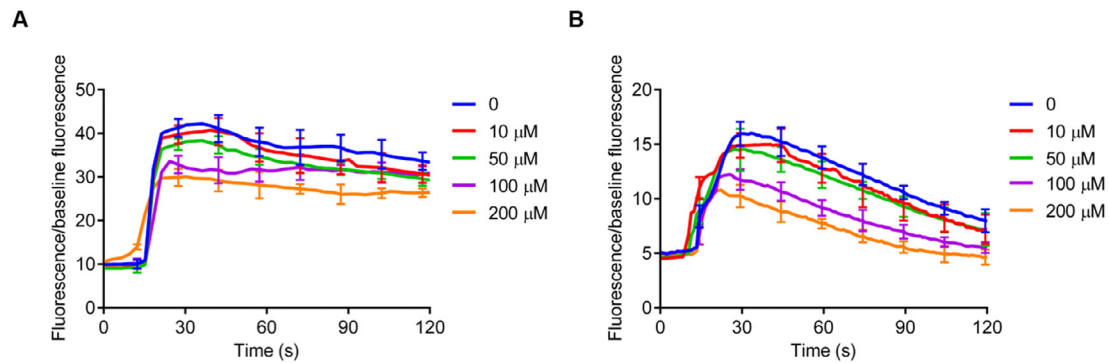


Fig. 2. Calcium imaging in MPMCs and LAD2 cells treated with HSYA and C48/80. (A) Representative Ca^{2+} imaging traces in MPMCs in response to 0, 50, 100 or 200 μM HSYA and 30 $\mu\text{g/ml}$ C48/80. (B) Representative Ca^{2+} imaging traces in LAD2 cells in response to 0, 50, 100 or 200 μM HSYA and 30 $\mu\text{g/ml}$ C48/80. ($n = 3$ per concentration; 50 cells were counted per condition).

Statistical analysis

Group data are expressed as the means $\pm$ SD. Independent sample variance analysis was used to determine significance in statistical comparisons using SPSS. Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Results

HSYA attenuated C48/80-triggered calcium flux in MPMCs and LAD2 cells

Ca^{2+} influx has a prominent influence on mast cell degranulation and HA and $\text{TNF-}\alpha$ release. C48/80 can mobilize calcium stores in mast cells. MPMCs were treated with HSYA and incubated with Fluo-3 AM for 30 min. The cells were imaged before and after C48/80 stimulation to test whether HSYA affects Ca^{2+} flux. The fluorescence intensity of the cells was analyzed. HSYA induced a dose-dependent decrease in the C48/80-triggered calcium flux in MPMCs (Fig. 2A). Meanwhile, HSYA induced a reduction in C48/80-triggered calcium flux in LAD2 human mast cells in a dose-dependent manner (Fig. 2B). Therefore, HSYA can reduce mast cell activity.

HSYA decreased C48/80-triggered degranulation in LAD2 cells

To evaluate the anti-anaphylactoid activity of HSYA, the human mast cell line LAD2 were used for subsequent experiments. The effect of HSYA on the viability of LAD2 cells was assessed using the MTT assay to determine the toxicity of HSYA. Indeed, HSYA did not significantly affect cell viability even at 400 μM (Fig. 3A). Therefore, the concentrations of HSYA below 400 μM were used for subsequent experiments. The activation of mast cells can lead to the rapid secretion of preformed inflammatory mediators, such as β -hexosaminidase, HA, and synthesized cytokines, including $\text{TNF-}\alpha$, MCP-1 and IL-8, which are critical for allergic inflammation (Theoharides and Kalogeromitros, 2006). To verify whether HSYA can suppress the C48/80-induced activation of mast cells by promoting degranulation, LAD2 cells were used as a model to detect changes in the secretion of β -hexosaminidase, HA and $\text{TNF-}\alpha$, the latter two of which are mediators of increased vascular permeability. LAD2 cells were simultaneously treated with different concentrations of HSYA (0, 10, 50, 100 or 200 μM) and 30 $\mu\text{g/ml}$ of C48/80 for 40 min, after which the levels of secreted β -hexosaminidase, Histamine and $\text{TNF-}\alpha$ were measured. 0, 10 μM , 50 μM , 100 μM and 200 μM of HSYA inhibited the C48/80 induced secretion of β -hexosaminidase as $72.46\% \pm 0.0292$, $65.93\% \pm 0.0176$, $58.69\% \pm 0.0226$, $53.85\% \pm 0.0417$ and $48.76\% \pm 0.0561$ (Fig. 3B), histamine as 509.15 ± 31.41 ng/ml, 455.58 ± 20.51 ng/ml, 445.97 ± 18.57 ng/ml, 386.64 ± 26.59 ng/ml

