Original Research

Reconstitution of anti-allergic activities of PG102 derived from *Actinidia arguta* by combining synthetic chemical compounds

Donghyun Kim[1], Jinyong Choi[1], Mi-Jeong Kim[2], Seon Hee Kim[2], Sang Heon Cho[3] and Sunyoung Kim[1,2]

[1]School of Biological Sciences, Seoul National University, Seoul, 151–747, Korea; [2]Viromed Co., Ltd., Seoul, 151–747, Korea; [3]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, 110–799, Korea
Corresponding author: Sunyoung Kim. Email: sunyoung@snu.ac.kr

Abstract

PG102, a water-soluble extract from an edible fruit, *Actinidia arguta*, has previously been shown to control various factors involved in allergy pathogenesis. It was investigated whether the original activities of PG102 could be reconstituted by mixing chemical compounds present in PG102. Six compounds present in PG102 were, individually or in the form of mixtures, tested for their effects on the expression of various Th2 cytokines and inflammatory mediators in the cell-based assay. Each chemical inhibited IL-4 expression to varying degrees. The chemical compounds were combined at a ratio present in PG102, resulting in two formulations, CQMIIH and CQM, consisting of all or the first three of the following chemicals, citric, quinic, and malic acids, *myo*-inositol, isoquercitrin, and 5-hydroxymethyl-2-furaldehyde. The mixtures reconstituted original activities of PG102 to a significant level. In the murine asthma model, CQM ameliorated asthmatic symptoms and significantly decreased the level of IgE and IL-5. The decreased phosphorylation of ERK1/2 was observed in cells and mice treated with PG102 and the mixtures. Our data indicated that the substantial portion of PG102's anti-allergic activities could be reconstituted, *in vitro* and *in vivo*, by mixing six chemical compounds, suggesting the possibility of developing a new type of anti-allergic agent. This approach may be useful for developing chemically defined functional products from complex botanical extracts.

Keywords: PG102, *Actinidia arguta*, reconstitution, allergy, asthma, IgE, Th2 cytokine, ERK1/2

***Experimental Biology and Medicine* 2013; 238: 631–640. DOI: 10.1177/1535370213489455**

Introduction

Allergic diseases are highly prevalent, and their incidence is increasing, especially in developed countries.[1] Pharmacologic compounds capable of inhibiting inflammatory responses, such as glucocorticoids and leukotriene receptor antagonists, are available for the treatment of patients with allergic diseases.[2–5] In addition, a new therapy using humanized monoclonal antibody directed against IgE provides an effective and generally safe approach to treatment of patients with IgE-mediated allergies.[6] However, many patients are frustrated by the lack of curative therapies and have concerns about medicine-related side-effects or financial strains.[6–8] Therefore, there has been a strong need for the development of a safe, cost-effective, and efficacious agent for allergic diseases.

We have previously shown that PG102, a water-soluble extract derived from an edible portion of *Actinidia arguta*, may have potential as a safe and effective reagent for the prevention or treatment of various allergic diseases by controlling the expression of IgE and various Th2 cytokines, such as IL-4, IL-5, and IL-13.[9–12] Because PG102 is a complex mixture, various approaches have been taken to identify a biologically active compound(s) or fraction(s) responsible for its activities. As one approach, we examined the effects of various single compounds present in *A. arguta*. The fruit of *A. arguta* is known to contain a large amount of citric, quinic, and malic acids, as well as *myo*-inositol.[13–15] Kiwifruit is also rich in vitamins such as vitamin C and flavonoids including quercitrin.[16,17] Because 5-hydroxymethyl-2-furaldehyde (HMF) is produced in the heating process of a wide variety of foods,[18,19] HMF may also be present in PG102 as it is prepared by boiling the fruits of *A. arguta*. Our data indicated that six chemicals tested in this study have inhibitory activities on IL-4 expression in RBL-2H3 cells at varying degrees. Using several *in vitro* cell culture systems and an *in vivo* murine asthma model,

we demonstrated that the mixture of six chemical compounds could reconstitute a significant portion of the original bioactivities of PG102.

Materials and methods

Reagents

PG102 was prepared from hardy kiwifruits purchased from a company specializing in this fruit (Vital Berry Marketing S.A., Chile) as described by Park *et al.*[10] Synthetic citric, quinic, and malic acids, as well as *myo*-inositol, isoquercitrin, and HMF were purchased from Sigma (St. Louis, MO). All chemicals were dissolved in sterile distilled water (except for HMF which 95% ethanol was used for) to make 100 mg/mL stock. pH of citric acid, quinic acid, malic acid, *myo*-inositol, isoquercitrin, and HMF was 6.9, 6.53, 7.12, 7.29, 7.13, and 7.26, respectively, when they were added to culture media at their highest concentration. CQMIIH and CQM were prepared by mixing six or three compounds at a ratio present in PG102, namely C:Q:M:I:I:H = 5.94:5.38:0.84:1.25:0.01:0.66 or C:Q:M = 5.94:5.38:0.84. Both mixtures were dissolved in sterile distilled water to make X100/mL and X60/mL stocks for *in vitro* and *in vivo* assays. pH of both mixtures was 7.3 when added to culture media at their highest concentration. pH of X12 CQM dissolved in distilled water, used for the animal experiment, was 3.61. Antibodies used for Western blot analysis were as follows: anti-p38 MAPK and anti-ERK1/2 antibodies from Santa Cruz Biotechnology (Santa cruz, CA), anti-phosphorylated p38 MAPK and anti-phosphorylated ERK1/2 antibodies from Cell Signaling (Danvers, MA), anti-HO-1 antibody from Stressgen (Ann Arbor, MI), and anti-β-actin antibody from Sigma.

