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Inhibition of release of inflammatory mediators in primary and cultured cells by a Chinese herbal medicine formula for allergic rhinitis

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Abstract

Background: We demonstrated that a Chinese herbal formula, which we refer to as RCM-101, developed from a traditional Chinese medicine formula, reduced nasal and non-nasal symptoms of seasonal allergic rhinitis (SAR). The present study in primary and cultured cells was undertaken to investigate the effects of RCM-101 on the production/release of inflammatory mediators known to be involved in SAR.

Methods: Compound 48/80-induced histamine release was studied in rat peritoneal mast cells. Production of leukotriene B₄ induced by the calcium ionophore A23187 was studied in porcine neutrophils using an HPLC assay and lipopolysaccharide-stimulated prostaglandin E₂ production was studied in murine macrophage (Raw 264.7) cells by immune-enzyme assay. Expression of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) was determined in Raw 264.7 cells, using western blotting techniques.

Results: RCM-101 (1–100 µg/mL) produced concentration-dependent inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells and of lipopolysaccharide-stimulated prostaglandin E₂ release from Raw 264.7 cells. Over the range 1 – 10 µg/mL, it inhibited A23187-induced leukotriene B₄ production in porcine neutrophils. In addition, RCM-101 (100 µg/mL) inhibited the expression of COX-2 protein but did not affect that of COX-1.

Conclusion: The findings indicate that RCM-101 inhibits the release and/or synthesis of histamine, leukotriene B₄ and prostaglandin E₂ in cultured cells. These interactions of RCM-101 with multiple inflammatory mediators are likely to be related to its ability to reduce symptoms of allergic rhinitis.

Background

Allergic rhinitis, in particular seasonal allergic rhinitis (SAR) or hay fever, is a common allergic condition [1]. World-wide, SAR afflicts 10 – 40% of individuals [2], with approximately 20% affected in the United States, 13% in Western Europe [3] and 16.1% in Australia [4]. SAR is an immune response to a wide variety of pollens from grasses, weeds and trees. It involves the interaction of allergens with specific immunoglobulin E (IgE) antibodies bound to high affinity Fcε receptors on the surface of mast cells and basophils in the nasal mucosa [5]. This interaction causes degranulation of these cells, releasing a number of inflammatory mediators which are responsible for a cascade of symptoms. Histamine, tryptase, prostaglandin and bradykinin are responsible for the immediate allergic response of sneezing, nasal itch and rhinorrhoea [5]. The late phase response, usually 4 – 6 hours after the immediate response, involves a large increase of eosinophils, basophils and other leukocytes at the inflammatory sites, in response to chemoattractants. In the late phase response, it is likely that histamine and leukotrienes are released from basophils rather than from mast cells because there is no corresponding increase in tryptase which originates from mast cells [5].

The conventional management of SAR is usually symptomatic, with histamine H₁ receptor antagonists, sympathomimetic amine vasoconstrictors and corticosteroids. However, these treatments frequently have certain undesirable side effects and, often do not provide complete symptom relief [6]. Except corticosteroids, which have more significant side-effects, conventional treatments usually target a single inflammatory mediator, which probably explains their limited effectiveness [7].

Complementary/alternative therapies are becoming increasingly used in Western countries for the treatment of allergic diseases, with growing perceptions that such treatments are effective and that they are associated with fewer and less severe side effects [8]. Certain Chinese herbal formulae have been reported to be beneficial for the treatment of asthma and allergic rhinitis, including SAR, with some results showing that their effectiveness is comparable to prednisolone [8]. Recently, we conducted a randomized placebo-controlled clinical trial on a Chinese herbal formula which was developed from a traditional Chinese medicine formula for the treatment of symptoms associated with rhinitis. The formula was optimized on the basis of Chinese medicine syndrome theory for the treatment of SAR. We demonstrated that, after eight weeks of treatment, the herbal medicine formula, which we refer to as RCM-101, was effective in reducing the nasal and non-nasal symptoms of SAR [9].

