



Imperatorin ameliorates mast cell-mediated allergic airway inflammation by inhibiting MRGPRX2 and CamKII/ERK signaling pathway

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ABSTRACT

Background: Allergic asthma is a common inflammatory lung disease associated with complex pathogenesis. Mast cell (MC) is one of the key drivers of allergic asthma, Mas-related G protein-coupled receptor X2 (MRGPRX2) on the MC could mediate MC activation and trigger a pseudo-allergic reaction. Imperatorin (IMP), the main active compound of *Radix Angelicae Dahuricae*, has been reported to exert various pharmacological effects. In this study, we focused on the therapeutic mechanism of IMP on MRGPRX2-induced pseudo-allergy and allergic asthma.

Methods: We examined the effect of IMP on MRGPRX2 related mast cell activation in mouse peritoneal MC (MPMC), Human Laboratory of Allergic Disease 2 MCs (LAD2 cells) and *Mrgprx2*-expressing HEK293 cells. Molecular docking and Surface plasmon resonance (SPR) were taken to reveal the binding character between IMP and MRGPRX2. MRGPRX2 downstream proteins were also detected by western blotting. IgE-independent responses was evaluated by using passive cutaneous anaphylaxis (PCA) and active systemic anaphylaxis (ASA) models. The therapeutic effect of IMP on asthma was evaluated by a lung inflammation mouse model which was induced by ovalbumin (OVA).

Results: IMP was found to reduce substance P (SP) induced calcium flux and suppressed degranulation of MC. SP can promote the phosphorylation of ERK and CamKII, which regulates the synthesis of inflammatory factors such as MIP-2 and TNF- α in MC. *In vivo* assays revealed that IMP can mitigate SP-induced mouse PCA and ASA. IMP could also mitigate lung inflammation in an OVA induced mice model by inhibiting MC activation in the lung tissue. Furthermore, IMP binds well to MRGPRX2 protein. The binding constant (K_D) is $4.48 \pm 0.49 \times 10^{-7}$ M. The data suggests that IMP is a novel inhibitor of MRGPRX2 to treat allergic asthma.

1. Introduction

Asthma is a complex lung disease with an increasing global prevalence rate that affecting more than 300 million people worldwide [1]. Epidemiological studies have shown that allergic asthma accounts for around 13% of all allergic diseases [1]. It is characterized by airway hyperresponsiveness, chronic inflammation and accumulation of immune system cells, and is triggered by endogenous and environmental stimuli [2,3]. Allergic asthma can be caused by the inhalation of environmental allergens, insect stings, or the intake of allergens in food [4–7]. First-line treatments for allergic asthma include inhalation of corticosteroids, β -adrenergic agonists, and leukotriene modifiers [8,9], which control airway inflammation and relax airway smooth muscle

contraction; however, none of these therapies target the underlying pathology, but rather simply control symptoms. Furthermore, these drugs with side effects and drug resistance also cannot meet the clinical demand [10].

Mast cells (MCs) play a key role in the early stages of allergic asthma by migrating toward inflamed tissues and stimulating the contraction of bronchial smooth muscle bundles, which plays an important role in the pathophysiology of allergic asthma [11–13]. Inhaled allergens can bind to and activate specific receptors located on the plasma membranes of MCs, inducing MCs to release allergic mediators, such as histamine, interleukins, and prostaglandins, thus causing smooth muscle contraction, mucosal secretion, allergic inflammation, and inflammatory cell infiltration [14–16]. Since MCs can be activated by allergens cross-

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linking with IgE molecules and binding to the high-affinity IgE receptor (FcεRI) on the surface of MCs [17,18], they could represent a therapeutic target for treating allergic asthma; however, few drugs targeting MCs are currently available [19,20].

The Mas-related G protein-coupled receptor member X2 (MRGPRX2) is expressed in mast cells and it is one of the endogenous receptors responsible for the IgE-independent activation of human mast cell [21]. Mediate MRGPRX2 has also been reported as a potential biomarker of allergic asthma [22,23]. Since MRGPRX2 knockdown was shown to significantly reduce MC activation and pseudo-allergic drug reactions [22]; MRGPRX2 may represent a new therapeutic target for reducing allergic reactions

At present, the reagents used to treat asthma are mainly bronchospasm relieving drugs, such as β₂-receptor agonists and theophylline, or anti-inflammatory drugs which include hormones and leukotriene regulators [24]. Most of these drugs reveal several adverse reactions with long-term using [25]. There are a variety of antibody drugs, such as Reslizumab, Dupilumab and Omalizumab, that have significant effects in the treatment of severe asthma [26,27]. However, the financial burden on patients is heavy because of the high price of these medications [26,28]. Traditional Chinese medicine (TCM) has a long history of use in China, and *Astragalus propinquus* (also known as Huangqi) and *Ephedra sinica* (Chinese ephedra, also known as Mahuang) have been widely used in the treatment of asthma [29,30]. At the same time, botanicals are also important resources for the development of new, low-toxic drugs [31]. *Angelica Dahuricae radix*, a perennial herb belonging to the family Apiaceae, has been commonly used as a herbal, anti-inflammatory medicine for the treatment of respiratory diseases [32]. Imperatorin (IMP, 9-(3-methylbut-2-enyloxy)-7H-furo[3,2-g] chromen-7-one) is one of the active furocoumarins in *Angelica Dahuricae radix*. IMP is reported to have a variety of pharmacological activities, such as anti-inflammatory, antibacterial, antihypertensive, labor pain relief, and allergy relief [33–35]. Previous studies have shown that IMP inhibits IgE/antigen-induced bone marrow-derived MC activation *in vitro* [36]. IMP also exerts anti-inflammatory effects in inflammatory lung injury and an allergic rhinitis model [33]. Therefore, we believe that IMP may have a therapeutic effect on allergic diseases and may provide a safer and economical alternative in the treatment of allergic disease. Whether IMP can be used to treat allergic asthma through MRGPRX2 is unclear.

2. Materials and methods

2.1. Materials

Substance P (SP; purity ≥ 98%) was obtained from Aladdin Reagent Co. (Shanghai, China). IMP (purity ≥ 98%) was obtained from Puifeide Biotech (Chengdu, China). Dexamethasone (purity ≥ 98%), acetylcholine chloride (Ach, purity ≥ 99%), and mass spectrometry-grade formic acid and ovalbumin (OVA; grade V, purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3, AM ester, and pluronic F-127 were obtained from Biotium (Fremont, CA, USA). The Proteome Profiler Human Phospho-Kinase Array kit was purchased from R&D Systems (Minneapolis, MN, USA). The following antibodies were used in this study: GAPDH (Proteintech, Wuhan, China); STAT3, P-STAT3 (Ser727, AF3924), p70S6K, and CamKII (Abcam, Cambridge, UK); CREB, P-CREB (S133), ERK1/2, P-ERK1/2(T202/Y204, Y185/Y187), P-MSK1/2, and P-PYK2 (Y402, MAB6210-SP; R&D Systems, MN, USA); and P-p70S6K (T389/412) and P-CamKII (T286) (Cell Signaling Technology, Danvers, USA). All secondary antibodies were purchased from Proteintech (Wuhan, China). ELISA kits were provided by Tiangen Biotech (Beijing, China). HPLC-grade methanol and acetonitrile were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). The human MRGPRX2 ELISA kit was provided by Mybiosource, Inc (San Diego, CA, USA).

2.2. Mice

C57BL/6 (wild-type; WT) mice were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). All animals were housed at the Experimental Animal Center of Xi'an Jiaotong University and provided with food and water ad libitum. All experiments requiring identical treatment administration to the animals were conducted by investigators blinded to the conditions.

2.3. Cell lines

Human Laboratory of Allergic Disease 2 MCs (LAD2 cells) were provided by Dr. A. Kirshenbaum and Dr. D. Metcalfe (NIH, Bethesda, MD, USA). HEK293 cells were provided by Prof. Xu Li (Xian Jiaotong University, Xi'an, China). LAD2 cells were maintained in StemPro-34 medium supplemented with 10 mL/L of StemPro nutrient supplement, 1:100 penicillin–streptomycin, 2 mM L-glutamine, and 100 ng/mL of human stem cell factor at 37 °C in a 5% CO₂ atmosphere. The medium was replaced every 2 weeks, and cells were maintained at 2 × 10⁶ cells/mL. Human MRGPRX2-expressing HEK293 cells were constructed using HIV-1-based lentiviral vectors and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

2.4. Mouse peritoneal MC (MPMC) purification

Eight-week-old adult male C57BL/6 mice were sacrificed by CO₂ inhalation and subjected to two sequential peritoneal lavages with 12 mL of ice-cold MC dissociation medium (MCDM: 1.5 mL of 10 × HBSS, 450 μL fetal bovine serum, 150 μL of 1 M HEPES, and 12.9 mL sterile water for a total volume of 15 mL [pH, 7.2]). MPMCs were separated by centrifugation at 200 × g for 10 min at 4 °C, re-suspended in 2 mL of MCDM, and overlaid on 4 mL isotonic 70% Percoll suspension (2.8 mL Percoll, 320 μL of 10 × HBSS, 40 μL of 1 M HEPES, and 830 μL of MCDM). After centrifugation at 500 × g for 20 min at 4 °C, MCs were re-suspended, transferred to a new tube, and diluted with MCDM at twice the volume of the suspension. The solution was mixed and centrifuged at 200 × g for 10 min at 4 °C. MCs were retrieved from the pellet with >95% purity, as confirmed by morphological analysis, re-suspended in DMEM with 100 ng/mL of recombinant mouse stem cell factor, and seeded into 96-well plates.

