Inhibition of COX-2 and PGE_2 in LPS-stimulated RAW264.7 cells by lonimacranthoide VI, a chlorogenic acid ester saponin

FUQIN GUAN[1,2], HAITING WANG[2], YU SHAN[2], YU CHEN[2], MING WANG[2], QIZHI WANG[2], MIN YIN[2], YOUYI ZHAO[2], XU FENG[1,2] and JIANHUA ZHANG[1]

[1]Jiangsu Key Laboratory for Bioresources of Saline Soils, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences/Nanjing Botanical Garden Memorial Sun Yat-Sen, Nanjing, Jiangsu 210014; [2]College of Life Science, Nanjing Agricultural University, Nanjing, Jiangsu 210095, P.R. China

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Abstract. Lonimacranthoide VI, first isolated from the flower buds of *Lonicera macranthoides* in our previous study, is a rare chlorogenic acid ester acylated at C-23 of hederagenin. In the present study, the anti-inflammatory effects of lonimacranthoide VI were studied. Lipopolysaccharides (LPS) induced an inflammatory response through the production of prostaglandin E_2 (PGE_2), and these levels were reduced when lonimacranthoide VI was pre-administered. Additionally, the mechanism of the anti-inflammatory effects of lonimacranthoide VI was investigated by measuring cyclooxygenase (COX) activity and mRNA expression. The results showed that lonimacranthoide VI inhibited mRNA expression and *in vitro* activity of *COX-2* in a dose-dependent manner, whereas only the higher lonimacranthoide VI concentration possibly reduced *COX-1* expression and *in vitro* activity. Taken together, these results indicate that lonimacranthoide VI is an important anti-inflammatory constituent of *Lonicera macranthoides* and that the anti-inflammatory effect is attributed to the inhibition of PGE_2 production through COX activity and mRNA expression.

Introduction

Inflammation is a complex process mediated by the activation of various immune cells. Macrophages play a central role in mediating a number of different immunopathological phenomena during inflammation by the overproduction of inflammatory mediators, known as prostaglandins (PGs) (1). Cyclooxygenase (COX) catalyzes the synthesis of PGs from arachidonic acid (AA). There are two isoforms of COX. *COX-1* is a reference gene that is expressed constitutively in the majority of tissues. *COX-2* is an immediate, early-response gene that is highly inducible by inflammatory stimuli, including endotoxin lipopolysaccharides (LPS) (2). This indicates that targeted inhibition of COX-2 is a promising approach in preventing inflammation and inflammation-associated cancer.

Lonimacranthoide VI (Fig. 1), which was first isolated from the flower buds of *Lonicera macranthoides* (Caprifoliaceae) in our present study, is a rare chlorogenic acid ester acylated at C-23 of hederagenin (3). Shan *et al* (4) reported that chlorogenic acid significantly decreased LPS-induced upregulation of COX-2 at protein and mRNA levels in RAW264.7 cells and consequently inhibited PGE_2 release from LPS-treated RAW264.7 cells, indicating that chlorogenic acid exerted anti-inflammatory effects. Previously, several studies have reported that triterpene saponins can also inhibit COX-2 expression (5,6). However, thus far, the effects of chlorogenic acid ester saponin on the cyclooxygenase isoforms (COX-1 and COX-2) have not been analyzed. In the present study, the effects of lonimacranthoide VI were observed on PGE_2 synthesis, *in vitro* activity of COX-1 and COX-2 and the gene expression of COX-1 and COX-2. These data provide a mechanistic basis for the chemopreventive and anti-inflammatory properties of lonimacranthoide VI.

Materials and methods

Reagents and chemicals. Lonimacranthoide VI was isolated from the flowers of *Lonicera macranthoides Hand.-Mazz.* and the structure is shown in Fig. 1. Lonimacranthoide VI was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as a stock solution stored at -20˚C and was subsequently diluted with medium prior to each experiment. The final DMSO concentration did not exceed 0.1% DMSO throughout the study (all the control groups comprised 0.1% DMSO).