In a previous investigation of the possible mechanism(s) of the anti-inflammatory/anti-allergic activity of RCM-101 in SAR, we found that the herbal formula inhibited histamine release from isolated guinea-pig tracheal preparations and the production of nitric oxide and prostaglandin E₂ by cultured macrophages [10]. In the present study, as an extended investigation into the pharmacological activities of RCM-101 in reducing the symptoms of SAR, we have investigated its effects on histamine release, leukotriene B₄ (LTB₄) and prostaglandin E₂ (PGE₂) production, and the expression of two enzymes involved in inflammatory processes, namely cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).

Methods

All experimental procedures involving animals were approved by RMIT University Animal Ethics Committee and were conducted in compliance with the Australian National Health and Medical Research Council guidelines.

Histamine, LTB₄ and PGE₂ are three key inflammatory mediators in allergic conditions such as SAR. To investigate the effects of RCM-101 on the synthesis/release of these mediators, we used three well characterized cell-based models, namely rat peritoneal mast cells for histamine [11], porcine neutrophils for LTB₄ [12] and murine macrophage cells (Raw 164.7) for PGE₂ [13,14].

Preparation and extraction of RCM-101

RCM-101 is a herbal formula with 18 herbal ingredients, modified from a traditional Chinese medicine formula. Each herb for the formula was supplied in a granulated form produced under Good Manufacturing Practices by Min Tong Pharmaceutical Company (Taichong, Taiwan) which holds certification from the Australian Therapeutic Goods Administration (TGA-GMP No: 1888). Authenticated, quality-certified raw herbs were first tested to ensure that they were free of heavy metals. They were then washed, dried and extracted in boiling water for 1 – 1.5 hour. The aqueous extract was separated by filtration (100 mesh) and the water content was reduced to 60% by heating (50 – 60°C) under reduced pressure (50 – 70 mmHg) for 2 – 5 hours. The concentrated extract of each herb was combined with starch as an excipient and the product was dried and ground into fine granules. For each preparation, 1 g of granulated product was equivalent to 5 g of the raw herb. The granulated herbal preparations were sterilised and sealed in plastic bottles. In our laboratory, the granulated preparations of the herbs were combined in the proportions given in Table 1 to produce the herbal formula. All herbal ingredients of RCM-101 are approved in the Australian Register of Therapeutic Goods as active raw herbs for use in medicines.

Table 1: Herbal constituents of RCM-101 (% of granulated herbs by weight*)

Scientific name	Botanical name	Chinese name	%
<i>Flos Magnoliae</i>	<i>Magnolia liliflora</i> (Desr.)	Xin Yi	3.81
<i>Frutus Schisandrae Chinensis</i>	<i>Schisandra Chinensis</i> (Turcz.)	Wu Wei Zi	2.25
<i>Frutus Terminaliae Chebulae</i>	<i>Terminalia chebula</i> Retz.	He Zi	13.87
<i>Frutus Xanthii Sibirici</i>	<i>Xanthii Sibirici</i> Patr. Ex Widd.	Cang Er Zi	7.11
<i>Herba Asari</i>	<i>Asarum sieboldii</i> Miq.	Xi Xin	3.81
<i>Herba Menthae Haplocalysis</i>	<i>Mentha haplocalyx</i> Briq.	Bo He	4.68
<i>Herba Schizonepetae Tenuifoliae</i>	<i>Schizonepeta Tenuifolia</i> Briq.	Jing Jie	14.21
<i>Pericappium Citri Reticulatae</i>	<i>Citrus reticulata</i> Blanco	Chen Pi	9.36
<i>Radix Angelicae Sinensis</i>	<i>Angelica sinensis</i> (Oliv.) Diels	Dang Gui	4.68
<i>Radix Astragali Membranaceus</i>	<i>Astragalus membranaceus</i> (Fisch.) Bge	Huang Qi	4.68
<i>Radix Bupleuri</i>	<i>Bupleurum chinense</i> D.C	Chai Hu	3.81
<i>Radix Codonopsis pilosulae</i>	<i>Codonopsis pilosula</i> (Franch.) Nannf.	Dang Shen	2.25
<i>Radix Glycyrrhizae Uralensis</i>	<i>Glycyrrhiza uralensis</i> (Fisch.)	Gan Cao	4.68
<i>Radix Saposhnikovia Divaricata</i>	<i>Saposhnikovia divaricata</i> (Turcz.)	Fang Feng	4.51
<i>Rhizoma Atractylodis Macrocephalae</i>	<i>Atractylodes macrocephala</i> Koidz	Bai Zhu	4.68
<i>Rhizoma Cimicifugae</i>	<i>Cimicifuga foetida</i> L.	Sheng Ma	4.68
<i>Rhizoma Ligustici Chuanxiong</i>	<i>Ligusticum chuanxiong</i> (Hort.)	Chuan Xiong	4.68
<i>Semen Plantaginis</i>	<i>Plantago asiatica</i> L. Wild.	Che Qian Zi	2.25