High-performance liquid chromatography chromatogram (HPLC)

HPLC analysis was performed by using the Chromelon™ chromatography data system equipped with a P680 pump and UVD 340U detector (Dionex, Sunnyvale, CA). To analyse the organic acid content in the PG102, PG102 and/or authentic organic acids were injected into the Allure Organic Acids column (5 μm, 4.6 mm I.D. × 300 mm; Restek, Bellefonte, PA). The mobile phase consisted of 100 mM KH_2PO_4 and the flow rate was 1.0 mL/min. The peaks were detected by 200 nm UV-detector. Isoquercitrin and HMF were determined by using a CAPCELL PAK C18 MG column (5 μm, 4.6 mm I.D. × 250 mm or 150 mm; Shiseido, Tokyo, Japan). The mobile phase of 10% AcCN in water (v/v) and 16% AcCN in 20 mM KH_2PO_4 (v/v) was pumped at a flow rate of 1.0 mL/min for respective chemicals. Each eluate was monitored at 354 nm and 280 nm. The authentic chemicals were subsequently diluted to obtain the desired concentrations in the various ranges and prepare a calibration curve. The curve was constructed by plotting the peak area of the HPLC against the corresponding concentration.

High-performance anion exchange chromatography-pulsed amperometric detector (HPAEC-PAD)

Carbohydrates in PG102 were analysed by HPAEC-PAD using the Dionex ICS3000 chromatography system (Sunnyvale, CA). Samples (25 μL) were injected on a CarboPac PA-1 guard column coupled to a CarboPac PA-1 anion exchange column. Isocratic elution conditions (15 mM NaOH, 1 mL/min, 15 min) were used for the analysis of *myo*-inositol. For quantification, a standard curve was created using diluted authentic *myo*-inositol solutions and their peak area of the PAD response against the known concentration.

Measurement of the level of IL-4, IL-5, IL-13, NO, and PGE_2

Effects of single chemicals or their mixtures, (CQMIIH and CQM), were measured in RBL-2H3 cells (a rat mast cell line), and EL4 cells (a mouse T lymphoma line), as previously described.[9,12] Briefly, to examine the effect on IL-4 and IL-13, RBL-2H3 cells were incubated in the presence of various chemicals, chemical mixtures, or PG102 for 30 min, and then stimulated with A23187 (1 μM) (Sigma). For IL-5, EL4 cells were treated with chemical mixtures or PG102 for 30 min before stimulation with PMA (10 ng/mL) and N^6, O^2-dibutyryl cAMP (bt2cAMP) (1 mM) (Sigma). Culture supernatants were collected after 12 h, followed by ELISA for respective cytokines (Pierce, Rockford, IL, USA and Biosource, GrandIsland, NY, USA). To measure the effect on inflammatory mediators, Raw264.7 cells (a mouse macrophage cell line) were plated on 24 well culture plates (2.5×10^5 cells/well) using 0.5 mL of DMEM supplemented with 10% FBS at 37°C under 5% CO_2. Six hours later, the cells were treated with various concentrations of chemical mixtures or PG102 and 100 ng/mL of LPS (Sigma). After 24 h, the supernatants were collected to measure the level of NO and PGE_2 by Griess assay and ELISA, respectively (BD Sciences).

Analysis of the level and status of MAPKs and HO-1 protein using Western blot hybridization

To analyse the MAPKs and HO-1 proteins, total cellular proteins were extracted at appropriate time points, as previously described.[9,20] To prepare lung lysates from mice, whole lung tissues were pooled according to respective experimental groups, subjected to mechanical disruption under liquid nitrogen by using a mortar and pestle, and lysed with RIPA lysis buffer. Cell and lung lysates were probed with antibodies specific for mouse p38, phosphorylated p38, ERK1/2, phosphorylated ERK1/2, HO-1, and β-actin.

Murine asthma model

Murine asthma model was prepared as previously described.[9] One group (OVA/CQM) of mice was administrated with CQM at X12/mouse/day (X600/kg/day) by gavage, while another group (OVA/PG102) of mice was fed with PG102 at 6 mg/mouse/day (300 mg/kg/day). As positive and negative controls, two groups (OVA/Water

and Normal) of mice were maintained by being intragastrically fed with 200 μL of water. Various asthmatic symptoms, including airway hyperresponsiveness (AHR), the infiltration of inflammatory cells, and the level of IgE and cytokines, were analysed as previously described.[9,21] All experimental procedures were approved by the University Animal Care and Use Committee at Seoul National University (SNU-061229-1) and carried out in accordance with their guidelines set.

Statistics

Results were expressed as the mean ± SD or SEM. Statistical significance between the mean values was determined by Student's *t*-test. Differences were considered significant when P values were < 0.05 or < 0.01.

Results

Quantification of chemical compounds present in PG102

To determine the amount of various chemical components present in PG102, HPLC or HPAEC-PAD analyses were performed using respective synthetic chemicals as standards. Synthetic citric, quinic, and malic acids were detected as a single peak with a retention time of 24, 9, and 12 min, respectively, at 200 nm, and the peaks with identical retention times were observed in the elution profile of PG102. In addition, co-chromatography of PG102 with respective synthetic chemicals confirmed that the peaks found in PG102 represented citric, quinic, and malic acids. For quantification, the isocratic HPLC method was used. A standard curve was created using known concentrations of standard chemicals, and then the area of respective peaks observed in the PG102 sample was calculated to determine the amount of each compound. The data indicated that citric, quinic, and malic acids are present in 5.94, 5.38, and 0.84% (w/w) of PG102, respectively (Table 1). In the same way, isoquercitrin, detected in 354 nm with a retention time of 27 min, was measured to be contained in 0.01% of PG102. HMF was separated in 280 nm with a retention time of 4 min and calculated to be present in 0.66% of PG102 (Table 1). In the case of *myo*-inositol, HPAEC-PAD was used to determine its presence and amount by comparing PG102 with a standard synthetic chemical and it was found to be present in 1.25% of PG102 (Table 1).