and 323.27 ± 46.33 ng/ml (Fig. 3C), and of $\text{TNF-}\alpha$ as 192.31 ± 18.40 pg/ml, 191.52 ± 11.50 pg/ml, 172.29 ± 10.20 pg/ml, 142.19 ± 15.39 pg/ml and 112.39 ± 9.67 pg/ml (Fig. 3D), respectively. Additionally, LAD2 cells were treated with different concentrations of HSYA (0, 50, 100 or 200 μM) and 30 $\mu\text{g/ml}$ of C48/80 for 6 h, after which the levels of MCP-1 and IL-8 were measured. 0, 10 μM , 50 μM , 100 μM and 200 μM of HSYA inhibited the C48/80 induced secretion of MCP-1 as 1122.62 ± 26.27 pg/ml, 960.65 ± 55.24 pg/ml, 806.62 ± 55.04 pg/ml, 674.98 ± 75.25 pg/ml and 475.04 ± 76.10 pg/ml (Fig. 3E) and of IL-8 as 82.52 ± 7.89 pg/ml, 81.35 ± 4.94 pg/ml, 82.18 ± 3.89 pg/ml, 61.01 ± 6.60 pg/ml and 48.22 ± 4.15 pg/ml (Fig. 3F), respectively. Therefore, HSYA attenuated the release of β -hexosaminidase, HA, $\text{TNF-}\alpha$, MCP-1 and IL-8 in LAD2 cells treated with C48/80 in a dose-dependent manner, indicating that HSYA can inhibit C48/80-induced mast cell degranulation.

HSYA attenuated LAD2 mast cell degranulation triggered by endogenous and exogenous substances

It has been reported that some small-molecule therapeutic drugs, such as tetrahydroisoquinoline (THIQ), and some peptidergic drugs, such as ciprofloxacin (McNeil et al., 2015) and LL-37 (Elenius et al., 2017), can induce pseudo-allergic drug reactions. Thus, we detected β -hexosaminidase release from LAD2 cells triggered by ciprofloxacin or LL-37. LAD2 cells were simultaneously treated with different concentrations of HSYA (0, 50, 100 or 200 μM) and 200 $\mu\text{g/ml}$ of ciprofloxacin or 30 $\mu\text{g/ml}$ of LL-37 for 40 min. 0, 10 μM , 50 μM , 100 μM and 200 μM of HSYA inhibited the ciprofloxacin induced secretion of β -hexosaminidase as $72.45\% \pm 0.0881$, $67.29\% \pm 0.0911$, $62.46\% \pm 0.0680$, $57.10\% \pm 0.0534$ and $54.30\% \pm 0.0508$ (Fig. 4A), inhibited the LL-37 induced secretion of β -hexosaminidase as $72.44\% \pm 0.0816$, $64.95\% \pm 0.0537$, $57.70\% \pm 0.0537$, $48.73\% \pm 0.0122$ and $43.35\% \pm 0.0092$ (Fig. 4B), inhibited the IgE induced secretion of β -hexosaminidase as $72.47\% \pm 0.0352$, $67.50\% \pm 0.0443$, $64.96\% \pm 0.0410$, $57.89\% \pm 0.0650$ and $50.79\% \pm 0.0570$ (Fig. 4C). 0, 10 μM , 50 μM , 100 μM and 200 μM of HSYA inhibited the ciprofloxacin induced secretion of Histamine as 466.38 ± 29.63 ng/ml, 457.57 ± 16.35 ng/ml, 447.96 ± 16.35 ng/ml, 406.81 ± 19.01 ng/ml and 347.37 ± 16.65 ng/ml (Fig. 4D), inhibited the LL-37 induced secretion of Histamine as 467.31 ± 51.57 ng/ml, 394.75 ± 35.73 ng/ml, 375.52 ± 37.62 ng/ml, 354.35 ± 19.99 ng/ml and 321.63 ± 30.09 ng/ml (Fig. 4E), inhibited the IgE induced secretion of Histamine as 489.31 ± 42.97 ng/ml, 498.92 ± 17.06 ng/ml, 486.10 ± 12.37 ng/ml, 461.64 ± 22.41 ng/ml and 404.15 ± 17.64 ng/ml (Fig. 4F).