* 1 g of each granulated herb is equivalent to 5 g of the raw herb (dry weight).

The herbal formula was extracted with ethanol (120 mg/mL) at room temperature with continuous agitation for 4 hours. The ethanol extract was collected by centrifugation (5000 rpm for 10 minutes) and vacuum filtration. The extract was dried using a rotary evaporator (Büchi Rotavapor, Brinkman Company, Westbury, NY, USA) and stored below -20°C. It was diluted to the required concentrations on the day of use.

Reagents

Compound 48/80, histamine hydrochloride, O-phthalaldehyde, spermidine hydrochloride, bovine serum albumin (BSA), phosphate buffer saline, heparin, disodium ethylenediaminetetraacetic acid (EDTA), lipopolysaccharide (LPS) *E. Coli*, calcium ionophore A23187, Hanks' balanced salt solution, RPMI 1640 medium, fetal bovine serum, phenylmethylsulfonyl fluoride, gentamycin, leupeptin, pepstatin A and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Company (St Louis, MO, USA). Monoclonal mouse anti-rat cyclooxygenase 2 antibodies and mouse macrophage lysate were obtained from Transduction Laboratories (Lexington, KY, USA). Goat anti-rabbit horseradish peroxidase conjugated immunoglobulin G was obtained from Dako Corporation (CA, USA). The Coomassie blue protein assay kit was purchased from Bio-Rad (USA). Polyclonal rabbit anti-mouse cyclooxygenase-1 antibody, immune-enzyme analysis PGE₂ kit, arachidonic acid, prostaglandin B₂, LTB₄, 6-trans LTB₄, 6 trans-12 epi LTB₄, 5-hydroxyeicosatetraenoic acid (5-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). HPLC-grade methanol was sup-

plied by Selby-Biolab (Clayton, Victoria, Australia). All other analytical reagents were obtained from Merck Pty Ltd (Kilsyth, Victoria, Australia).

Histamine release from rat peritoneal mast cells

Rat peritoneal mast cells were collected in Tyrode buffer as previously described [11]. Briefly, rats (Sprague-Dawley, 200 – 300 g) of either sex were killed and 10 mL of Tyrode buffer (NaCl, 137 mM; KCl, 2.7 mM; HEPES, 10 mM; MgCl₂, 1 mM; CaCl₂, 1.0 mM; NaH₂PO₄, 0.41 mM), containing 0.3% BSA and 5 units/mL heparin was injected into the peritoneal cavity. The abdomen was gently massaged for about 90 seconds, and then carefully opened and the cell-containing peritoneal fluid collected with a transfer pipette. The cell-containing fluid was centrifuged at 4°C at 800 rpm for 5 minutes. The cells were collected, washed in 10 mL of Tyrode buffer and centrifuged again. This procedure was repeated twice [11]. The cells were then suspended in the concentration of 1 × 10⁶ cells/mL in 10 mM HEPES-Tyrode buffer (pH 7.4) containing 0.1% BSA.

The 200 mL peritoneal cell suspensions were incubated with various concentrations of RCM-101 for 10 minutes at 37°C and then exposed to compound 48/80 for 10 minutes. Aliquots of 100 µL of rat peritoneal mast cells in Tyrode buffer were combined with 100 µL aliquots of RCM-101 extract in Tyrode buffer such that 5 × 10⁵ cells/mL were incubated with RCM-101 at concentrations of 1, 10 and 100 µg/mL for 10 minutes immediately prior to stimulation of the cells with compound 48/80. The cell suspensions were then centrifuged at 4°C at 4800 rpm

and the supernatant collected. As an internal standard, 10 μ L of spermidine (1 mg/mL) was added to 200 mL aliquots of the supernatant, followed by 20 μ L of 30% perchloric acid (HClO_4). The mixture was then filtered and 100 μ L was transferred into HPLC vials for histamine determination [11]. The Ca^{2+} chelating agent, EDTA, (100 μ M) was used as a positive control.

