

Anti-inflammatory Activity of Prosapogenin Methyl Ester of Platycodin D via Nuclear Factor-kappaB Pathway Inhibition

Ji Won CHUNG,^{a,#} Eun Jung NOH,^{a,#} Hai Lin ZHAO,^a Joon-Soo SIM,^{a,b} Young Wan HA,^a
Eun Myoung SHIN,^a Eun Bang LEE,^a Choon Sik CHEONG,^c and Yeong Shik KIM^{*,a}

^aNatural Products Research Institute, College of Pharmacy, Seoul National University; Seoul 151–742, Korea; ^bNational Institute of Agricultural Biotechnology; Suwon 441–707, Korea; and ^cCollege of Pharmacy, Duksung Women's University; Seoul 132–714, Korea. Received June 25, 2008; accepted August 20, 2008; published online August 22, 2008

Platycodin D (PD) isolated from Platycodi Radix has been reported to have anti-inflammatory and anti-tumor activities. In this study, we have investigated anti-inflammatory activities of prosapogenin D (PrsD) and prosapogenin D methyl ester (PrsDMe) of PD. The results indicated that PrsDMe concentration-dependently inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E₂ (PGE₂) production, however, PrsD did not inhibit NO production in LPS-induced macrophages. Furthermore, PrsDMe inhibited the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) without appreciable cytotoxic effects. In the transfectant RAW 264.7 cells, PrsDMe was observed to reduce the level of nuclear factor- κ B (NF- κ B) activity. PrsDMe also inhibited the degradation of an inhibitory protein called inhibitor κ B (I κ B). Therefore, it was suggested that PrsDMe inhibited the expression of LPS-induced iNOS and COX-2 genes by suppressing NF- κ B activation at the transcriptional level. Also, PrsDMe showed carrageenan-induced acute anti-inflammatory activity and the adjuvant-induced anti-arthritis activity in mice. In conclusion, we suggest that these compounds exert an anti-inflammatory effect through the regulation of the NF- κ B pathway. The different activities of PD, PrsD and PrsDMe are based on the structure of the sugar substituent or methyl group at the C₂₈-carboxyl position.

Key words platycodin D; prosapogenin methyl ester; anti-inflammation; nuclear factor- κ B; animal model

Immune responses to infection or to localized injury or trauma are among the main causes of local or systemic inflammation. Despite the beneficial effect of inflammation in limiting responses to cellular and organ damage, a breakdown in the regulation of the inflammatory response may result in a wide range of chronic diseases such as arthritis, inflammatory bowel diseases, asthma and others.¹⁾

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, sulindac and indomethacin, are widely believed to have anti-inflammatory effects due to their ability to inhibit prostanoid production, and relieve inflammation by inhibiting cyclooxygenase (COX).

COX are the rate-limiting enzymes that catalyze the formation of prostaglandins from arachidonic acid.^{2–5)} Levels of prostaglandins increase early in the course of the inflammatory process. The constitutive isoform, COX-1, is expressed in most tissues predominantly in platelets, gastrointestinal tract, kidney, and liver.^{2–4)} The inducible isoform, COX-2, is activated in response to pro-inflammatory cytokines and growth factors.^{3,4)} Because COX-2 protein is induced by several kinds of stimuli in inflammatory cells, inhibitors of COX-2 protein induction might be candidates for the new-type NSAIDs. Also, inflammation is associated with inducible nitric oxide synthase (iNOS). Especially, nitric oxide (NO) produced by activated macrophages via iNOS was initially considered a component of innate immunity in the fight against infections.⁶⁾

It has been well accepted that nuclear-factor- κ B (NF- κ B) signaling pathway plays important roles in the inflammation, control of cell growth, apoptosis, stress response, and many other physiological processes.^{7–11)} There are several important molecules such as NF- κ B, inhibitor κ B (I κ B), IKK, within NF- κ B signaling pathway.¹²⁾ NF- κ B is a key protein in the pathway, and has been described as a major culprit and

a therapeutic target in inflammation and cancer.^{13–16)} It is activated by lipopolysaccharide (LPS), which is a mammalian transcription factor that controls various genes that are important for immunity and inflammation.¹⁷⁾ NF- κ B is mainly composed of p50 and p65.¹⁷⁾ In unstimulated cells, NF- κ B is present in the cytoplasm through interactions with an inhibitory protein, I κ B. NF- κ B is activated by I κ B α degradation following phosphorylation of I κ B α .¹⁸⁾

Platycodi Radix is the root of *Platycodon grandiflorum* A. DE (Campanulaceae).¹⁹⁾ It is edible and the principal herb in Oriental medicine for diseases of the lungs and throat, and is commonly used for the inflammatory conditions of the eyes, ears, and sinuses.¹⁹⁾ Saponins (PS) are the primary constituents of Platycodi Radix,^{20–22)} and they are responsible for diverse effects including anti-inflammation,^{23–26)} anti-allergy,²³⁾ anti-tumor,²⁷⁾ augmentation of immune response,^{28–30)} and stimulating the apoptosis in skin cells.²⁷⁾ In the last decade, PS has generated renewed interests due to their pharmacological potentials for healing the diseases of adulthood such as hyperlipidemia or anti-obesity.^{31–36)} In the previous study, platycodin D (PD) inhibited the induction of COX-2 by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), with suppression of the production of prostaglandin E₂ (PGE₂) in rat peritoneal macrophages.²⁶⁾ Also, we demonstrated that 2''-*O*-acetyl polygalacin D, platycodin A, platycodin D, and polygalacin D inhibited LPS-induced iNOS and COX-2 through suppression of NF- κ B activation in RAW cells.²⁴⁾ Their structures are mainly characteristic of sugar moieties coupled to C-3 and C-28 of aglycone. Because platycodin D is a main saponin component of Platycodi Radix and is easily available, as opposed to other platycodi saponins, the sugars on C-28 can be removed to make prosapogenin (PrsD) and then esterified (PrsDMe) for better bioavailability and safety.

In this study, we focused on the anti-inflammatory activity

* To whom correspondence should be addressed. e-mail: kims@snu.ac.kr

These authors contributed equally to this work.

of PrsDMe, *in vitro* and *in vivo* in a murine macrophage cell line, RAW 264.7. The results demonstrated that PrsDMe inhibits LPS-induced NO production, COX-2 and iNOS expression through inactivation of NF- κ B signaling pathway.

