

ORIGINAL ARTICLE

Gomisin N has anti-allergic effect and inhibits inflammatory cytokine expression in mouse bone marrow-derived mast cells

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Abstract

Gomisin N is a bioactive compound and a prominent anti-allergic agent found in the fruits of tree *Schizandra chinensis*. However, its effects on the bone marrow-derived mast cell (BMMC)-mediated allergy and inflammation mechanism remain unknown. In this study, the biological effects of gomisin were evaluated while focusing on its effects on the allergic mediator in PMA + A23187-stimulated BMDCs. The anti-allergic effect of gomisin has shown that inhibited PMA + A23187-induced interleukin-6 (IL-6) production. An investigation was also conducted to determine its effects on the production of several allergic mediators including prostaglandin D₂ (PGD₂), leukotriene C₄ (LTC₄), β -hexosaminidase (β -Hex), and cyclooxygenase-2 (COX-2) protein. The results revealed that gomisin inhibited the PMA + A23187-induced production of IL-6, PGD₂, LTC₄, β -Hex, and COX-2 protein. Taken together, these findings indicate that gomisin N has the potential for use in the treatment of allergy.

Keywords: Gomisin, bone marrow-derived mast cell, allergy, interleukin-6, *Schizandra chinensis*

Introduction

Gomisin N is an active compound found in the fruit of *Schizandra chinensis*, which is native to Asia. *S. chinensis* is traditionally used in herbal medicine in these countries as a treatment for rheumatism and arthritis.⁽¹⁾ *S. chinensis* have been proven to exert various biological activities. Of this plant, the schizandrin, gomisin A, and gomisin N were studied for their cytotoxic activity.⁽²⁾ However, its direct molecular targets on mast cell-mediated inflammation such as allergic disease are not known.

Mast cells are one of the most important cells involved in disease related to allergic responses. When activated, mast cells release a number of biologically active molecules through various processes. Histamine, serotonin, and serine proteases are released through exocytosis, whereas lipid mediators such as eicosanoids are released through activation of the cyclooxygenase (COX)-2 and

5-lipoxygenase pathways. As such, the pattern of cytokine expression largely determines the nature of the inflammatory response.^(3,4) Calcium (Ca²⁺) acts as a second messenger during cell activation, and an increase Ca²⁺ level has been proposed as an essential trigger for mast cell activation.⁽⁵⁾

Interleukin-6 (IL-6) is an interleukin that acts both as a proinflammatory and as an anti-inflammatory cytokine.⁽⁶⁾ IL-6 is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation.⁽⁷⁾ Further, IL-6 modulates a variety of physiological events that occur in the nervous system, endocrine system, and bone metabolism.^(8,9)

Bone marrow-derived mast cells (BMDCs) exhibit prostaglandin D₂ (PGD₂) biosynthetic responses over time, in addition to COX-1-dependent immediate and

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COX-2-dependent delayed responses. This cell model also appears suitable for assessing the effect of 5-LOX inhibitors, since the immediate leukotriene C_4 (LTC_4) generation elicited by the IgE-dependent or cytokine-initiated stimulus occurs in BMMC through 5-LOX.⁽¹⁰⁾ COX has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells and is responsible for the production of PGD that maintain homeostasis; however, COX-2 is up-regulated in inflammatory cells in response to an inflammatory stimulus and is responsible for the production of prostaglandins at the site of inflammation.^(11–13) Therefore, there is an increasing interest in the usefulness of COX-2 inhibitors.

In the present study, gomisin N was investigated to determine if it suppresses PMA + A23187-induced IL-6, PGD_2 , and LTC_4 production and COX-2 expression in BMMCs. The results show that gomisin N has anti-allergic activity.

Materials and methods

Animals

Male Balb/cJ mice (20–30 g) were purchased from DAMUL, Korea. All animals were kept in a temperature-controlled room under a 12-h light/12-h dark cycle. The animals were provided with free access to commercial solid food (SCF Co., Ltd., Korea) and water *ad libitum*. Mice were acclimatized for at least 1 week prior to beginning the experiments.