The HPLC system (Shimadzu, Kyoto, Japan), which included a fluorescent detector (Shimadzu RF10XL), C-10ATvp pumps, SIL-10ADvp auto-injector and STRODS-II reversed phase column, equipped with post-column derivatisation was set up as previously described [11]. Samples of the peritoneal cell supernatant/internal standard solution were injected into the HPLC system using an autosampler. Histamine and spermidine were detected with excitation and emission wavelengths of 360 nm and 440 nm, respectively. Four-point standard curves for histamine were prepared, ranging from 50 – 2500 ng/mL in 10 mM HEPES-Tyrode buffer.

Leukotriene B₄ production

Synthesis of LTB_4 was induced in neutrophils as previously described [15], with slight modification. Porcine blood was collected from a local abattoir. Neutrophils were isolated using a Percoll gradient and suspended in Hanks' buffer, containing 5 mM HEPES. Suspended neutrophils (2.8×10^6 cells/mL) were incubated (37°C) with RCM-101 extract, NDGA (as a positive control, 0.1, 1 or 10 μ g/mL) or vehicle (ethanol), for 5 minutes before the addition of arachidonic acid (2.5 μ M) substrate. Porcine neutrophils were suspended in Hanks' buffer in concentration of 2.8×10^6 cells/mL. RCM-101 (0.1, 1, 100 μ g/mL) was added 10 minutes before the calcium ionophore A23187 (2.5 μ M). After 5 minute incubation, production of LTB_4 was initiated by the addition of the calcium ionophore A23187 (2.5 μ M) and 5 minutes later the reaction was terminated by adjusting the pH to 3 with citric acid. PGB_2 (45 ng) and 15-HETE (83 ng) were then added as internal standards. The reaction mixture was extracted with 5 mL of chloroform/methanol (7:3 v/v) and dried under vacuum. The residue was dissolved in 120 μ L of HPLC mobile phase (methanol-water-acetic acid, 76/34/0.08, v/v/v, pH 3.0) and leukotriene metabolites were assayed using a Waters HPLC system equipped with an auto sampler, a multi-solvent delivery system and a Waters 996 Photodiode Array Detector. Standard curves were prepared by the addition of LTB_4 (10 – 200 ng) and 5-HETE (500 – 800 ng) to neutrophil suspensions. Data were analysed using Water Millennium Software, Version 3.2, results being expressed as percentage of the vehicle control which was taken as 100%.

Prostaglandin E₂ production

Murine macrophages (Raw 264.7 cells, American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated FBS, 100 μ g/mL gentamycin, 1.5 g/L sodium bicarbonate and 10 mM HEPES, at 37°C, in an atmosphere containing 5% CO_2 . Cells were sub-cultured once a week by harvesting them with trypsin/EDTA and seeding them in 75 cm^2 flasks. Once confluent, murine macrophages were suspended in serum-free RPMI medium at concentration of 2×10^5 cells/mL, and the cells were seeded in 24-well plates (1×10^5 cells/well), in serum-free RPMI medium. Cells were then treated with RCM-101 (1, 10, or 100 μ g/mL) or vehicle 10 minutes before the addition of LPS (1 μ g/mL). The supernatant and the cells were separated. PGE_2 was assayed in the supernatant using an immune-enzyme analysis kit. The assay depends on competition between PGE_2 and PGE_2 acetylcholinesterase conjugate (PGE_2 -tracer) for a limited amount of monoclonal PGE_2 antibody. The assays were carried out according to the manufacturer's protocol, in triplicate. PGE_2 release was calculated using software supplied by the kit manufacturer.