MATERIALS AND METHODS

Materials Platycodin D (Fig. 1) was isolated from the root of *Platycodon grandiflorum* (Platycodi Radix) as previously described.³⁶⁾ The ester type glycoside, PD, is susceptible to acidic or basic-catalyzed hydrolysis and its PrsDMe was prepared according to a published procedure by Christie³⁷⁾ with a slight modification. In brief, PD (100 mg) was added to 1 M potassium methoxide in anhydrous methanol (total volume 15 ml) under the conditions of 60 °C and N₂ atmosphere for 1 h to allow the trans-esterification reaction. The reaction mixture was neutralized with 0.5 N HCl to precipitate the product. The pellet was recrystallized in methanol to get the white compound. The compounds of PrsD and PrsDMe (Fig. 1) were identified on the basis of ¹H-, ¹³C-NMR spectra^{20,38)} and electrospray ionization mass spectrometry and their purity was checked by HPLC analysis.^{35,36)}

Chemicals Protease inhibitors cocktails, Dulbecco's

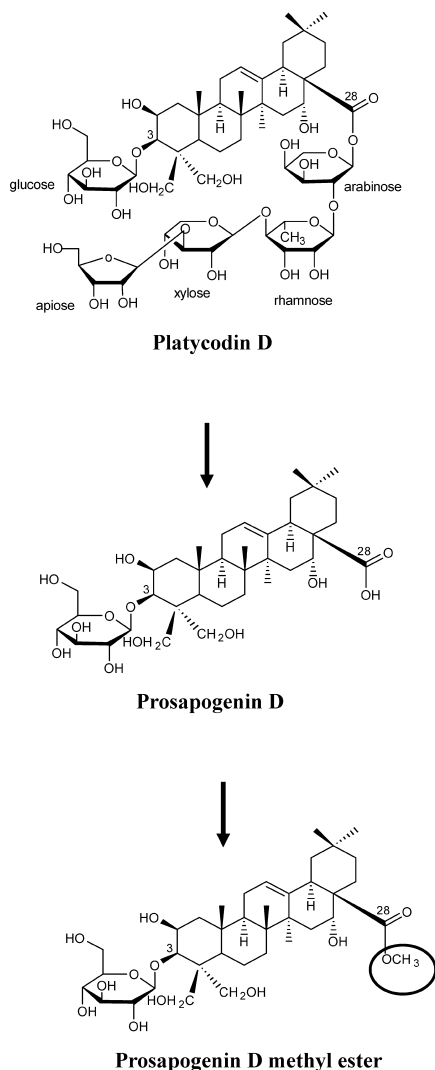


Fig. 1. Chemical Structures of Platycodin D Derivatives

phosphate buffered saline (D-PBS, pH 7.4), Dulbecco's Modified Eagle Medium (DMEM), lipopolysaccharide (LPS), Trypan Blue, dimethyl sulfoxide (DMSO), penicillin-streptomycin, sulfanilamide, naphthylethylenediamine dihydrochloride, 2-amino-5-mercapto-1,3,4-thiadiazole (AMT), penicillin-streptomycin solution, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), indomethacin (IM), Tween 20, 4-methylumbelliferyl phosphate (MUP), Easy Blue reagent (iNtRON Biotechnology, Korea), HEPES and sodium bicarbonate were purchased from Sigma Chemical Co. Ltd (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was from South Pacific (New Zealand). Cell Counting Kit (CCK-8) was purchased from Dojindo Laboratories (Tokyo, Japan). ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.), polyvinylidene difluoride (PVDF) membranes (Millipore, MA, U.S.A.), Protein Assay Reagent (Bio-Rad, Vancouver, Canada), anti-iNOS, COX-2, I κ B α , p65, α / β -tubulin, β -actin, and horseradish peroxidase (HRP)-conjugated secondary antibody (Santacruz, CA, U.S.A.), ECL Plus detection kit (Amersham, U.K.), ONE-STEP RT-PCR Pre Mix kit (Intron Biotechnology, Korea) were used.

Animals Male ICR mice (Samtako, Kyungki-do, Korea) weighing 25–30 g were used in this study. All animal studies were carried out in a pathogen-free barrier zone of Seoul National University Animal Laboratory with the procedure outlined in the Guide for the Care and Use of Laboratory Animals. All animals were acclimated at least for one week, caged in five groups or less, and fed with a diet of animal chow and water *ad libitum*. They were housed at 23 \pm 0.5 °C and 10% humidity in a 12 h light–dark cycle.

Cell Culture RAW 264.7 cells, murine macrophages, were obtained from the American Type Culture Collection. These cells were maintained at subconfluence in 95% air and 5% CO₂ humidified atmosphere at 37 °C. The medium used for routine subculture was DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μ g/ml). Cells were counted with a hemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

Cell Viability Cytotoxicity of PD, PrsD and PrsDMe were evaluated by Cell Counting Kit (CCK-8) purchased from Dojindo Laboratories (Tokyo, Japan). RAW 264.7 cells were plated at a density of 1 \times 10⁴ cells/well in a 96-well plate and incubated at 37 °C for 24 h and then various concentrations of PD, PrsD and PrsDMe were treated in a 96-well plate. After additional 24 h incubation at 37 °C, 10 μ l of CCK solution was added in the wells and additional incubation was needed for 3 h. The resulting was assayed at 450 nm using microplate fluorometer (Molecular Devices, Sunnyvale, CA, U.S.A.).

Nitrite Assay RAW 264.7 cells were plated at a density of 1 \times 10⁵ cells/well in a 24-well plate with 500 μ l of culture medium, and incubated at 37 °C for 24 h. The cells were treated with PD, PrsD and PrsDMe (5, 7.5, 10, 20 μ M) for 2 h and stimulated with LPS (0.1 μ g/ml) for 18 h. The nitrite concentrations of supernatants (100 μ l/well) in medium were measured by adding 100 μ l of griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water). To quantify nitrite concentration, standard nitrite solutions were prepared and absorbance of the mixture was determined with a microplate

fluorometer (Molecular Devices) at 540 nm.

Determination of PGE₂ Concentration RAW 264.7 cells were seeded in wells and incubated for 24 h. After incubation, the cells were incubated with PrsDMe of different concentration in the presence or absence of LPS (0.1 μg/ml). The PGE₂ concentration in the culture medium was determined by ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.).