Drugs and chemicals

Gomisin was purchased from (Osaka, Japan). RPMI 1640, penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Hyclone (Hyclone Labs, Logan, UT). Bovine serum albumin (BSA), PMA, and A23187 were purchased from Sigma (St. Louis, MO).

Preparation and activation of BMMCs

BMMCs from male Balb/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% FBS) and 50% WEHI-3 cell-conditioned medium as a source of interleukin-3 (IL-3). After 3 weeks, >98% of the cells were BMMCs.⁽⁹⁾

MTT assay for cell viability

The cell viability was examined by MTT assay. BMMCs were seeded at 1×10^5 /mL densities in 96-well plates (Nunc, Denmark). Each group had non-treated group as control. Gomisin N (20 μ g/mL) was added to each well and incubated for 24 h at 37°C, 5% CO_2 . MTT solutions (5 mg/mL) were added to each well and then cells were cultured for another 4 h. The supernatant was discarded and 100 μ L of dimethyl sulfoxide (DMSO) was added to each well. The optical density was read at 590 nm. Cytotoxicity was calculated by subtracting from one the ratio of the mean absorbance value for treated cells over the mean absorbance value for untreated cells.

Enzyme-linked immunosorbent assay

Cells were seeded at a density of 1×10^6 cells/mL in 24-well tissue culture plates and then pretreated with various concentrations of gomisin N (10 or 20 μ g/mL) for 30 min prior to PMA (50 nM) + A23187 (1 μ M) stimulation. Enzyme-linked immunosorbent assay (ELISA) plates (Falcon, Becton Dickinson Labware) were then coated overnight at 4°C with anti-mouse IL-6 antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Nonspecific protein-binding sites were then blocked by incubating the plates with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. Immediately following the incubation, each sample and IL-6 standard was added to the wells, and the plates were then incubated for 2 h. Next, working detector (biotinylated anti-mouse IL-6 monoclonal antibody and streptavidin-HRP reagent) was added to each well and the plates were incubated for an additional 1 h. Substrate solution (tetramethylbenzidine [TMB]) was then added to the wells, after which the plates were incubated in the dark for 30 min. Next, the reaction was stopped using stop solution (2 N H_3PO_4) and the absorbance at 450 nm was measured. All standards and samples were assayed in duplicate.

Determination of PGD_2 levels

To measure the inhibitory activity of gomisin N on COX-2, cells were suspended in enriched medium at 1×10^6 cells/mL and preincubated with aspirin (10 g/mL) for 2 h to irreversibly inactivate any pre-existing COX-1. After washing, the BMMCs were activated with PMA (50 nM) + A23187 (1 μ M) at 37°C for 2 h in the presence or absence of gomisin N dissolved in PBS. All reactions were quenched by centrifugation at 890 g at 4°C for 5 min. The supernatant and cell pellet were frozen immediately in liquid N_2 and stored at –80°C until needed for further analysis. Under these conditions, the COX-2-dependent phase of PGD_2 generation reached 2.2 ng/ 10^6 cells. The data are reported as the arithmetic mean of triplicate determinations.

Determination of LTC_4 levels

BMMCs suspended in enriched medium at 1×10^6 cells/mL were pretreated with gomisin N for 30 min at 37°C and then stimulated with PMA (50 nM) + A23187 (1 μ M). After 15 min of stimulation, the supernatants were isolated for further analysis by enzyme immunoassay (EIA). LTC_4 was determined using an EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Under the conditions employed, LTC_4 reached 20 ng/ 10^6 cells. All data are the arithmetic means of triplicate determinations.

