Antitumor effects of dammarane-type saponins from steamed Notoginseng

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ABSTRACT

Six dammarane-type saponins were extracted from steamed *Panax notoginseng*. Their chemical structures were identified spectroscopically as ginsenosides Rh_1 (1), Rg_1 (2), 20 (S)-Rg_3 (3), 20 (R)-Rg_3 (4), Rb_3 (5), and Rb_1 (6). Compounds (0.1-10 μM) were tested for inhibition of tumor necrosis factor-α (TNF)-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) luciferase reporter activity using a human kidney 293T cell-based assay. Ginsenoside Rb_3 (5) showed the most significant activity with an IC_{50} of 8.2 μM. This compound also inhibited the induction of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) messenger Ribonucleic acid (mRNA) in a dose-dependent manner after HepG2 cells had been treated with TNF-α (10 ng/mL).

Key words: Antitumor, dammarane-type ginsenoside, inhibitory activity, *Panax notoginseng*

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INTRODUCTION

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) reportedly plays an important role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS).[1] Thus, NF-κB activation has been considered a molecular target for screening the anti-inflammatory activity of various compounds. To date, many natural products, including polyphenols, sterols, and sesquiterpenes, have been found to be NF-κB inhibitors.[2-4]

Notoginseng, which is the root of *Panax notoginseng* (Burk.) F. H. Chen (Araliaceae), is cultivated primarily in the Yunnan province of China and is a traditional Chinese medicinal herb used to promote blood clotting, relieve swelling, and alleviate pain.[5,6] Chemical constituents in P. notoginseng roots include saponins, amino acids, polysaccharides and flavonoids. Dammarane-type triterpenoidal saponins are the major bioactive constituents of notoginseng and more than 30 such saponins have been isolated.[7,8] Furthermore, many studies have reported that traditional processing of ginseng, to make red and black ginseng as a means of preserving and enhancing its efficacy, in fact results in changes in the chemical composition.[9-11] There is no reported bioactivity study concerning processed notoginseng with regard to its traditional uses. The current study describes the isolation of six dammarane-type saponins from steamed notoginseng. Molecular structures were determined through a combination of spectroscopic analyses, including 1H and 13C Nuclear Magnetic Resonance (NMR), Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Correlation (HMQC), Heteronuclear Multiple Bond Correlation (HMBC), and Mass spectrometry (MS) spectral data, and literature data. The effects of the isolated compounds (0.1-10 μM) on the inhibition of tumor necrosis factor-α (TNFα)-induced NF-κB luciferase reporter activity using a human kidney 293T cell-based assay are reported here.

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RESULTS AND DISCUSSION

The nuclear transcription NF-κB cell-reporter system was used to evaluate and compare the anti-inflammatory activity of isolated compounds. As a means of measuring the promoter response in cells, the luciferase assay is a simple, straightforward, and effective technique. After

transfection of the luciferase vector, the cells were lysed and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg and excess Adenosine-5'-triphosphate (ATP). Under these conditions, the luciferase enzyme expressed by the reporter vector catalyzes the oxidative carboxylation of luciferin. The NF-κB luciferase assay was designed to monitor the activity of NF-κB-regulated signal transduction pathways in cultured cells. The NF-κB-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal cytomegalovirus promoter and tandem repeats of the NF-κB transcriptional response element. Using this assay, the activity of NF-κB in response to various chemical compounds and natural products can be monitored readily. Pro-inflammatory agents such as TNF-α activate the NF-κB pathway.[12] 293T cells transfected with the NF-κB luciferase reporter plasmid exhibited an approximately four-fold increase in luciferase signal after treatment with TNF-α (10 ng/mL), representing an increase in transcriptional activity versus untreated cells. All of the isolated compounds except ginsenosides 20 (S)-Rg_3 (3) and 20 (R)-Rg_3 (4) exhibited inhibitory effects on TNFα-induced NF-κB transcriptional activity in transfected 293T cells. Of the compounds tested, ginsenoside Rb_3 (5) was the most active (IC_{50} = 8.2 μM). From a structure-activity perspective, compounds 5 and 6, which each have two sugar moieties at position 20, were highly active. No activity was observed with compound 1-4, which lack a sugar moiety or contain only a single glucose molecule at position 20. Furthermore, compound 5 with its 20-O-glc (1-6)-xyl moiety was more active than 6 with its 20-O-glc (1-6)-glc moiety [Figure 1].