Determination of COX-1 and COX-2 protein expression in Raw 264.7 cells

Cultured Raw 264.7 cells prepared as described above for determination of PGE_2 production, with and without incubation with RCM-101, were washed twice with ice-cold phosphate buffer saline then lysed with 100 μ L/well of lysis buffer (50 mM Tris base, pH 7.6, 2 mM MgCl_2 , 1 mM EGTA, 1% TritonX, 1 mM phenyl PMSF, 1 mM pepstatin, 1 mM aprotinin, 1 mM leupeptin) for 5 minutes. The cells and the supernatant were collected and centrifuged for 5 minutes at 14000 rpm. The cell debris was discarded and the supernatant was assayed for protein concentration using Coomassie Protein Assay Kit (Bio-Rad Laboratories Pty Ltd, California, USA) and the UV-visible spectrophotometer (Cintra 5, GBC Scientific Equipment Pty Ltd, Illinois, USA)

COX-1 and COX-2 protein was measured by Western blotting as previously described [16] with a slight modification. Aliquots of 20 μ g of total protein were loaded to each lane of 7.5% SDS-polyacrylamide gels. The proteins were then electrically transferred to nitrocellulose membranes which were incubated overnight with a polyclonal anti-rabbit COX-1 antibody or a monoclonal mouse anti-rat COX-2 antibody (diluted 1:500 and 1:2500 respectively) in 5% non-fat milk in Tris-buffered saline (Tris base 25 mM, glycine 19 mM, methanol 20%). On the next day, membranes were washed with Tris-buffered saline (Tris base 20 mM, NaCl 137 mM, Tween-20 0.1%, pH 7.5) for 40 minutes with constant agitation, during which time the buffer was changed every 5 minutes. The mem-

branes were then incubated with swine anti-rabbit or goat anti-mouse secondary conjugated to horseradish peroxidase diluted 1:5000 with blocking buffer (Tris base 20 mM, NaCl 137 mM, Tween-20 0.1%, pH 7.5 and 5% non-fat milk). The results were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means \pm standard deviation (SD). The statistical significance of differences between means was determined by unpaired, two-tailed Student's t-test or, for more than two groups, by first testing for global differences by one- or two-way analysis of variance (ANOVA) and then testing for differences between predetermined pairs of means by Dunnet's test. The differences with probability levels less than 0.05 ($P < 0.05$) were considered to be statistically significant.

Results

Inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells

The amount of histamine released from rat unstimulated peritoneal mast cell preparations was 46.3 ± 37.1 ng/mL ($n = 14$). When the cells were stimulated with compound 48/80 (1 μ g/mL) histamine release increased markedly to 638.3 ± 308 ng/mL ($n = 14$). As shown in Figure 1, compound 48/80-induced histamine release was inhibited by RCM-101 (1 – 100 μ g/mL) in a concentration dependent manner. Compound 48/80-induced histamine release was also reduced by 10 μ M EDTA (Figure 1).

Inhibition of leukotriene B₄ production in porcine neutrophils

In the absence of calcium ionophore A23187 incubation, the production of LTB₄ by porcine neutrophils was 9.58 ± 4.6 ng/mL. In vehicle (ethanol) control experiments, A23187 incubation increased LTB₄ production to 167.77 ± 70.4 ng/mL ($n = 8$).

As shown in Figure 2, LTB₄ production in A23187-incubated neutrophils was inhibited by RCM-101 at concentrations of 1 and 10 μ g/mL. NDGA (1 and 10 μ g/mL) also inhibited A23187-induced LTB₄ production.

Inhibition of prostaglandin E₂ production in LPS-stimulated Raw 264.7 cells

Unstimulated Raw 264.7 cells incubated in serum-free RPMI medium for 24 hours produced a baseline concentration of PGE₂ of 52 ± 24.4 pg/mL ($n = 6$). Incubating the cells with LPS (1 μ g/mL) increased the PGE₂ level to 3874 ± 818.13 pg/mL ($n = 6$). This induced production of PGE₂ was reduced in a concentration-dependent manner by RCM-101 (1 – 100 μ g/mL), when present during incubation with LPS. Indomethacin (1, 10 and 100 μ M), when