Therefore, the secretion of β -hexosaminidase and Histamine from LAD2 cells triggered by ciprofloxacin, LL-37 or IgE decreased in a dose-dependent manner in response to HSYA treatment, indicating that

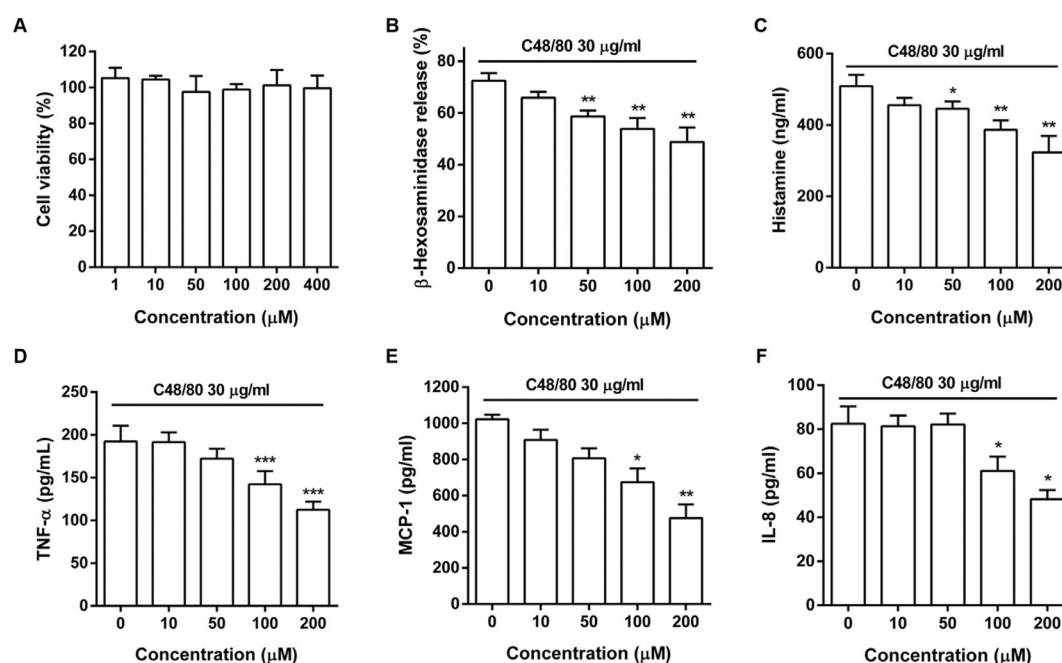


Fig. 3. C48/80 induced mast cells degranulation while treated with HSYA. (A) Viability of LAD2 cells treated with HSYA for 24 h. (B, C) Release of β -hexosaminidase (B) and Histamine (C) from LAD2 cells treated with 0, 10, 50, 100 or 200 HSYA and 30 μ g/ml C48/80 for 30 min. (D, E, F) Release of TNF- α (D), MCP-1 (E) and IL-8 (F) from LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA and 30 μ g/ml C48/80 for 6 h. Data are presented as the means $\pm$ SD ($n = 3$). Two-tailed unpaired Student's t -tests were used to determine significance in statistical comparisons, and statistical significance was defined at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