RNA extraction and reverse transcription–polymerase chain reaction

Total cellular RNA was isolated using the easy-BLUE™ RNA extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Total RNA (2 μ g) was converted to cDNA by treatment with 200 units of reverse

transcriptase and 500 ng of oligo-dT primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 1 mM dNTPs at 42°C for 1 h. The reaction was stopped by heating at 70°C for 15 min and 3 µL of the cDNA mixture was used for enzymatic amplification. Polymerase chain reaction (PCR) was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 µM each of primers for iNOS, COX-2, and β-actin. Amplification conditions were: denaturation at 94°C for 3 min for the first cycle and for 45 sec starting from the second cycle, annealing of COX-2 at 53°C for 45 sec, for 30 cycles. Final extension was performed at 72°C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Primers used were 5'-CATGTTCTCTGGGAAATCGTGG-3' (sense) and 5'-AACGCACTAGGTTTGCCGAGTA-3' (antisense) for IL-6; 5'-CACTCAGTTTGTGAGTCATTC-3' (sense) and 5'-GATTAGTACTGTAGGGTTAATG-3' (antisense) for COX-2; and 5'-ATGAAGATCCTGACCGAGCGT-3' (sense) and 5'-AACGCAGCTCAGTAACAGTCCG-3' (antisense) for β-actin.

Assay of β-hexosaminidase release

β-Hexosaminidase (β-Hex), a marker of mast cell degranulation, was quantified by spectrophotometric analysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside hydrolysis (Sigma). In brief, after harvesting the supernatant, cells were lysed in the same volume of medium by three freeze-thaw cycles. Ten milliliters of the BMMC lysate or supernatant samples was then mixed with 50 µL of β-Hex substrate solution (1.3 mg/mL *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 100 mM sodium citrate, pH 4.5) in the wells of a 96-well plate, after which the samples were incubated at 37°C for 90 min. The reaction was stopped by the addition of 140 µL of 0.2 M glycine-NaOH (pH 10.7) and the absorbance was then measured at 410 nm using a microplate reader. The percentage of β-Hex released into the supernatant was calculated by the following formula: $[S/(S + P)] \times 100$, where *S* and *P* are the β-Hex contents of the supernatant and the cell pellet, respectively.

Statistical analysis

The data from the experiments are presented as the mean ± SEM. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. *P*-values <0.05 were considered to be significant.

Results

The effects of gomisin N on PMA + A23187-induced IL-6 expression

To evaluate the effects of gomisin N on the production of IL-6, we pretreated cells with gomisin A, gomisin N, and schizandrin before stimulation with PMA (50 nM) + A23187 (1 µM) for 6 h, after which the samples were analyzed by ELISA. As shown in Figure 1, the level of IL-6 was

considerably higher after stimulation with PMA + A23187 in BMMCs. Pretreatment of cells with schizandrin, gomisin A, and gomisin N (5, 10, and 20 µg/mL) inhibited these increases in a concentration-dependent and statistically significant manner. The cytotoxic effects of gomisin N were evaluated in BMMCs by MTT assay. Treatment with gomisin N at concentrations of up to 20 µg/mL did not affect the viability of BMMCs (data not shown).

The effects of gomisin N on PMA + A23187-induced PGD₂ production

After stimulation with PMA + A23187, cell-cultured media were harvested to measure the level of PGD₂ production. PGD₂ production was clearly detected 2 h after stimulation with PMA + A23187. PGD₂ production also peaked in PMA (50 nM) + A23187 (1 µM) treated samples. Next, an investigation of the inhibitory effect of gomisin N on the PMA + A23187-induced PGD₂ production in BMMCs was performed. gomisin N significantly inhibited PMA + A23187-induced PGD₂ production in a dose-dependent manner (Figure 2). These results show that gomisin N could inhibit PMA + A23187-induced PGD₂ production in BMMCs.