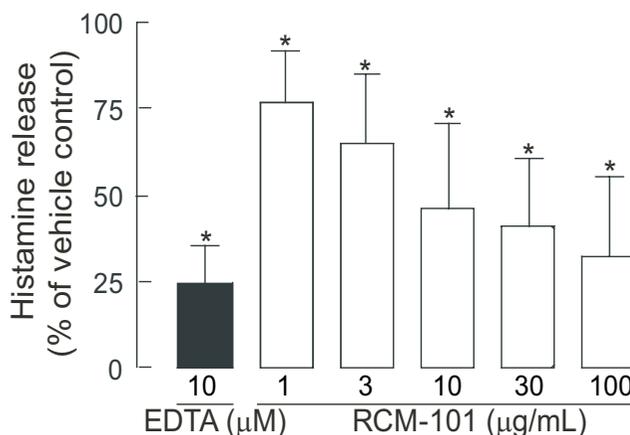


Figure 1

Inhibition of compound 48/80-stimulated histamine release from rat peritoneal mast cells by RCM-101. EDTA was used as a positive control. Data are plotted as means \pm SD, ($n = 8$). * $P < 0.05$, One-way ANOVA and Dunnet's test

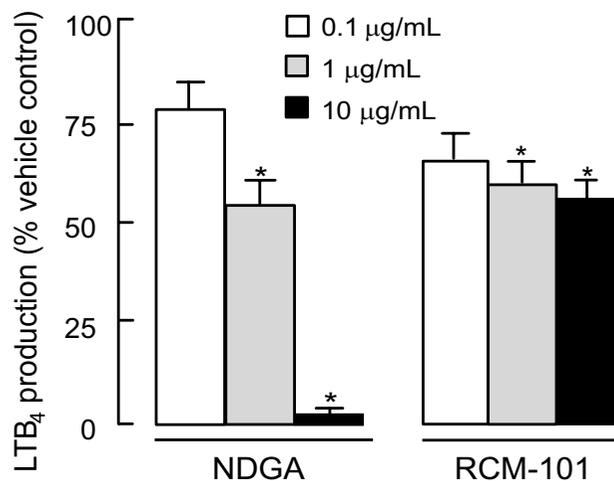


Figure 2

Inhibition of LTB₄ formation in porcine neutrophils by RCM-101 and nordihydroguaiaretic acid (NDGA). Data are plotted as means \pm SD, ($n = 8$ in each case). * $P < 0.05$, One-way ANOVA and Dunnet's test

present during LPS incubation, completely blocked PGE₂ production. The data are shown in Figure 3.

Effects of RCM-101 on COX-1 and COX-2 protein expression in LPS-stimulated Raw 267.4 cells

As shown in Figure 4, immunoreactivity bands corresponding to COX-1 and COX-2 (70 kDa) were detected by Western blot analysis of the supernatant of lysed Raw 264.7 cells. Densitometric analysis of the marker chemiluminescence indicated that expression of COX-1 protein was unaffected by RCM-101 (10 and 100 µg/mL). Similarly, dexamethasone (10 and 100 µM) also did not alter COX-1 protein expression. In contrast, the expression of COX-2 protein was significantly ($P < 0.05$, one-way ANOVA, Dunnet's test) reduced by 100 µg/mL RCM-101 and also by 100 µM dexamethasone. Figure 4 shows examples of the visualised bands on nitrocellulose membranes corresponding to COX-1 and COX-2 proteins.

Discussion

This study was undertaken to extend our previous investigation of possible pharmacological mechanisms for the effects of the herbal formula RCM-101 in reducing SAR symptoms [10]. The main findings of the present study are that RCM-101 inhibits compound 48/80-induced release of histamine from isolated rat peritoneal mast cells and inhibits the production of LTB₄ by porcine neutrophils and of PGE₂ by Raw 264.7 cells. Histamine, PGE₂ and LTB₄ are well known mediators of inflammatory/allergic responses. Taken together with our previous findings in isolated tissues from rats and guinea-pigs, it seems

that RCM-101, a herbal formula with 18 constituent Chinese herbs, has activity directed to inhibition of the synthesis or release of multiple key inflammatory mediators.