Cell culture and cell viability assay. RAW264.7 murine macrophages were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy

Correspondence to: Professor Xu Feng, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences/Nanjing Botanical Garden Memorial Sun Yat-Sen, P.O. Box 1435, 1 Qianhu Houcun Road, Zhongshanmen Wai, Nanjing, Jiangsu 210014, P.R. China
E-mail: fengxucnbg@cnbg.net

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of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2. The effect of lonimacranthoide VI on cell viability was assessed by the 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, the RAW264.7 cells were seeded at $5x10^4$ cells/well in a 96-well plate and treated with various concentrations of lonimacranthoide VI or vehicles. Each treated or control group contained six parallel wells. After incubation for 24 h at 37˚C in a humidified incubator, cell viability was determined. MTT (5 mg/ml in phosphate-buffered saline) was added to each well and incubated for 4 h; subsequently, 100 μl of the solubilization solution (10% sodium dodecyl sulphate in 0.012 M HCl) was added into each well and the plate remained in the incubator overnight. Absorbance was recorded on a microplate reader (Tecan Austria GmbH, Salzburg, Austria) at a wavelength of 570 nm (reference wavelength, 690 nm). The percentage of cell proliferation was calculated as a ratio of the optical density (OD) value of the sample to the OD value of the control. All the experiments were performed under the same conditions at least three times. The cell inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = (1-average absorbance of treated group/average absorbance of control group) x100%.

Measurement of PGE_2. RAW264.7 cells were plated at a density of $2.5x10^5$/ml cells in a 24-well plate with 1 ml of culture medium per well and cultured overnight. The cells were pre-incubated for 2 h with various doses of lonimacranthoide VI and stimulated for 24 h with 100 ng/ml LPS. The cell culture supernatants were collected immediately following treatment and centrifuged at 1,000 x g for 15 min to remove the particulate matter. PGE_2 was determined using an enzyme immunoassay (EIA) kit (catalog no. ADI-900-001, Enzo Life Sciences, Switzerland). The medium and PGE_2 EIA conjugate was added to a 96-well plate pre-coated with goat anti-mouse IgG and left to react for 2 h, followed by a final wash to remove any unbound antibody-enzyme reagent. A substrate solution was added and the intensity of the color produced was measured at 405 nm (correction wavelength set at 570-590 nm).

In vitro COX inhibition assay. The ability of lonimacranthoide VI to inhibit ovine COX-1 and COX-2 was determined using an enzyme immunoassay (EIA) kit (catalog no. 560101; Cayman Chemical Co., Ann Arbor, MI, USA). COX catalyzes the first step in the biosynthesis of AA to PGH_2. $PGF_{2\alpha}$, produced from PGH_2 by reduction with stannous chloride, was measured by EIA (ACE™ competitive EIA, Cayman Chemical, Ann Arbor, MI, USA). Briefly, to a series of supplied reaction buffer solutions [960 μl 0.1 M Tris-HCl (pH 8.0) containing 5 mM EDTA and 2 mM phenol] with either COX-1 or COX-2 (10 μl) enzyme in the presence of heme (10 μl), 10 μl of various concentrations of test drug solutions (1, 10, or 100 μM in a final volume of 1 μl) were added. These solutions were incubated for 5 min at 37˚C and subsequently 10 μl AA solution (100 μM) was added. The COX reaction was stopped by the addition of 50 μl 1 M HCl after 2 min. Then 100 μl of stannus chloride was added to produce $PGF_{2\alpha}$, which was measured by EIA. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of the PG tracer is held at a constant while the concentration of PGs varies. The specific antiserum-PG complex bound to a mouse anti-rabbit IgG that had been previously attached to the well. The plate was washed to remove any unbound reagents and 200 μl Ellman's reagent, which contains the substrate to acetylcholine esterase, was added to the well. The product of this enzymatic reaction generates a distinct yellow color that absorbs at 406 nm. The intensity of this color, determined by spectrophotometry, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation. Percent inhibition was calculated by the comparison of the compounds treated to the various control incubations.

Quantification of mRNA by reverse transcription-polymerase chain reaction (PCR). Total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and the concentration of RNA was determined at 260 nm. cDNA was synthesized by extension of oligo (dT) primers with 10 units of avian myeloblastosis virus reverse transcriptase in a mixture containing 1 μg total RNA. The cDNA amplification was performed using the PCR kit (Takara Bio, Inc., Shiga, Japan), denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 1 min. The primer sequences used were as follows: *COX-2* sense, 5'-GGGAAGCCTTCTCCAACC-3' and antisense, 5'-GAA CCCAGGTCCTCGCTT-3'; and *GADPH* sense, 5'-AACGAC CCCTTCATTGACC-3' and antisense, 5'-TCAGATGCC TGCTTCACC-3', which was used as an internal control. The PCR products (10 μl) were separated on 2% agarose gel and visualized by ethidium bromide staining. The gel image was captured and analyzed using Quantity One software (Tanon Science and Technology Co., Shanghai, China).