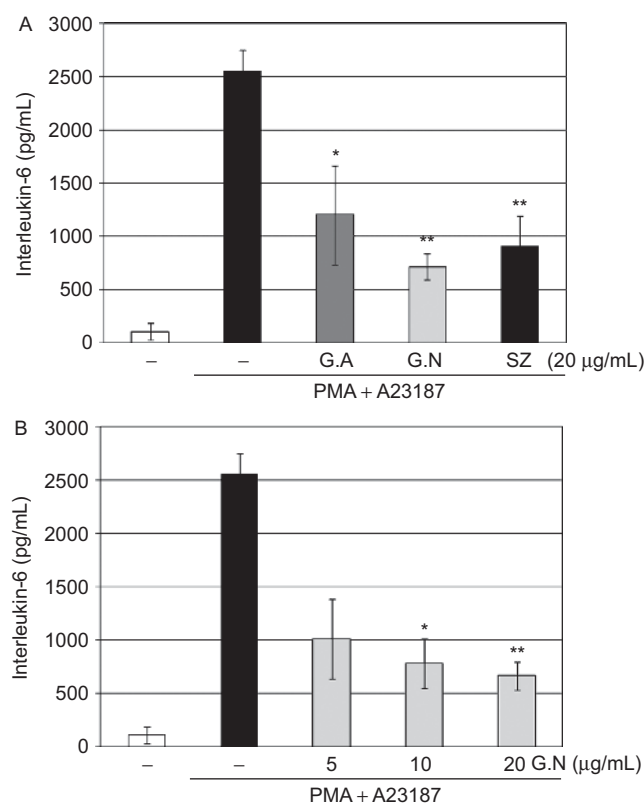


Figure 1. Effects of gomisin N on the secretion of IL-6 in PMA + A23187-stimulated bone marrow-derived mast cells (BMMCs). The cells (1×10^6 cells/mL) were pretreated with gomisin N (10 or 20 µg/mL) 30 min prior to stimulation with PMA (50 nM) + A23187 (1 µM). Six hours after PMA + A23187 stimulation, the IL-6 level of the supernatants was measured by enzyme-linked immunosorbent assay (ELISA). Statistical significance: **P* < 0.05 and ***P* < 0.005, when compared with the PMA + A23187-treated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean ± SE of duplicate determinations from three separate experiments.

The effects of gomisin N on PMA + A23187-induced LTC₄ production

After stimulation with PMA + A23187, cell-cultured media were harvested to measure the level of LTC₄ production. LTC₄ production was clearly detected 15 min after stimulation with PMA + A23187. LTC₄ production also peaked in PMA (50 nM) + A23187 (1 μ M)-treated samples. Next, an investigation of the inhibitory effects of gomisin N on the PMA + A23187-induced LTC₄ production by BMMCs was conducted. Gomisin N significantly inhibited the PMA + A23187-induced LTC₄ production in a dose-dependent manner (Figure 3). These results show that gomisin N could inhibit PMA + A23187-induced LTC₄ production in BMMCs.

The effects of gomisin N on PMA + A23187-induced COX-2 expression

RT-PCR analysis was performed to determine if the inhibitory effects of gomisin N on PGs were related to modulation of the COX-2 expression. In unstimulated BMMCs, COX-2 protein was undetectable. However, COX-2 mRNA was strongly expressed in response to PMA + A23187, and gomisin N significantly inhibited COX-2 expression (Figure 4).

The inhibitory effects of gomisin N on PMA + A23187-induced degranulation

When mast cells are activated by various stimuli, the release of histamine occurs in a fashion similar to that of β -Hex, which is a degranulation marker. An investigation of the effects of gomisin N on the PMA + A23187-induced β -Hex release using ELISA was performed. Pretreatment of BMMCs with gomisin N at concentrations of 10 or 20 μ g/mL reduced β -Hex production (Figure 5).