2.5. Intracellular calcium ion (Ca²⁺) mobilization assay *in vitro*

MPMC, LAD2, or MRGPRX2-HEK293 cells were plated in a 96-well plate (1 × 10⁴ cells/well) with 100 μL of DMEM and incubated for 24 h at 37 °C. Various IMP doses were added to each well and incubated for 1 h. All drugs were diluted with calcium imaging buffer (CIB: 125 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO₃, and 20 mM sucrose, with the pH adjusted to 7.4 using NaOH). Cells were washed twice with CIB and loaded with the incubation buffer (3.5 mM Fluo-3 AM, 0.1% [w/v] F-127, diluted with CIB) at 37 °C for 30 min. Cells were washed twice with CIB and imaged immediately. Two hundred images of the cells were captured using blue light (1 s per image) under an inverted fluorescence microscope (Nikon, Ti-U, Japan). Cells were identified as responding if [Ca²⁺]_i increased by at least 50% after drugs were added.

2.6. β-hexosaminidase assay *in vitro*

LAD2 cells (2 × 10⁴/well) were incubated with IMP (25, 50, or 100 μM) for 1 h, followed by incubation with SP (4 μg/mL) for 30 min at 37 °C. The supernatants were collected, and cells were lysed with 0.1% Triton X-100 in TM buffer. β-hexosaminidase released into the supernatant and in cell lysates was quantified by 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 adding a stop

buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11) and the percentage of β -hexosaminidase released was assessed by measuring the absorption of the samples at 405 nm using a microplate spectrophotometer and calculated as the absorbance of the culture supernatant at 405 nm $\times$ 100/absorbance of the total cell lysate supernatant at 405 nm.

2.7. Histamine release assay *in vitro*

LAD2 cells were seeded in 96-well plates (2×10^4 /well) and incubated overnight at 37 °C with 5% CO₂. After the culture medium had been removed, KN93, MEK162, or various IMP doses were added at the indicated concentrations for 1 h, followed by SP stimulation for 30 min. The 96-well plate was centrifuged at $200 \times g$ for 5 min at 4 °C and subjected to LCESI-MS/MS using an LCMS 8040 mass spectrometer (Shimadzu Corp., Kyoto, Japan). Histamine was evaluated using an HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 μ m; Agela Technologies, Tianjin, China) and isocratic elution with acetonitrile–water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 mL/min.

2.8. Mouse passive cutaneous anaphylaxis (PCA) assay

Adult male mice were randomly divided into groups and a vehicle, 3 mg/kg, 6 mg/kg or 12 mg/kg IMP was orally administrated. After 1 h, 0.2 mL of Evans blue dye (0.4%) solution was injected into the tail vein of the mouse. The thickness of the paw was measured using a vernier caliper. Five minutes later, substance P (SP) (30 μ g/mL) was injected into the left paw with a microinjector. An equal volume of PBS was injected into the right paw as a negative control, and paw thickness was measured 15 min after the injection. Mice were sacrificed, the paw tissues were collected and dried for 24 h at 50 °C, then cut into pieces and weighed. Evans blue was extracted by adding 450 μ L acetone-saline (7:3) mixture at 37 °C for 12 h. The supernatant was collected into 96-well plate (200 μ L/well) and the optical density (OD) was measured at 620 nm with a spectrophotometer (Bio-Rad, CA, USA).

Another group of mice were given the same procedure but not injected with Evan's blue solution. Mice were sacrificed 15 min after the paw challenge, paw skin was taken for H&E staining, and the severity of blood vessel swelling and exudation were observed under the light microscope.

2.9. Inflammatory factor secretion *in vivo*

Systemic hypersensitivity occurs when exposed to large doses of allergenic substances. SP was injected through the tail vein to simulate active systemic allergy (ASA). Similarly, adult male mice were randomly divided into the following groups (six mice per group): control and experimental groups (3 mg/kg, 6 mg/kg, 12 mg/kg IMP orally administrated). Mice were injected with SP (30 μ g/mL) 30 min after gavage. Blood was collected for histamine detection 2 h after challenge, and for MIP-2, CCL-2 and TNF- α detection, blood was collected 6 h after SP challenge. Mouse TNF- α , CCL-2, and MIP-2 ELISA Kits were purchased from Sino Biological Inc. (Beijing, China) and TNF- α , CCL-2, and MIP-2 concentrations in the blood were detected with ELISA kits according to the manufacturer's instructions.

2.10. Western blot analysis

CamKII, STAT3, ERK, CREB, MSK1/2, and P70S6K phosphorylation levels were measured in LAD2 cells by western blot analysis, as described previously [37].

2.11. Molecular docking assay

The molecular docking assay was carried out using the Surflex-DockMode of SYBYL-X 2.0 program package (Tripos, St.Louis, MO,

USA). Possible tertiary structures for MRGPRX2 were predicted using I-TASSER: <http://zhanglab.ccmb.med.umich.edu/I-TASSER/>. We used the predicted MRGPRX2 structure for the docking assay of with IMP and MRGPRX2.

2.12. Surface plasmon resonance (SPR) assay

To assess SPR, MRGPRX2 protein with a 6*his tag (30 μ g/mL) was fixed on a carboxyl sensor chip (Nicoya Lifesciences, Waterloo, Canada) by capture-coupling, and IMP (0.38, 0.75, 1.5, or 3 μ M) was sequentially injected into the chamber in PBS running buffer. Interactions between MRGPRX2 and the fixed small molecules were detected using Open SPRTM (Nicoya Lifesciences, Waterloo, Canada) at 25 °C. Binding time and disassociation time were both 250 s, the flow rate was 20 μ L/s, and the chip was regenerated using hydrochloric acid (pH 2.0). A one-to-one diffusion-corrected model was fitted to the wavelength shifts corresponding to the varying drug concentrations. Data were retrieved and analyzed using TraceDrawer (Reichert Technologies Life Sciences, Newyork, USA).

2.13. Induction of allergic inflammation and airway hyper-reactivity (AHR) in mice

Mice were sensitized on days 1, 3, 5, and 7 by intraperitoneal injection with 200 μ L of vehicle (0.1 M phosphate buffered saline (PBS)) or OVA (50 μ g) and challenged with atomized vehicle (PBS, 4 mL) or OVA (800 μ g) on days 22, 24, 26, and 28. Airway inflammation and AHR were assessed 24 h after the last atomized challenge. IMP (6 mg/kg in 0.5% carboxymethylcellulose sodium; CMC-Na) or the same volume of 0.5% CMC-Na was intragastrically administered 1 h before SP challenge.

2.14. Measurement of airway reactivity

Mice were anesthetized, ventilated, and baseline lung function was measured using a flexiVent apparatus (SCIREQ, Quebec, Canada), as described previously [38]. Mice were exposed to Acetylcholine (Ach), and Newtonian resistance (Rn), respiratory resistance (Rrs), tissue damping (G), and respiratory compliance (Crs) were determined using the flexiVent software.

2.15. Bronchoalveolar lavage fluid (BALF) collection

BALF was collected by lavaging the lungs of the mice twice with saline (0.75 mL). Cells were collected by means of centrifugation ($200 \times g$) and were resuspended in 200 μ L of PBS. Total cell numbers were determined, cytospin specimens were prepared and stained with Diff-Quik Stain, and the proportions of different cell types (eosinophils, neutrophils, and lymphocytes) were quantified by counting at least 200 cells per cytospin preparation. The supernatants from BAL samples were collected by means of centrifugation ($200 \times g$).

2.16. Cytokine and chemokine measurements

ELISA kits were used to measure the levels of mice macrophage inflammatory protein 1 β (MIP-1 β), C-C motif chemokine ligand 2 (CCL-2), tumor necrosis factor α (TNF- α), interleukin 4 (IL-4), IL-5, and IL-13 in BALF and human IL-8 and TNF- α in LAD2 cell supernatants, according to the manufacturer's instructions.

2.17. Lung histology

Mouse lungs were inflated via the trachea, removed, and fixed in 10% neutral buffered formalin. Formalin-fixed tissues were embedded in paraffin, and 5 μ m-thick sections were stained with hematoxylin and eosin (H&E), Masson, or periodic acid–Schiff (PAS) to quantify the number of infiltrated cells, mucus production, and collagen deposition,

respectively. Inflammatory cells, PAS-positive bronchial epithelial cells, and collagen deposition area were quantified as described previously [39]. To identify MCs, paraffin-embedded lung sections were stained with FITC-avidin. Non-degranulated MCs contained characteristic granules and de-granulated MCs were characterized by the appearance of extracellular green fluorescence granules and lower levels of condensed intracellular granules.

2.18. Statistical analysis

Quantitative data were analyzed using Student's *t*-test (to compare two groups) and Analysis of Variance (ANOVA, to compare three or more groups). *P* values of < 0.05 were considered statistically different.