Table 1 Content of chemical compounds contained in PG102 and their IL-4 inhibitory activities

Compound	Amount in PG102 (w/w)*	SC_{50}† [μg/mL (μM)]	Specific activity (units/mg)‡	Total activity (units)§
Citric acid	5.94%	144.8 (754)	6.9	410.2
Quinic acid	5.38%	1084.0 (5641)	0.9	49.6
Malic acid	0.84%	90.6 (675)	11.0	92.8
myo-Inositol	1.25%	6293.0 (34930)	0.2	2.0
Isoquercitrin	0.01%	1.9 (4)	513.6	51.4
HMF	0.66%	82.2 (652)	12.2	80.3

*The amount of respective chemicals was determined by HPLC or HPAEC-PAD, depending on the compounds.
†SC_{50} is the concentration of respective chemicals that can suppress the A23187-stimulated production of IL-4 by 50%.
‡The amount (mg) of each chemical present in its SC_{50} value was defined as one unit. A specific activity is the number of units contained in 1 mg of respective chemical compounds.
§Total activity = specific activity × amount (mg) of respective chemicals present in 1 g of PG102.

Determination of biological activities of respective chemical compounds

It was previously reported that PG102 controls the expression of IgE and various Th2 cytokines at the RNA and protein levels, *in vivo* as well as *in vitro*.[9–12] One important biological activity of PG102 is the inhibition of IL-4 expression as this cytokine plays a key role in the regulation of the Th1 and Th2 responses.[22,23] Therefore, it was first tested whether six chemical compounds had such an IL-4 inhibitory activity, using RBL-2H3 cells a rat mast cell line. Cells were treated with respective synthetic chemicals, and 30 min later, stimulated with calcium ionophore, A23187. Culture supernatants were taken after 12 h, followed by an ELISA to determine the level of IL-4. IL-4 was not detectable in unstimulated RBL-2H3 cells, but treatment with A23187 highly increased its level. The A23187-mediated increase of IL-4 production was inhibited by all chemicals in a dose-dependent manner, to a varying degree (Figure 1). In the case of isoquercitrin, for example, the concentration that can suppress the A23187-stimulated production of IL-4 by 50% (here defined as SC_{50}; Suppressive concentration, 50%) was 4 μM or 1.9 μg/mL, while it was 34.9 mM or 6.3 mg/mL for *myo*-inositol. The MTT assay showed that the highest concentrations of respective chemicals used in the above experiments did not have any effect on the viability of the RBL-2H3 cells (data not shown). These results suggested that all six chemicals contributed to the original biological activities found in PG102, but that the individual magnitude of contribution of the respective chemicals varied.

To quantitatively analyse the data, the amount of chemical compound present in 1 mL, that could inhibit IL-4 expression by 50%, was defined as one unit. A specific activity was then calculated as the number of units contained in 1 mg of chemical compound. For example, the SC_{50} value of isoquercitrin was 1.9 μg/mL (= 1 unit/mL), and therefore its specific activity was calculated as 513.6 units/mg by dividing 1 unit by 1.9 μg (Table 1). In the same way, the specific activities of citric, quinic, and malic acids, *myo*-inositol, and HMF were calculated, and they were 6.9, 0.9, 11.0, 0.2, and 12.2 units/mg, respectively (Table 1). Using this method, the contribution of each chemical to the original bioactivity of PG102 could be estimated by multiplying respective specific activity (units/mg) by the amount (mg) of specific chemical present in 1 g of PG102, which was defined as the total activity (units) of the chemical. For instance, the total activity of citric acid was calculated to be 410.2 units by multiplying its specific activity (6.9 units/mg) by its amount (59.4 mg) in 1 g of PG102. The values of specific and total activities of respective chemical compounds are summarized in Table 1. These

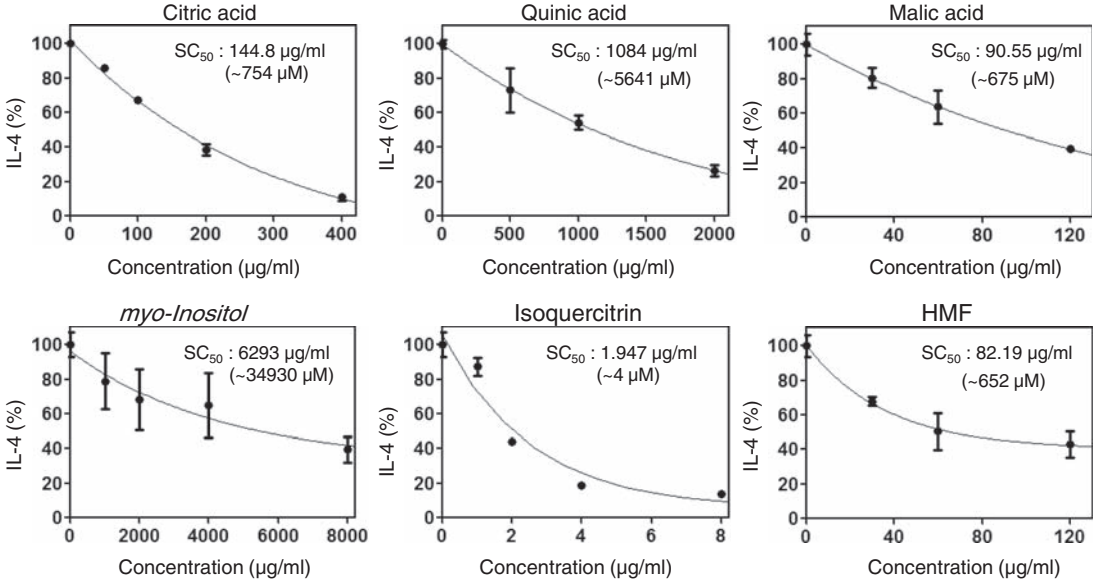

Figure 1 Effect of individual chemical compounds present in PG102 on IL-4 expression. Rat RBL-2H3 mast cells were treated with various concentrations of chemical compounds contained in PG102, and 30 min later, stimulated with A23187 (1 μM). Twelve hours later, cell culture supernatants were taken, and the level of IL-4 was determined using an ELISA kit. Data represent the mean ± SD from more than two separate experiments, each containing triplicate samples

analyses showed that isoquercitrin has the highest IL-4 inhibitory activity in RBL-2H3 cells, while citric acid as a whole contributing the most to the biological activity of PG102. It has to be noted that the precise SC_{50} value can vary depending on experimental conditions such as stimulators, cell lines, and cell maintenance method among many other parameters. In this work, therefore, SC_{50} value was used as a relative guidance for further experiments, rather than as an absolute number.