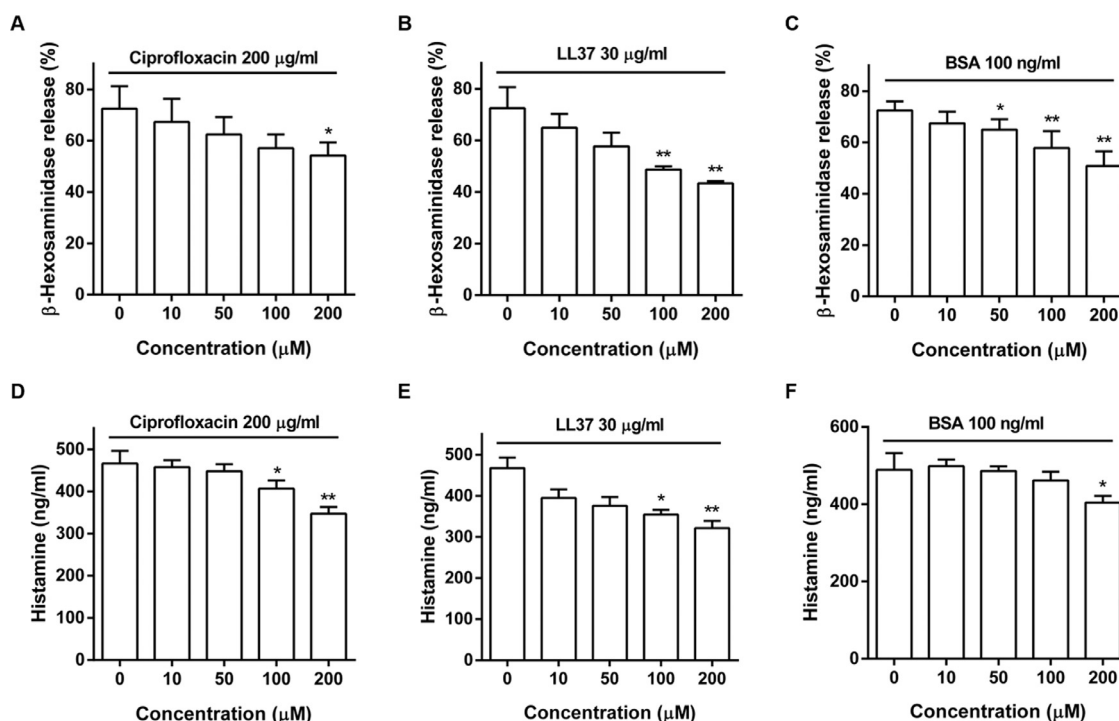


Fig. 4. Ciprofloxacin, LL37 or BSA-IgE induced mast cells degranulation under the treatment of HSYA. (A) β -hexosaminidase release of LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA and 200 μ g/ml ciprofloxacin for 30 min. (B) β -hexosaminidase release of LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA and 30 μ g/ml LL-37 for 30 min. (C) β -hexosaminidase release of LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA or 100 ng/ml BSA for 30 min after IgE incubation. (D) Histamine release of LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA and 200 μ g/ml ciprofloxacin for 30 min. (E) Histamine release of LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA and 30 μ g/ml LL-37 for 30 min. (F) Histamine release of LAD2 cells treated with 0, 10, 50, 100 or 200 μ M HSYA or 100 ng/ml BSA for 30 min after IgE incubation. Data are presented as the means $\pm$ SD ($n = 3$). Two-tailed unpaired Student's t -tests were used to determine significance in statistical comparisons, and statistical significance was defined at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