2.19. Study approval

All animal experiments were conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mouse experimental protocols were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019-711).

3. Results

3.1. IMP-inhibited MRGPRX2 induced cell activation

SP is known to increase MC Ca^{2+} flux and activate MCs via

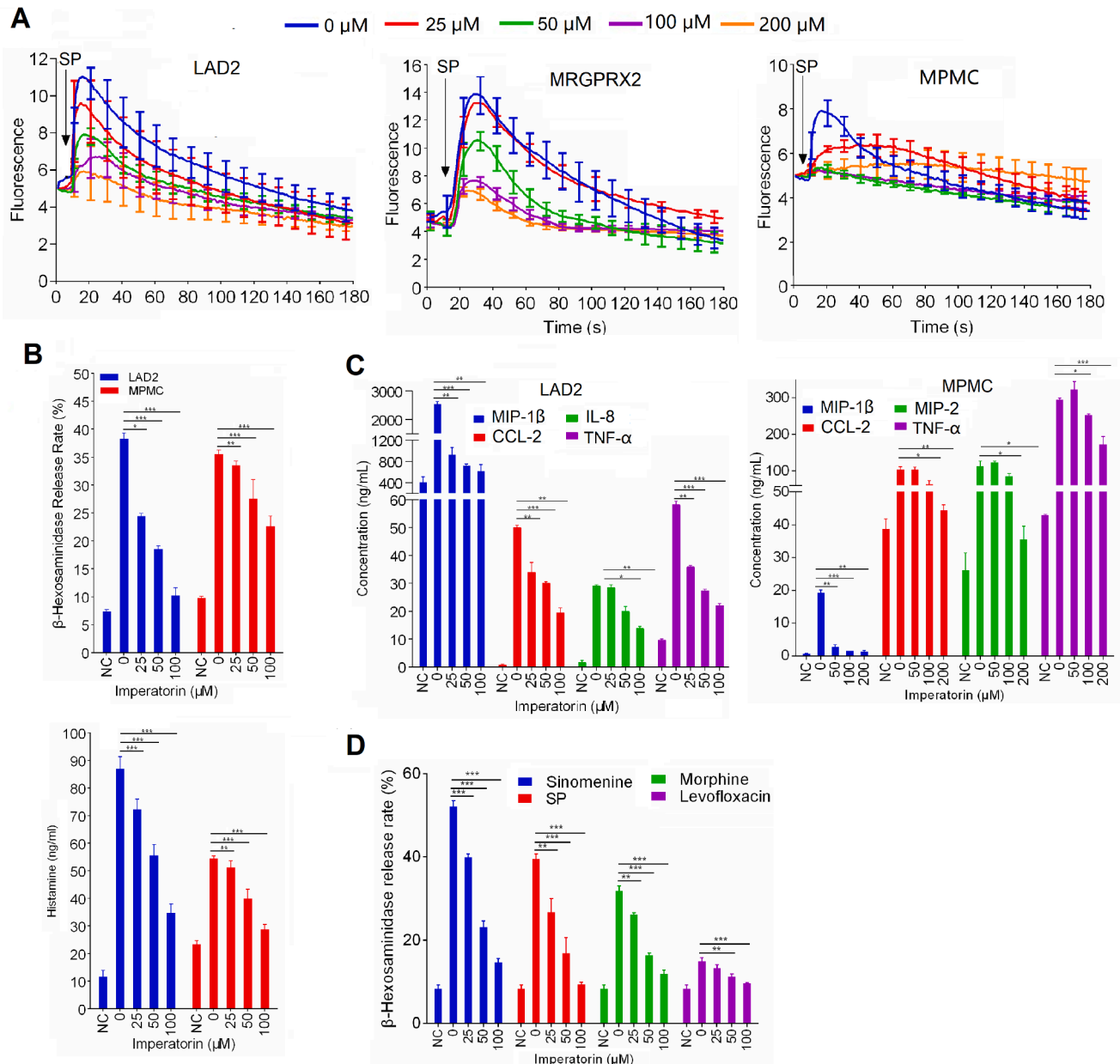


Fig. 1. Imperatorin (IMP) inhibits Substance P (SP)-induced cell activation *in vitro*. (A) Calcium (Ca^{2+}) flux change in Human Laboratory of Allergic Disease 2 MCs (LAD2 cells), Mas related G protein-coupled receptor X2 (MRGPRX2)-expressing HEK293 cells, and mouse peritoneal mast cells (MPMCs) with various doses of IMP after SP stimulation. (B) and (C) LAD2 cells and MPMC cells were treated with different IMP concentrations and stimulated with SP (4 $\mu\text{g/mL}$). Levels of β -hexosaminidase, histamine, and cytokine (MIP-1 β , TNF- α , CCL-2, and IL-8 or MIP-2) were detected. Data are presented as mean $\pm$ standard deviation (SD). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to the normal control (NC) (*n* = 5). (D) LAD2 cells were pre-incubated with different IMP doses for 30 min and stimulated with several agonists of MRGPRX2 for 30 min. Levels of β -hexosaminidase were detected. ***P* < 0.01, ****P* < 0.001. Data are presented as mean $\pm$ SD (*n* = 5).

MRGPRX2[40]; therefore, we sought to determine the effect of IMP on MC activation. We incubated LAD2 cells, mouse peritoneal mast cells (MPMCs), and HEK293 cells expressing high MRGPRX2 levels with IMP for 1 h before stimulating them with SP. IMP dose-dependently decreased Ca^{2+} levels in LAD2, MRGPRX2 expressed HEK293T and MPMC cells (Fig. 1A), suggesting that IMP inhibits SP-induced MC activation. Since degranulation is a hallmark of activated MCs, we detected the release of β -hexosaminidase, histamine, and inflammatory factors (such as MIP-1 β , CCL-2, IL-8, and TNF- α) in LAD2 cells and MPMCs. IMP significantly decreased the degranulation of LAD2 cells and MPMCs, as well as MIP-1 β , CCL-2, IL-8, and TNF- α levels (Fig. 1B, C), indicating that IMP may inhibit SP-induced MC activation via MRGPRX2. To confirm the inhibitory effect of IMP, we used three other reported MRGPRX2 agonists [22,41], finding that IMP dose-dependently inhibited the MC degranulation induced by those agonists (Fig. 1D). These results demonstrate that IMP displays good anti-pseudo-allergy effects related to MRGPRX2.

3.2. IMP inhibits SP induced pseudo-allergy

To test whether IMP could modulate SP-induced skin inflammation

in vivo, we investigated the effect of IMP on responses during passive cutaneous anaphylaxis (PCA). In a mouse model of SP-induced skin inflammation, mice were intragastrically administrated with IMP. Thirty minutes later, Evan's blue was injected into the tail and SP was subcutaneously injected into the paws to investigate the anti-allergic effect of IMP. IMP application dose-dependently suppressed PCA (Fig. 2A–C). The paw skin tissue was also collected and H&E staining was taken to show the vessel hemangiectasis (Fig. 2D). Prominent dilated vessels were observed after treatment with SP, which was prevented by IMP treatment (Fig. 2D). For the active systemic anaphylaxis (ASA) assay, mouse serum was collected to detect the cytokine concentration changes with IMP treatment. The serum histamine, IL-8, CCL-2, and TNF- α were increased remarkably after SP stimulation; while mice were orally administered IMP, the histamine, IL-8, CCL-2, and TNF- α level in the serum were decreased obviously after SP stimulation in a dose dependent manner (Fig. 2E).