As a confirmation of NF-κB inhibition in the reporter assay, ginsenosides Rb_3 (5), and Rb_1 (6) were evaluated against the expression of NF-κB target genes by reverse transcription-polymerase chain reaction (RT-PCR). HepG2 cells treated with TNF-α (10 ng/mL) significantly up-regulated the messenger Ribonucleic acid (mRNA) expression of the NF-κB target genes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), by approximately 2.2- and 3.5-fold, respectively. Ginsenoside Rb_3 (5) significantly inhibited the induction of COX-2 and iNOS mRNA in a dose-dependent manner [Figure 2].

Experimental

General experimental procedures

Melting points were measured using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. The electrospray ionization mass spectra were recorded on an AGILENT 1100 LC-MSD trap spectrometer. Column chromatography was conducted using silica gel 60 (40-63 and 63-200 μm particle size) and RP-18 (40-63 μm particle size), which were both obtained from Merck.

Plant materials

The roots of 6-year-old *Panax notoginseng* were provided by the Dong Jin Corporation (Geumsan, Korea) in October 2008 and were identified by one of us (Prof. Gyu Yong Song). A voucher specimen (CNU08203) was deposited at the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Extraction and isolation

The raw materials were crushed into small grains and then steamed at 120°C for 4 h directly, without mixing

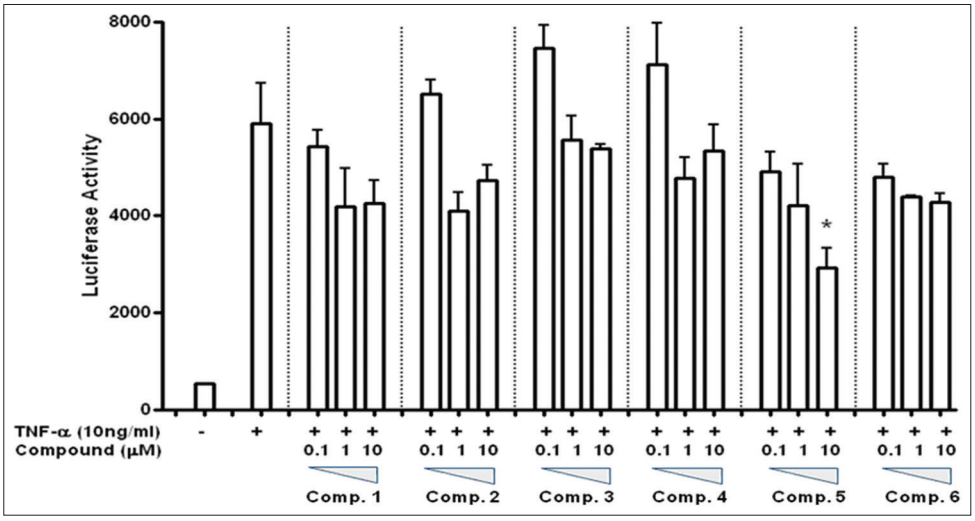

Figure 1: Effects of isolated compounds on TNF α-induced NF-κB luciferase reporter activities in 293T cell lines. 293T cells transiently transfected with pNF-κB-Luc and pSV-β-galactosidase were pretreated for 1 h with either vehicle (DMSO) and compounds, prior to 1 h of treatment with TNF-α (10 ng/mL). Unstimulated 293T cells were used as a negative control. Cells were then harvested and luciferase activities were assessed. Results are expressed as relative luciferase activity