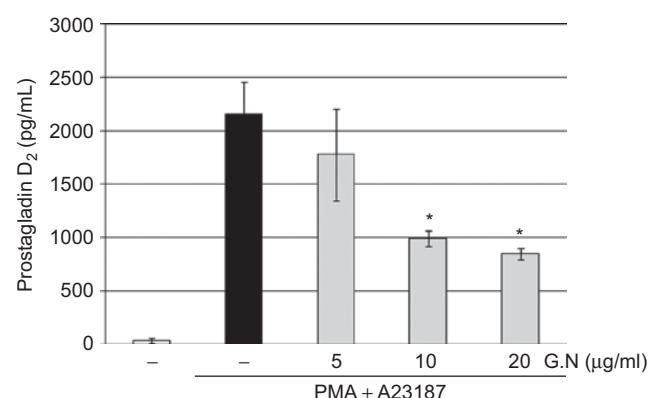


Figure 2. Effects of gomisin N on the production of prostaglandin D₂ (PGD₂) in PMA + A23187-stimulated bone marrow-derived mast cells (BMMCs). The cells (1×10^6 cells/mL) were pretreated with gomisin N (10 or 20 μ g/mL) 30 min prior to stimulation with PMA (50 nM) + A23187 (1 μ M). Two hours after PMA + A23187 stimulation, the PGD₂ levels of the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Statistical significance: * $P < 0.05$, as compared with the PMA + A23187-treated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

Discussion

BMMCs exhibit PGD₂ biosynthetic responses over time, in addition to COX-1-dependent immediate and COX-2-dependent delayed responses. This cell model also appears suitable for assessing the effect of 5-LOX inhibitors, since the immediate LTC₄ generation elicited by the IgE-dependent or cytokine-initiated stimulus occurs in BMMC through 5-LOX.⁽⁹⁾ Allergic reactions occur in response to normally harmless environmental substances known as allergens, and these reactions are acquired, predictable, and rapid. Allergy occurs in one of four forms of hypersensitivity and is known as type I hypersensitivity. Allergy is characterized by excessive activation of mast cells and basophils by a type of antibody known as IgE, which results in an extreme inflammatory response.⁽¹⁴⁾

Mast cells play an important role in the induction and expression of allergic responses and have long been implicated in the pathology and mortality of anaphylaxis and other allergic disorders by virtue of their ability to be activated through Fc ϵ RI-bound antigen-specific IgE and their concentration at surfaces that interface with the external environment.⁽³⁾ Mast cells may also be activated by various cytokines through each cytokine receptor. Activation through any of these receptors leads to the release of a number of biologically active molecules, including histamine, serotonin, proteoglycans, and neutral proteases. Among these molecules, histamine is one of the most important chemical mediators in the pathological allergic reaction.⁽¹⁵⁾

The inhibitory effects of gomisin N on PGD₂ production were examined to determine if they occur via a direct effect of the COX-2 mRNA or are mediated by some other mechanism. As shown in Figure 4, the COX-2 protein was

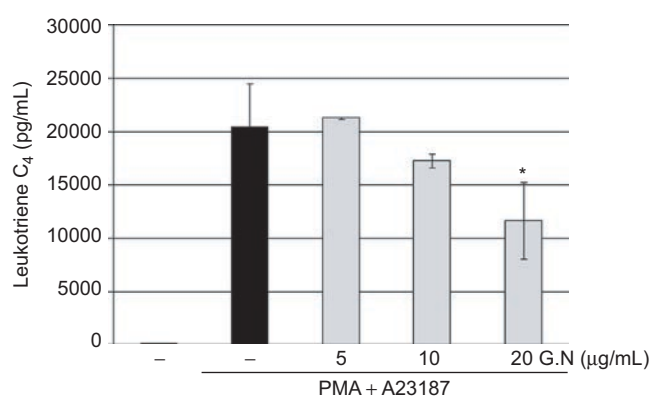


Figure 3. Effects of gomisin N on the production of leukotriene C₄ (LTC₄) in PMA + A23187-stimulated bone marrow-derived mast cells (BMMCs). The cells (1×10^6 cells/mL) were pretreated with gomisin N (10 or 20 μ g/mL) 30 min prior to stimulation with PMA (50 nM) + A23187 (1 μ M). At 15 min after PMA + A23187 stimulation, the LTC₄ levels of the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Statistical significance: * $P < 0.05$, as compared with the PMA + A23187-treated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

