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Tussilagone inhibits MRGPRX2-mediated mast cell degranulation and suppresses pseudo-allergic reactions

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ABSTRACT

Mas-related G protein-coupled receptor X2 (MRGPRX2) is a crucial target in pseudo-allergic reactions. Tussilagone (Tus), the main bioactive component derived from *Tussilago farfara*, has anti-inflammatory effects, but its potential inhibitory effects on pseudo-allergic responses remain unclear. This research aimed to evaluate the inhibitory role of Tus on pseudo-allergic reactions and its underlying mechanism. In vivo Systemic pseudo-allergic reactions and passive cutaneous anaphylaxis (PCA) models were established to assess the effects of Tus. In vitro, mast cell (LAD2) degranulation, inflammatory cytokine release, and signaling pathway protein expression were assessed. Calcium influx was measured in MRGPRX2-expressing HEK293 cells. The results showed that Tus significantly attenuated Tween 80- and substance P (SP)-induced systemic pseudo-allergy and PCA reactions. It also suppressed mast cell degranulation and decreased production of tumor necrosis factor-alpha (TNF-α), Interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1). In MRGPRX2-expressing HEK293 cells, Tus suppressed Tween 80- and SP-induced Ca^{2+} influx. Mechanistically, Tus inhibited tolimidone-induced Lyn kinase activation and suppressed SP-and Tween 80-induced β-hexosaminidase release, exhibiting an inhibitory profile comparable to that of the Lyn/Btk antagonist bosutinib. Additionally, Tus attenuated the phosphorylation levels of MRGPRX2 downstream signal molecules, including Btk, PLCγ1, PKC, p38 MAPK, IκB-α and NF-κB (p65). In conclusion, Tus attenuates SP-and Tween 80-induced mast cell activation and pseudo-allergic reactions by targeting the Lyn/Btk/PLCγ1 and p38/NF-κB pathways, highlighting its therapeutic potential for pseudo-allergy.

1. Introduction

Non-IgE-mediated anaphylaxis, also known as pseudoallergy, is estimated to account for nearly 60% of adverse drug reactions and poses a significant health risk (Böhm and Cascorbi 2016). The characteristic manifestations of pseudo-allergic reactions resemble those of allergic reactions, including asthma, urticaria, angioedema, hypotension, bronchospasm, and potentially life-threatening shock (McNeil et al. 2015; Pichler and Hausmann 2016). However, unlike allergic reactions, which require an induction (sensitization) phase and an effector phase, Pseudo-allergic responses can be triggered upon initial drug exposure without prior sensitization (Demoly et al. 2014; Pichler 2019). The close relationship and similarities between allergic and pseudo-allergic symptoms present challenges in diagnosing and treating pseudoallergy (Kumar et al. 2021).

Mast cells are effector cells in allergy disease and participate in innate immune regulation (Reber and Frossard 2014). Mast cell degranulation in pseudoallergy is triggered without IgE involvement. (Kumar et al. 2021).Various stimuli, such as basic secretagogues, antimicrobial drugs, neuromuscular blocking drugs and opiates can directly activate mast cells (Roy et al. 2021). Following activation, mast cells degranulate, releasing preformed mediators including heparin, tryptase,

Abbreviations: Btk, Bruton's Tyrosine Kinase; CIB, calcium imaging buffer; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; LAD2 cells, the Laboratory of Allergic Disease 2 (LAD2) human mast cells; IκB-α, Inhibitor of Nuclear Factor Kappa B Alpha; ILs, interleukins; IP3R, Inositol 1,4,5-trisphosphate receptor; Lyn, Lck/Yes novel tyrosine kinase; MCP-1, monocyte chemotactic protein-1; MRGPRX2, Mas-related G protein–coupled receptor X2; NF-κB, nuclear factor kappa-B; OVA, ovalbumin; PBS, phosphate-buffered saline; PCA, passive cutaneous anaphylaxis; PKC, protein kinase C; PLCγ, phospholipase C-γ; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; Tus, tussilagone; SMILES, Simplified Molecular Input Line Entry System; SP, Substance P.

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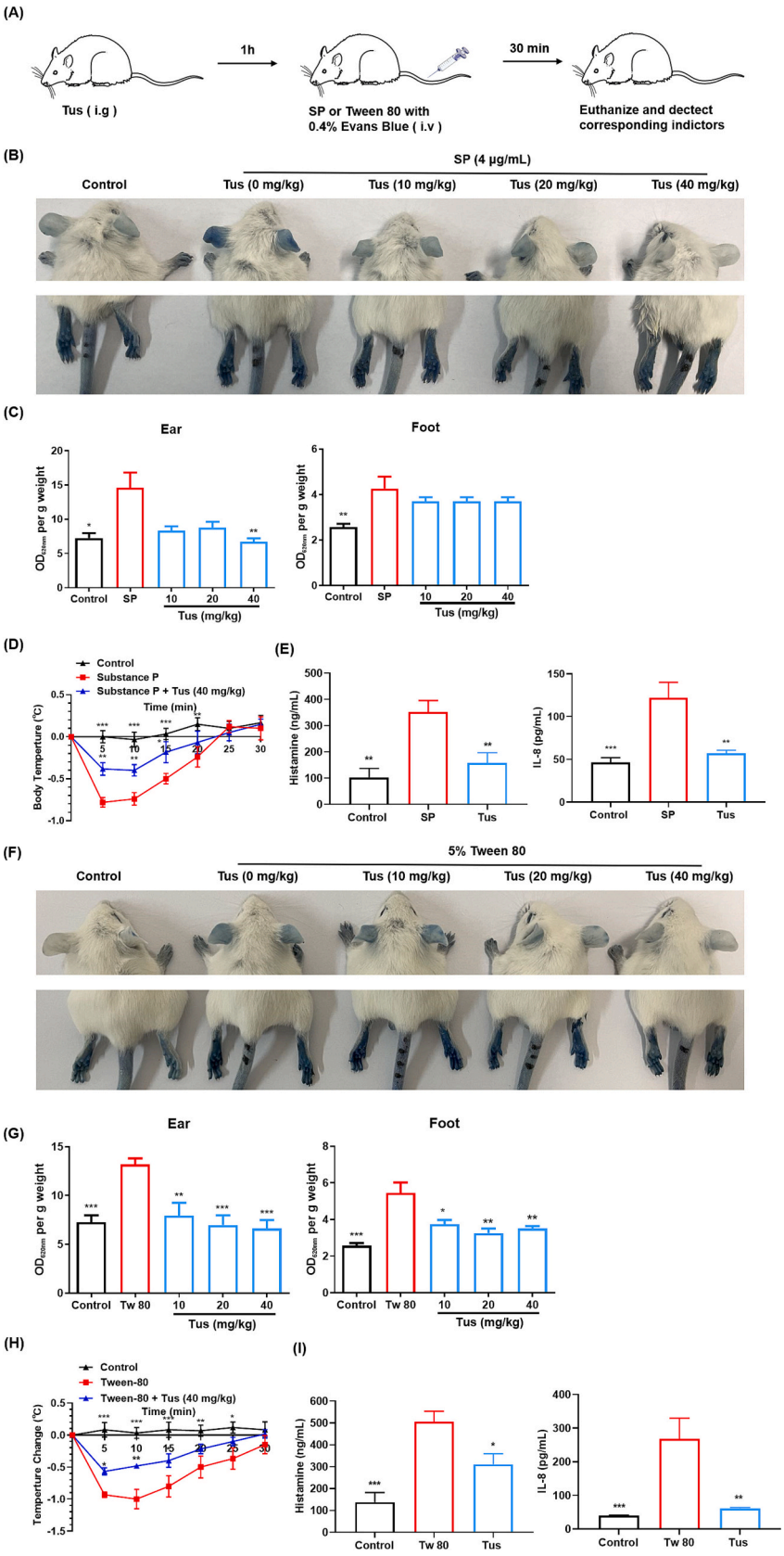

Fig. 1. Tus alleviated Tween 80 or SP-induced systemic anaphylaxis. (A) The process of Systemic Anaphylaxis. (B, F) Representative images showing Evans Blue extravasation, $n = 6$. (C, G) Quantification of Evans Blue leakage into the ears and paws, n = 6. (D, H) The change in body temperature within 30 min, n = 6. (E, I) The effect of Tus on SP or Tween 80-induced serum concentrations of histamine and IL-8, n = 6. Values represent the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs SP or Tween 80 group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and histamin (He et al. 2013). Histamine induces increased vascular permeability and vasodilation (Gao et al. 2022); trypsin promotes the release of β-Hexosaminidase (Vliagoftis et al. 2004); and heparin increases vascular permeability (Oschatz et al. 2011).