with water, to give the steamed notoginseng. Air-dried steamed notoginseng (3 kg) was powdered and extracted with 95% EtOH (12 L) three times. The 95% EtOH extract was concentrated under reduced pressure to afford a residue (308 g), which was then suspended in H_2O. This solution was extracted with EtOAc (3 × 3 L) to give 135 g of a EtOAc soluble fraction and 150 g of a H_2O soluble fraction. The H_2O soluble fraction (130 g) was subjected to a highly porous synthetic resin column chromatography using a stepwise gradient of H_2O-EtOH (25%, 50%, and 95% EtOH), to yield three fractions (1-3). The fraction 3 was subjected to silica gel column chromatography eluted with $CHCl_3$–MeOH (7:1) to afford nine fractions (3A-3I). Fraction 3C was repeatedly chromatographed over silica gel ($CHCl_3$–MeOH–H_2O, 7:3:0.1), YMC-18 columns MeOH-H_2O (2:1) to afford 1 (70 mg) and 2 (60 mg). In a similar way, compounds 3 (120 mg) and 4 (58 mg) from fraction 3D, compound 5 (350 mg) from fraction 3F, and compound 6 (210 mg) from fraction 3H were obtained, respectively, through repeated column chromatography. Six compounds [Figure 3] were identified by comparing their physical and spectroscopic data with those reported in the literature.[13]

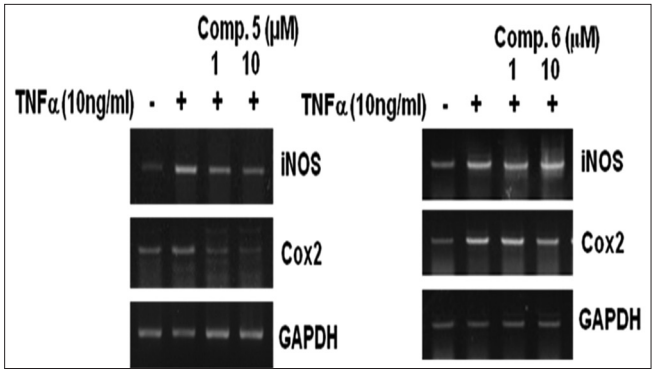

Figure 2: Effects of compounds 5 and 6 on COX-2 and iNOS mRNA expression in HepG2 cell lines. HepG2 cells were pretreated in the absence and presence of compounds 5 and 6 for 1 h before TNF-α treatment (10 ng/mL), then exposed to TNF-α for 6 h. Total mRNAs were prepared from the cell pellets using TRIzol. The relative levels of mRNAs were assessed by RT-PCR

Ginsenoside Rh_1 (1): White powder; m.p.: 192-194°C; [α]: +19 (c 0.2, MeOH); ESI-MS: m/z 639 $[M + H]^+$;

Ginsenoside Rg_1 (2): White powder; m.p.: 194-196°C; [α]: +32 (c 0.2, MeOH); ESI-MS: m/z 801 $[M + H]^+$;

20 (S)-ginsenoside Rg_3 (3): White powder; m.p.: 298-301°C; [α]: +17 (c 0.2, MeOH); ESI-MS: m/z 785 $[M + H]^+$;

20 (R)-ginsenoside Rg_3 (4): White powder; m.p.: 315-318°C; [α]: –11 (c 0.2, MeOH); ESI-MS: m/z 785 $[M + H]^+$;

Ginsenoside Rb_3 (5): White powder; m.p.: 193-195°C; [α]: +18 (c 0.5, MeOH); ESI-MS: m/z 1079 $[M + H]^+$;

Ginsenoside Rb_1 (6): White powder; m.p.: 197-199°C; [α]: +12 (c 0.5, MeOH); ESI-MS: m/z 1109 $[M + H]^+$.