Western Blot Analysis RAW 264.7 cells were plated at a density of 1×10⁶ cells/well in a 6-well plate with 2 ml of culture medium, and incubated at 37 °C for 24 h. The cells were treated with PrsD and PrsDMe (5, 10, 20 μM) for 2 h and stimulated with LPS (0.1 μg/ml) for 18 h. After incubation, the total cytosolic extraction were lysed as described previous report.⁶⁾ Cytosolic fractions were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE) (8% for iNOS and 10% for COX-2, β-actin and IκBα), and transferred to PVDF membranes. After being blocked by 5% skim milk for 1 h, the membrane was incubated with first antibody and then added second antibody. For developing the membrane, enhanced chemiluminescence (ECL) detection kit was used.

RNA Extraction and Reverse Transcriptase RT-PCR RAW 264.7 cells were plated at a density of 1×10⁶ cells/well in a 6-well plate with 2 ml of culture medium, and incubated at 37 °C for 24 h and the cell were treated with PrsD and PrsDMe (5, 10, 20 μM) for 2 h and stimulated with LPS (0.1 μg/ml) for 5 h. RT-PCR was performed with total RNA. RNA was extracted using Easy Blue reagent (iNtRON Biotechnology, Korea) according to the manufacturer's recommendations. The purity of RNA preparation was checked by measuring the absorbance ratio at 260/280 nm. The sense and anti-sense primers for COX-2, iNOS and β-actin mRNA were used. RT-PCR was performed using ONE-STEP RT-PCR PreMix kit (Intron Biotechnology, Korea), according to the manufacturer's instructions. 2% agarose gel stained with ethidium bromide was used to identify amplified DNA. The gels were viewed with UV transillumination.

Reporter Gene Assay Reporter enzyme activity was measured by cell-based assay system for monitoring NF-κB activity. The pNF-κB-SEAP-NPT plasmid that permits expression of the secretory alkaline phosphatase (SEAP) reporter gene in response to the NF-κB activity and contains the neomycin phosphotransferase (NPT) gene for geneticin resistance in host cells was constructed and transfected into murine macrophages.³⁹⁾ Transfected RAW 264.7 cells were plated at a density of 1×10⁵ cells/well in a 24-well plate with 500 μl of culture medium including geneticin, and incubated at 37 °C for 24 h. The cells were treated with PD, PrsD and PrsDMe (5, 10, 20 μM) for 2 h and stimulated with LPS (0.1 μg/ml) for 16 h. Fluorescence from the product of the SEAP/MUP was measured using a 96 well microplate fluorometer by excitation at 360 nm and measuring light emission at 449 nm.^{39–41)}

Preparation of Nuclear Extracts Nuclear proteins were extracted by using a modification of the Andrew's method.⁴²⁾ In brief, RAW 264.7 cells were plated at the density of 2.0×10⁶ cells on T-25 flask and incubated for 24 h. They were pretreated with the compound for 2 h and incubated with LPS for 1 h. Cells were scrapped and washed with D-PBS and resuspended in 80 μl of lysis buffer. They were in-

cubated for 5 min on ice and then vortexed to disrupt cell membranes for 10 min. After centrifugation at 12000×g for 30 min at 4 °C, pellets containing crude nuclei were washed with buffer A (20 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 3000×g for 10 min at 4 °C. Pellets were resuspended in extraction buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DDT, 0.5 mM PMSF, and Protease Inhibitor Cocktail) and incubated at 4 °C for 40 min with a constant shaking. They were centrifuged at 12000×g for 30 min to obtain the supernatant containing nuclear extracts.

Electrophoretic Mobility Shift Assay (EMSA) The oligonucleotide probe used for EMSA contained the NF-κB consensus sequence. Double-stranded NF-κB consensus oligonucleotide (AGT TGA GGG GAC TTT CCC AGGC) and mutant one (AGT TGA GGC GAC TTT CCC AGG C) were purchased from Promega (Madison, WI, U.S.A.). The probe was 5'-end-labeled with [γ-³²P]-ATP using T4 polynucleotide kinase (Promega) and separated from the unincorporated label. Binding reaction was performed for 30 min with 10 μg of nuclear protein in 20 μl of binding buffer (100 mM Tris-HCl, pH 7.9, 250 mM NaCl, 10 mM MgCl₂, 5 mM EDTA, 0.5% NP-40, 2.5% BSA, 50% glycerol) containing 1 μg of Poly (dI-dC) and 2 μl of 5'-labeled probe (15000–30000 cpm). In the competition experiment, a 40-fold molar excess of the unlabeled probe or unlabeled mutated probe (sc-2511, Santa Cruze) was added before the labeled probe. DNA-protein complex was separated from the unbound probe on 6% polyacrylamide gel in 0.25×TBE running buffer at 100 V. After electrophoresis, the gels were dried and visualized by autoradiography. The intensity of each band was quantitatively determined using a UN-SCAN-IT™ software (Silk Scientific, UT, U.S.A.) and the density ratio represents the relative intensity of each band against those of controls in each experiment.

Carrageenan-Induced Paw Edema Test in Mice Edema was induced in the right hind paw of mice by subcutaneous (s.c.) injection of 0.05 ml/mouse of 1.0% λ-carrageenan (Sigma) in saline according to the previous reports.^{43,44)} The test substances were given orally 30 min before carrageenan injection. The paw thickness was measured using a dial thickness gauge (No. 2046F, Mitutoyo, Kawasaki, Japan). The percent increase of paw thickness was calculated based on the volume of the pre injection paw.

Adjuvant-Induced Arthritis Test in Mice Adjuvant arthritis was induced in mice by injection (0.05 ml/mouse s.c.) of Freund's complete adjuvant (Sigma) in the right hind paw, and thickness of the hind paw was measured just before and at intervals of days after adjuvant injection using a dial thickness gauge.^{43–45)} The test substances were given orally once a day.

Data Analysis The results were expressed as means± standard deviation (S.D.). Analysis of variance (ANOVA) with Bonferroni's test was used for the statistical analysis of multiple comparisons of data. *p*-values less than 0.05 were considered statistically significant.