Effect of chemical mixtures on Th2 cytokines in RBL-2H3 and EL4 cells

The above data showed that tested compounds contained biological activities, but to a varying degree. Therefore, it was tested whether mixing these chemicals could reconstitute the original biological activities of PG102. Two mixtures were prepared, one containing all six compounds (CQMIIH: citric acid, quinic acid, malic acid, isoquercitrin, *myo*-inositol, and HMF) and the other having three organic acids (CQM; citric, quinic, and malic acids). Chemicals were mixed at a ratio present in PG102, namely C:Q:M:I:I:H = 5.94:5.38:0.84:1.25:0.01:0.66 or C:Q:M = 5.94:5.38:0.84. When the dose effect was tested, the amount of CQMIIH or CQM present in 1 mg of PG102 was defined as X1, and then the levels of Th2 cytokines were plotted against X0.125/mL – X4/mL of each mixture (Figure 2). Note that X1 CQMIH contains 59.4 μg of citric acid, 53.8 μg of quinic acid, 8.4 μg of malic acid, 12.5 μg of *myo*-inositol, 0.1 μg of isoquercitrin, and 6.6 μg of HMF, while X1 CQM has the same amount of the first three chemicals.

RBL-2H3 cells were treated with PG102, CQMIIH, or CQM, and 30 min later, stimulated with A23187. After 12 h, culture supernatants were taken, followed by an ELISA for IL-4. As shown in Figure 2(a), CQMIIH and CQM inhibited the level of IL-4 in a dose-dependent manner, with their SC_{50} values being X0.76/mL and X1.24/mL, respectively. PG102, used as a control, showed its SC_{50} value to be 0.938 mg/mL (Figure 2a). Similar experiments were performed for IL-13, using RBL-2H3 cells. The SC_{50} value for PG102 was 1.559 mg/mL, while those for CQMIIH and CQM were X2.13/mL and X1.89/mL, respectively (Figure 2b).

For IL-5, a murine T cell line, EL4, was treated with PG102, CQMIIH, or CQM, and stimulated by N^6, O^2-dibutyryl cAMP (bt2cAMP), and PMA. The production of IL-5 was barely detected in unstimulated EL4 cells, but its level was markedly increased to 3914 pg/mL after the 12 h of treatment with PMA and bt2cAMP (Figure 2c). PG102, CQMIIH, and CQM inhibited the production of IL-5 in a dose-dependent manner. The SC_{50} value of PG102 was 2.178 mg/mL, while the production of IL-5 was affected by two chemical mixtures, to a much lesser extent (Figure 2c). This result suggested that IL-5 expression might be controlled by different compounds present in PG102. Taken together, PG102 and the two chemical mixtures appear to have inhibitory activities on the expression of selective Th2 cytokines.

Regulation of the MAPK phosphorylation by mixtures in RBL-2H3 cells

It has been reported that three MAP (mitogen-activated protein) kinase signalling cascades, namely, those of ERK1/2, p38 MAPK, and JNK, play important roles in the expression of various Th2 cytokines.[24–26] To elucidate the molecular mechanism underlying the effect of PG102 and chemical mixtures on Th2 cytokines, it was investigated whether these chemicals could regulate the MAPK signalling pathways in RBL-2H3 cells. Cells were treated with PG102, CQMIIH, or CQM, stimulated by A23187 after 30 min, and the cell lysates were taken after 2 h, followed