Cell lines and culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37°C and 5% CO_2. Human TNF-α was purchased from ATgen (Seoul, Korea).

NF-κB luciferase assay

Cells were seeded at a concentration of 1×10^5 cells/mL (1.5 mL) in a 12-well plate and grown for 24 h. All cells were then transfected with Pnf-κB-luc plasmid (0.5 μg/well), kindly provided by Dr. Kyoon E. Kim (Chungnam National

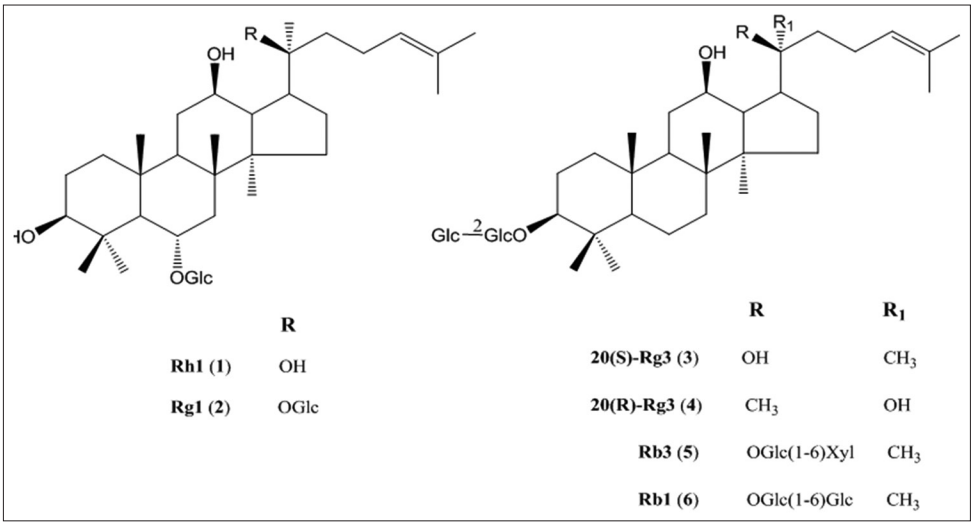

Figure 3: Structures of compounds 1-6

University, Daejeon, Korea). Transfection were performed by the lipofectamine LTX (Invitrogen, Carlsbad, CA) as described by the manufacturer. After 23 h of transfection, medium was changed to assay medium (Charcoal 0.5% FBS + non-essential amino acid + phenol red free DMEM). After 24 h of transfection, cells were treated with test compounds for another 24 hours. After 25 h of transfection, cells were treated with 10 ng/mL of TNF-α for another 23 hours. The luciferase activity of cell lysates was assayed with 100 μL of by luciferase assay kit using an LB 953 Autolumat (EG and G Berthold, Nashua, NH). Transfections were performed in triplicate, and activation was normalized against α-galactosidase activity.

Reverse transcription polymerase chain reaction

Total RNA was isolated using the Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Total RNA (~2 μg) was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega, Madison, WI) for 1 h at 42°C. PCR of synthetic complementary Deoxyribonucleic acid (cDNA) was performed using a Taq polymerase pre-mixture (TaKaRa, Japan). PCR products were subjected to electrophoresis on 1% agarose gels and stained with Ethidium bromide (EtBr). PCR was conducted with the following primer pairs: iNOS sense 5'-TCATCCGCTATGCTGGCTAC-3', iNOS antisense 5'-CTCAGGGTCACGGCCATTG-3', COX-2 sense 5'-GCCCAGCACTTCACGCATCAG-3', COX-2 antisense 5'-GACCAGGCACCAGACCAAAGACC-3', GAPDH sense 5'-TGTTGCCATCAATGACCCCTT-3', and GAPDH antisense 5'-CTCCACGACGTACTCAGCG -3'. The specificity of products generated by each set of primers was examined using gel electrophoresis and further confirmed by a melting curve analysis.

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