Mast cell-derived mediators, particularly histamine are considered to be responsible for the acute (early stage) allergic symptoms of SAR [17]. These mediators act on the smooth muscle cells of small blood vessels, blood platelets, mucous glands and sensory nerve endings to produce or contribute to symptoms such as nasal congestion, nasal and throat itching, sneezing and hypersecretion of mucus [18]. The release of mast cell-derived histamine is inhibited by RCM-101. While the inhibition mechanisms are not clear, RCM-101 has been shown to contain several herbal ingredients that inhibit the release or action of histamine. For example, *Rhizoma Cimicifugae* was reported to exert a potent inhibitory action on histamine-mediated contractions in guinea pig ileum [19] and *Flos Magnoliae* inhibits mast cell-mediated allergic reactions by preventing mast cell degranulation and IgE-mediated histamine release [20]. *Herba Schizonepetae* was also reported to reduce compound 48/80-induced histamine release [21]. Moreover, the Chinese herbal formula *Xiao Chai Hu Tang*, which contains several of the herbal ingredients of RCM-101, has also been shown to inhibit histamine release from rat peritoneal mast cells [11].

Limited information is available about the chemical constituents of the herbs in RCM-101 responsible for inhibition of the release or action of histamine or their action mechanisms. However, glycyrrhetic acid, which is

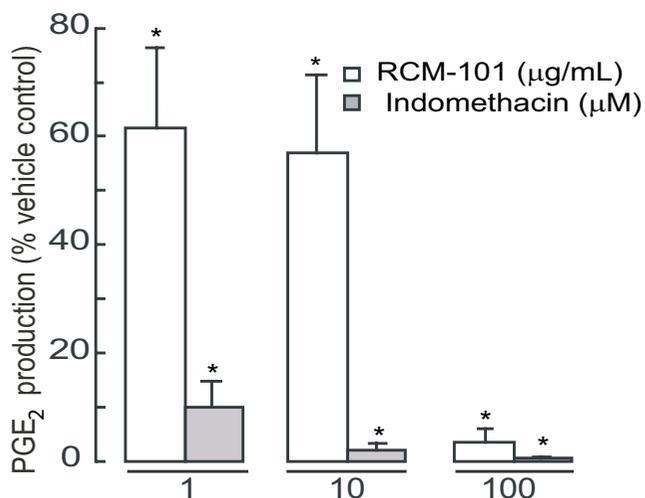


Figure 3
Inhibition of PGE₂ production in LPS-stimulated Raw 264.7 cells by RCM-101 and indomethacin. Data are plotted as means ± SD of percentage control PGE₂ production (n = 6 in each case). * $P < 0.05$, One-way ANOVA and Dunnet's test

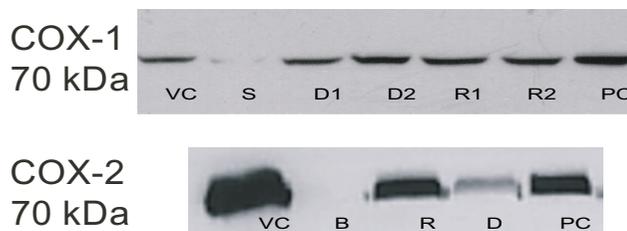


Figure 4
Western blot assay of COX-1 and COX-2 expression by macrophage (Raw 264.7. 20) cells after LPS stimulation. For each lane of SDS polyacrylamide gels, 20 µg of protein was loaded. COX-1 and COX-2. The proteins were detected on nitrocellulose membranes using specific antibodies and visualized by enhanced chemiluminescence. (A) **COX-1**: VC = vehicle control; S = separation lane (not loaded with protein); D1 = dexamethasone (100 µM); D2 = dexamethasone (10 µM); R1 = RCM-101 (100 µg/mL); R2 = RCM-101 (10 µg/mL); PC = positive control (COX-1 lysate). (B) **COX-2**: VC = vehicle control; B = cells not stimulated with LPS; R = RCM-101 (100 µg/mL); D = dexamethasone (100 µM); PC = positive control (COX-2 lysate).

present in *Radix Glycyrrhizae*, a herbal component of RCM-101, was shown to inhibit the release of histamine by targeting protein kinase C- β (nPKC β) [22]. Conjugated linoleic acid, identified in *Rhizoma Chuanxiong*, another herbal component of the formula, is known to inhibit immediate anaphylaxis, histamine release and the synthesis of arachidonic acid metabolites [23].