RESULTS

Inhibition of LPS-Induced NO Production by Platy-

codin D, Prosapogenin D, and Prosapogenin D Methyl Ester in RAW 264.7 Macrophages To investigate the inhibitory effects of PD, PrsD and PrsDMe on LPS-induced NO production in RAW 264.7 macrophages, the accumulated nitrite was determined by the Griess reaction in culture medium. NO production was detected in RAW 264.7 cells stimulated by LPS in the presence or absence of samples for 20 h. LPS (0.1 $\mu\text{g/ml}$) significantly increased the concentration of nitrite as compared to basal level without LPS. Although PD and PrsDMe (5, 7.5, 10, 20 μM) significantly inhibited LPS-induced NO production (Fig. 2B), PrsD (5, 7.5, 10, 20 μM) did not inhibit LPS-stimulated NO production (Fig. 2B). PrsDMe did not affect cell viability at the same concentrations on incubation with LPS (Fig. 2A), but PD showed cytotoxicity at 20 μM (Fig. 2A). Thus, we only evaluated the anti-inflammatory activity of PrsDMe at the above concentrations.

Inhibition of LPS-Induced PGE₂ Secretion by Prosapogenin D Methyl Ester on RAW 264.7 Macrophages We investigated the inhibitory effect of PrsDMe on LPS-induced PGE₂ secretion in RAW 264.7 cells. PGE₂ concentration was determined by ELISA assay. LPS (0.1 $\mu\text{g/ml}$) significantly increased the concentration of PGE₂ as compared to basal level without LPS. PrsDMe (5, 10, 20 μM) significantly inhibited LPS-induced PGE₂ production in a concentration-dependent manner (Fig. 3).

Inhibition of LPS-Induced Protein Expression of iNOS and COX-2 by Prosapogenin D Methyl Ester The effects of PrsDMe on iNOS and COX-2 protein expression in RAW 264.7 cells were examined by Western blot analysis. As shown in Fig. 4A, these compounds inhibited expression of iNOS and COX-2 proteins.

Inhibition of LPS-Induced mRNA Expression of iNOS and COX-2 by Prosapogenin D Methyl Ester Studies were extended to determine whether the expression of iNOS and COX-2 proteins paralleled their mRNAs. RAW 264.7 cells treated with LPS (0.1 $\mu\text{g/ml}$) dramatically induced the iNOS and COX-2 mRNA expression while their mRNAs were not detectable in unstimulated macrophages. RT-PCR analysis showed that PrsDMe inhibited LPS-induced increase in the iNOS and COX-2 mRNA expression (Fig. 4B).

Effects of PrsDMe on LPS-Induced NF- κ B Activation in Transfectant RAW 264.7 Cells To investigate the inhibition of NF- κ B activation by PrsDMe, a pNF- κ B-SEAP-NPT plasmid containing NF- κ B binding sites in the enhancer element was transfected into RAW 264.7 cells. The level of NF- κ B activation in transfectant RAW 264.7 cells was tested using a fluorescence method. In stimulated transfectant RAW 264.7 cells with LPS (0.1 $\mu\text{g/ml}$), the NF- κ B activity (relative fluorescence units: RFU) was increased compared to the basal level without LPS. PrsDMe reduced NF- κ B activation by LPS in a dose-dependent manner (Fig. 5A).

Inhibition of LPS-Induced NF- κ B Nuclear Protein-DNA Binding Activity by Prosapogenin D Methyl Ester EMSA was conducted to determine whether PrsDMe changed NF- κ B DNA binding activity. NF- κ B is activated by LPS and other inflammatory stimuli in macrophages. As NF- κ B is a transcription factor, its activation is an essential step in gene expression including iNOS and COX-2 in RAW 264.7 cells. LPS (0.1 $\mu\text{g/ml}$) increased the binding activity of

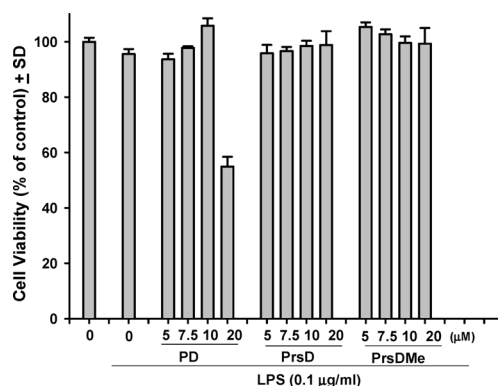


Fig. 2A. Cell Viability by PD, PrsD and PrsDMe in RAW 264.7 Cells

Cell viability was determined by Cell Counting kit assay and expressed as a percentage of the control.

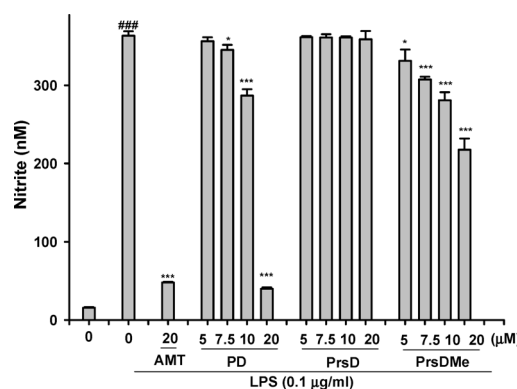


Fig. 2B. Effect of PD, PrsD and PrsDMe on LPS-Induced Nitrite Production in RAW 264.7 Cells

The nitrite production was measured by the Griess reaction. Control values were obtained in the absence of LPS or sample. Data were obtained from three independent experiments and expressed as means \pm S.D. * p < 0.05 and *** p < 0.001 indicate significant differences from the LPS-treated group; ### p < 0.001 indicates significant differences from the unstimulated control group. AMT = 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; PD = platycodin D; PrsDMe = prosapogenin D methyl ester.

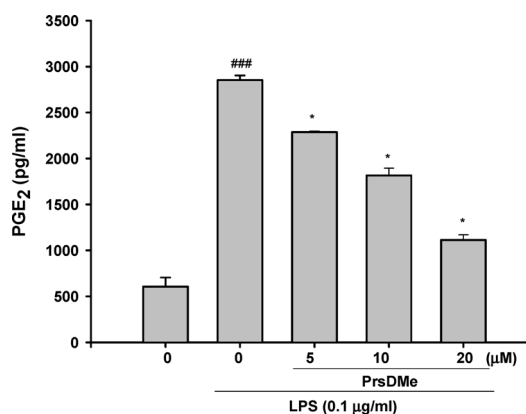


Fig. 3. Effect of the PrsDMe on LPS-Induced PGE₂ Synthesis in RAW 264.7 Macrophages

The PGE₂ concentration in the culture medium was measured by ELISA as described in Materials and Methods. The values are expressed as means \pm S.D. of triplicate tests. * p < 0.05 indicates differences versus LPS treatment; ### p < 0.001 indicates significant differences from the unstimulated group.

nuclear extracts to the NF- κ B DNA consensus sequence. PrsDMe inhibited LPS-induced NF- κ B binding ability (Fig. 5B).