3.3. IMP inhibits MRGPRX2-related LAD2 activation via the CamKII/ERK pathway

To elucidate the downstream pathways through which IMP inhibits

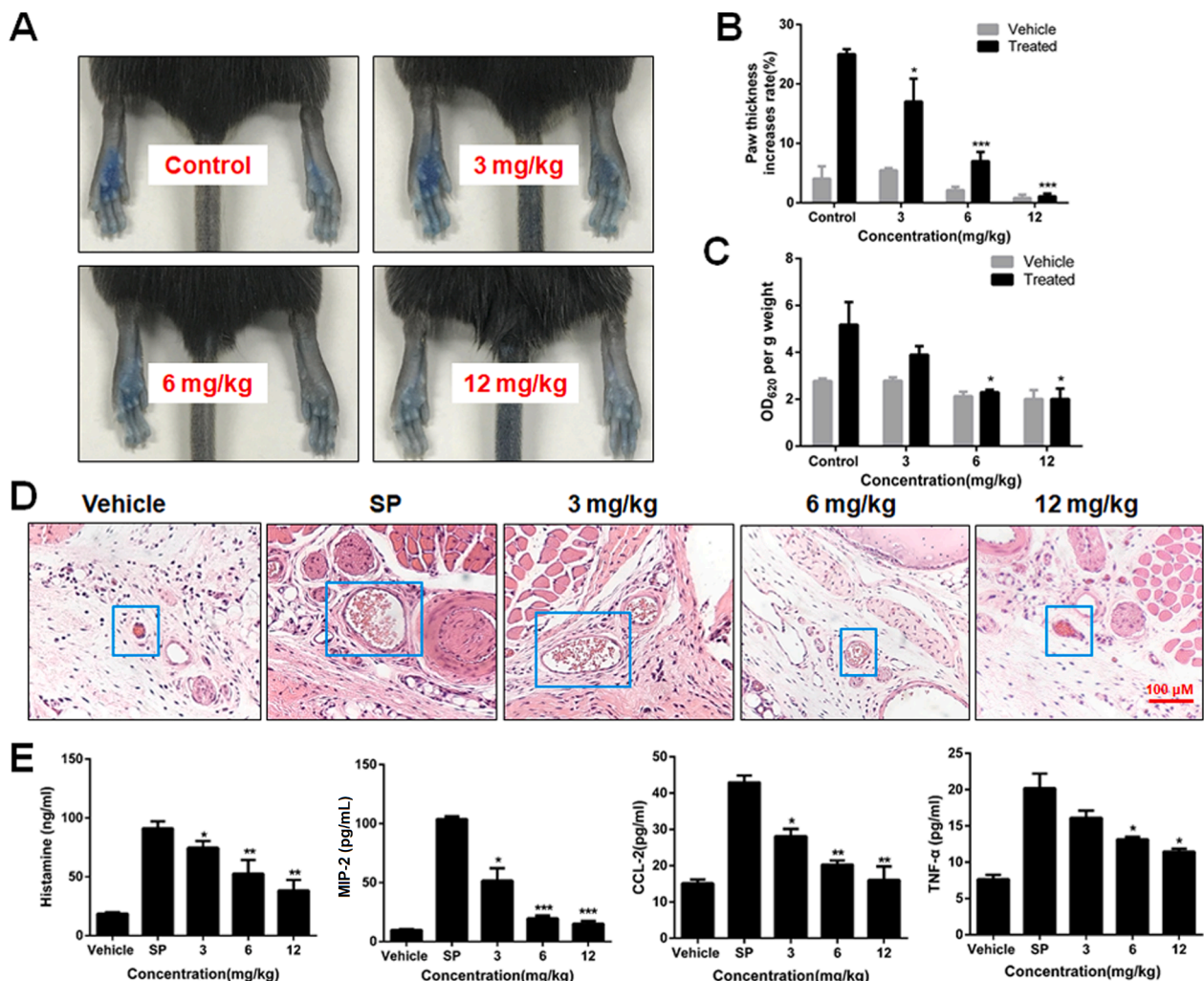


Fig. 2. Imperatorin (IMP) suppresses Substance P (SP)-induced passive cutaneous anaphylaxis (PCA) and active systemic anaphylaxis (ASA) in mice. (A) Evan's Blue leakage into the paw; left paw was injected with SP, while the right paw was injected with the vehicle. (B) and (C), Evan's blue leakage into the paw; paw thickness was analyzed 15 min after injection. (D) IMP inhibited SP-induced hemangiectasis showed by hematoxylin and eosin (H&E) stains. The part in the light blue box is the representative hemangiectasis in the picture. (E) For the ASA assay, mouse serum was collected to detect histamine, IL-8, CCL-2 and TNF- α levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean $\pm$ SD ($n = 5$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MC activation via MRGPRX2, the phosphorylation level of several proteins was detected by western blot. SP upregulated p70S6K, ERK1/2, CREB (S133), PRAS40 (T246), STAT3 (S727) and MSK1/2 phosphorylation levels (Fig. 3A); however, this effect was significantly reduced upon pretreatment with IMP. STAT3 and p70S6K phosphorylations have been reported to induce MC degranulation, and ERK is known to positively regulate these kinases. Since MRGPRX2 activation mobilizes cellular Ca^{2+} that may activate calmodulins (such as CamKII, which is known to regulate ERK phosphorylation), we used KN93 (CamKII inhibitor) and MEK162 (an ERK pathway inhibitor) to evaluate whether CamKII and ERK participate in MRGPRX2 downstream pathways. Both inhibitors prevented LAD2 activation (Fig. 3B) and cell degranulation, suggesting that SP significantly upregulates CamKII α phosphorylation, whereas IMP can effectively inhibit this effect (Fig. 3C). We further evaluated the relationship between CamKII and ERK, finding that when CamKII was inhibited by KN-93, ERK phosphorylation almost completely disappeared, followed by the loss of MSK1/2, p70S6K, and STAT3 phosphorylation downstream (Fig. 3D). In addition, CamKII phosphorylation was also downregulated when ERK was inhibited by MEK162 (Fig. 3E). Together, these results indicate that the CamKII/ERK pathway participates in signaling downstream of MRGPRX2 and that IMP inhibits the MRGPRX2-related MC activation via the CamKII/ERK pathway.

3.4. The binding character of IMP and MRGPRX2

Since MRGPRX2 participates in asthma occurrence and

development, we screened numerous compounds from traditional Chinese medicine and found that IMP is a potential MRGPRX2 inhibitor. Therefore, five possible tertiary structures for MRGPRX2 were predicted using I-TASSER (Fig. 4A), and SYBYL 2.0 software was used to molecularly dock known compounds reported to act against the MRGPRX2 receptor with these five possible structures. Regression curves were produced using the pEC₅₀ values and docking scores of each compound, indicating Model 3 as the optimal model; therefore, Model 3 was used for subsequent molecular docking (Fig. 4B). The sphere space field model revealed that IMP had a good fit within the active cavity of MRGPRX2, with the furan oxygen in IMP forming a critical hydrogen bond by binding to GLN181 at a distance of 1.99 Å (Fig. 4C). Furthermore, we used SPR to confirm the binding between IMP and MRGPRX2, revealing a binding constant (K_D) of $4.48 \pm 0.49 \times 10^{-7}$ M (Fig. 4D).

3.5. Therapeutic effect of IMP in a mouse model of lung inflammation

To investigate the anti-allergic asthma effect of IMP *in vivo*, we used OVA-induction models with an IMP dosing regimen (Fig. 5A). Mouse BALF were collected, we measured changes of total cell levels in the BALF to determine the effect of IMP on lung inflammation. OVA challenge significantly induced the infiltration of inflammatory cells into BALF compared with the vehicle group (Fig. 5B). Treatment with IMP markedly reduced numbers of eosinophils, neutrophils, and total cells compared with the OVA group. The Rn, Rrs, and G values all increased and Crs decreased in both vehicle groups, whereas IMP treatment significantly improved airway resistance and airway compliance.