Prostaglandins and leukotrienes were found to be involved in the pathophysiology of SAR [24] and LTB₄ is released by infiltrating neutrophils during the immediate phase of allergic responses [25]. The present study found that RCM-101 inhibits the production/release of LTB₄ induced by the calcium ionophore A23187 in porcine neutrophils. Previous studies showed that extracts of the herb *Radix Glycyrrhizae* inhibit A23187-induced release of arachidonic acid from cell membranes by inhibiting phospholipaseA₂ and that they also inhibit 5-lipoxygenase, acting together to suppress the production of LTC₄ and LTB₄ [26]. Glycyrrhetic acid and caffeic acid, present in *Radix Glycyrrhizae*, *Herba Menthae*, *Rhizoma Ligusticum Chuanxiong* and *Rhizoma Cimicifugae* [27], both were shown to inhibit arachidonic metabolite formation [28,29]. These findings suggest a possible action of RCM-101 on SAR through the inhibition of the release of LTB₄.

PGE₂ is released in both the early phase (from mast cells) and late phase (from basophils and eosinophils) responses of SAR [17]. We found that LPS-induced production of PGE₂ by murine macrophages was inhibited by RCM-101. The findings are consistent with previous studies on individual herbal components of RCM-101. There is also evidence indicating that *Radix Glycyrrhizae* inhibits PGE₂ production in rats tissues and *Rhizoma Cimicifugae* blocks LPS-induced production of PGE₂ [19]. In addition, both topical and oral administration glycyrrhetic acid was reported to prevent ear oedema and to inhibit PGE₂ and LTC₄ formation induced by arachidonic acid in mice [30]. These findings suggest a possible action of RCM-101 on SAR through the inhibition of PGE₂ production.

The inhibition of prostaglandin production by RCM-101 is most likely due to inhibition of COX-2 protein expression, because we observed that COX-2 protein expression was markedly reduced by RCM-101 whereas the expression of COX-1 protein was unaffected. It is known that COX-2 is responsible for prostaglandin production in Raw 264.7 cells [14]. Previous studies also observed that *Rhizoma Cimicifugae* and *Radix Glycyrrhizae* inhibited COX-2 activity [26].

Conclusion

The results obtained in this study indicate that RCM-101 has inhibitory actions on multiple inflammatory mediators, including the release of histamine from mast cells,

and production of LTB₄ and PGE₂ by neutrophils and Raw 264.7 cells, respectively. In addition, RCM-101 also selectively inhibits the expression of the inducible enzyme COX-2. These actions of RCM-101 may contribute to its efficacy in SAR. The exact mechanisms of these actions and the contributions by individual herbal ingredients of RCM-101 require further investigation.

Abbreviations

- 5-HETE: 5-hydroxyeicosatetraenoic acid
- 15-HETE: 15-hydroxyeicosatetraenoic acid
- ANOVA: Analysis of variance
- BSA: Bovine serum albumin
- COX-1: Cyclooxygenase-1
- COX-2: Cyclooxygenase-2
- EDTA: Disodium ethylenediaminetetraacetic acid
- EGTA: Ethylene glycol tetraacetic acid
- HPLC: High performance liquid chromatography
- IgE: Immunoglobulin E
- LPS: Lipopolysaccharide
- LTB₄: Leukotriene B₄
- LTC₄: Leukotriene C₄
- NDGA: Nordihydroguaiaretic acid
- PGE₂: Prostaglandin E₂
- PMSF: Phenylmethanesulphonyl fluoride
- SAR: Seasonal allergic rhinitis
- SD: Standard deviation

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

GBL conducted the experiments, contributed to the interpretation of the findings and preparation of the manuscript. CCLX contributed to the design of the study, the interpretation of the findings and preparation of the manuscript. DFS contributed to the interpretation of the find-

ings and critically revised the manuscript. FCKT contributed to the design of the study and preparation of the manuscript. SM conducted some of the experiments, contributed to the interpretation of the findings and preparation of the manuscript. CGL contributed to the design and conduct of the study, the interpretation of the findings and preparation of the manuscript. All authors approved the final manuscript.

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