Inhibition of LPS-Induced I κ B α Degradation by Pro-

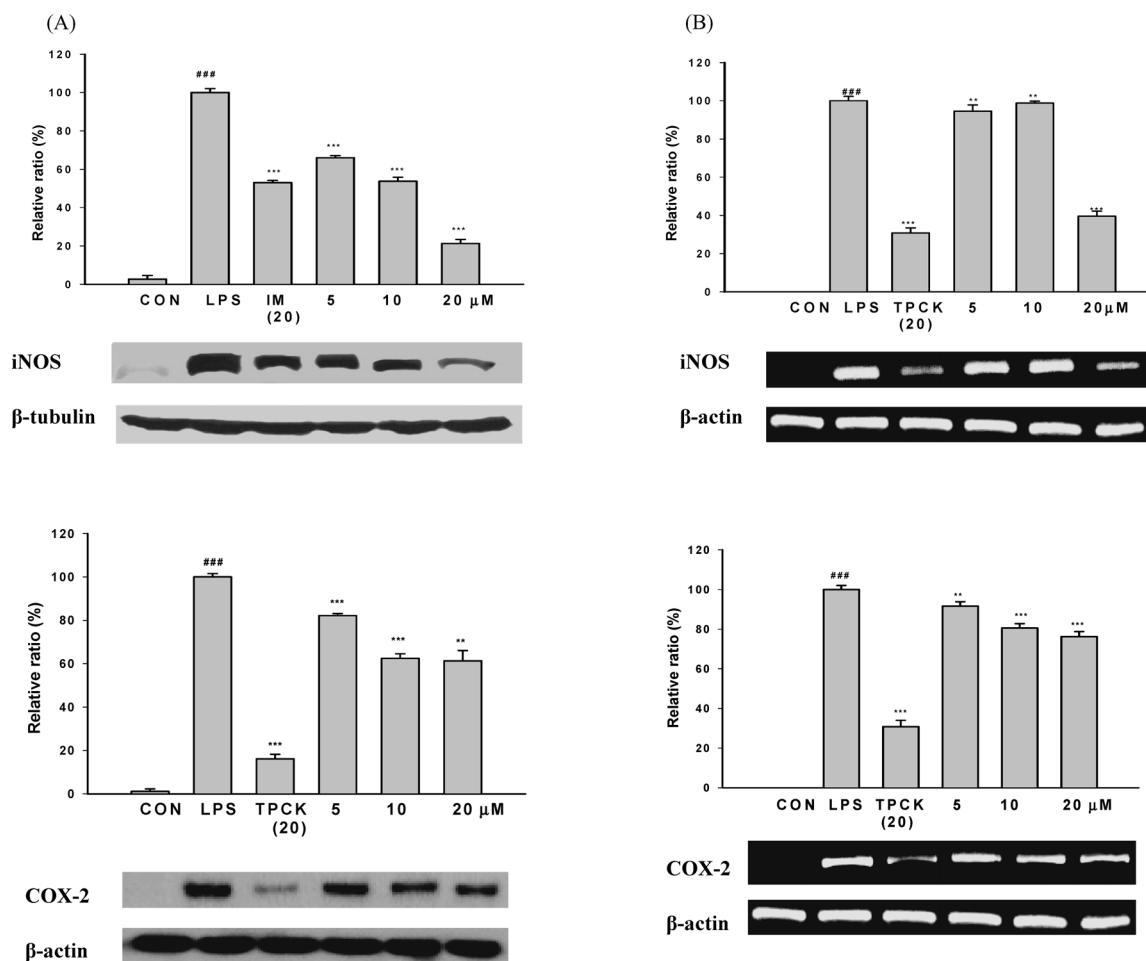


Fig. 4. Effect of LPS-Induced iNOS and COX-2 Protein Levels (A) and mRNA Expression (B) by PrsDMe in RAW 264.7 Macrophages

(A) Equal amounts of total proteins (80 μ g/lane) were subjected to 8% (for iNOS) and 10% (for COX-2, β -actin and β -tubulin) SDS-PAGE and expression of iNOS, COX-2, β -actin and β -tubulin was detected by Western blotting analysis. β -Actin and β -tubulin were used as an internal control. (B) Total RNA of lysed cells was prepared for the RT-PCR analysis. PCR of β -actin was performed to control for similar initial cDNA content of sample.

sapogenin D Methyl Ester The phosphorylation of $I\kappa B\alpha$ and then degradation is an important step in NF- κ B activation by stimuli. The effect of PrsDMe on LPS-induced $I\kappa B\alpha$ degradation in macrophages was investigated by Western blot analysis. LPS reduced $I\kappa B\alpha$ level by degradation of phosphorylated $I\kappa B\alpha$, while PrsDMe prevented degradation of $I\kappa B\alpha$ in RAW 264.7 cells (Fig. 6). The result suggests that PrsDMe inhibited NF- κ B to enter nuclear where NF- κ B regulate genes including pro-inflammatory cytokines, chemokines, and inducible enzymes.

Effect on Carrageenan-Induced Paw Edema in Mice by Prosapogenin D Methyl Ester The carrageenan rat paw model has been used for testing the COX-2 inhibitors against the acute inflammation.^{43,45} In the mouse paw edema experiment, inflammation was induced by carrageenan injection. The effect of PrsDMe on carrageenan-induced paw edema is shown in Fig. 7A. Oral administration of the PrsDMe resulted in inhibition of carrageenan-induced paw edema at the dose of 20 mg/kg in mice. In particular, treatment with PrsDMe suppressed edema formation from 1 to 4 h after edema injection. As a positive control, Ibuprofen (200 mg/ml) also inhibited paw edema.