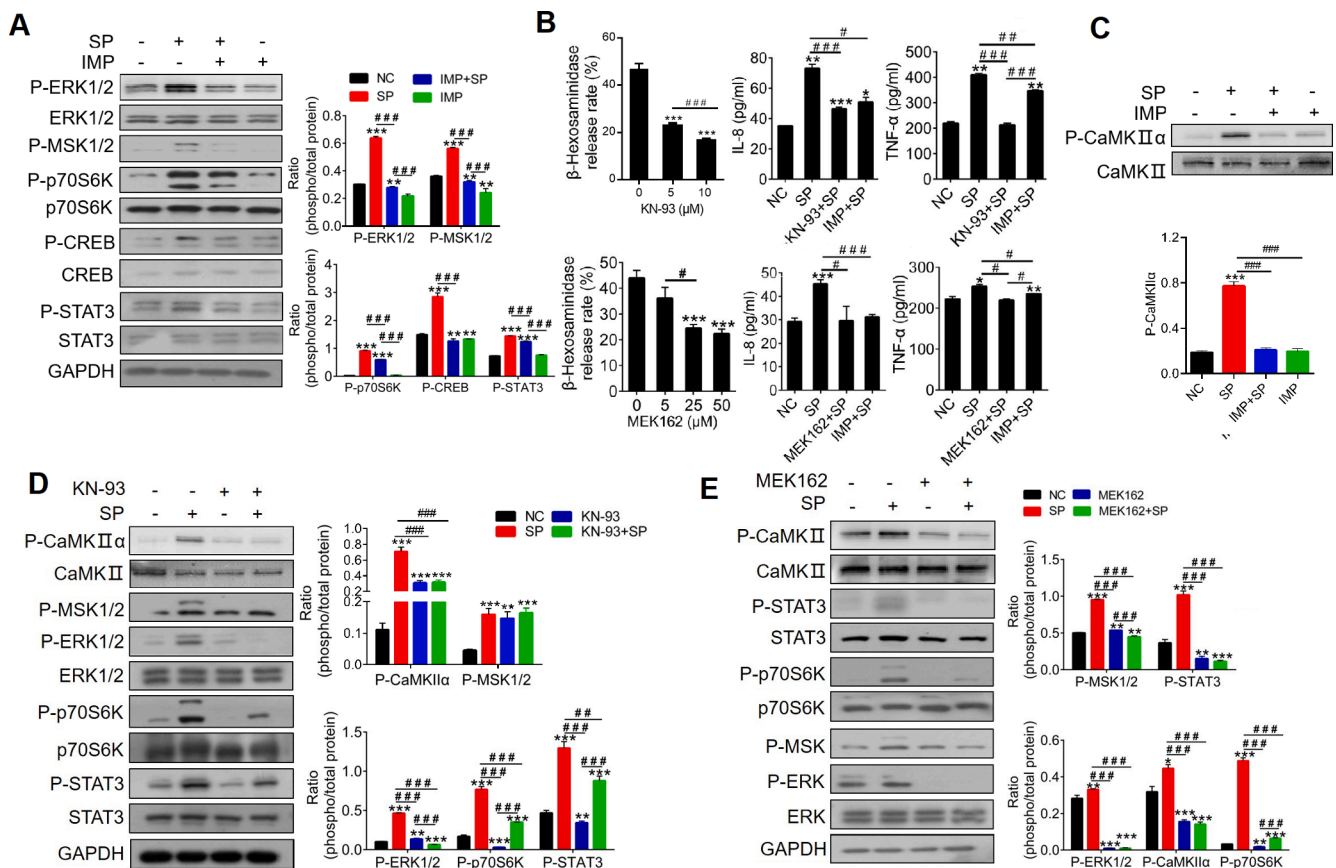


Fig. 3. Imperatorin (IMP) inhibits MRGPRX2-related mast cell (MC) activation via the CamKII/ERK pathway. (A) Kinase phosphorylation after Substance P (SP) stimulation and IMP treatment in LAD2 cells ($n = 3$). (B) Western blotting band quantitation data for P-ERK, P-MSK, P-CREB, and P-STAT3 after SP stimulation and IMP treatment in LAD2 cells ($n = 3$). (C) P-CaMKII α level changes after treatment with IMP and SP stimulation ($n = 3$). (D) SP-induced LAD2 degranulation was inhibited by both KN-93 and MEK162 ($n = 3$). (E) KN-93 inhibits the ERK pathway in SP-induced LAD2 activation ($n = 3$). (F) MEK162 inhibits the CamKII pathway in SP-induced LAD2 activation ($n = 3$). Data are presented as mean $\pm$ standard deviation (SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the related group.

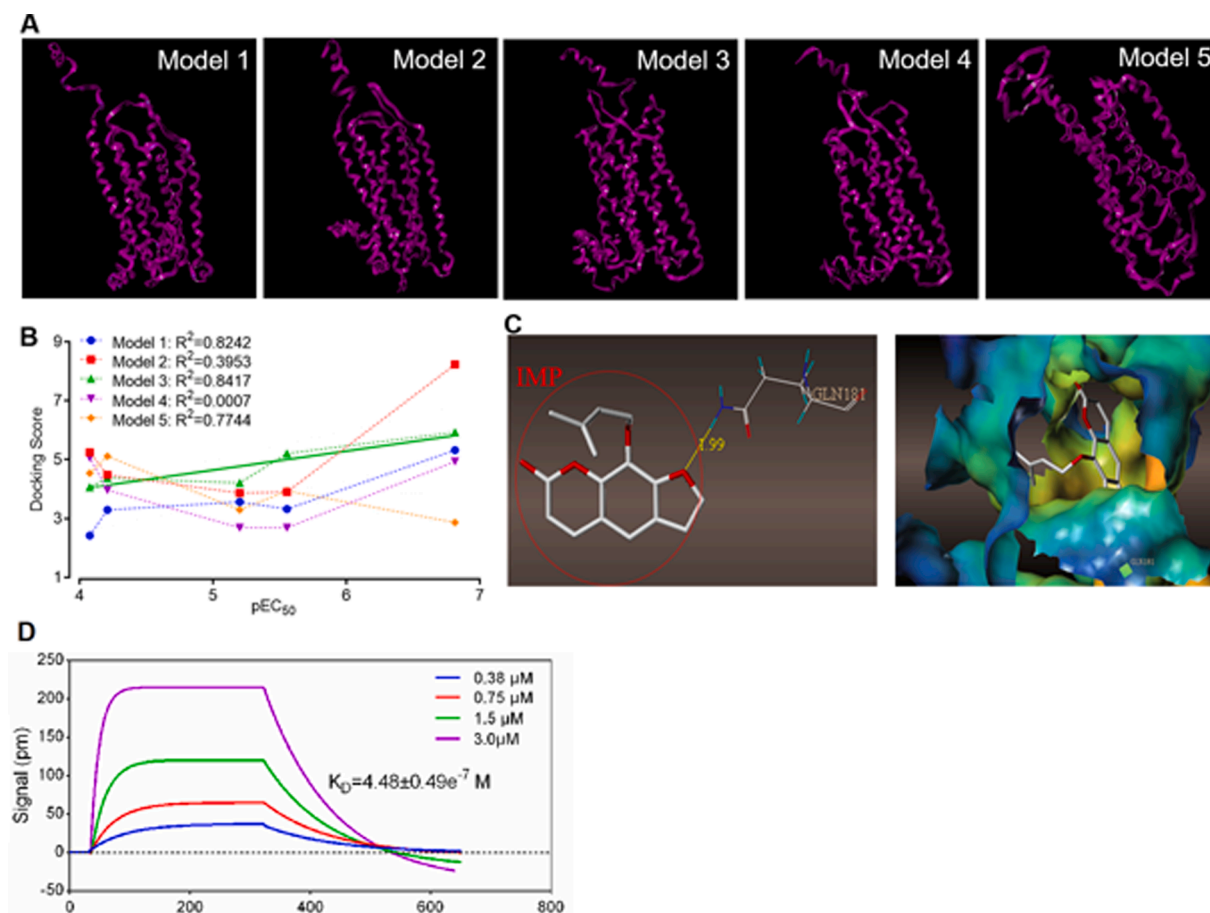


Fig. 4. The binding characters between Imperatorin (IMP) and MRGPRX2. (A) Five possible tertiary structures of MRGPRX2. (B) Correlation analysis of the activity of five agonists (pEC_{50}) and docking scores for five possible tertiary structures of MRGPRX2. The five agonists are substance P, sinomenine, morphine, levofloxacin and ofloxacin, the green straight line is the linear regression curve of the pEC_{50} of the agonists and Model 3. (C) The molecular modelling for IMP with MRGPRX2 Model 3. The left figure is the binding interactions model. IMP structure is shown in the red circle. The oxygen of furan in IMP form a critical hydrogen bond by binding with GLN181 of MRGPRX2 with distances of 1.99 Å. The right figure is the cavity model of IMP and MRGPRX2. (E) Surface plasmon resonance analysis of IMP and MRGPRX2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, the therapeutic effect improved as the ACh concentration increased (Fig. 5C). Next, we stained lung tissue with H&E, PAS, Masson, and FITC-avidin, finding that IMP significantly reduced lung tissue inflammation in both OVA-challenged and SP-induced mice with asthma, including in terms of inflammatory infiltration of lung tissue, mucus secretion, collagen content, and the degranulated MC ratio (Fig. 5D). In addition, IMP significantly inhibited chemokine and cytokine levels in BALF, including pulmonary allergic inflammation-associated IL-4 and IL-13, eosinophil-associated IL-5, and TNF- α , which are involved in regulating the interaction between MCs and airway smooth muscle cells (Fig. 5E) and contribute to the relief of lung inflammation.

4. Discussion

Allergic asthma, a chronic respiratory disease, resembles a complex syndrome with many pathological features involving MCs, lymphocytes and many other kinds of immune cells [42]. MCs release various inflammation mediators which act on smooth muscle, the vasculature, and inflammatory cells to induce immune cell inflammation and tissue remodeling in asthma patients. In this study, we aimed to elucidate a possible mechanism for the beneficial effects of IMP on mast cell associated lung inflammation. We evaluated the therapeutic effect of IMP, an inhibitor of MRGPRX2, on allergic asthma by inhibiting MC activation via MRGPRX2 through ERK/CamKII pathway.

MC is one of the important effector cells in allergic asthma.

Therefore, for our *in vitro* study, we used LAD2 cell and MPMC to study the protective effect of IMP. Our findings revealed that IMP, one of the main active ingredients in *Angelicae dahuricae radix*, can alleviate allergic inflammation and IgE-independent allergy both *in vitro* and *in vivo*. SP is reported to be an agonist of MRGPRX2 to activate MC activation. In our study, IMP inhibited SP induced MC activation and degranulation dose dependently, we used we used an MRGPRX2 expressing HEK293T cells to further confirm its anti-allergy effect is related to MRGPRX2. IMP inhibited SP induced cell Ca^{2+} influx increasing.