Statistical analysis. All the experiments were repeated at least three times. Data are reported as means ± standard deviation. The statistically significant differences of the test compounds compared to the untreated control were calculated using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of lonimacranthoide VI on the cell viability. To evaluate the anti-inflammatory effects of lonimacranthoide VI, a murine RAW264.7 macrophages *in vitro* model was used. RAW264.7 cells were treated with various concentrations of lonimacranthoide VI and cell viability was measured using the MTT assay. As shown in Fig. 2, the resulting survival curve shows that lonimacranthoide VI does not have cytotoxic effects on the proliferation of cells. As lonimacranthoide VI showed no cytotoxicity at concentrations ≤100 μM in RAW264.7 macrophages, lonimacranthoide VI was used at a concentration of 0-100 μM for the remaining experiments.

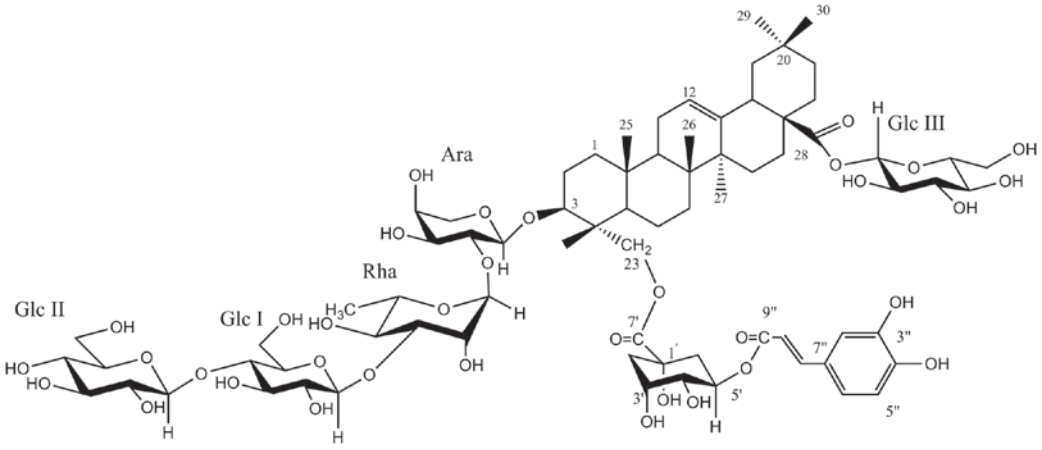

Figure 1. Chemical structure of lonimacranthoide VI.

Effect of lonimacranthoide VI on serum PGE_2 concentration. Since PGE_2 is one of the most important inflammatory mediators, the effects of lonimacranthoide VI on the LPS-induced release of PGE_2 from RAW264.7 cells were observed. The cells were pretreated with lonimacranthoide VI for 2 h followed by incubation with 100 ng/ml LPS. One day after LPS treatment, the PGE_2 contents in the culture medium were detected. The LPS-induced PGE_2 secretion level was inhibited by treatment with the compound at all the doses examined (IC_{50}=0.25 μM) and the maximum inhibition was observed at a dose of 100 μM (Fig. 3). Indomethacin (IM) was used as a positive control.

In vitro COX inhibition. COX-1 and COX-2 catalyze the biosynthesis of PGH_2 from the AA substrate. The inhibition of COX-1 results in certain undesirable side-effects, whereas COX-2 inhibition provides therapeutic effects in pain, inflammation, cancer, glaucoma, Alzheimer's and Parkinson disease (7). Therefore, the present study aimed to examine the COX-1 and COX-2 inhibitory activity of lonimacranthoide VI on purified enzymes as a mechanism of topical anti-inflammatory action. The compound showed inhibitory effects on COX-1 and COX-2 (Tables I and II). Furthermore, 10 μM lonimacranthoide VI inhibited COX-2, whereas the dose had no effect on COX-1.