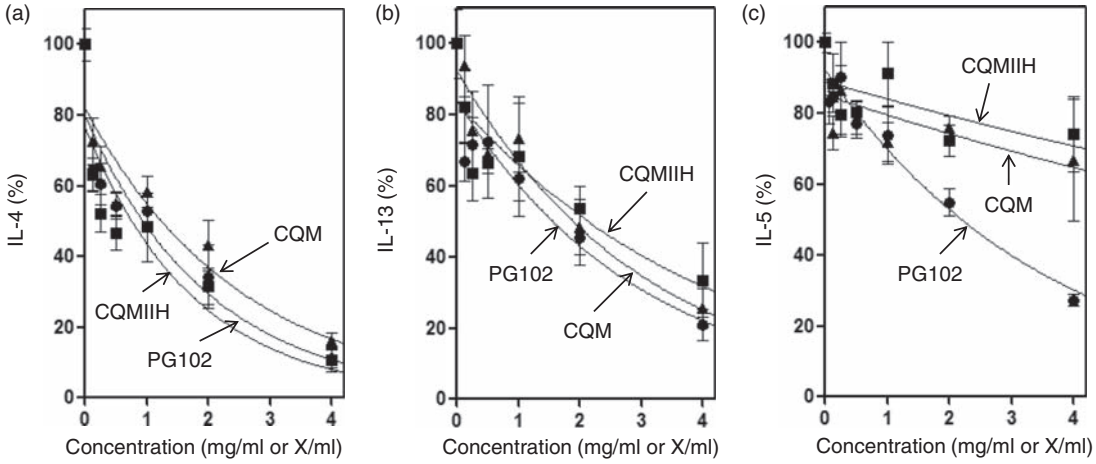

Figure 2 Effect of chemical mixtures derived from PG102 constituents, CQMIIH and CQM, on IL-4, IL-13, and IL-5. (a and b) RBL-2H3 cells were treated with PG102 (0.125, 0.25, 0.5, 1, 2, 4 mg/mL), CQMIIH, or CQM (X0.125, X0.25, X0.5, X1, X2, X4/mL), and 30 min later, stimulated with A23187 (1 μM). Twelve hours later, the level of IL-4 and IL-13 was measured by ELISA kits. Here, X1 indicates the amount of respective chemicals contained in 1 mg of PG102. For example, X1 CQM contains 59.4 μg of citric acid, 53.8 μg of quinic acid, and 8.4 μg of malic acid. CQMIIH: mixture of citric, quinic, and malic acids, *myo*-inositol, isoquercitrin, and HMF; CQM: mixture of citric, quinic, and malic acids. (c) Mouse EL4 T cells were treated with PG102, CQMIIH, or CQM, and then stimulated with bt2cAMP (1 mM) and PMA (10 ng/mL) for 12 h. The supernatant level of IL-5 was determined by an ELISA kit. Data represent the mean ± SD from more than two separate experiments, each containing triplicate samples

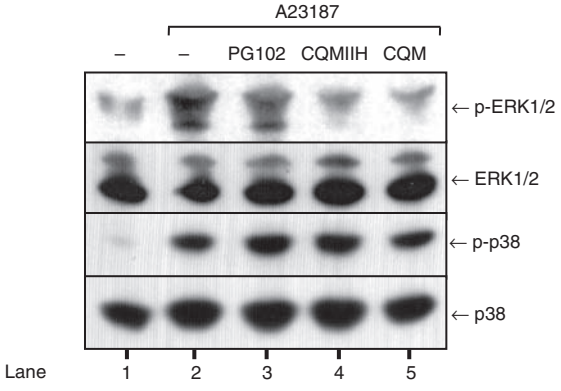

Figure 3 Effect of CQMIIH and CQM on phosphorylation of MAPKs. RBL-2H3 cells were treated with PG102 (2 mg/mL), CQMIIH (X4/mL), or CQM (X4/mL), and 30 min later, stimulated with A23187 (1 μM). Two hours later, total proteins were prepared followed by Western blot hybridization using specific antibodies for total or phosphorylated ERK1/2 and total or phosphorylated p38 MAPK

by Western blot analysis, using antibodies specific for total or phosphorylated MAPKs. The amount of PG102 used in this experiment was 2 mg/mL as has previously been published.[9] For CQMIIH and CQM, X4/mL was used because these chemical mixtures contained minimum 50% of PG102's bioactivities in the cell-based bioassay (Figure 2a and b), depending on cytokines measured. The total amounts of ERK1 and ERK2 were unaffected by the treatment with PG102, CQMIIH, CQM as well as A23187 (Figure 3). However, stimulation with A23187 highly increased the phosphorylation of ERK1/2. In the presence of the CQMIIH or CQM, however, the amount of phosphorylated ERK1/2 was markedly decreased, almost to the level found in unstimulated cells (Figure 3; compare lanes 2, 4, and 5). Treatment with PG102, used as a control, suppressed A23187-induced phosphorylation of ERK1/2, but to a much lesser extent (Figure 3; compare lanes 2 and 3). The phosphorylation status of p38 MAPK and JNK was not affected by PG102, CQMIIH, or CQM (Figure 3 and data not shown). The above data suggested that PG102 and the two chemical mixtures might control the expression of IL-4, selectively using the ERK1/2 pathway.

Effect of chemical mixtures on inflammatory mediators in Raw264.7 cells

It was previously reported that PG102 contains anti-inflammatory activities in several *in vivo* murine models.[9,11,12] To investigate the effect of two chemical mixtures on various inflammatory mediators, Raw264.7 cells, a mouse macrophage cell line, were treated with PG102, CQMIIH, or CQM in the presence of LPS, and, after 24 h, the culture supernatant was analysed by a Griess assay and an ELISA. LPS treatment highly increased the level of nitrite and PGE_2. The production of NO and PGE_2 was inhibited by PG102 in a dose-dependent manner. However, the production of NO and PGE_2 was affected by two chemical mixtures, to a much lesser extent (Figure 4a and b).

PG102 has also been reported to activate the expression of HO-1, a potent anti-inflammatory enzyme, in RBL-2H3 cells as well as in the lung tissue of OVA-induced asthmatic mice.[9] To examine whether CQMIIH or CQM could increase the expression of HO-1, Raw264.7 cells were treated with PG102, CQMIIH, or CQM in the absence or presence of LPS for 9 h, and total cell lysates were analysed by Western blot analysis to measure the level of HO-1. As shown in Figure 4(c) (compare lanes 1 and 2 and lanes 5 and 6), unstimulated Raw264.7 cells barely produced HO-1, but the treatment with PG102 highly increased its level. The presence of CQMIIH or CQM did not have significant effects on its expression, regardless of co-treatment with LPS (Figure 4c; compare lanes 2, 3, and 4, and lanes 6, 7, and 8). Taken together, these chemical mixtures did not seem to contain the anti-inflammatory activities of PG102.