Both Tween 80 (a common food and pharmaceutical stabilizer) and substance P (SP, a. neuropeptide) have been confirmed to trigger pseudo-allergy (McNeil et al. 2015; Mi et al. 2019). Research show that SP-induced pseudo-allergic responses are mediated by the Mas-related G protein-coupled receptor X2/B2 (MRGPRX2/B2), establishing SP as a typical MRGPRX2 agonist(Xue et al. 2021). MRGPRX2 (human ortholog of mouse MRGPRB2) is a newly characterized receptor found on mast cell surfaces, playing an active role in itch sensation and allergic signaling (McNeil et al. 2015). It is considered to be the key target for inducing pseudo-allergic reactions (Misery et al. 2023). An immediate increase in Ca^{2+} mobilization following MRGPRX2 activation is essential for triggering inflammatory mediator secretion (Occhiuto et al. 2019). Therefore, inhibiting MRGPRX2 or the signaling pathways involving MRGPRX2 effectively suppresses the occurrence of pseudoallergy. Currently, pharmacotherapy primarily involves norepinephrine, glucocorticoids and antihistamines to improve or alleviate some symptoms. However, their scope and efficacy remain limited (Cardona et al. 2020). Tussilagone (Tus), a *sesquiterpene* compound derived from *Tussilago farfara* flower buds, has the chemical name of tussilagone is 1 (10), 4a, 8-trimethyl-7-isopropyl-1, 2, 3, 4, 4a, 5, 6, 8-octahydronaphthalene-1-carboxylic acid lactone. This compound exhibits a molecular mass of 390.5 Da and possesses the molecular formula $C_{23}H_{34}O_5$. It has a variety of pharmacological effects and exhibits good antioxidant (Sun et al. 2023), anti-inflammatory (Hwangbo et al. 2009), anti-cancer (Nam and Kim 2020) and neuroprotective effects. Furthermore, Tus has inhibitory effects on 12-O-tetradecanoylphorbol-13-acetate (TPA)-triggered cutaneous inflammation in murine models (Lee et al. 2016), attenuates ovalbumin (OVA)-induced allergic rhinitis in guinea pigs, and inhibits IgE-mediated activation in RBL-2H3 mast cells (Jin et al. 2020). Despite all the above-stated evidence, the role of Tus in regulating pseudo-allergic reactions remains unclear. We hypothesized that Tus inhibits mast cell activation and pseudo-allergic responses by suppressing downstream MRGPRX2-associated signaling pathways. To test this hypothesis, we established both in vivo and in vitro models of pseudoallergy to investigate the anti-pseudoallergic effects of Tus and elucidate its molecular mechanisms.

2. Materials and methods

2.1. Chemicals

Tussilagone (CAS: 104012–37-5, purity ≥98% by HPLC) was acquired from Pufeide Biotech (Chengdu, China). Tween 80 was acquired from Sigma-Aldrich (USA) and SP was acquired from MedChem Express (Shanghai, China). For cell experiments, Tus was prepared by initial dissolution in DMSO followed by dilution with Tyrode's solution, while SP and Tween 80 were dissolved directly in Tyrode's solution. For animal experiments, Tus was prepared by initial dissolution in DMSO followed by dilution with saline, whereas SP and Tween 80 were dissolved in saline. The concentration of SP (4 μg/mL) was selected based on previous literature (Xue et al. 2021), and the concentrations of Tween 80 (0.03% for cell experiments and 5% for animal experiments) were determined according to preliminary experimental studies.

2.2. Cell preparation

The human mast cell line LAD2 was generously provided by Drs. A. Kirshenbaum and D. Metcalfe (NIH, USA). The cells were grown at 37 °Cwith 5% CO_2 in StemPro-34 complete medium containing: penicillin-streptomycin (1%; Solarbio, Beijing, China), StemPro nutrient supplement (10 mL/L; Gibco, Grand lsland, USA), human SCF (100 ng/mL; SinoBiological, Beijing, China) and L-glutamine (2 mM; Gibco, Grand lsland, USA). Cell concentrations were kept at 5×10^5 cells/mL. Separately, MRGPRX2-expressing HEK293 cells and NC- HEK293 cells were grown in DMEM (Solarbio) containing1% penicillin-streptomycin and 10% FBS under the same culture parameters.

2.3. Animals

All animal procedures complied with NIH animal care guidelines and were approved by Xi'an Jiaotong University's IACUC (Protocol No. XJTUAE2023–1258). Male Kunming and C57BL/6 mice (18–22 g) were purchased from the Experimental Animal Center at Xi'an Jiaotong University. Animals were housed under specific pathogen-free conditions (22 ± 2 °C, 55 ± 15% humidity, 12 h light/dark cycle) with ad libitum access to food and water.

2.4. Systemic pseudo-allergic reactions in mice

In studies of systemic pseudo-allergic reactions, we conducted the Evans blue extravasation assay in Kunming mice (Liang et al. 2024; Yi et al. 2018; Zhao et al. 2021). Kunming mice were randomly assigned to 10 groups, with 6 animals in each group. Systemic pseudo-allergic reactions were triggered with SP in mice of the first 5 groups and Tween-80 in the other 5. In each of the allergic reaction-triggered group, 4 treatment groups orally received Tus (0, 10, 20, and 40 mg/kg) in saline ，while control animals were given the same amount of saline. After 1 h, the SP-triggered groups were intravenously administered 0.1 mg/kg SP in 0.4% Evans blue via tail vein injection (Sigma-Aldrich, St Louis, MO, USA), while the Tween 80-triggered groups received 5% Tween 80 (Sigma-Aldrich) in 0.4% Evans blue at the same injection volume. Control group animals were treated with 0.4% Evans blue alone. After 30 min, mice'euthanasia was carried out by cervical dislocation (Fig. 1A). Tissue samples (ears and paws) were photographed and collected in 1.5 mL Eppendorf tubes, desiccated at 65 °C for 24 h, and individually weighed. Evans blue extraction was performed by incubating tissues in 500 μL of saline-acetone- solution (3:7, *v*/v) for 24 h at room temperature. After mechanical homogenization (TissueLyser, 10 min), 200 μL of supernatant was transferred to 96-well plates for spectrophotometric quantification at 620 nm. Optical density values were normalized to tissue dry weight (per gram).

2.5. Body temperature

Kunming mice were randomly assigned to 6 groups, with 6 animals in each group. Systemic pseudo-allergic reactions were triggered with SP in mice of the first 3 groups and Tween-80 in the other 3. In each of the allergic reaction-triggered group, 2 treatment groups orally received Tus (0 and 40 mg/kg) in saline, while control animals were given the same amount of saline. After 1 h, the SP- triggered groups were intravenously administered 0.1 mg/kg SP (20 mL/kg) via tail vein, while the Tween 80- triggered groups received 5% Tween 80 at the same injection volume. Control group animals were treated with saline alone. Rectal temperature measurements were obtained by inserting a thermocouple probe and recorded every 5 min over a 30-min observation period.

2.6. Analysis of serum

Following body temperature measurement, mice were given 3% pentobarbital sodium (10 mL/kg) to induce anesthesia, blood was extracted from the orbital sinus of each mouse into 1.5 mL Eppendorf tubes at 0 and 6 h. The samples were centrifuged to obtain serum, with the 0 h serum used for histamine measurement and the 6 h serum for IL-8 detection. The release of histamine was quantified using o-phthalaldehyde (sigma-Aldrich) as an indicator. Briefly, a black 96-well plate was filled with 50 μL of serum. Each well was then filled with 10 μL of 0.5 M NaOH and 2 μL of a 0.1% o-phthalaldehyde methanol solution. The mixture was then incubated at room temperature for 3 min. The reaction

was terminated by adding 6 μL of 3 M HCl per well. After 30 min, a microplate reader was used to quantify the fluorescence using 450 nm emission filters and 360 nm excitation. Histamine concentrations were calculated by extrapolating values from a histamine dihydrochloride standard calibration curve. The measurement of human IL-8 was performed using ELISA kits (RuiXin Biotech, Fujian, China) in accordance with the manufacturer's guidelines.