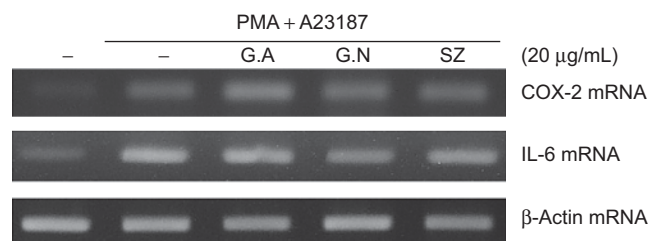


Figure 4. Effects of gomisin N on PMA + A23187-induced COX-2 protein expressions in bone marrow-derived mast cells (BMMCs). BMMCs were pretreated with the indicated concentrations of gomisin N for 30 min prior to being incubated with PMA (50 nM) + A23187 (1 μ M) for 6 h. Equal amounts of mRNA (2 μ g) were then separated by agarose gel. The β -actin mRNA was carried out in parallel to confirm equivalency of cDNA preparation.

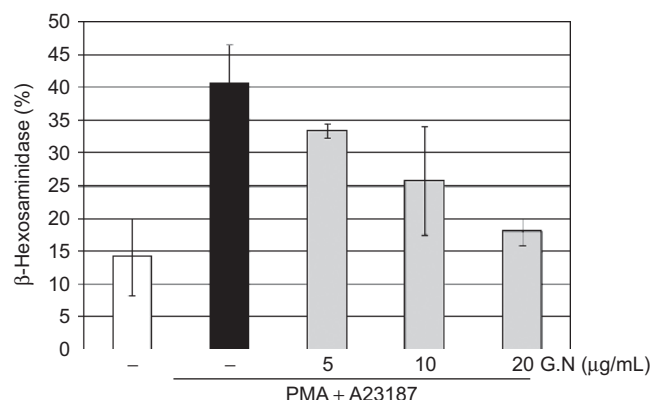


Figure 5. Effect of gomisin N on the release of β -hexosaminidase in PMA + A23187-stimulated bone marrow-derived mast cells (BMMCs). BMMCs (1×10^6 cells/mL) were pretreated with gomisin N (10 or 20 μ g/mL) 30 min prior to stimulation with PMA (50 nM) + A23187 (1 μ M). At 90 min after PMA + A23187 stimulation, the β -Hex released into the supernatant cell lysate was measured by enzyme-linked immunosorbent assay (ELISA). The procedure is described in detail in "Materials and methods" section. All data are the arithmetic mean of triplicate determinations. Statistical significance: $*P < 0.05$, as compared with the PMA + A23187-treated group. Significant differences between treated groups were determined using the Dunnett's t -test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

not detected in unstimulated BMMC, whereas treatment with a combination of PMA + A23187 strongly induced the formation of detectable COX-2 protein and COX-2 protein expression was inhibited in a dose-dependent manner by gomisin N.

Arachidonic acid can also be converted to leukotrienes (LTs) by the action of 5-LOX in BMMCs. The inhibition of 5-LOX is believed to be the ideal treatment for allergic diseases and asthma.⁽¹⁶⁾ Therefore, the inhibitory activity of gomisin N on the generation of LTC₄ in BMMCs was examined. As shown in Figure 3, the BMMCs stimulated with PMA + A23187 for 15 min produced ~ 20 ng/mL LTC₄, and preincubation of the BMMC with gomisin N resulted in the dose-dependent suppression of this LTC₄ biosynthesis.

The results of these investigations indicate that gomisin N can exhibit inhibitory effects on PMA + A23187-induced IL-6, PGD₂, and LTC₄ production and

COX-2 mRNA expression in BMMCs. Based on these findings, gomisin N could represent a new drug treatment for various allergic diseases. Therefore, further studies are necessary to examine its clinical application.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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