Effects of lonimacranthoide VI on mRNA expression of COX-1 and COX-2. To investigate the effects of lonimacranthoide VI on mRNA expression of *COX-1* and *COX-2*, the RAW264.7 macrophage cells were pre-treated with the compound at various concentrations ranging 1-100 μM and were stimulated with 100 ng/ml LPS for 24 h. The COX-2 non-selective inhibitor, IM, was used as a standard drug for comparing the ability of lonimacranthoide VI in modulating the pro-inflammatory genes, *COX-1* and *COX-2*. As shown in Fig. 4, 100 ng/ml LPS can stimulate *COX-2* mRNA expression, whereas it had no significant effects on the *COX-1* mRNA expression. In addition, 100 μM lonimacranthoide VI can significantly suppress the mRNA expression of *COX-1* and *COX-2* in the LPS-stimulated RAW264.7 macrophage cells as compared to LPS-treated cells alone. However, the presence of IM only produced a significant reduction of *COX-2* mRNA expression in LPS-treated cells.

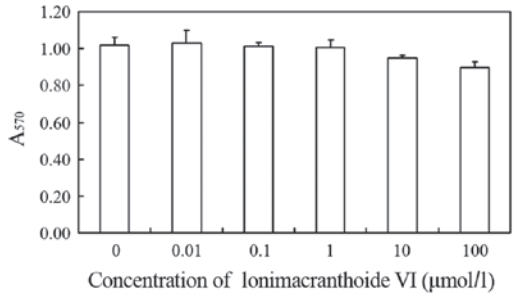

Figure 2. Effect of lonimacranthoide VI on the viability of RAW264.7 cells, as determined by the MTT assay.

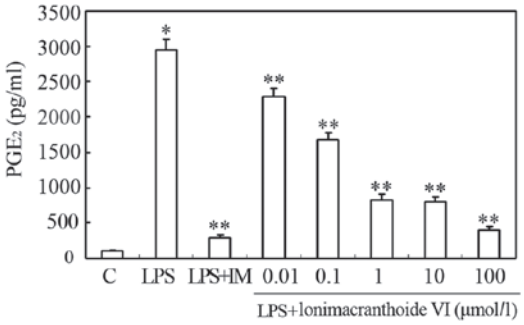

Figure 3. Inhibitory effects of lonimacranthoide VI on LPS-induced PGE_2 production in RAW264.7 macrophage cells. *P<0.01 vs. control; **P<0.01 vs. LPS. C, control; LPS, lipopolysaccharides; PGE_2, prostaglandin E_2.

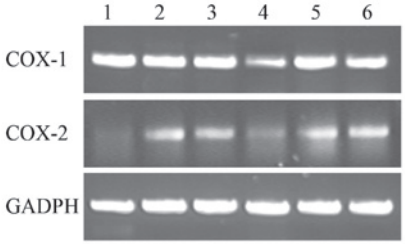

Figure 4. Effects of lonimacranthoide VI on *COX-1* and *COX-2* mRNA expression in LPS-induced RAW264.7 macrophage cells. Lane 1, control; lane 2, LPS (100 nmol/l); lane 3, LPS+IM (100 nmol/l); lane 4, LPS+lonimacranthoide VI (100 μmol/l); lane 5, LPS+lonimacranthoide VI (10 μmol/l); and lane 6, LPS+lonimacranthoide VI (1 μmol/l). *COX*, cyclooxygenase; LPS, lipopolysaccharides; IM, indomethacin.

Table I. Inhibitory effects of lonimacranthoide VI on *in vitro* COX-1 enzyme activity.

Group	$PGF_{2\alpha}$, pg/ml	Inhibitory rate, %
COX-1 inhibitor tubes	3.5±0.3	-
COX-1 100% initial activity tubes	151.4±21.5	-
IM, μmol/l		
10	3.5±0.5	97.68
Lonimacranthoide VI, μmol/l		
100	15.0±0.8	90.08
10	143.1±45.5	5.45
1	205.5±3.0	-35.80

COX-1, cyclooxygenase-1; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; IM, indomethacin.

Table II. Inhibitory effects of lonimacranthoide VI on *in vitro* COX-2 enzyme activity.