2.7. Hind paw swelling and extravasation

C57BL/6 mice were used in research on passive cutaneous anaphylaxis (PCA) for two reasons: one is that MRGPRB2 (the orthologue of MRGPRX2 in mice knockout mice are commonly based on C57BL/6 mice; another reason is the use of two animal models in vivo experiments to further verify the inhibitory effect of tussilagone on pseudo-allergic reactions. C57BL/6 mice were randomly assigned to 4 groups, with 6 animals in each group. Passive cutaneous anaphylaxis was triggered with SP in mice of the first 2 groups and Tween-80 in the other 2. In each of the allergic reaction-triggered group, groups orally received Tus (0 and 40 mg/kg). Then, both groups received a tail vein injection of 0.4% Evans Blue. After 1 h of treatment initiation, 5 μL of either 4 μg/mL SP or 5% Tween 80 was injected into right hind paws, with left paws receiving equivalent volumes of saline as internal controls. Paw perimeter was measured using a vernier caliper both before and 15 min after compound administration. After 30 min, mice'euthanasia was carried out by cervical dislocation. Tissue samples (paws) were photographed and collected in 1.5 mL Eppendorf tubes, desiccated at 65 °C for 24 h, and individually weighed. Evans blue extraction was performed by incubating tissues in 500 μL of saline-acetone- solution (3:7, *v*/v) for 24 h at room temperature. After mechanical homogenization (TissueLyser, 10 min), 200 μL of supernatant was transferred to 96-well plates for spectrophotometric quantification at 620 nm. Optical density values were normalized to tissue dry weight (per gram).

2.8. Cell viability assay

LAD2 human mast cells were plated at 3×10^4 cells per well in 96-well culture plates, followed by 24 h treatment with Tus at concentrations ranging from 0 to 200 μM. 10 μL of CCK-8 solution was added per well and the plate was incubated for 2 h to assess cellular viability The absorbance at 450 nm (A_{450}) was measured using a microplate reader, and the viability of LAD2 cells was determined according to the following formula $[(A_{Treated} - A_{Blank}) / (A_{N.C.} - A_{Blank})] \times 100\%$.

2.9. B-hexosaminidase release assay

LAD2 human mast cells were plated at 3×10^4 cells per well in 96-well culture plates and treated with Tus (0, 3, 10, 30 and 100 μM) in Tyrode's solution for different time periods. The blank control group received Tyrode's solution only. Subsequently, the cells were sensitized with 0.03% Tween 80 or SP (4 μg/mL) for 30 min at 37 °C in 5% CO_2. Total β-hexosaminidase levels were measured after cell lysis using Tyrode's solution containing 0.1% Triton X-100. After centrifugation, all supernatants were collected. Subsequently, 50 μL of 1 mM β-hexosamine in 0.1 M citrate buffer (pH 4.5) was added to each well, followed by 2 h incubation at 37 °C. The reaction was terminated with 200 μL of 0.1 M carbonate/bicarbonate buffer, and absorbance was measured at 405 nm. β-Hexosaminidase release was calculated as: (A of culture supernatant /A of total cell lysate supernatant) × 100%.

2.10. Toluidine blue staining

LAD2 human mast cells were plated at 3×10^4 cells per well in 96-well culture plates. Subsequently, the Tween 80 group cells were treated for 2 h with Tus at concentrations of 0, 3, 10, and 30 μM in Tyrode's solution, while the SP group cells were treated for 4 h with Tus at concentrations of 0, 10, 30 and 100 μM in Tyrode's solution. The blank control group received Tyrode's solution only. After incubation, each well received 0.03% Tween 80 or SP (4 μg/mL) for 30 min, while the control group received the same volume of Tyrode's solution. Then the supernatants were centrifuged and discarded. The cells were rinsed twice with PBS, stained with toluidine blue (Solarbio; 5 min), then incubated with an equal volume of distilled water for 15 min. Cell supernatants were discarded, and cells were washed three times with distilled water before adding 50 μL of distilled water to each well. Finally, cell morphology was examined and recorded using an inverted microscope.

2.11. Annexin V staining

LAD2 human mast cells were plated at 3×10^4 cells per well in 96-well culture plates Cells were incubated and grouped according to the protocol described in Section 2.9. After stimulation with 0.03% Tween 80 or SP (4 μg/mL) for 30 min, all experimental groups were rinsed twice with cold PBS followed by treatment with 5 μL of Annexin V-FITC (Abbkine Scientific, Wuhan, China). Following 5 min of dark incubation, cellular fluorescence was examined and imaged using a fluorescence microscope.

2.12. Measurement of annexin V positive rate

Cells were incubated and grouped according to the protocol described in Section 2.9. After stimulation with 0.03% Tween 80 or 4 μg/mL SP, all experimental groups were rinsed twice with cold PBS prior to final resuspension in binding buffer (1× concentration). Next, 100 μL from the prepared cell suspension (containing $\geq 1.2 \times 10^4$ cells) was transferred into 5 mL culture tubes. Cells were stained with 5 μL of PE Annexin V prior to a 15-min incubation in the dark at room temperature. Following incubation, each tube received 400 μL of binding buffer (1× concentration), and the Annexin V fluorescence was quantified by flow cytometry within1 h.

2.13. Cytokines release assay

LAD2 human mast cells were plated at 3×10^4 cells per well in 96-well culture plates. Subsequently, the Tween 80 group cells were treated for 2 h with Tus (0 and 30 μM) in Tyrode's solution, while the SP group cells were treated for 4 h with Tus (0 and 100 μM) in Tyrode's solution. The blank control group received Tyrode's solution only. After incubation, 0.03% Tween 80 or SP (4 μg/mL) was added to each well for 30 min, while the control group received the same volume of Tyrode's solution. After centrifugation, all supernatants were collected and subjected to measurement of Human TNF-a, IL-8, and MCP-1 using ELISA kits (RuiXin Biotech, FuJian, China). The protocols were strictly conducted according to the manufacturer's instructions.

2.14. Ca^{2+} concentration assay

MRGPRX2-expressing HEK293 cells and NC-HEK293 cells were plated at 3×10^4 cells per well in 96-well culture plates and incubated overnight at 37 °C. Subsequently, MRGPRX2-expressing HEK293 cells were treated with Tus at different concentrations (3, 10, 30, and 100 μM) for either 2 or 4 h at 37 °C. Following this, the culture medium of cells was removed and the cells were rinsed with PBS. Next, each well was supplemented with 100 μL Fluo-4 staining solution (Beyotime Biotechnology Beijing, China) and incubated at 37 °C for 30 min in the dark. After the cells were washed with PBS again, the NC-HEK293 cells were stimulated with either Tus (30 μM or 100 μM), 4 μg/mL of SP, or 0.03% Tween 80. Meanwhile, the MRGPRX2-expressing HEK293 cells were stimulated with either 4 μg/mL of SP or 0.03% of Tween-80. Immediately after stimulation, the cells were observed using a fluorescence microscope.