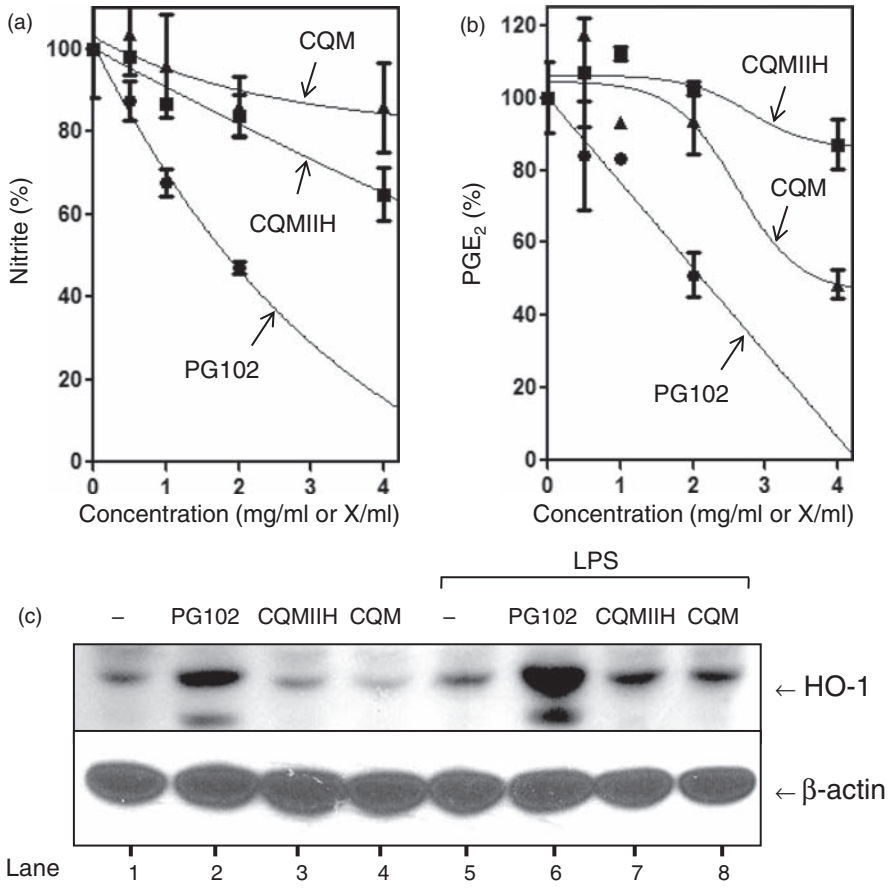

Figure 4 Effect of CQMIIH and CQM on NO, PGE_2, and HO-1. Raw264.7 cells, a murine macrophage line, were treated with PG102 (0.5, 1, 2 mg/mL), CQMIIH, or CQM (X0.5, X1, X2, X4/mL) and then stimulated with LPS (100 ng/mL) for 24 h. (a) The supernatant level of nitrite was measured by Griess assay. (b) The level of PGE_2 was analysed, using the same supernatant, by an ELISA kit. (c) Raw264.7 cells were treated with PG102 (2 mg/mL), CQMIIH (X4/mL), or CQM (X4/mL) in the absence or presence of LPS (100 ng/mL). Total proteins were prepared after 9 h, followed by Western blot analysis using specific antibodies for HO-1 or β-actin. β-actin was used as a loading control

Anti-allergic effects of CQM mixture in the OVA-induced murine asthma model

Because CQM, consisting of only three organic acids, could reconstitute a large portion of original PG102 activities *in vitro*, it was tested whether the CQM mixture contained anti-allergic effects of PG102 in the asthma mouse model. In the animal model, 6 mg PG102 was routinely used on a daily basis.[9] Because CQM contained approximately half of original activities in the cell-based assays (Figures 2 and 4), X12 CQM (equivalent to 6 mg PG102) was used per mouse on a daily basis to test its possible anti-allergic effect.

When immunized with OVA, mice developed severe AHR, as indicated in the enhanced pause (Penh) value, whereas mice fed with PG102 or CQM suffered less AHR at 60 mg/mL of methacholine (Figure 5a). The total number of infiltrating cells in OVA-immunized mice was dramatically increased as compared to that in normal mice. When mice were orally administrated with PG102 or CQM, however, it was decreased by 27% or 41%, respectively (Figure 5b). In particular, the number of eosinophils in PG102 or CQM-treated mice was decreased by 37% or 50%, respectively, as compared to that in OVA-immunized and water-fed mice (Figure 5b). The number of monocytes between all groups was comparable, while that of lymphocytes were slightly increased in the PG102-treated group (Figure 5b). Histological analysis confirmed that the infiltration of eosinophils was increased around perivascular and peribronchiolar regions of the lung in OVA-immunized mice, while it was clearly decreased in PG102 or CQM-fed mice (Figure 5c). These data showed that oral intake of X12 CQM could produce anti-asthmatic effects as effectively as 6 mg PG102 in this murine model.

During allergic cascades, IgE and Th2 cytokines activate various cells including mast cells, basophils, and Th2 cells, leading to various allergic responses.[22] To analyse the effect of PG102 and CQM on these mediators, we measured the IgE level in the serum, IL-13 level in the BALF, and IL-5 and IL-13 levels in the supernatant of OVA-restimulated splenocytes. The serum level of IgE was increased by approximately 10-fold in the OVA-immunized mice, as compared to that in the normal mice (Figure 6a). When mice were administrated with PG102 or CQM, however, the level was decreased by 45% and 74%, respectively (Figure 6a). Furthermore, the treatment with PG102 or CQM significantly decreased the level of OVA-specific IgE (Figure 6b). The level of IL-13 in the BALF was also increased by the immunization with OVA, but was significantly lowered in the BALF samples when treated with PG102 (Figure 6c).