Effect on Adjuvant-Induced Arthritis in Mice by Prosapogenin D Methyl Ester In chronic test of developing inflammatory arthritis, PrsDMe showed an inhibitory activity

against adjuvant-induced arthritis in mice on oral administration of 20 mg/kg (Fig. 7B).

DISCUSSION

It has been reported that platycodin D (PD) inhibits TPA-induced PGE₂ production and COX-2 expression.²⁶ We also demonstrated that 2'-O-acetyl polygalacin D, platycodin A, platycodin D, and polygalacin D inhibits LPS-induced iNOS and COX-2 activity through the suppression of NF- κ B activation in RAW cells.²⁴

In considering the structure of PD, sugars are attached at two positions, C₃-hydroxyl (R₁) and C₂₈-carboxyl (R₂) groups (Fig. 1). For the purpose of lowering polarity and toxicity of PD, sugar linkage at C₂₈-carboxyl position (R₂) was hydrolyzed to produce PrsD and the resulting carboxyl group was methylated to yield the methyl ester of PrsD (PrsDMe). In addition, any anti-inflammatory activities of PrsD and PrsDMe have not been reported. We detected the NO production in macrophages exposed to PD, PrsD and PrsDMe. Nitrite assay revealed that PD and PrsDMe inhibited the production of NO. However, in this investigation, PrsD was not active in inhibiting LPS-induced NO production at concentrations of 5, 7.5, 10 and 20 μ M. Similar results have been reported about other types of plant saponins such as those

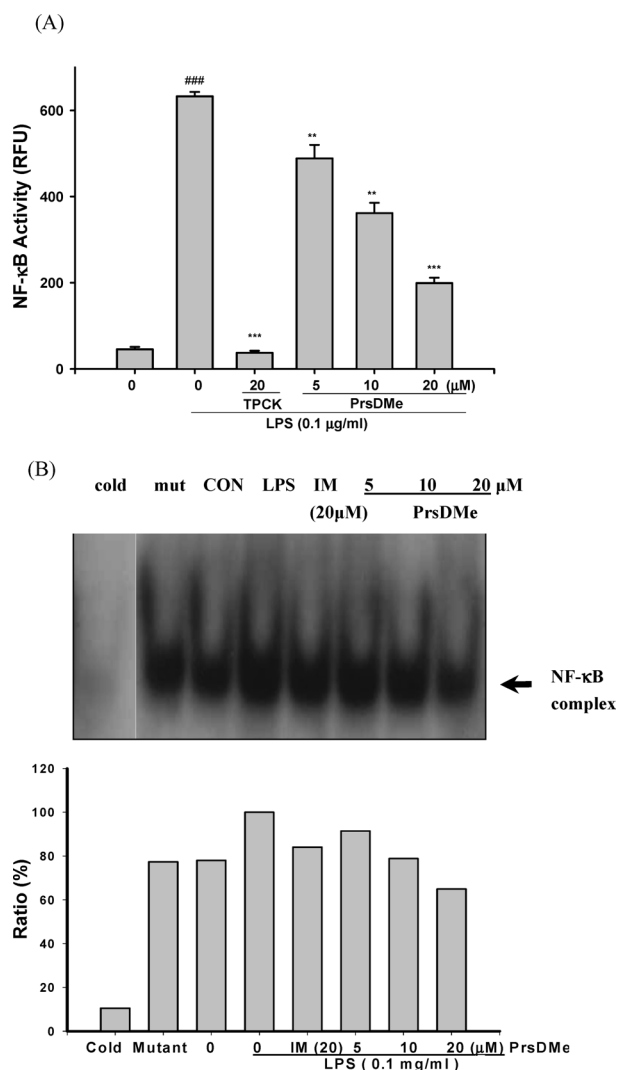


Fig. 5. Suppression of LPS-Induced NF-κB Activity (A) and DNA Binding (B) by PrsDMe in RAW 264.7 Macrophages

(A) Fluorescence from the product of the SEAP/MUP was measured using a fluorometer (Molecular Devices, Gemini XS). The data were obtained from three independent experiments and expressed as means ± S.D. ****p* < 0.01 indicates significant differences from the LPS-treated group; ###*p* < 0.001 indicates significant differences from the unstimulated group. (B) Nuclear protein from the cells was pretreated with 5, 10 and 20 μM of prosapogenin D methyl ester and 20 μM of TPCK for 2 h, and then incubated with LPS (0.1 μg/ml) or LPS only for 1 h. Detection of NF-κB binding activity was performed by electromobility shift assay (EMSA). Specificity of NF-κB complex formation was verified in LPS-only sample by displacement with a 50-fold excess of the unlabeled mutated oligonucleotide (mut) and a 50-fold excess of the unlabeled consensus oligonucleotide (cold).

found in *Panax ginseng*, a well-known herbal medicine containing various saponins that can be distinguished from each other by the number or position of sugar groups, and which can exhibit various pharmacological effects depending on the number of sugar present.^{46,47} Another study reported that saponin methyl ester from *Kalopanax pictus* shows higher anti-inflammatory,⁴⁴ while sapogenins that have free carboxyl group at C₂₈ are lack of anti-inflammatory activity. From this point of view, we speculate that the different pharmacological activities of saponins are based on the structure. And we suggest that the glycosylation or esterification of the C₂₈-carboxyl group of PrsD is responsible for their strong anti-inflammatory properties and the oligosaccharides moiety is not so critical at R₂ position of the prosapogenin moiety. In this study, PrsDMe was investigated for anti-inflam-

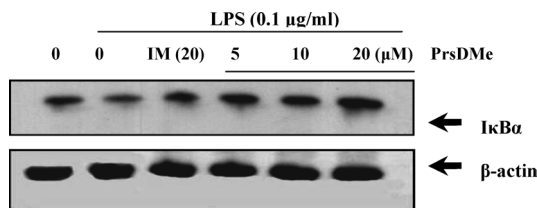


Fig. 6. Effect of PrsDMe on LPS-Induced IκBα Degradation in RAW 264.7 Macrophages

Cells were pretreated with 5, 10 and 20 μM of prosapogenin D methyl ester and 20 μM of indomethacin for 2 h, and then incubated with LPS (0.1 μg/ml) or LPS only for 1 h. Detection of IκBα expression was determined by Western blot analysis.