A previous study has reported that MC MRGPRX2 is the key receptor of pseudo-allergy, and serum MRGPRX2 is a new marker for asthma [43]. The ERK pathway is involved in the downstream pathway of MRGPRX2 activation [44]. In our study, SP induced ERK phosphorylation in LAD2 cells, after the cells were pre-incubated with IMP, the phosphorylation of ERK was significantly inhibited. At the same time, its downstream signals such as MSK, p70S6K, CREB and STAT3 phosphorylation were inhibited. Calcium/calmodulin kinase (CamK) belongs to the threonine/serine family, which can catalyze the calmodulin kinase cascade reaction, amplify calmodulin-mediated calcium ion signal transduction, and activate calcium ion-related signal pathways. CamKII is reported to be upstream protein of ERK and can cause ERK phosphorylation [45]. In renal fibers, CamKII activation can induce ERK phosphorylation, and CamKII is upstream of the ERK pathway [46]. A previous study has shown that Stress hormone receptors such as GC receptor are activated by CamKII and CREB to mediate memory

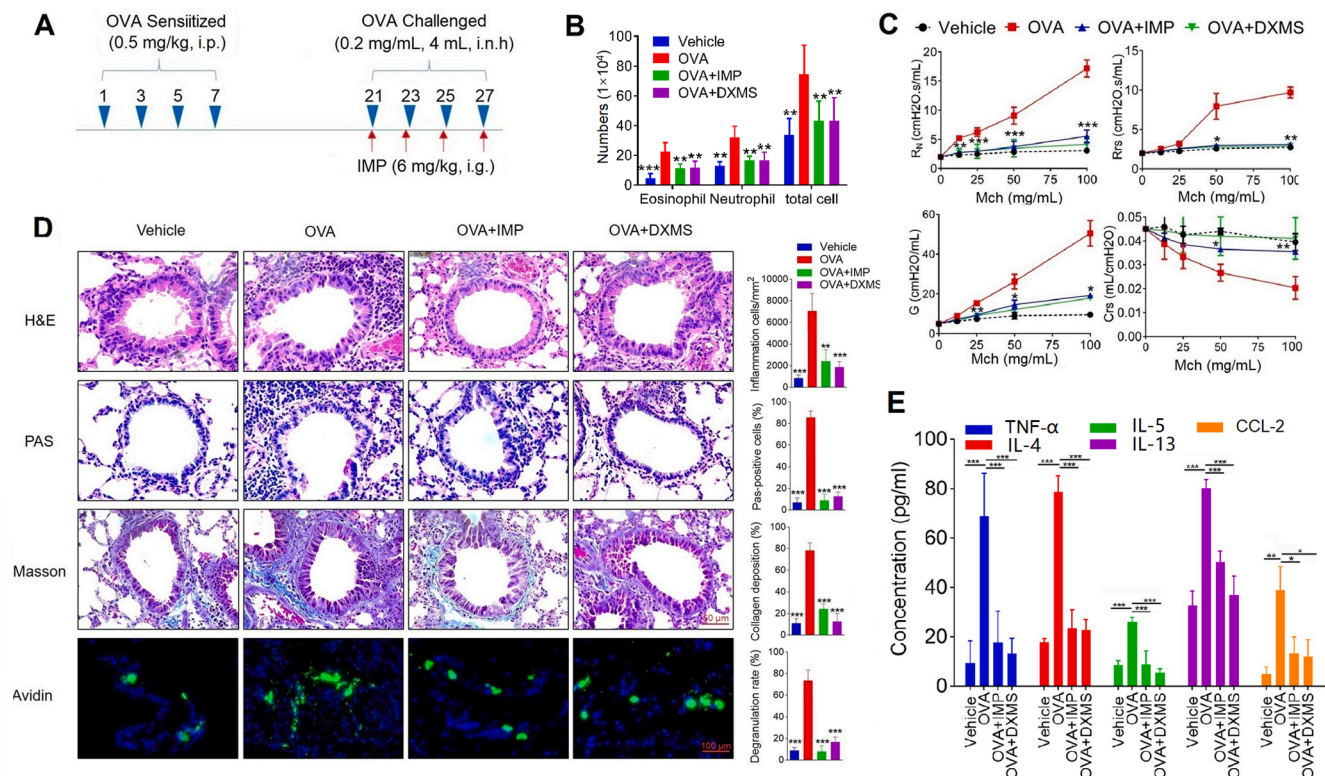


Fig. 5. Mouse lung inflammation in ovalbumin (OVA)-induced asthma. (A) Protocol for sensitization and challenge in mice with induced asthma. (B) 24 h after the last challenge, BAL fluid was collected, and the numbers of eosinophils, neutrophils, and total cells were determined. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with OVA group. (C) Airway responses to methacholine measured on day 12 and 24 h after the last OVA or phosphate buffered saline (PBS) challenge. (D) On day 29, lung sections were stained with Hematein Eosin (H&E), periodic acid-Schiff (PAS), Masson, or FITC-avidin. (E) TNF- α , IL-4, IL-5, and IL-13 were detected in bronchoalveolar lavage fluid (BALF) on day 29. Data are presented as mean $\pm$ standard deviation (SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 7$).

consolidation [47], and IMP can ameliorate learning and memory deficits through BDNF/TrkB and ERK/CamKII α /CREB signaling in prenatally-stressed female offspring [48], but whether these pathways were included in mast cells and MRGPRX2 downstream signals is still unclear.

In this study, we found that CamKII is also involved in the MRGPRX2 signaling pathways, and CamKII was phosphorylated after SP stimulation in LAD2 cells. To further verify the relationship between CamKII and ERK in MRGPRX2 downstream pathway, we used KN-93, a CamKII inhibitor, to investigate the role of CamKII downstream of MRGPRX2. After CamKII was inhibited, degranulation was significantly inhibited, indicating that CamKII was involved in MRGPR22-mediated LAD2 cell activation. When CAMKII was inhibited, phosphorylation of MSK and ERK was significantly inhibited, and phosphorylation of p70S6K and nuclear factor STAT3 was also affected. It can be inferred that CamKII, ERK, p70S6K and STAT3 all participate in downstream CamKII signals.

At the same time, MEK162 (an MEK inhibitor) was used to inhibit the ERK pathway, and it was found that SP-induced degranulation was significantly inhibited, while further protein analysis revealed that the ERK pathway was blocked, and we also found an interesting phenomenon that after MEK inhibition, CamKII phosphorylation was limited. This result indicates that ERK is may not only regulated by CamKII, but is also regulated by the ERK pathway. In other words, there may be crosstalk between the CamKII and ERK pathway. It has been reported that MEK162 can inhibit calcium/calmodulin kinase IV [49], MEK162 may directly inhibit CamKII, but this will require further study. Furthermore, protein kinase A (PKA) and CamKII are activated stress kinases, which are known to influence many Ca^{2+} regulatory proteins (Wagner S., et al. Antioxid Redox Signal 18(9):1063–1077, 2013). PKA activity has been reported to participate in SP/corticotrophin-releasing factor-induced MCs activation, and inhibition of PKA protects MCs from

degranulation (Sur B., et al. Int J Mol Sci 18(1):190, 2017). PKA is also known to activate src, which inhibits PP1 and leads to CamKII activation (Tsiurulnikov E, et al. Theriogenology 128:54–61, 2019). In our study, CamKII has been demonstrated to be involved in MRGPRX2-mediated LAD2 cell activation. [50]. Therefore, SP/MRGPRX2 axis in MCs may modulate the phosphorylation of CamKII by modulating the action of PP1 and PKA, which needs further research.

Since IMP can inhibit the MRGPRX2 downstream signals in MC, we further investigated whether IMP could bind to MRGPRX2. Due to the complex structure of the G protein coupled receptor and the small molecular weight of MRGPRX2, until now, the spatial structure of MRGPRX2 has been unclear. In this study, we predicted the possible tertiary structures of MRGPRX2 and provided some crucial information to clarify its spatial structure. We found that IMP has a good fit within the active cavity of MRGPRX2. We further evaluated the binding by using SPR, to find that IMP binds well with MRGPRX2. All of the above confirmed that IMP is an antagonist of MRGPRX2.

Asthma is an airway inflammatory disorder mediated by multiple immune cells [1]. Mast cells are activated from the early to the late stage of asthma, and they secrete allergic mediators and various cytokines, including TNF- α , IL-4, IL-5 and CCL-2 [12]. TNF- α contributes to the inflammatory response and airway constriction [51]. Activated Th2 cells secrete high levels of IL-4, IL-5 and IL-13, causing airway inflammation, including hyper-responsiveness, goblet cell hyperplasia, eosinophil infiltration, and excessive mucus secretion [52]. CCL-2 is a potent chemotactic molecule that plays a fundamental role in inflammation by recruiting inflammatory cells to specific areas [53]. In the OVA-induced allergic airway inflammation in mice, the IMP treatment suppressed airway inflammatory responses such as the infiltration of immune cells, activation of mast cells and cytokine release in the BALF. Because IMP produced a good inhibition effect of MRGPRX2-mediated mast cell

activation, and mast cells are one of the key mediators of allergic asthma, we further evaluated the anti-asthmatic effects of IMP using an OVA-induced allergic airway inflammation model. IMP significantly attenuated AHR and pulmonary inflammation in mice, as well as reduced MC activation in lung tissue. We also examined the association between IMP and MRGPRX2 *in vitro*, finding that IMP inhibited MRGPRX2-mediated MC activation and pseudo-allergy via MRGPRX2. Therefore, the effect of IMP on lung inflammation in mice appears to be closely related to MRGPRX2; however, further studies are required to investigate other IMP-dependent targets to treat mouse lung inflammation. It is also possible that IMP may have curative effect on other allergic or MRGPRX2 related disease.

In conclusion, this study identifies IMP as an MRGPRX2 antagonist that can be a potential therapeutic agent for treating asthma through the CamKII/ERK pathway. In addition, we propose MRGPRX2 as a novel drug target for allergic asthma.

CRedit authorship contribution statement

Nan Wang: Conceptualization, Methodology, Writing - original draft. **Jue Wang:** Methodology, Formal analysis, Writing - original draft. **Yongjing Zhang:** Methodology, Formal analysis, Data curation. **Yingnan Zeng:** Methodology, Formal analysis, Writing - original draft. **Shiling Hu:** Methodology, Formal analysis. **Haoyun Bai:** Methodology, Formal analysis. **Yajing Hou:** Software, Validation. **Cheng Wang:** Software, Validation. **Huaizhen He:** Software, Validation. **Langchong He:** Supervision, Resources, Conceptualization, Methodology.