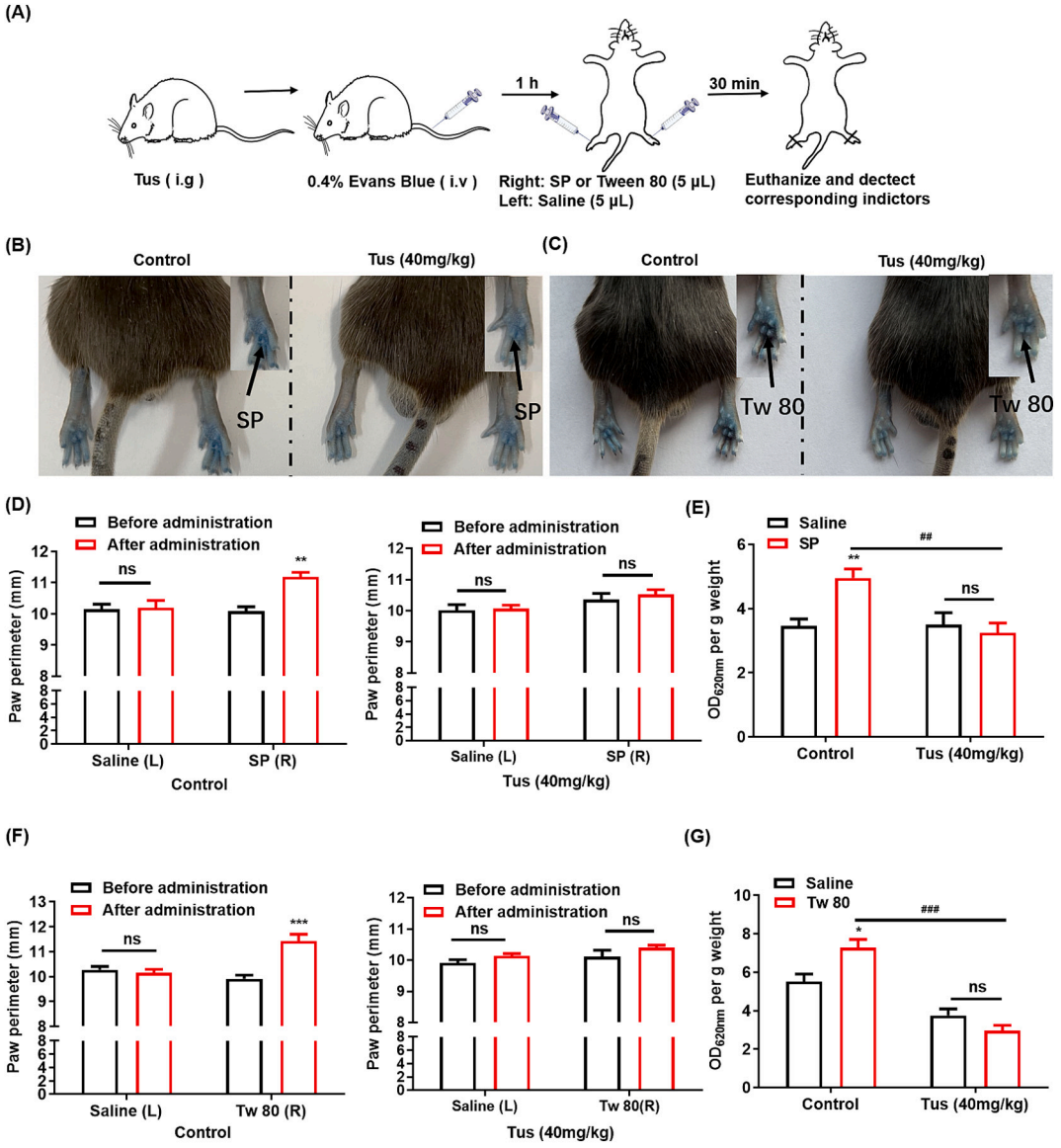

Fig. 2. Tus suppressed Tween 80 or SP-induced local inflammatory reactions. (A) The process of local inflammatory reactions. (B, C) Representative images showing Evans Blue extravasation (black arrows indicate edema, n = 6). (D, F) Quantification of paw perimeter change 15 min after injection, n = 6. (E, G) Quantification of Evans blue leakage into the paws, n = 6. Values represent the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Before administration or Saline; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs SP or Tween 80 group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.15. Molecular docking

To investigate potential modes of interaction, molecular docking was employed as a hypothesis-generating approach to predict possible binding poses of Tus within the MRGPRX2 receptor. The Simplified Molecular Input Line Entry System (SMILES) form of Tus chemical structure was converted into a 3D structure to minimize energy for subsequent docking analysis. The Cryo-EM structure of MRGPRX2 (PDB ID: 7VV6) and the homology model of Lyn (PDB ID: 3A4O) were utilized in this study. The respective template structures were retrieved from the RCSB Protein Data Bank. The molecular docking procedure was conducted using SYBYL-X 2.0 software. Following docking completion, PyMOL was employed to visualize the most favorable receptor-ligand binding conformation. Notably, this computational method only generates mechanistic hypotheses and does not establish binding affinity or direct pharmacological antagonism.

2.16. Western blotting analysis

Total proteins from untreated and Tus-treated LAD2 cells stimulated or not with 0.03% Tween 80 or SP (4 μg/mL). The cell lysate was prepared by ice-cold extraction for 30 min using RIPA lysis buffer supplemented with 10% protease inhibitor cocktail and phosphatase inhibitors. Protein concentrations were subsequently quantified using a bicinchoninic acid (BCA) assay kit (Beyotime, Beijing, China). The proteins of interest were separated by gel electrophoresis and then transferred onto a PVDF membrane. The membranes were initially incubated overnight with primary antibodies including Lyn, p-Lyn, Btk, p-Btk, PLCγ, p-PLCγ, PKC, p-PKC, p38 MAPK, p-p38 MAPK, p-NF-κB (pP65) (1:1000, CST), NF-κB (P65, 1:1000, Affinit), IκB-α (1:1000, Immounway), p-IκB-α (1:1000, Immounway), GAPDH (1:1000, Proteintech) and β-actin (1:1000, Proteintech). Then they were incubated with second antibodies (1:10,000 dilution in TBST) on the following day. Protein bands were subsequently visualized using enhanced

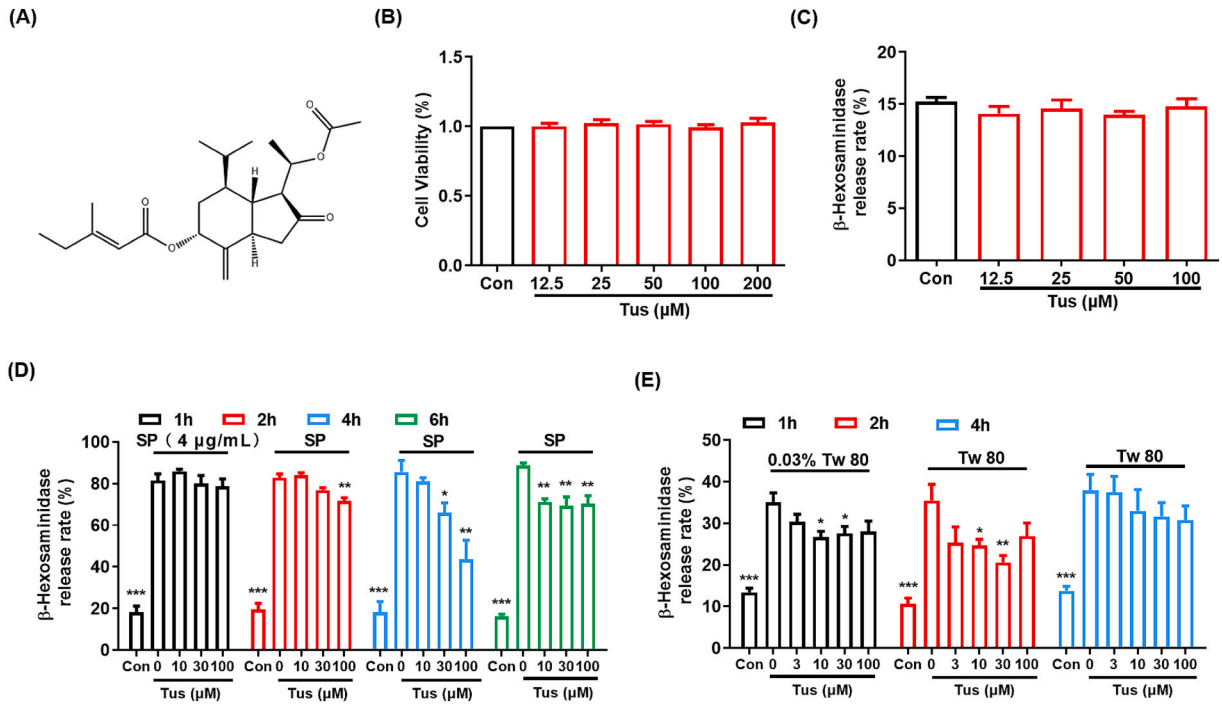

Fig. 3. Tus inhibited SP or Tween 80-induced degranulation. (A) The structure of tussilagone. (B) The viability of LAD2 cells treated with Tus (0–200 μM) for 24 h, $n = 4$. (C) Tus (0–100 μM) did not induce LAD2 cells degranulation and release of β-hexosaminidase, n = 4. (D-E) The time-dependence manner graph of Tus for SP or Tween 80-induce degranulation, n = 4–6. Values represent the mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control or Tus (0 μM) group.

chemiluminescence (ECL) substrate (Abbkine, Wuhan, China) and quantitatively analyzed through densitometry with ImageJ software. For Western blot analyses, we calculate the phosphorylated-to-total protein ratio for each sample. This value is then normalized to the control group mean and expressed as a fold change.

2.17. Statistical analysis

All data in this study are presented as the mean ± SE. Statistical significance among multiple groups was assessed using one-way analysis of variance (ANOVA) followed by a least significant difference test (LSD) or Dunnett T3 test. P value <0.05 was considered statistically significant.

3. Results

3.1. Tus alleviated substance P- and tween 80-induced systemic pseudo-allergic reactions

As shown in Fig. 1B-C and F-G, Evans Blue extravasation was significantly enhanced in mouse ears and legs following Tween 80 or SP challenge compared to untreated controls The Tus treatment demonstrated a dose-dependent reduction in Evans Blue extravasation compared to the model group. In the body temperature experiment (Fig. 1D and H), compared to the control group, the temperature of the mice in the model group decreased by 0.78 ± 0.06 °C following the injection of SP, and decreased by 1.0 ± 0.15 °C following Tween 80 injection. When mice were pretreated with Tus, the effects of both SP and Tween 80 on reducing body temperature were weakened significantly. Furthermore, the analysis of mice serum revealed that Tus decreased histamine and IL-8 levels, which had been elevated due to stimulation by SP and Tween 80 (Fig. 1E and I). These findings indicate that Tus may suppress systemic anaphylaxis triggered by either SP or Tween 80.