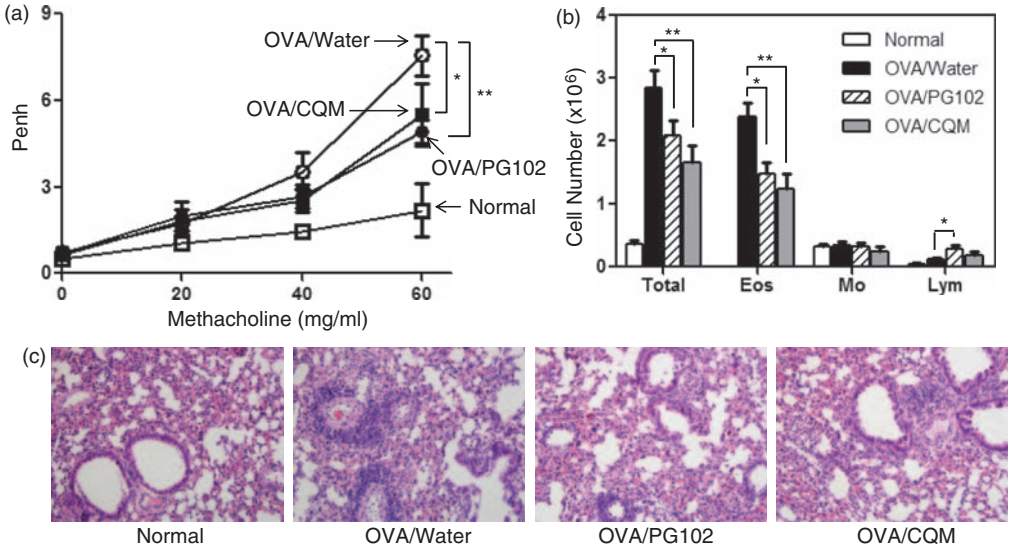

Figure 5 Effects of CQM in asthmatic mice. (A) AHR (airway hyperresponsiveness) was measured after inhalation with methacholine (0, 20, 40, and 60 mg/mL). Penh was used to measure the degree of AHR in conscious mice. Normal, naïve mice; OVA/Water, animals immunized with OVA and fed with water only; OVA/PG102, animals immunized with OVA and intragastrically administrated with PG102 (6 mg/mouse/day); OVA/CQM, animals immunized with OVA and fed with CQM (X12/mouse/day). (b) The number of total cells (Total), eosinophils (Eos), monocytes/macrophages (Mo), and lymphocytes (Lym) in BALF were counted according to stained morphological characteristics. The results are shown as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. OVA/Water group (Student's *t*-test). (c) Lung tissues from mice of respective groups were stained with hematoxylin and eosin, and inflammatory cells were studied by light microscopy. The original microscope magnification is ×200. (A color version of this figure is available in the online Journal)

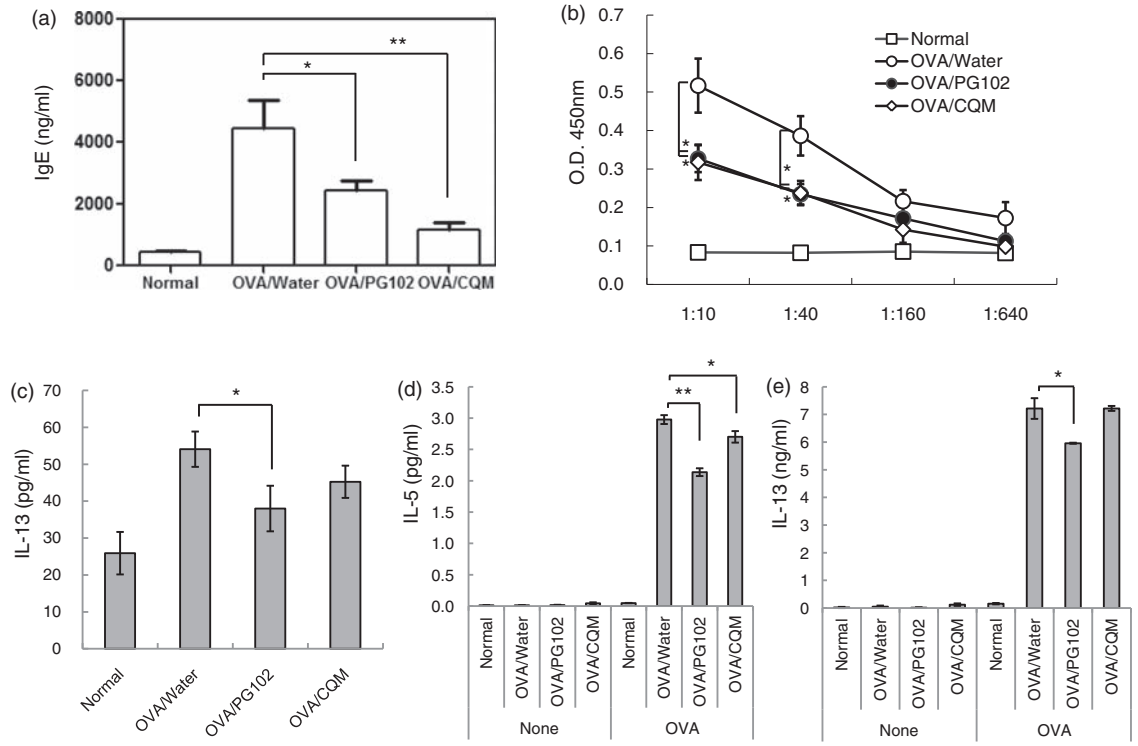

Figure 6 Effects of CQM on the level of IgE and cytokines in asthmatic mice. (a) The serum level of total IgE was determined using an ELISA kit. (b) Because OVA-specific IgE antibody was unavailable, the relative level of OVA-specific IgE was represented as absorbance at O.D.450 nm, using a manual ELISA method. (c) The level of IL-13 in the BALF was measured by an ELISA kit. (d, e) Isolated splenocytes were cultured in the absence or presence of OVA (100 μg/mL) for three days. The culture supernatants were taken to measure the level of IL-5 (d) and IL-13 (e). Values are shown as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. OVA/Water mice (Student's *t*-test)

The magnitude of decrease was lowered in CQM-fed mice (Figure 6c).