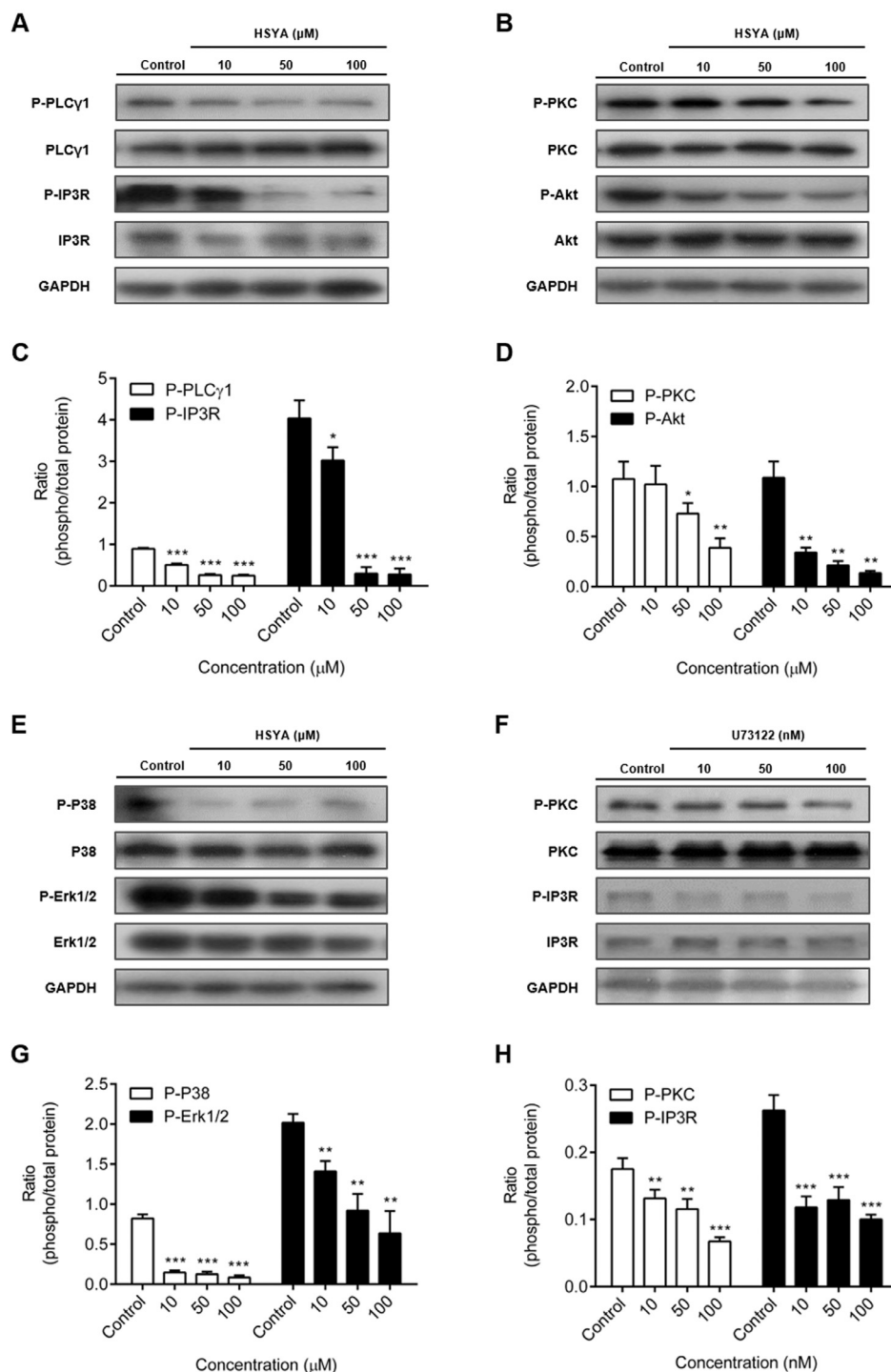


Fig. 5. Effect of HSYA and C48/80 on the expression of PLC γ , IP3R, PKC, Akt, P38 and Erk1/2 in LAD2 cells. (A, B, E) Western blot analysis of the expression levels of PLC γ 1, P-PLC γ 1, IP3R and P-IP3R (A), PKC, P-PKC, Akt, P-Akt (B), P38, P-P38, Erk1/2, P-Erk1/2 (C) in LAD2 cells treated with 0, 10, 50 or 100 μ M HSYA and 30 μ g/ml C48/80 for 24 h. (C, D, G) Quantification of (A, B, E) by densitometric analysis. (F) Western blot analysis of the expression levels of PKC, P-PKC, IP3R, P-IP3R in LAD2 cells treated with 0, 10, 50 or 100 nM U73122 and 30 μ g/ml C48/80 for 24 h. (H) Quantification of (F) by densitometric analysis. Data are presented as the means $\pm$ SD ($n = 3$). Two-tailed unpaired Student's t -tests were used to determine significance in statistical comparisons, and statistical significance was defined at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

HSYA inhibited the release of inflammatory mediators by mast cells.

HSYA decreased the activation of the PLC γ -PKC-IP3 signaling pathway regulating calcium fluctuations in mast cells

Activated mast cells can activate the PI3K pathway and the enzyme phospholipase C γ 1 (PLC γ 1) (Kalesnikoff and Galli, 2008). PLC γ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane to produce the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which result in the activation of protein kinase C (PKC) and the rise in intracellular calcium ($[Ca^{2+}]_i$),

respectively. Binding of IP3 to IP3 receptors (IP3R) in the ER (endoplasmic reticulum) membrane causes the release of Ca^{2+} from ER stores, resulting in a decrease in the concentration of free Ca^{2+} in the ER. The activation of mast cells also stimulates the ERK and P38 MAP kinase pathways. MAP kinases are important signaling mediators between the cytosol and the nucleus, which regulate a variety of transcription factors. Compared to those in LAD2 cells that were treated with C48/80 alone, the levels of phosphorylated PLC γ 1 and IP3R (Fig. 5A, C), PKC and Akt (Fig. 5B, D), P38 and Erk1/2 (Fig. 5E, G) analyzed by Western blotting were decreased in a dose-dependent manner in LAD2 cells that were simultaneously treated with 10 μ M,