3.2. Tus suppressed substance P and tween 80-induced local inflammatory reactions

For evaluating the anti-pseudo-allergy activity of Tus, mice with SP- and Tween 80-induced cutaneous inflammation were used. Both Evans Blue extravasation and paw swelling were significantly increased in the paws injected with SP or Tween 80 (Fig. 2B-C). However, treatment with Tus led to a noticeable reduction in Evans Blue extravasation and paw swelling compared to the model group (Fig. 2D-G).

3.3. Tus inhibited substance P and tween 80-induced LAD2 cells degranulation

The in vitro anti-viability assay revealed that Tus did not exhibit any significant cytotoxicity toward LAD2 cells even at concentrations up to 200 μM (Fig. 3B). Additionally, there was no induction of β-hexosaminidase release on LAD2 cells upon exposure to Tus (Fig. 3C). To determine the optimal pre-incubation time and concentration, LAD2 cells were treated with Tus at various concentrations (0, 3, 10, 30, and 100 μM) for different time intervals (1, 2, 4, and 6 h), followed by stimulation with either Tween 80 or SP for 30 min. As shown in Fig. 3D-E, pretreatment with Tus (10, 30, and 100 μM) for 4 h significantly attenuated the SP-induced release of β-hexosaminidase from LAD2 cells in a dose-dependent manner, while Tween-80-induced β-hexosaminidase from LAD2 cells, was attenuated in a dose-dependent manner, after 2 h of pre-treatment with Tus (3, 10 and 30 μM). In Tween 80-stimulated cells treated with Tus (100 μM for 2 h), the inhibitory efficacy observed at 100 μM was lower than that at 30 μM. This effect was not attributable to cytotoxicity (Fig. 1B), suggesting the possible involvement of compensatory signaling pathways. Therefore, in the following in vitro assays, Tus (3, 10 and 30 μM) was incubated for 2 h in Tween 80-stimulated cells and Tus (10, 30, and 100 μM) was incubated for 4 h in SP-stimulated LAD2 cells. Moreover, SP induces mast cell activation through the MRGPRX2 receptor (Fujisawa et al. 2014; McNeil et al. 2015), and the mast cell activation by tween 80 is also related to MRGPRX2 (Fig. S1). These findings demonstrate that Tus exhibits no

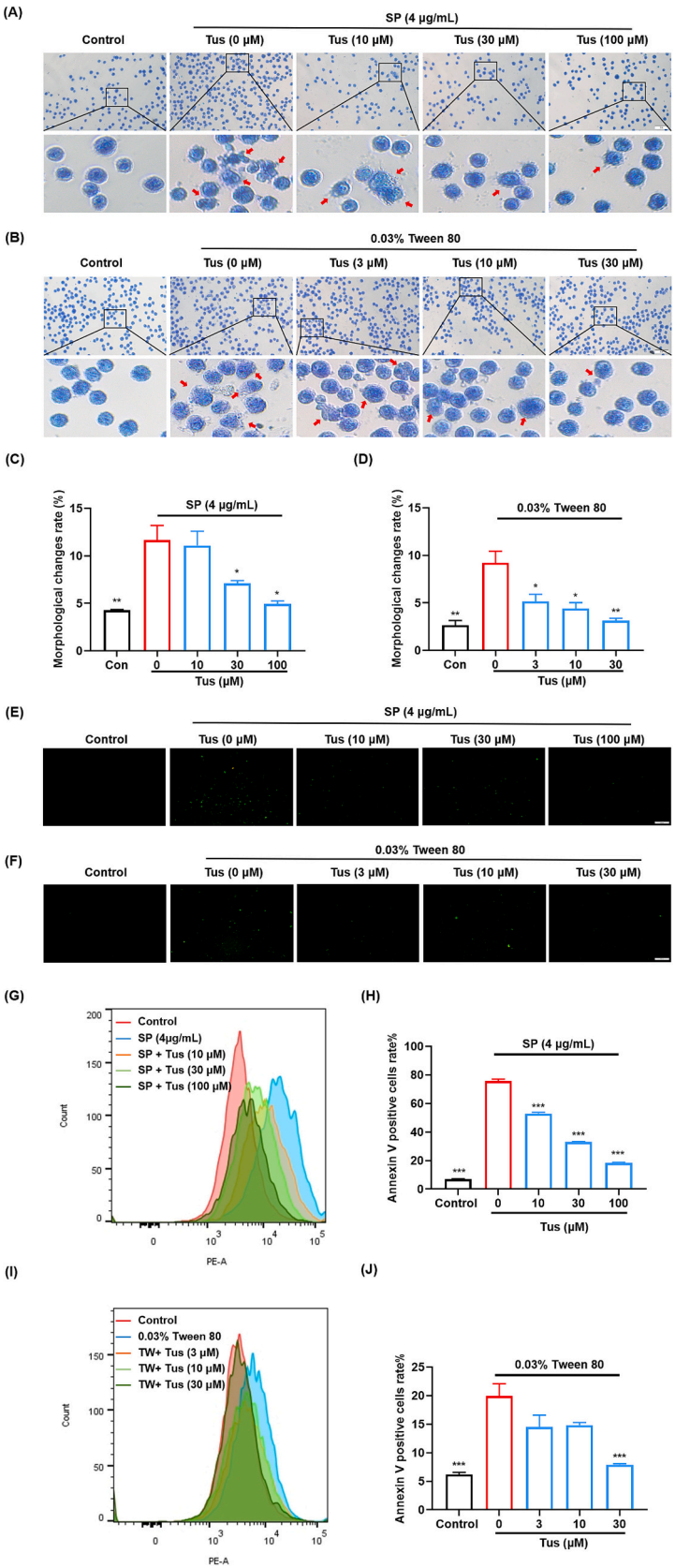

Fig. 4. Tus reduced morphological changes in mast cells during degranulation. (A, B) Effects of Tus on morphology of LAD2 cells and release of granules sensitized by SP or Tween 80 (Toluidine blue staining, scale bar = 50 μm, $n = 3$). (C, D) The morphological changes rate, $n = 3$. (E, F) Effects of Tus on release of granules sensitized by SP or Tween 80 (Annexin V staining, scale bar, 50 μm, n = 3). (G, I) The fluorescence intensity of Annexin V, n = 3. (H, J) The Annexin V positive cells rate, n = 3. Values represent the mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Tus (0 μM) group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

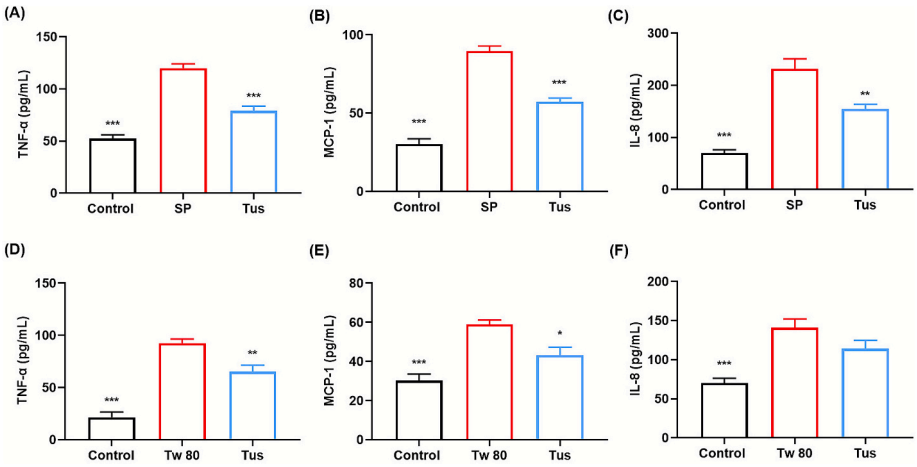

Fig. 5. Tus inhibited SP or Tween 80-induced cytokines release. (A-C) Tus (100 μM) inhibited SP triggered TNF-α, MCP-1 and IL-8 secretion, $n = 5$. (D—F) Tus (30 μM) inhibited Tween 80-triggered TNF-α, MCP-1 and IL-8 secretion, n = 5. Values represent the mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs SP or Tween 80 group.

discernible toxicity on LAD2 cells and significantly inhibits SP-and Tween 80-induced mast cell degranulation.