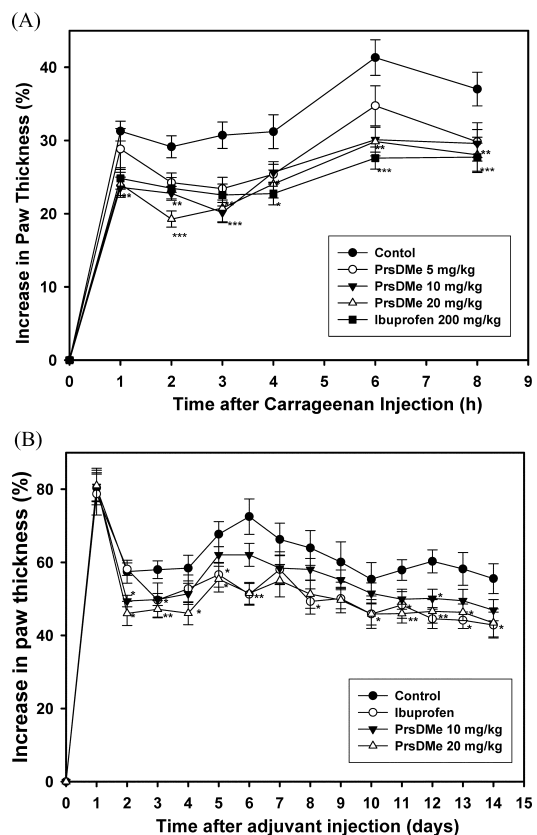


Fig. 7. Effect of PrsDMe on Carrageenan-Induced Paw Edema (A) and Freund's Complete Adjuvant Arthritis in Mice (B)

(A) PrsDMe was administered 30 min before 1% carrageenan injection into mice for alleviation of acute inflammation. Paw thickness was measured immediately prior to injection and each time postinjection. The percent increase of paw thickness was calculated based on the thickness of preinjection. (B) Freund's complete adjuvant (FCA)-induced arthritic rodent model was used for testing PrsDMe in sub-chronic or chronic inflammation. Paw thickness was measured immediately prior to injection and at intervals of days postinjection. The percent index of paw thickness was calculated based on values of pre-injection. Each value represents the mean ± S.D. (*n* = 10, **p* < 0.05, ***p* < 0.01).

matory properties by studying nitric oxide and PGE₂ secretion, iNOS and COX-2 gene expression in LPS-induced RAW 264.7 cells. PrsDMe inhibited LPS-induced nitric oxide and PGE₂ production *via* inhibition of iNOS and COX-2 gene expression in a concentration-dependent manner. This compound decreased the protein levels of iNOS and COX-2 by reducing expression of iNOS and COX-2 mRNA. At the gene levels, expression of iNOS and COX-2 are largely regulated by transcriptional activation of NF-κB. Through results from Western blot analysis, EMSA and NF-κB-SEAP cell-based assays, we demonstrated that PrsDMe prevented acti-

vation of NF- κ B by LPS in macrophage cells *via* inhibition of I κ B α degradation. Members of the protein tyrosine kinase family play roles in macrophage activation in response to LPS.⁴⁸⁾ Phosphorylation of I κ B α in cells stimulated by LPS is considered to be mediated with the NF- κ B-inducing kinase and the subsequent I- κ B kinase complexed with other proteins in the plasma membrane.⁴⁹⁾ The pathways of NF- κ B-inducing kinase and MEKK1 regulate the phosphorylation of I κ B α *via* IKK.⁵⁰⁾ It is likely that the inhibition of I κ B α degradation by PrsDMe is mediated with the suppression of IKK.

These results suggest that PrsDMe inhibit LPS-induced expression of the iNOS and COX-2 genes through blocking NF- κ B activation and I κ B α degradation. Also, above *in vitro* data could be demonstrated in adjuvant-induced arthritis as well as carrageenan-induced edema rodent models. Carrageenan injection in the hind paw of the rat is one of the most commonly used models of inflammation and inflammatory pain.⁵¹⁾ The initial phase of edema (0—1 h), has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin. And the second accelerating phase of swelling (1—6 h), has been correlated with the elevated production of prostaglandins.⁵²⁾ Because of strong inhibition of COX-2, treatment with PrsDMe dose-dependently suppressed carrageenan-induced paw edema in second phase from 1 to 4 h. And also, Freund's complete adjuvant (FCA)-induced arthritis test has been extensively used as a model of sub-chronic or chronic inflammation in rodents. By this *in vivo* test, PrsDMe effect on sub-chronic or chronic anti-inflammatory activity.

In conclusion, PrsDMe can be offered as a leading compound for developing anti-inflammatory agents based on the inhibition of *in vivo* animal models as well as suppression of COX-2 and iNOS and LPS-induced NF- κ B activation in macrophages.

Acknowledgement This work was supported by the Korea Research Foundation Grant (Basic Research Promotion Fund KRF-2007-E0016) funded by the Korean Government (MEST) and the grant from National Institute of Agricultural Biotechnology, Suwon, Korea.