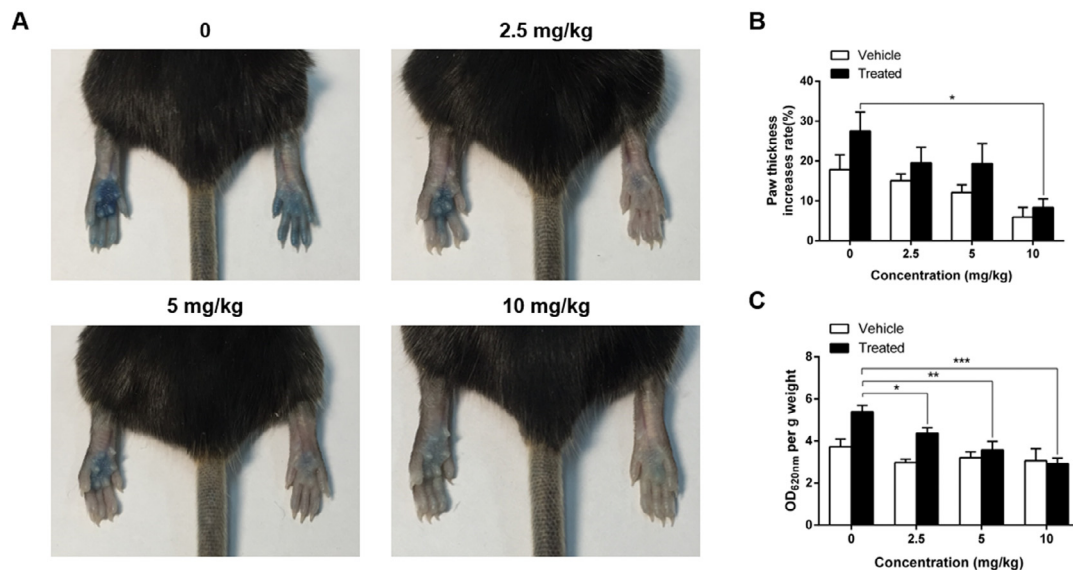


Fig. 6. Cutaneous flare responses in mice treated with 10 µg/ml C48/80 after 30 min of tail vein injection with HSYA. (A) Representative images of Evans blue extravasation 15 min after the intraplantar injection of 10 µg/ml C48/80 in the left paw or saline in the right paw after 30 min of tail vein injection of 0, 2.5 mg/kg, 5 mg/kg or 10 mg/kg HSYA. (B) Quantification of increase in paw thickness after 15 min. (C) Quantification of Evans blue extravasation into the paw after 15 min. Statistical significance was defined at $p < 0.05$. Data are presented as the means $\pm$ SD ($n = 5$). Two-tailed unpaired Student's t -tests were used to determine significance in statistical comparisons. Statistical significance was defined at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

50 µM or 100 µM HSYA and 30 µg/ml of C48/80 for 24 h. For making sure the potential inhibit effect of HSYA for C48/80 activated PLC γ -PKC-IP3R signaling pathway, PLC inhibitor U73122 was applied in to validate the PLC-PKC-IP3R signaling pathway. The results showed that phosphorylation of PKC and IP3R were attenuated under PLC inhibitor treatment (Fig. 5F, H)

In vivo assessment of the anti-anaphylactoid effect of HSYA on C48/80-induced cutaneous flare in mice

To validate whether HSYA can inhibit drug-induced pseudo-allergic reactions, C57BL/6 mice were used to generate a C48/80-induced cutaneous flare model to evaluate the impact of HSYA on paw edema in mice. After 30 min of tail vein injection with HSYA and Evans blue, followed by the subcutaneous injection of C48/80 for 15 min, paw edema and Evans blue extravasation were detected. The representative images of Evans blue extravasation demonstrated a weakened cutaneous flare in response to 0, 2.5 mg/kg, 5 mg/kg or 10 mg/kg HSYA (Fig. 6A). In response to 0, 2.5 mg/kg, 5 mg/kg or 10 mg/kg of HSYA, paw thickness increased by $27.51\% \pm 0.0956$, $19.59\% \pm 0.0774$, $19.39\% \pm 0.0857$, and $8.34\% \pm 0.0376$ (Fig. 6B) and the relative Evans blue extravasation was 5.39 ± 0.0061 , 4.37 ± 0.0052 , 3.58 ± 0.0070 and 2.92 ± 0.0046 (Fig. 6C), respectively. Therefore, HSYA inhibited cutaneous flare in mice in a dose-dependent manner, which is indicative of its anti-pseudo-allergic effect.