Group	$PGF_{2\alpha}$, pg/ml	Inhibitory rate, %
COX-2 inhibitor tubes	0.9±0.1	-
COX-2 100% initial activity tubes	144.5±29.3	-
NS-398, μmol/l		
10	37.3±4.1	74.19
Lonimacranthoide VI, μmol/l		
100	40.5±7.0	71.99
10	116.5±5.6	19.39
1	143.5±6.5	0.66

COX-2, cyclooxygenase-2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

Discussion

Natural products play a significant role in drug discovery and development. The search for natural products with anti-inflammatory activity has increased in recent years (8). The dried flower buds of *Lonicera macranthoides Hand.-Mazz.*, a plant of *Lonicera* in Caprifoliaceae, are commonly used in traditional Chinese medicine in the Southwest of China (9). *Lonicera* plants have antipyretic and detoxification properties and have been widely used to treat carbuncles and boils, toxins in blood, fever and colds (9). The study by Liu *et al* (10) reported that the total saponins of *Lonicera fulvotnetosa Hsu et S. C. Cheng. Ms* could inhibit the mouse ear edema provoked by croton oil and also the carrageenan-induced hind paw edema in rats. Following this, loniceroside A and loniceroside C, isolated from *Lonicera japonica*, were shown to possess anti-inflammatory activity in a croton oil-induced ear edema model *in vivo* (11,12). In the present study, lonimacranthoide VI was demonstrated to inhibit the LPS-induced production of PGE_2, indicating anti-inflammatory effects. Therefore, lonimacranthoide VI is an important anti-inflammatory constituent of *Lonicera macranthoides*.

Inflammation is the response towards the presence of pathogens, chemicals or mechanical injury. The inflammatory response is induced by inflammatory mediators generated via a series of inducible genes that have critical functions in the host immune defence, signal transduction pathways and vascular regulation. The cyclooxygenase isoforms (COX-1 and COX-2) are among the most thoroughly studied mammalian oxygenases involved in the inflammation response pathway (13). The results of Fig. 4 and Table II indicated that lonimacranthoide VI appeared to produce a significant dose-dependent reduction on the mRNA expression and *in vitro* activity of *COX-2*. By contrast, lonimacranthoide VI (concentration range, 1-10 μM) did not suppress the mRNA expression and *in vitro* activity of *COX-1* (Fig. 4, Table I) in the LPS-stimulated RAW264.7 macrophage cells as compared to LPS-treated cells alone. However, the COX-1 gene expression and *in vitro* activity of 100 μmol/l lonimacranthoide VI was significantly lower compared to LPS-treated cells alone (Fig. 4, Table I). This indicates that higher concentrations of lonimacranthoide VI may induce inhibition of COX-1 expression and activity. The decrease in PGE_2 production following lonimacranthoide VI treatment corresponded with the decrease in *COX* (*COX-1* and *COX-2*) mRNA expression and *in vitro* activity, particularly for *COX-2*.

A number of studies have demonstrated that the expression of *COX-2* is largely regulated by transcriptional activation (14,15). Lipopolysaccharide and other pro-inflammatory cytokines activate NF-κB, which is a mammalian transcription factor that regulates several genes important in immunity and inflammation. NF-κB binding sites have been identified on the murine COX-2 promoter, which plays a role in LPS-mediated induction of COX-2 in macrophages. In addition, binding of CCAAT-enhancer-binding proteins (C/EBPs), c-AMP response element binding proteins (CREBs) and c-Jun to the COX-2 promoter enhances its transcriptional activation (16). The present study is limited to understanding the gene expression and *in vitro* activity of COX-1 and COX-2 and the production of PGE_2, therefore, it may be noteworthy to understand the effect of lonimacranthoide VI at the transcriptional activation level involving the NF-κB, C/EBP, CREB and c-Jun proteins.

In conclusion, lonimacranthoide VI was found to inhibit mRNA expression and *in vitro* activity of *COX-2* and PGE_2 production in a dose-dependent manner. Although lower concentrations of lonimacranthoide VI did not significantly reduce the mRNA expression and *in vitro* activity of *COX-1* in the LPS-stimulated RAW264.7 macrophage cells, a higher concentration may possibly reduce *COX-1* expression and *in vitro* activity further. To the best of our knowledge, this is the first study explaining the anti-inflammatory pathway of lonimacranthoide VI, which provides support to the traditional utilization of this plant in pain and inflammation. The study clearly indicates that lonimacranthoide VI inhibits the production of PGE_2 via the inhibition of *COX-2* expression and activity; however, caution is recommended as LMS4-1 may inhibit the *COX-1* expression at higher doses.

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