REFERENCES

- Han J., Ulevitch R. J., *Nat. Immunol.*, **6**, 1198—1205 (2005).
- Mitchell J. A., Akarasereenont P., Thiemermann C., Flower R. J., Vane J. R., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 11693—11697 (1993).
- Dubois R. N., Abramson S. B., Crofford L., Gupta R. A., Simon L. S., Van De Putte L. B., Lipsky P. E., *FASEB J.*, **12**, 1063—1073 (1998).
- Wallace J. L., *Am. J. Med.*, **107**, 11S—16S; discussion 16S—17S (1999).
- Chandrasekharan N. V., Dai H., Roos K. L., Evanson N. K., Tomsik J., Elton T. S., Simmons D. L., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 13926—13931 (2002).
- Zhou H. Y., Shin E. M., Guo L. Y., Zou L. B., Xu G. H., Lee S. H., Ze K. R., Kim E. K., Kang S. S., Kim Y. S., *Eur. J. Pharmacol.*, **572**, 239—248 (2007).
- Storz P., Toker A., *Cell Cycle*, **2**, 9—10 (2003).
- Lin A., Karin M., *Semin. Cancer Biol.*, **13**, 107—114 (2003).
- Li Q., Verma I. M., *Nat. Rev. Immunol.*, **2**, 725—734 (2002).
- Yamamoto Y., Gaynor R. B., *Curr. Mol. Med.*, **1**, 287—296 (2001).
- Karin M., Cao Y., Greten F. R., Li Z. W., *Nat. Rev. Cancer*, **2**, 301—310 (2002).
- Sarkar F. H., Li Y., *Mutat. Res.*, **555**, 53—64 (2004).
- Bharti A. C., Aggarwal B. B., *Biochem. Pharmacol.*, **64**, 883—888 (2002).
- Biswas D. K., Dai S. C., Cruz A., Weiser B., Graner E., Pardee A. B., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 10386—10391 (2001).
- Haefner B., *Drug Discov. Today*, **7**, 653—663 (2002).
- Orlowski R. Z., Baldwin A. S. Jr., *Trends Mol. Med.*, **8**, 385—389 (2002).
- Barnes P. J., Karin M., *N. Engl. J. Med.*, **336**, 1066—1071 (1997).
- May M. J., Ghosh S., *Immunol. Today*, **19**, 80—88 (1998).
- Bensky D., Gamble A., "Chinese Herbal Medicine," Eastland Press, Seattle, 1986.
- Ishii H., Tori K., Tozoy T., Yoshimura Y. S., *J. Chem. Soc., Perkin Trans. 1*, **1984**, 661—668 (1984).
- Ishii H., Tori K., Yoshimura Y., *J. Chem. Soc., Perkin Trans. 1*, **1981**, 1928—1933 (1981).
- Tada A., Kaneiwa Y., Shoji J., Shibata S., *Chem. Pharm. Bull.*, **23**, 2965—2972 (1975).
- Shin C. Y., Lee W. J., Lee E. B., Choi E. Y., Ko K. H., *Planta Med.*, **68**, 221—225 (2002).
- Ahn K. S., Noh E. J., Zhao H. L., Jung S. H., Kang S. S., Kim Y. S., *Life Sci.*, **76**, 2315—2328 (2005).
- Choi S. S., Han E. J., Lee T. H., Han K. J., Lee H. K., Suh H. W., *Am. J. Chin. Med.*, **32**, 257—268 (2004).
- Kim Y. P., Lee E. B., Kim S. Y., Li D., Ban H. S., Lim S. S., Shin K. H., Ohuchi K., *Planta Med.*, **67**, 362—364 (2001).
- Ahn K. S., Hahn B. S., Kwack K., Lee E. B., Kim Y. S., *Eur. J. Pharmacol.*, **537**, 1—11 (2006).
- Choi C. Y., Kim J. Y., Kim Y. S., Chung Y. C., Hahn K. S., Jeong H. G., *Cancer Lett.*, **166**, 17—25 (2001).
- Choi C. Y., Kim J. Y., Kim Y. S., Chun Y. C., Seo J. K., Jeong H. G., *Int. Immunopharmacol.*, **1**, 1141—1151 (2001).
- Wang C., Schuller Levis G. B., Lee E. B., Levis W. R., Lee D. W., Kim B. S., Park S. Y., Park E., *Int. Immunopharmacol.*, **4**, 1039—1049 (2004).
- Xu B. J., Han L. K., Zheng Y. N., Lee J. H., Sung C. K., *Arch. Pharm. Res.*, **28**, 180—185 (2005).
- Han L. K., Zheng Y. N., Xu B. J., Okuda H., Kimura Y., *J. Nutr.*, **132**, 2241—2245 (2002).
- Han L. K., Xu B. J., Kimura Y., Zheng Y., Okuda H., *J. Nutr.*, **130**, 2760—2764 (2000).
- Zhao H. L., Kim Y. S., *Arch. Pharm. Res.*, **27**, 1048—1052 (2004).
- Zhao H. L., Cho K. H., Ha Y. W., Jeong T. S., Lee W. S., Kim Y. S., *Eur. J. Pharmacol.*, **537**, 166—173 (2006).
- Zhao H. L., Sim J. S., Shim S. H., Ha Y. W., Kang S. S., Kim Y. S., *Int. J. Obes. (London)*, **29**, 983—990 (2005).
- Christie W. W., "Advances in Lipid Methodology," 3rd ed., Vol. VII, ed. by Christie W. W., Oily Press, Dundee, 1993, pp. 69—111.
- Ishii H., Tozoy T., Yoshimura Y., *J. Chem. Soc., Perkin Trans. 1*, **1981**, 1928 (1981).
- Moon K. Y., Hahn B. S., Lee J., Kim Y. S., *Anal. Biochem.*, **292**, 17—21 (2001).
- Moon K. Y., Ahn K. S., Lee J., Kim Y. S., *Arch. Pharm. Res.*, **24**, 307—311 (2001).
- Ahn K. S., Moon K. Y., Lee J., Kim Y. S., *J. Dermatol. Sci.*, **31**, 193—201 (2003).
- Andrews N. C., Faller D. V., *Nucleic Acids Res.*, **19**, 2499 (1991).
- Morri C. J., "Inflammation Protocols," ed. by Paul G. W., Derek A. W., Chan C.-C., Isabelle B., Kathryn J. W., Human Press, Totowa, New Jersey, 2003, pp. 115—121, 321—328.
- Li da W., Hyun J. E., Jeong C. S., Kim Y. S., Lee E. B., *Biol. Pharm. Bull.*, **26**, 429—433 (2003).
- Winter C. A., Risley E. A., Nuss G. W., *Proc. Soc. Exp. Biol. Med.*, **111**, 544—547 (1962).
- Jeon B. H., Kim C. S., Kim H. S., Park J. B., Nam K. Y., Chang S. J., *Acta Pharmacol. Sin.*, **21**, 1095—1100 (2000).
- Kiefer D., Pantuso T., *Am. Fam. Physician*, **68**, 1539—1542 (2003).
- Geng Y., Zhang B., Lotz M., *J. Immunol.*, **151**, 6692—6700 (1993).
- Stancovski I., Baltimore D., *Cell*, **91**, 299—302 (1997).
- Pan M. H., Lin-Shiau S. Y., Lin J. K., *Biochem. Pharmacol.*, **60**, 1665—1676 (2000).
- Nantel F., Denis D., Gordon R., Northey A., Cirino M., Metters K. M., Chan C. C., *Br. J. Pharmacol.*, **128**, 853—859 (1999).
- Di Rosa M., Giroud J. P., Willoughby D. A., *J. Pathol.*, **104**, 15—29 (1971).