Discussion

Here, we have shown that HSYA can inhibit drug-induced anaphylactoid reactions by suppressing Ca^{2+} mobilization and degranulation in mast cells. The activity of HSYA *in vitro* and *in vivo* in drug-induced anaphylactoid animal and cell models confirms that HSYA has a clear and efficient anti-allergic effect. These results demonstrate the potential of HSYA as a novel anti-allergic agent.

The pathogenesis of pseudo-allergic drug reactions is mediated by non-immune-mediated mechanisms that can arise from the direct activation of inflammatory effector cells or mast cells by certain

substances (Pichler and Hausmann, 2016). Mast cells are a class of human immune cells that are primarily present in tissues exposed to the external environment such as the skin, lungs, and gastrointestinal tract (Kleinschmidt et al., 2010), and these cells participate in various pathologic and physiologic processes such as anaphylactic reactions, asthma, hypertrophy, and HA release (Jensen et al., 2014; Singh et al., 2016). LAD2 cells are mast cells derived from connective tissues in the human skin (Guhl et al., 2010). HSYA suppressed C48/80-induced calcium mobilization in MPMCs and LAD2 cells and prevented LAD2 cell degranulation. Furthermore, HSYA reduced the phosphorylation of proteins involved in these processes.

Drugs that directly trigger mast cell degranulation act by producing DAG and IP3 via the PKC pathway, which causes the release of calcium ions from the ER (Hoth and Penner, 1992; Putney and McKay, 1999). Consequently, the calcium sensor Stim1 on the ER interacts with the membrane protein Orail, thereby opening the calcium ion channel CRAC (Baba et al., 2008), which further increases $[\text{Ca}^{2+}]_i$ and induces cytoskeletal reorganization and subsequent degranulation. DAG can activate PKC, interact with calmodulin to activate calcineurin, and induce the translocation of nuclear factors (NFATs) into the nucleus, which regulate the secretion and release of HA and other pro-inflammatory mediators (Hoth and Penner, 1992; Neher, 1988).

Inhibition of PLC γ has been reported to lead to the formation of filopodium-like structures by inhibiting Rac and Cdc42 in antigen-stimulated RBL-2H3 cells (El-Sibai and Backer, 2007). Phosphorylation of PLC γ leads to the activation of more downstream molecules, such as IP3R, PKC, and MAPK, triggering the degranulation of mast cells and the generation of pro-inflammatory cytokines (Gilfillan and Tkaczyk, 2006). In our study, HSYA inhibited the phosphorylation of PLC γ 1, IP3R, PKC, Akt, P38 and ERK1/2, indicating that HSYA inhibits C48/80-induced PLC γ -PKC-IP3 signaling pathway activation and mast cell activation. These observations were also applicable to other drugs that cause anaphylactoid reactions, such as ciprofloxacin and LL-37, indicated by a suppression of β -hexosaminidase and HA release.

Han and Huang (Han et al., 2015; Huang et al., 2013) have demonstrated the anti-allergic effect of HSYA on RBL-2H3 cells, but they did not extend their investigation in human mast cell models or animal

models. RBL-2H3 cells are generally described in the literature as a mast cell line which were derived from rat (Passante and Frankish, 2009), while LAD2 cells were established from human (Guhl et al., 2010), which expressed tryptase and chymase and were the most reliable markers of human mature mast cells.

We are the first to use a mouse model of pseudo-allergic reactions to explore the anti-anaphylactoid effects of HSYA, and our results demonstrated a marked inhibitory effect of HSYA on allergic inflammatory responses. Drug-induced anaphylaxis is a complex and uncontrolled process with differences among individuals, which make it very difficult to be completely suppressed. Appropriate medications and the time of administration are important in treating this condition. HSYA could be combined with potential anaphylactoid drugs when there is a lack of therapeutic efficacy from these agents. However, the molecular targets of HSYA responsible for its anti-allergic effects are still unknown. These targets should be investigated in the future.

In conclusion, the pharmacological regulation of mast cell activation is an effective strategy for treating allergic diseases, and HSYA exerts an inhibitory effect on drug-induced anaphylactoid reactions.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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