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References

- [1] A. Papi, C. Brightling, S.E. Pedersen, H.K. Reddel, Asthma, *Lancet* 391 (10122) (2018) 783–800, [https://doi.org/10.1016/S0140-6736\(17\)33311-1](https://doi.org/10.1016/S0140-6736(17)33311-1).
- [2] R. Wadhwa, T. Aggarwal, V. Malyla, N. Kumar, G. Gupta, D.K. Chellappan, H. Dureja, M. Mehta, S. Satija, M. Gulati, P.K. Maurya, T. Collet, P.M. Hansbro, K. Dua, Identification of biomarkers and genetic approaches toward chronic obstructive pulmonary disease, *J. Cell. Physiol.* 234 (10) (2019) 16703–16723, <https://doi.org/10.1002/jcp.28482>.
- [3] D. Lelli, A. Sahebkar, T.P. Johnston, C. Pedone, Curcumin use in pulmonary diseases: State of the art and future perspectives, *Pharmacol. Res.* 115 (2017) 133–148.
- [4] O. Enilari, S. Sinha, The Global Impact of Asthma in Adult Populations, *Annals of global health* 85(1) (2019).
- [5] Y. Hassoun, C. James, D.I. Bernstein, The effects of air pollution on the development of atopic disease, *Clinic Rev. Allerg. Immunol.* 57 (3) (2019) 403–414, <https://doi.org/10.1007/s12016-019-08730-3>.
- [6] N. Shrine, M.A. Portelli, C. John, M. Soler Artigas, N. Bennett, R. Hall, J. Lewis, A. P. Henry, C.K. Billington, A. Ahmad, R.J. Packer, D. Shaw, Z.E.K. Pogson, A. Fogarty, T.M. McKeever, A. Singapur, L.G. Heaney, A.H. Mansur, R. Chaudhuri, N.C. Thomson, J.W. Holloway, G.A. Lockett, P.H. Howarth, R. Djukanovic, J. Hankinson, R. Niven, A. Simpson, K.F. Chung, P.J. Sterk, J.D. Blakey, I. M. Adcock, S. Hu, Y. Guo, M. Obeidat, D.D. Sin, M. van den Berge, D.C. Nickle, Y. Bossé, M.D. Tobin, I.P. Hall, C.E. Brightling, L.V. Wain, I. Sayers, Moderate-to-severe asthma in individuals of European ancestry: a genome-wide association study, *The Lancet, Respirat. Med.* 7 (1) (2019) 20–34.
- [7] Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017, *Lancet (London, England)* 392(10159) (2018) 1789–1858.
- [8] J.L. McCracken, S.P. Veeranki, B.T. Ameredes, W.J. Calhoun, Diagnosis and Management of Asthma in Adults: A Review (vol 318, pg 279, 2017), *Jama-J Am Med Assoc* 318(16) (2017) 1615–1615.
- [9] E. Heffler, L.N.G. Madeira, M. Ferrando, F. Puggioni, F. Racca, L. Malvezzi, G. Passalacqua, G.W. Canonica, Inhaled corticosteroids safety and adverse effects in patients with asthma, *J. Allergy Clin. Immunol.: In Practice* 6 (3) (2018) 776–781, <https://doi.org/10.1016/j.jaip.2018.01.025>.
- [10] A. KWDA, P. GLDC, B. BAUI, K. KASR, H. US, W. S. L. Kantha, d.S. KSH, Effect of long term inhaled corticosteroid therapy on adrenal suppression, growth and bone health in children with asthma, *BMC Pediatr.* 19 (1) (2019), <https://doi.org/10.1186/s12887-019-1760-8>.
- [11] K.N. Cahill, H. Katz, J. Cui, J. Lai, S. Kazani, A. Crosby-Thompson, D. Garofalo, M. Castro, N.N. Jarjour, E. DiMango, S.C. Erzurum, J.L. Trevor, K. Shenoy, V. M. Chinchilli, M.E. Wechsler, T.M. Laidlaw, E. Israel, J.A. Boyce, Effect of KIT inhibition by imatinib on airway mast cells in patients with severe refractory Asthma (KIA), *J. Allergy Clin. Immunol.* 139 (2) (2017) AB169, <https://doi.org/10.1016/j.jaci.2016.12.553>.
- [12] H. Virk, G. Arthur, P. Bradding, Mast cells and their activation in lung disease, *Transl. Res.* 174 (2016) 60–76, <https://doi.org/10.1016/j.trsl.2016.01.005>.
- [13] L.P. Boulet, Airway remodeling in asthma: update on mechanisms and therapeutic approaches, *Curr. Opin. Pulm. Med.* 24 (1) (2018) 56–62.
- [14] M. Kubo, Innate and adaptive type 2 immunity in lung allergic inflammation, *Immunol. Rev.* 278 (1) (2017) 162–172, <https://doi.org/10.1111/imr.12557>.
- [15] S.J. Galli, S. Nakae, M. Tsai, Mast cells in the development of adaptive immune responses, *Nat. Immunol.* 6 (2) (2005) 135–142, <https://doi.org/10.1038/ni1158>.
- [16] Z.G. Gao, K.A. Jacobson, Purinergic Signaling in Mast Cell Degranulation and Asthma, *Front Pharmacol* 8 (2017).
- [17] D.P. Potaczek, M. Kabesch, Current concepts of IgE regulation and impact of genetic determinants, *Clin. Exp. Allergy* 42 (6) (2012) 852–871, <https://doi.org/10.1111/j.1365-2222.2011.03953.x>.
- [18] D. Fujisawa, J.-I. Kashiwakura, H. Kita, Y. Kikukawa, Y. Fujitani, T. Sasaki-Sakamoto, K. Kuroda, S. Nunomura, K. Hayama, T. Terui, C. Ra, Y. Okayama, Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria, *J. Allergy Clin. Immunol.* 134 (3) (2014) 622–633.e9, <https://doi.org/10.1016/j.jaci.2014.05.004>.
- [19] J.R. Ingelfinger, T.C. Theoharides, P. Valent, C. Akin, Mast cells, mastocytosis, and related disorders, *N Engl. J. Med.* 373 (2) (2015) 163–172, <https://doi.org/10.1056/NEJMra1409760>.
- [20] K.N. Cahill, H.R. Katz, J. Cui, J. Lai, S. Kazani, A. Crosby-Thompson, D. Garofalo, M. Castro, N. Jarjour, E. DiMango, S. Erzurum, J.L. Trevor, K. Shenoy, V. M. Chinchilli, M.E. Wechsler, T.M. Laidlaw, J.A. Boyce, E. Israel, KIT inhibition by Imatinib in patients with severe refractory asthma, *N Engl. J. Med.* 376 (20) (2017) 1911–1920, <https://doi.org/10.1056/NEJMoa1613125>.
- [21] T. Karhu, K. Akiyama, O. Vuolteenaho, U. Bergmann, T. Naito, K. Tatemoto, K.-H. Herzog, Mast cell degranulation via MRGPRX2 by isolated human albumin fragments, *Biochimica et Biophysica Acta (BBA) – General Subjects* 1861 (11) (2017) 2530–2534, <https://doi.org/10.1016/j.bbagen.2017.08.013>.
- [22] B.D. McNeil, P. Pundir, S. Meeker, L. Han, B.J. Undem, M. Kulka, X. Dong, Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions, *Nature* 519 (7542) (2015) 237–241, <https://doi.org/10.1038/nature14022>.
- [23] J. An, J.H. Lee, H.K. Won, Y. Kang, W.J. Song, H.S. Kwon, Y.S. Cho, H.B. Moon, T. B. Kim, Clinical significance of serum MRGPRX2 as a new biomarker in allergic asthma, *Allergy* (2019).
- [24] T.E. Albertson, M.E. Sutter, A.L. Chan, The acute management of asthma, *Clin. Rev. Allerg. Immunol.* 48 (1) (2015) 114–125, <https://doi.org/10.1007/s12016-014-8448-5>.
- [25] C.I. Wi, E.A. Krusemark, G. Voge, S. Sohn, H. Liu, E. Ryu, M.A. Park, J.A. Castro-Rodriguez, Y.J. Juhn, Usefulness of asthma predictive index in ascertaining asthma status of children using medical records: An explorative study, *Allergy* 73 (6) (2018) 1276–1283.
- [26] M. Caminati, D. Bagnasco, L.J. Rosenwasser, A. Vianello, G. Senna, Biologics for the treatments of allergic conditions, *Immunol. Allergy Clin. North Am.* 40 (4) (2020) 549–564, <https://doi.org/10.1016/j.jiac.2020.07.003>.
- [27] A.M. Moran, S. Ramakrishnan, C.A. Borg, C.M. Connolly, S. Couillard, C. M. Mwasuku, I.D. Pavord, T.S.C. Hinks, L. Lehtimäki, Blood eosinophil depletion with mepolizumab, benralizumab, and prednisolone in eosinophilic asthma, *Am. J. Respir. Crit. Care Med.* 202 (9) (2020) 1314–1316, <https://doi.org/10.1164/rccm.202003-0729LE>.
- [28] G.W. Canonica, G.L. Colombo, P. Rogliani, P. Santus, C. Pitotti, S. Di Matteo, C. Martinotti, G.M. Bruno, Omalizumab for Severe Allergic Asthma Treatment in Italy: A Cost-Effectiveness Analysis from PROXIMA Study, *Risk Manag Healthc P* 13 (2020) 43–53.
- [29] Q.-Q. Xing, L.-W. Liu, X. Zhao, Y. Lu, Y.-M. Dong, Z.-Q. Liang, Serum proteomics analysis based on label-free revealed the protective effect of Chinese herbal formula Gu-Ben-Fang-Xiao, *Biomed. Pharmacother.* 119 (2019) 109390, <https://doi.org/10.1016/j.biopha.2019.109390>.
- [30] W.F. Gan, Q. Huang, M.J. Wang, J.D. Wang, Y.X. Hui, K. Zhao, N. Huang, T.T. Liao, A randomized controlled trial study protocol of modified Mahuang-Fuzi-Xixin decoction in the treatment of patients with mild bronchial asthma during acute exacerbation, *Medicine* 99(35) (2020).
- [31] P. Liu, H. Yang, F. Long, H.-P. Hao, X. Xu, Y. Liu, X.-W. Shi, D.-D. Zhang, H.-C. Zheng, Q.-Y. Wen, W.-W. Li, H. Ji, X.-J. Jiang, B.-L. Zhang, L.-W. Qi, P. Li, Bioactive equivalence of combinatorial components identified in screening of an herbal medicine, *Pharm. Res.* 31 (7) (2014) 1788–1800, <https://doi.org/10.1007/s11095-013-1283-1>.
- [32] K. Lee, M.S. Shin, I. Ham, H.Y. Choi, Investigation of the mechanisms of Angelica dahurica root extract-induced vasorelaxation in isolated rat aortic rings, *BMC Complement. Altern. Med.* 15 (2015) 395.