3.4. Tus reduced morphological changes in LAD2 cells during degranulation

Mast cell degranulation is an important process in the development of pseudo-allergic reactions (Yang et al. 2023). The morphological changes associated with LAD2 cell degranulation were assessed using toluidine blue and Annexin V-FITC stain. Normal LAD2 cells exhibited intact cell membranes with purple granules stored within the cytoplasm. Conversely, SP or Tween 80-stimulated LAD2 cells displayed irregular shapes and extracellular release of purple granules (Fig. 4A-B). In Tus treatment groups, a dose-dependent reduction in the number of extracellular purple granules was observed (Fig. 4C-D). Annexin V, a phospholipid-binding protein, exhibits specific binding affinity for phosphatidylserine. Under normal states, phosphatidylserine is primarily located in the inner side of the cell membrane but translocates to the outer surface during cell degranulation where it can be fluorescently labeled by Annexin V-FITC binding (Demo et al. 1999). Minimal green fluorescence labeling was observed in control groups, while SP group and Tween 80 group exhibited a significant increase in labeled cells. Pretreatment with Tus reduced phosphatidylserine translocation in LAD2 cells (Fig. 4E-F). Furthermore, Annexin V fluorescence intensity and the percentage of positive cells in LAD2 cells were quantified by flow cytometry. The results showed that both fluorescence intensity and the number of positive cells were significantly elevated in the SP and Tween 80 groups. Pretreatment with Tus significantly decreased both the fluorescence intensity and the number of positive cells in LAD2 cells (Fig. 4G-J). In conclusion, Tus ameliorated the morphological changes and inhibited phosphatidylserine translocation in LAD2 cells.

3.5. Tus inhibited substance P and tween 80-induced cytokines release in LAD2 cells

Cytokines play a pivotal role in pseudo-allergic reactions. To evaluate the modulatory effects of Tus, we measured TNF-α, IL-8, and MCP-1 concentrations in culture supernatants using ELISA. Compared to the control group, model groups (stimulated with SP or Tween 80) showed significantly elevated levels of TNF-α, IL-8 and MCP-1(Fig. 5). However, Tus treatment led to a reduction in the release of these cytokines compared to the model groups. These findings indicated that Tus effectively suppresses the production of inflammatory factors induced by SP or Tween 80.

3.6. Tus acted on MRGPRX2 downstream signaling pathway

An elevation in intracellular Ca^{2+} levels is necessary for mast cell degranulation(Penner 1988). Upon stimulation, the intracellular Ca^{2+} concentration significantly increase. As shown in Fig. 6A and D, SP, Tween 80 and Tus did not cause an increase in Ca^{2+} concentration in NC-HEK293 cells. In MRGPRX2-expressing HEK293 cells, both SP and Tween 80 induced an elevation of Ca^{2+} concentration, which later exhibited a downward trend in response to Tus treatment (Fig. 6B-C and 6E-F). This result further supports Tus inhibitory effect on SP- or Tween 80-induced Ca^{2+} release and indicates that its suppression of pseudo-allergic responses may occur via signaling pathways associated with MRGPRX2.

The ligand–protein interactions between MRGPRX2, Lyn and Tus were then investigated by molecular docking. The final docking results were shown in Fig. 6G and H. MRGPRX2 and Tus formed hydrogen bonds at GLN-20 (2.2 Å) with a binding affinity score of 5.3253. In addition, Lyn and Tus formed hydrogen bonds at SER-95 (1.8 Å) with a binding score of 7.3347. Tus demonstrates stronger predicted binding affinity for Lyn than for MRGPRX2, suggesting that its direct target may not be the MRGPRX2 receptor.

3.7. Tus inhibited activity of Lyn and Btk to regulate MRGPRX2 downstream signaling pathway phosphorylation

Tus (100 μM) significantly suppressed Lyn kinase activation induced by the potent selective agonist tolimidone (10 μM) (MedChem Express), as shown in Fig. 7A and B. Treatment with various concentrations of Tus alone (0, 30, and 100 μM) led to the inhibition of phosphorylated Lyn and Btk expression (Fig. 7E-F). Furthermore, Tus inhibited β-hexosaminidase release induced by SP and Tween 80, similar to the effect of Bosutinib (10 μM, MedChem Express), a Lyn/Btk antagonist (Fig. 7C-D). Compared to SP or Tween 80 alone, Tus exhibited concentration-dependent reduction in the phosphorylation levels of Lyn and Btk. Tus also decreased the phosphorylation levels of PLCγ1, PKC, p38 and NF-κB (p65) induced by SP and Tween 80, which are part of the downstream signaling pathway of MRGPRX2 (Fig. 7G-J and Fig. 8). Additionally, Tus inhibited the phosphorylation of IκB-α, a key regulator of NF-κB (p65) phosphorylation. It was demonstrated that Tus could inhibit pseudo-allergic reactions by suppressing the Lyn-Btk-PLCγ and p38-NF-κB signaling pathways in mast cells.

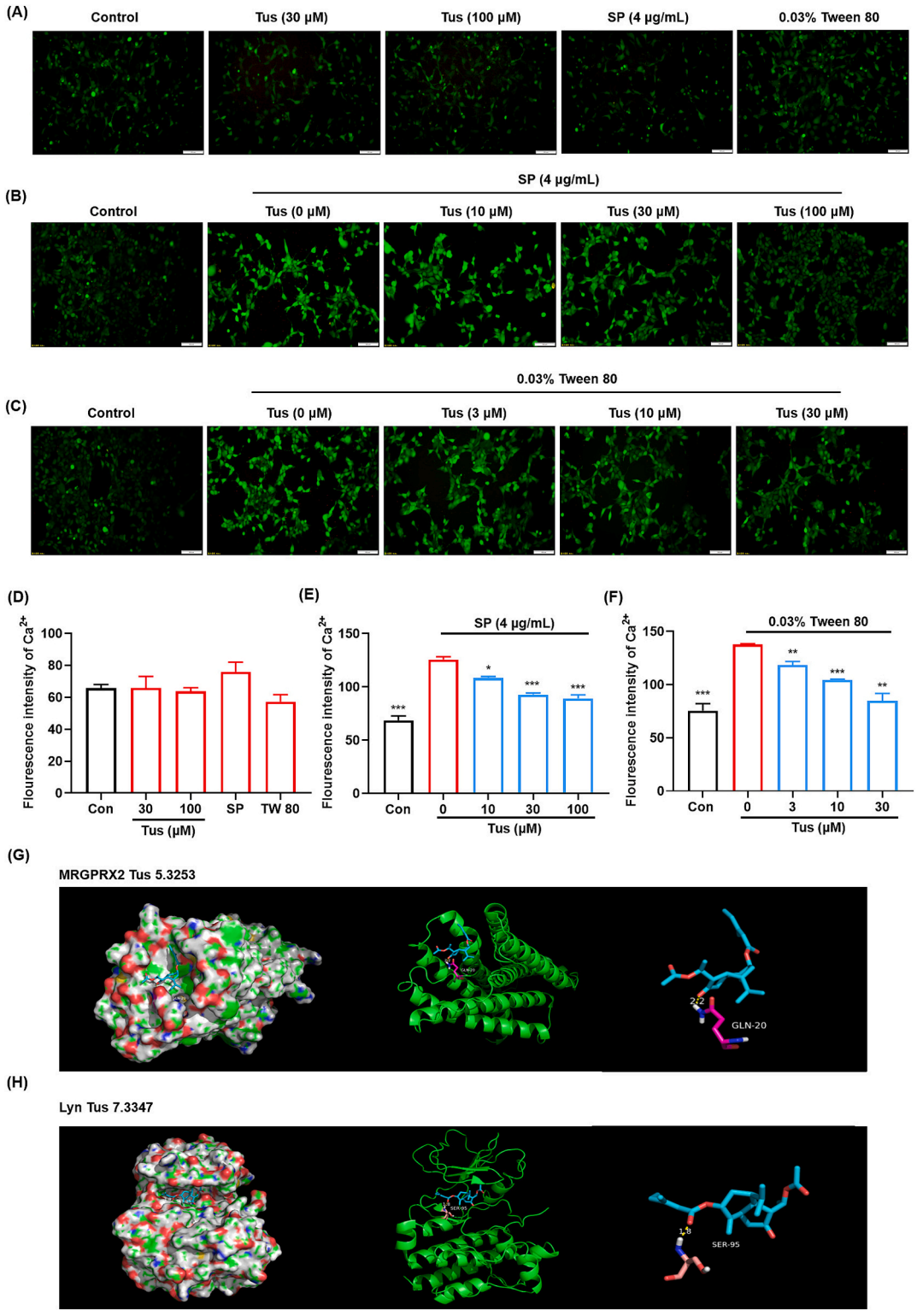

Fig. 6. The interaction of Tus with MRGPRX2 and Lyn. (A) The effect of Tus, SP and Tween 80 on the calcium concentration in NC-HEK293 cells (scale bar 100 μm, n = 3). (B) Inhibitory effect of Tus on the calcium concentration of MRGPRX2-expressing HEK293 cells induced by SP (scale bar 100 μm, n = 3). (C) Inhibitory effect of Tus on the calcium concentration of MRGPRX2-expressing HEK293 cells induced by Tween 80 (scale bar 100 μm, n = 3). (D) The quantification of the fluorescence intensity of NC-HEK293 cells with Tus, SP and Tween 80, n = 3. (E) The quantification of the fluorescence intensity of SP-sensitized MRGPRX2-expressing HEK293cells with Tus pretreatment, n = 3. (F) The quantification of the fluorescence intensity of Tween 80-sensitized MRGPRX2-expressing HEK293 cells with Tus pretreatment, n = 3. (G) Docking results of MRGPRX2 and Tus. (H) Docking results of Lyn and Tus. Values represent the mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Tus (0 μM) group.