When splenocytes from OVA-immunized mice were isolated and cultured in the presence of OVA for three days, the level of IL-5 and IL-13 was highly increased, as compared with the control group. The level of IL-5 and IL-13 was lowered when splenocytes from PG102-treated animals were used (Figure 6d and e). Treatment with the CQM

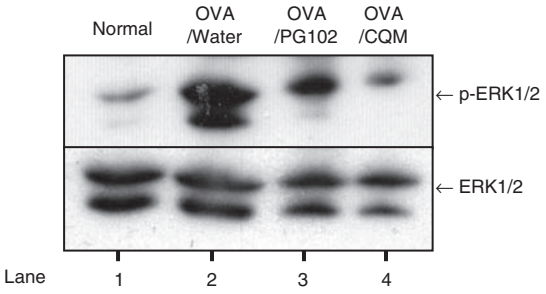

Figure 7 Effect of CQM on phosphorylation of ERK1/2 in the lung tissue of asthmatic mice. Whole lysates were taken by mechanically grinding the lung tissues of mice. Immunoblotting was performed by using specific antibodies for total or phosphorylated ERK1/2

mixture significantly suppressed an OVA-mediated increase in the level of IL-5, while exerting little influence on IL-13. These results showed that CQM could partially reconstitute anti-allergic effects of PG102 in this murine model, and its activities might have resulted mainly from the inhibition of IgE and IL-5 production.

To confirm that CQM controls the ERK pathway in this animal model, the phosphorylation status of ERK1/2 was also investigated in the lung of OVA-induced asthmatic mice. The lung tissue was taken from the above asthmatic mice and mechanically ground, and then the total lung lysates were analysed by Western blot analysis, using antibodies specific for total or phosphorylated ERK1/2. The level of phosphorylated ERK1/2 was highly increased in the lungs of OVA-immunized and water-fed mice, as compared with that in normal mice (Figure 7; compare lanes 1 and 2), but the treatment with CQM markedly inhibited the phosphorylation of ERK1/2 (Figure 7; compare lanes 2 and 4). PG102 decreased the level of ERK1/2 phosphorylation to a lesser extent than CQM (Figure 7; compare lanes 2 and 3). These data suggested that the CQM mixture could downregulate the ERK signalling cascade *in vivo* as well as *in vitro*, and this activity might be a key factor contributing to various anti-allergic effects observed in the above animal experiment.

Discussion

We have previously demonstrated that PG102, prepared from an edible fruit known as *A. arguta*, not only inhibits the production of Th2 cytokines and IgE in various *in vitro* cell culture systems, but also ameliorates allergic symptoms in several allergic disease animal models.[9–12] In an effort to identify biologically active compound(s), it was first tested whether chemical compounds previously reported to be present in *A. arguta* are actually contained in PG102, and then whether these compounds individually had any biological activities shown by PG102. Our data indicated that six single compounds were indeed present in PG102 and exerted an inhibitory influence on the expression of IL-4 to varying degrees.

In efforts to reconstitute the biological activities of PG102, these chemicals were mixed in an appropriate ratio, resulting in two formulations (CQMIIH and CQM), and their biological activities were tested both *in vitro* and *in vivo*. CQMIIH and CQM contained a high level of inhibitory activities on the production of IL-4, IL-5, and IL-13 cytokines. In particular, the CQM mixture produced significant anti-asthmatic activities in the mouse model. Its suppressive effect on the serum level of IgE was especially prominent, indicating that IgE might be one of the major targets of CQM. These results indicated that the original activities of PG102 could be reconstituted, *in vitro* and *in vivo*, by mixing selective chemicals present in PG102.

To unravel the mechanism(s) underlying the inhibitory activity on Th2 cytokines, the effects of CQMIIH and CQM were investigated on MAPKs, which are well known to be involved in the expression of these cytokines.[25,27] These two mixtures suppressed A23187-mediated ERK phosphorylation in RBL-2H3 cells without affecting either p38 or JNK phosphorylation. Consistent with these *in vitro* data, ERK1/2 phosphorylation was highly suppressed in the lung samples taken from mice orally administered with CQM. In the same experiments, treatment with CQM was found to produce significant therapeutic effects and influence the profiles of cell biological and biochemical factors associated with asthma. Our data are in good agreement with the previous reports showing the amelioration of asthmatic symptoms and regulation of relevant biochemical markers by the use of ERK inhibitors in two different murine asthma models.[28,29]

The ERK signalling pathway is known to be involved in the control of the production of leukotrienes that play important roles in allergic disorders including CD8+ T cell-mediated allergic inflammation.[30–33] Therefore, anti-asthmatic effects of CQM may be the result of its combined abilities to control various factors regulated by the ERK signalling pathway as well as to downregulate the expression of Th2 cytokines. The ERK signal transduction cascade also plays important roles in the control of cell growth, proliferation, survival, and fate.[34] This MAPK pathway is used to induce various malignant tumours including acute lymphoblastic leukaemia, for example by providing signals necessary for the growth of malignant hematopoietic cells.[34,35] Indeed, a specific inhibitor of the ERK signalling pathway has been shown to produce anti-tumour effects.[36,37] It would be interesting to test whether CQM could contain anti-tumour effects as it shows such selective inhibitory effects on this pathway.

In summary, six chemicals present in PG102 acted as active compounds to varying degrees. When they were mixed in an appropriate ratio, the mixtures could reconstitute a significant level of PG102's original biological activities. In OVA-induced asthmatic mice, oral administration with one particular formulation, called CQM, ameliorated asthmatic symptoms, such as AHR and eosinophilia. Such therapeutic effect seems to have been achieved by lowering the level of IgE and Th2 cytokines, probably through the selective inhibition of the ERK signalling cascade. Taken together, the mixtures, especially CQM, of

chemical compounds present in PG102, may have potential as a safe and effective reagent for various allergic diseases. This type of approach may be useful for developing biologically active formulations from complex, but functional, botanical extracts.

Author contributions: DK, JC, SHK, and SK participated in the design, interpretation of the studies, and analysis of the data. DK, JC, and M-JK conducted the experiments. DK, S-HC, and SK wrote and reviewed the manuscript.

ACKNOWLEDGEMENTS

This work was supported in part by grants 2011 K000287 from the Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, in which ViroMed Co., Ltd. is a participating company. We thank Chung-Won Kim, Eun-Sil Hong, and Eun-Jung Kwon for contributions to the extraction of PG102 and the studies on the murine asthma model.

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(Received May 31, 2012, Accepted December 13, 2012)