- [33] Y.-Z. Li, J.-H. Chen, C.-F. Tsai, W.-L. Yeh, Anti-inflammatory property of imperatorin on alveolar macrophages and inflammatory lung injury, *J. Nat. Prod.* 82 (4) (2019) 1002–1008, <https://doi.org/10.1021/acs.jnatprod.9b00145>.
- [34] Y. Cao, Y. Zhang, N. Wang, L. He, Antioxidant effect of imperatorin from *Angelica dahurica* in hypertension via inhibiting NADPH oxidase activation and MAPK pathway, *J. Am. Soc. Hypertension* 8 (8) (2014) 527–536, <https://doi.org/10.1016/j.jash.2014.04.006>.
- [35] Z. Xian, G. Jin, H. Li, J. Jiang, C. Wang, L. Zhu, Z. Jin, L. Li, H. Piao, M. Zheng, G. Yan, Imperatorin suppresses anaphylactic reaction and IgE-Mediated allergic responses by inhibiting multiple steps of FcεRI Signaling in Mast Cells: IMP alleviates allergic responses in PCA, *BioMed. Res. Int.* 2019 (2019) 1–12, <https://doi.org/10.1155/2019/7823761>.
- [36] K.-T. Jeong, E. Lee, N.-Y. Park, S.-G. Kim, H.-H. Park, J. Lee, Y.J. Lee, E. Lee, Imperatorin suppresses degranulation and eicosanoid generation in activated bone marrow-derived mast cells, *Biomolecules Therapeut.* 23 (5) (2015) 421–427, <https://doi.org/10.4062/biomolther.2015.023>.
- [37] N. Wang, Y. Zhang, L. Wu, Y. Wang, Y. Cao, L. He, X. Li, J. Zhao, Puerarin protected the brain from cerebral ischemia injury via astrocyte apoptosis inhibition, *Neuropharmacology* 79 (2014) 282–289, <https://doi.org/10.1016/j.neuropharm.2013.12.004>.
- [38] J.A. Mathews, J. Ford, S. Norton, D. Kang, A. Dellinger, D.R. Gibb, A.Q. Ford, H. Massay, C.L. Kepley, P. Scherle, A.D. Keegan, D.H. Conrad, A potential new target for asthma therapy: A Disintegrin and Metalloprotease 10 (ADAM10) involvement in murine experimental asthma, *Allergy* 66 (9) (2011) 1193–1200.
- [39] C.C. Lee, C.N. Wang, Y.T. Lai, J.J. Kang, J.W. Liao, B.L. Chiang, H.C. Chen, Y.W. Cheng, Shikonin inhibits maturation of bone marrow-derived dendritic cells and suppresses allergic airway inflammation in a murine model of asthma, *Brit J Pharmacol* 161(7) (2010) 1496–1511.
- [40] J. Karhausen, S.N. Abraham, How mast cells make decisions, *J Clin Invest* 126(10) (2016) 3735–3738.
- [41] R. Liu, D. Che, T. Zhao, P. Pundir, J. Cao, Y. Lv, J. Wang, P. Ma, J. Fu, N. Wang, X. Wang, T. Zhang, X. Dong, L. He, MRGPX2 is essential for sinomenine hydrochloride induced anaphylactoid reactions, *Biochem. Pharmacol.* 146 (2017) 214–223, <https://doi.org/10.1016/j.bcp.2017.09.017>.
- [42] E. Mendez-Enriquez, J. Hallgren, Mast Cells and Their Progenitors in Allergic Asthma, *Front Immunol* 10 (2019).
- [43] J. An, J.-H. Lee, H.-K. Won, Y. Kang, W.-J. Song, H.-S. Kwon, Y.S. Cho, H.-B. Moon, T.-B. Kim, Clinical significance of serum MRGPX2 as a new biomarker in allergic asthma, *Allergy* 75 (4) (2020) 959–962, <https://doi.org/10.1111/all.14084>.
- [44] C. Qiao, S. Hu, D. Che, J. Wang, J. Gao, R. Ma, W. Jiang, T. Zhang, R. Liu, The anti-anaphylactoid effects of Piperine through regulating MAS-related G protein-coupled receptor X2 activation, *Phytotherapy Res.* 34 (6) (2020) 1409–1420, <https://doi.org/10.1002/ptr.6615>.
- [45] Q. Liu, J. Song, Y. Pan, D. Shi, C. Yang, S. Wang, B. Xiong, Wnt5a/CaMKII/ERK/CCL2 axis is required for tumor-associated macrophages to promote colorectal cancer progression, *Int. J. Biol. Sci.* 16 (6) (2020) 1023–1034, <https://doi.org/10.7150/ijbs.40535>.
- [46] J. Zhou, S. Liu, L. Guo, R. Wang, J. Chen, J. Shen, NMDA receptor-mediated CaMKII/ERK activation contributes to renal fibrosis, *BMC Nephrol.* 21 (1) (2020), <https://doi.org/10.1186/s12882-020-02050-x>.
- [47] D.Y. Chen, D. Bambah-Mukku, G. Pollonini, C.M. Alberini, Glucocorticoid receptors recruit the CaMKII alpha-BDNF-CREB pathways to mediate memory consolidation, *Nat. Neurosci.* 15 (12) (2012) 1707–+.
- [48] X.X. Zheng, K.Y. Zhang, Y.C. Li, Y.W. Chen, Y.S. Yue, S.Z. Xia, Y. Li, H.H. Deng, H. L. Jing, Y.J. Cao, Imperatorin ameliorates learning and memory deficits through BDNF/TrkB and ERK/CaMKII alpha/CREB signaling in prenatally-stressed female offspring, *Phytother. Res.* 34 (9) (2020) 2408–2418.
- [49] Assessment report. https://www.ema.europa.eu/en/documents/assessment-report/mektovi-epar-public-assessment-report_en.pdf.
- [50] M. Makhinson, J.K. Chotiner, J.B. Watson, T.J. O'Dell, Adenylyl cyclase activation modulates activity-dependent changes in synaptic strength and Ca²⁺/Calmodulin-Dependent Kinase II Autophosphorylation, *J. Neurosci.* 19 (7) (1999) 2500–2510, <https://doi.org/10.1523/JNEUROSCI.19-07-02500.1999>.
- [51] T. Mauad, E.H. Bel, P.J. Sterk, Asthma therapy and airway remodeling, *J. Allergy Clin. Immunol.* 120 (5) (2007) 997–1009, <https://doi.org/10.1016/j.jaci.2007.06.031>.
- [52] R. Afshar, B.D. Medoff, A.D. Luster, Allergic asthma: a tale of many T cells, *Clin. Exp. Allergy* 38 (12) (2008) 1847–1857.
- [53] S.C. Diesner, A. Olivera, S. Dillahun, C. Schultz, T. Watzlawek, E. Förster-Waldl, A. Pollak, E. Jensen-Jarolim, E. Untersmayr, J. Rivera, Sphingosine-kinase 1 and 2 contribute to oral sensitization and effector phase in a mouse model of food allergy, *Immunol. Lett.* 141 (2) (2012) 210–219, <https://doi.org/10.1016/j.imlet.2011.10.006>.