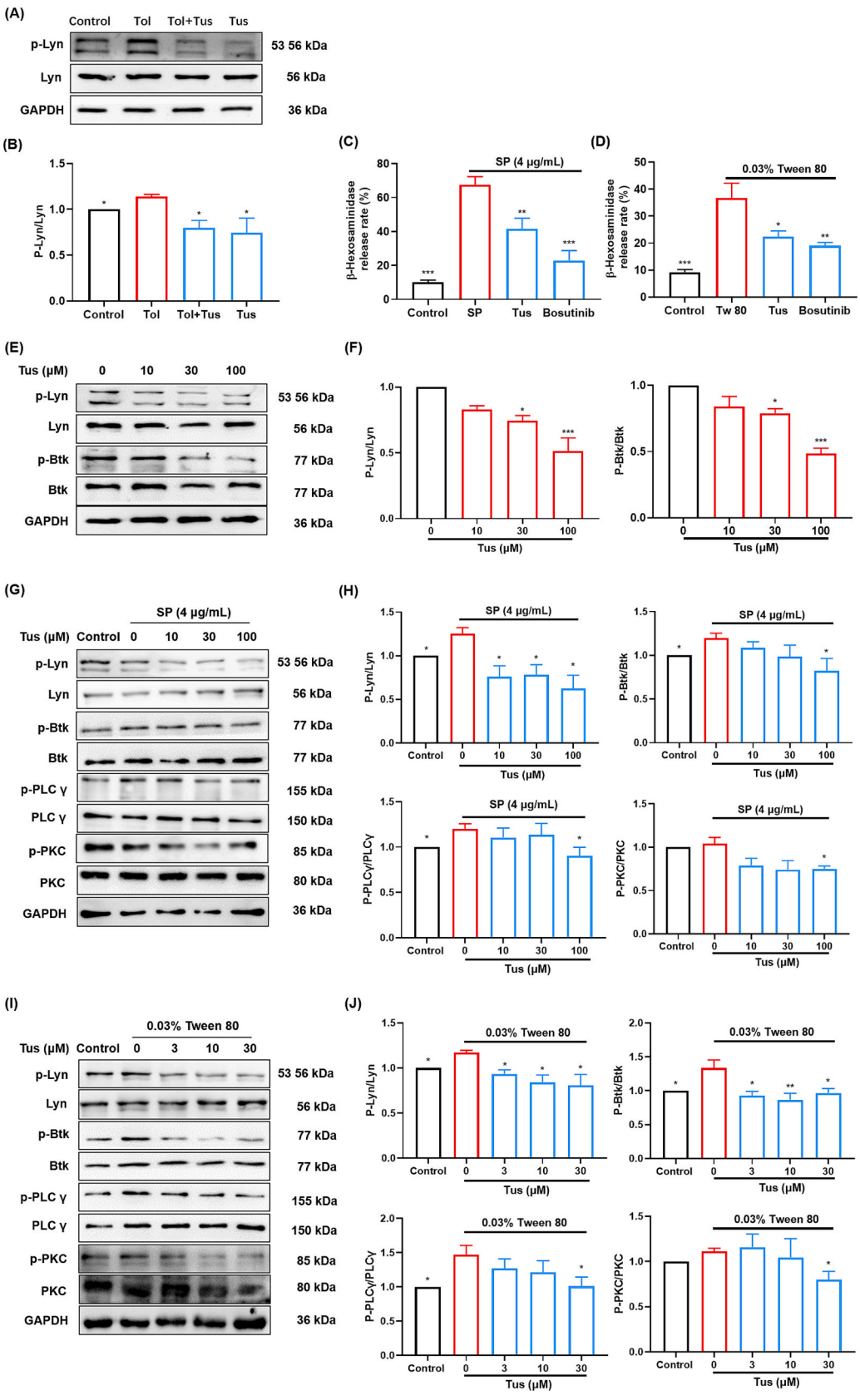

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Fig. 7. Tus decreased the Lyn/Btk/PLCγ/PKC signaling pathway. (A) Western blot analysis of the levels of Lyn, phosphorylation-Lyn in LAD2 cells treated with Tolimidone and Tus. (B) Quantification of Lyn, phosphorylation-Lyn protein expression in (A), n = 3. (C) The β-hexosaminidase release in LAD2 cells treated with 100 μM Tus or 10 μM Bosutinib combined with 4 μg/mL SP, n = 6. (D) The β-hexosaminidase release in LAD2 cells treated with 100 μM Tus or 10 μM Bosutinib combined with 0.03% Tween 80, n = 5. (E) Western blot analysis of the levels of Lyn, phosphorylation-Lyn, Btk and phosphorylation-Btk in LAD2 cells treated with 10, 30, and 100 μM Tus. (F) Quantification of Lyn, phosphorylation-Lyn, Btk, phosphorylation-Btk in (E), n = 3. (G) Western blot analysis of the levels of Lyn, phosphorylation-Lyn, Btk, phosphorylation-Btk, PLCγ, phosphorylation-PLCγ, PKC, phosphorylation-PKC in LAD2 cells treated with Tus and SP. (H) Quantification of Lyn, phosphorylation-Lyn, Btk, phosphorylation-Btk, PLCγ, phosphorylation-PLCγ, PKC, phosphorylation-PKC protein expression in (G), n = 3–6. (I) Western blot analysis of the levels of Lyn, phosphorylation-Lyn, Btk, phosphorylation-Btk, PLCγ, phosphorylation-PLCγ, PKC, phosphorylation-PKC in LAD2 cells treated with Tus and Tween 80. (J) Quantification of Lyn, phosphorylation-Lyn, Btk, phosphorylation-Btk, PLCγ, phosphorylation-PLCγ, PKC, phosphorylation-PKC protein expression in (I), n = 3–5. Values represent the mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Tus (0 μM) group.

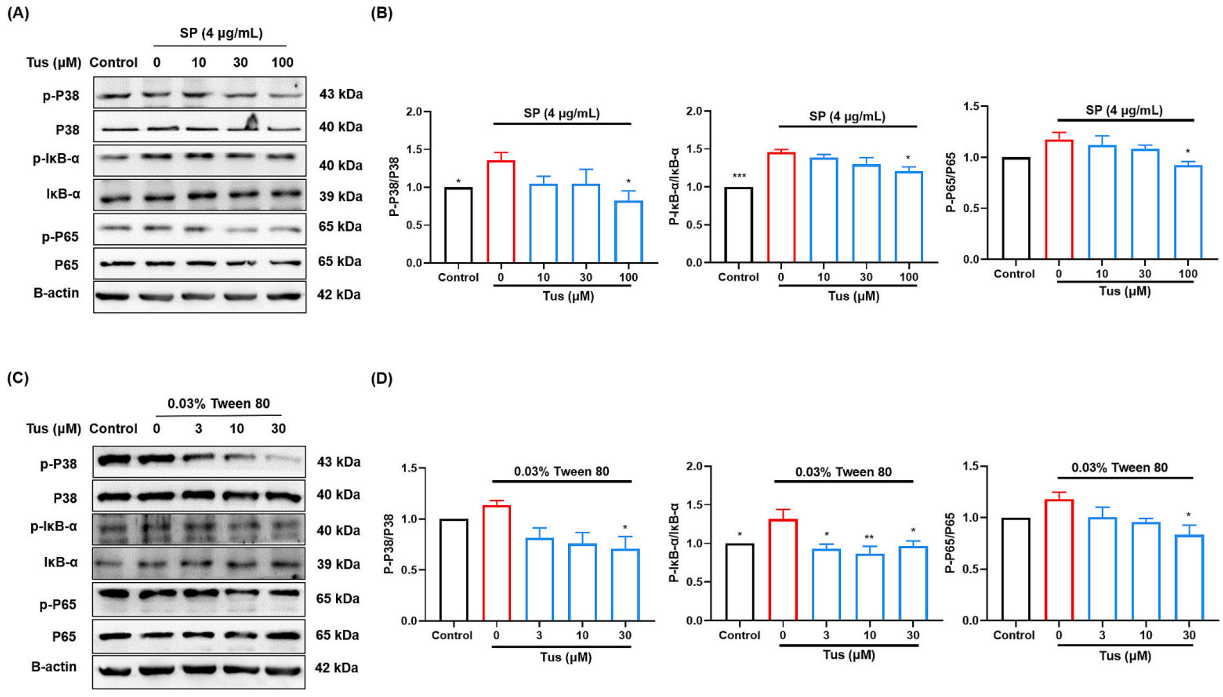

Fig. 8. Tus attenuated p38/ NF-κB (p65) of MRGPRX2 downstream signaling pathway. (A) Western blot analysis of the levels of p38, phosphorylation-p38, IκB-α, phosphorylation-IκB-α, NF-κB and phosphorylation-NF-κB in LAD2 cells treated with Tus and SP. (B) Quantification of p38, phosphorylation-p38, IκB-α, phosphorylation- IκB-α, NF-κB and phosphorylation- NF-κB in (A), n = 4–5. (C) Western blot analysis of the levels of p38, phosphorylation-p38, IκB-α, phosphorylation-IκB-α, NF-κB and phosphorylation-NF-κB in LAD2 cells treated with Tus and Tween 80. (D) Quantification of p38, phosphorylation-p38, IκB-α, phosphorylation- IκB-α, NF-κB and phosphorylation- NF-κB in (C), n = 4. Values represent the mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Tus (0 μM) group.

4. Discussion

Pseudoallergy is characterized by inflammation that occurs without involvement of the adaptive immune system, and it is triggered by the initial dose of medication (Pichler 2019). Mast cells express MRGPRX2, and its stimulation likely plays a role in pseudo-allergic reaction development(Roy et al. 2021). It is a target receptor for the activation of mast cells in pseudo-allergic reactions (McNeil et al. 2015). Furthermore, there is a need to develop safe, valid and economically viable therapeutic drugs to inhibit MRGPRX2-mediated pseudo-allergy. Some natural products have been confirmed to inhibit MRGPRX2-mediated mast cell activation, both directly and indirectly. For example, Imperatorin reduces mast cell-dependent airway inflammation through inhibition of the MRGPRX2 pathway (Wang et al. 2021). Other products have been reported to inhibit mast cell activation by inhibiting the downstream signaling of MRGPRX2. Callahan et al., showed that osthole inhibits mast cell activation by targeting activation of the calcium signaling pathway without directly affecting MRGPRX2 (Callahan et al. 2020).

Our results indicate that Tus suppressed Evans blue extravasation and vascular permeability during systemic pseudo-allergic and PCA responses, suggesting its ability to inhibit MRGPRX2 agonist-induced mast cells activity in tissues, specifically that triggered by SP and Tween 80. In vitro experiments showed that Tus concentrations up to 200 μM did not significantly affect cell viability. At lower concentrations (10–30 μM), Tus consistently inhibited mast cell degranulation. However, the reduced inhibitory efficacy observed at 100 μM compared to 30 μM suggests possible compensatory or off-target activation of alternative signaling pathways under Tween 80 stimulation. Accordingly, in subsequent assays, Tus was tested at 3, 10, and 30 μM in Tween 80-stimulated cells, and at 10, 30, and 100 μM in SP-stimulated LAD2 cells. Notably, the use of 100 μM Tus is precedented in the literature (Choi et al. 2018). In addition, Tus treatment significantly reduced LAD2 cells degranulation and the release of inflammatory factors. Furthermore, Tus significantly attenuated SP- and Tween 80-induced intracellular calcium mobilization in MRGPRX2-HEK293 cells. This assay is also a functional surrogate for MRGPRX2-dependent calcium signaling in mast cells. Molecular docking results, however, revealed that Tus exhibits a stronger binding affinity for Lyn than for MRGPRX2, suggesting that its mechanism may not involve direct interaction with MRGPRX2. Instead, it likely exerts its inhibitory effect by regulating MRGPRX2-associated signaling pathways. Therefore, it is important to clarify that "Tus inhibit MRGPRX2-mediated responses" refers to modulation of downstream signaling pathways, not direct binding to the MRGPRX2 receptor.

The findings of our study indicate that Tus binds to Lyn within a

cavity through the amino acid residue SER-95. This cavity is advantageous for the molecular design of novel Lyn-selective inhibitors (Miyano et al. 2009). Tus also exerted a significant inhibitory effect Lyn kinase, which was activated by the selective allosteric activator tolimidone. The kinase Lyn is essential for triggering both degranulation and cytokine secretion in mast cells following antigen stimulation (Conti et al. 2018). MRGPRX2 belongs to the family of G-protein coupled receptors (GPCRs) and research indicated an interaction between the neuropeptide (NEP)-GPCR signaling pathway and IGF-1R-Lyn-PI3K signaling (Sumitomo et al. 2005). Moreover, Ding et al., demonstrated that α-Linolenic acid and artemisinic acid effectively inhibit MRGPRX2-mediated degranulation and mast cell activation by suppressing the activity of Lyn kinase, showing Lyn's regulatory role in MRGPRX2-mediated pseudo-allergic reactions (Ding et al. 2022; Ding et al. 2021). Thus, Lyn likely modulates both MRGPRX2 and FcεRI signaling pathways, playing a key role in MRGPRX2-mediated pseudo-allergic responses.

Activated Lyn molecules can phosphorylate tyrosine 551 of Btk (Baba et al. 2001), which high expression is necessary for the cytokine production in response to FcεRI cross-linking in mast cells (Bernstein et al. 2024; Hata et al. 1998). In Lyn/Btk-deficient cells, mast cell activation is significantly reduced and the inhibition of the Lyn/Btk/PLCγ pathway activity led to reduced Ca^{2+} flux (Iwaki et al. 2005; Kawakami et al. 2000; Nishimoto et al. 2005). The Lyn-Syk-Btk-PLCγ-Ca^{2+} pathway is also involved in IgE-mediated anaphylaxis (Cao et al. 2020). In this study, we found that SP and Tween 80 triggered the Lyn-Syk-Btk-PLCγ-Ca^{2+} signaling pathway, independently of the IgE pathway. Tus also reduced the phosphorylation of Lyn, Btk, PLCγ and PKC. The phosphorylation of Lyn, Btk and PLCγ can induce the endoplasmic reticulum to release Ca^{2+}. The release of Ca^{2+} and the activation of PKC can further activate calcium channels on the cell membrane, leading to the influx of extracellular Ca^{2+} and an increase in intracellular Ca^{2+} concentration (Liu and Khalil 2018; Martínez-Martínez et al. 2022), thereby triggering mast cell degranulation. Calcium influx is a prerequisite for mast cell degranulation. In the present study, intracellular Ca^{2+} levels in LAD2 cells were not directly measured; therefore, we were unable to directly visualize the inhibitory effects of Tus on SP– or Tween 80–induced calcium influx in this cell model. This limitation is partially addressed by our findings that Tus significantly reduces β-hexosaminidase release and inflammatory mediator production in LAD2 cells, and suppresses the expression of key proteins involved in calcium signaling, including Lyn, Btk, and PLCγ. Taken together with the in vitro data, these results support the inference that Tus modulates MRGPRX2 agonist–induced mast cell activation through regulation of the Lyn/Btk/PLCγ signaling pathway.

Furthermore, PKC activation can stimulate both the p38 MAPK signaling pathway and the NF-κB pathway (Pu et al. 2019). p38-MAPK can modulate NF-κB-dependent transcription through activation IκB kinase (IKK) (Nie et al. 2019) Our findings also demonstrate that Tus can decrease phosphorylation of p38, IκB-α and NF-κB (P65). As downstream pathways of MRGPRX2, the p38 MAPK signaling pathway and NF-κB pathway, required in inflammation, are also involved in degranulation and the release of pro-inflammatory factors.

5. Conclusion

Tus suppresses MRGPRX2-mediated mast cell activation and pseudo-allergic reactions by inhibiting Lyn kinase activation, thereby attenuating the downstream Lyn-Btk-PLCγ-Ca^{2+} and p38/NF-κB pathways. These findings support the potential of Tus as a promising therapeutic candidate for pseudo-allergic and related inflammatory diseases.

CRediT authorship contribution statement

Ping-ping Yan: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ting-ting Huang:** Methodology, Investigation, Data curation. **Mawusse K.I. Attiogbe:** Methodology, Data curation. **Yan-ni Mi:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Yong-xiao Cao:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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All authors critically reviewed and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2026.117763.

Data availability

Data will be made available on request.

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