A New Triterpenoid from *Panax ginseng* Exhibits Cytotoxicity through p53 and the Caspase Signaling Pathway in the HepG2 Cell Line

Jian Huang¹,², Xiao-hui Tang¹, Takashi Ikejima², Xiu-jia Sun³, Xiao-bo Wang⁴, Rong-gang Xi⁴, and Li-jun Wu¹*  
¹Department of Phytochemistry, Shenyang Pharmaceutical University, ²China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, 110016, P. R. China; ³School of Chemistry, Qiqihaer University, 42 Wenhua Street, Qiqihaer, 161006, P. R. China, and ⁴School of Pharmacy, Department of traditional Chinese Medicine, 210th Hospital of People Liberation Army, 80 Shenli roa

(Received July 12, 2007)

A new triterpenoid, 20(R),22(ξ),24(S)-dammar-25(26)-ene-3β,6α,12β,20,22,24-hexanol (1), and three known triterpenoids, β-D-glucopyranoside,(3β,12β)-12,20-dihydroxydammar-24-en-3-yl,6-acetate (2), 20(R)-ginsenoside Rg3 (3), and 20(R)-ginsenoside Rh2 (4), were isolated from the leaves of *Panax ginseng*. Their structures were determined by chemical analysis and spectral methods (IR, 1D and 2D NMR, HR-ESI-MS). Compounds 1-4 were exhibited various degrees of cytotoxicity in the human hepatoma cell line, HepG2. Compound 1 had the highest cytotoxic potency, with an IC₅₀ value of 20.1 μM, by stimulating p53-mediated cell cycle arrest at the G1 to S phase transition, leading to apoptosis via activation of the caspase signaling pathway.

**Key words:** *Panax ginseng*, Triterpenoid, Cytotoxicity, HepG2, Cell cycle, p53, Caspase

INTRODUCTION

Apoptosis, or programmed cell death, and cell cycle arrest are two primary cellular events that cancer treatments are meant to induce. Apoptosis occurs when a cell initiates a series of biochemical and morphological events that result in a decrease in cell volume, dilatation of the endoplasmic reticulum, condensation and fragmentation of nuclear chromatin, and formation of membrane-bound apoptotic bodies (Hill et al., 1997; Mizukami et al., 1999; Kawazoe et al., 1999; Kim and Han, 2001). After DNA damage, which can be caused by a variety of stimuli, including chemotherapeutic agents, the cell cycle is arrested at the G1 to S phase or G2 to M phase transitions to allow time for repair of damaged DNA or direct cell apoptosis. An imbalance between DNA damage and DNA repair may affect cell viability (Waldman et al., 1995; Bunz et al., 1998; Wang et al., 1999; Cicirello et al., 2001).

*P. ginseng* is an ancient and famous herbal drug in traditional Chinese medicine, and has been used in Chinese folklore for more than 4000 years. It is mainly distributed in the Changbai Mountain region in northeast China. In this study, a new triterpenoid and three known triterpenoids were isolated from the total saponin fraction of its leaves. Based on chemical and spectral analysis, the structures of the four triterpenoids were determined to be 20(R),22(ξ),24(S)-dammar-25(26)-ene-3β,6α,12β,20,22,24-hexanol (1), β-D-glucopyranoside,(3β,12β)-12,20-dihydroxydammar-24-en-3-yl,6-acetate (2), 20(R)-ginsenoside Rg3 (3), and 20(R)-ginsenoside Rh2 (4). 20(R)-ginsenoside Rg3 is moderately cytotoxic against HeLa cells (Dou et al., 2001). We evaluated the cytotoxicity of compounds 1-4 against HepG2 cells. Compound 1 displayed the most potent cytotoxicity, leading to further investigation into the mechanisms responsible for this effect.

MATERIALS AND METHODS

Plant material

Plant material was collected in autumn in Jilin Province, in the northeast district of China. It was provided by...
Fusong County Natural Biotechnology Co., LTD. The specimen was identified by Professor Qishi Sun (Shenyang Pharmaceutical University, Shenyang, China).

General experimental procedures

The melting point was measured on a Yamaco-hot stage and was uncorrected. Optical rotation was measured in MeOH solution on a Perkin-Elmer 241 automatic polarimeter (Norwalk, CT, U.S.A.) at 25°C. ¹H-NMR (600 MHz), ¹³C-NMR (150 MHz), and 2D NMR (600 MHz) spectra were recorded on a Bruker ARX-600 spectrometer (Bruker, Switzerland) with TMS as an internal standard. The HR-ESI-MS was determined by Bruker APEX IV FT mass spectrometer (Bruker, Switzerland). IR spectra were recorded on a Nicolet 380 FT-IR spectrometer (Thermo Nicolet, Switzerland). 1H-NMR (600 MHz), 13C-NMR (150 MHz), and 2D NMR (600 MHz) spectra were recorded on a Bruker ARX-600 spectrometer (Bruker, Switzerland) with TMS as an internal standard. The HR-ESI-MS was determined by Bruker APEX IV FT mass spectrometer (Bruker, Switzerland). IR spectra were recorded on a Nicolet 380 FT-IR spectrometer (Thermo Nicolet, U.S.A.). Column chromatography was performed on macro-reticulated absorption resin of AB-8, Si gel G (200-300 mesh, Qingdao Ocean Chemical Inc., Qingdao, China), Sephadex LH-20 (Pharmacia, Kalamazoo, MI, U.S.A.), and reversed-phase Si gel column chromatography (Chromatex C₁₈, Fuji Silysia Chemical, Kasugai, Japan).

Extraction and isolation

Air-dried leaves of *P. ginseng* (1.5 kg) were extracted with 95% ethanol three times at 25°C, and the combined extracts were concentrated by vacuum. The ethanol extract was subjected to a macro-reticulated absorption resin of AB-8, Si gel G (200-300 mesh, Qingdao Ocean Chemical Inc., Qingdao, China), Sephadex LH-20 (Pharmacia, Kalamazoo, MI, U.S.A.), and reversed-phase Si gel column chromatography (Chromatex C₁₈, Fuji Silysia Chemical, Kasugai, Japan).

### 20(R),22(β),24(S)-Dammar-25(26)-ene-3β,6α,12β,20,22,24-hexanol (1)

White amorphous powder (MeOH); m.p. 125-127°C; [α]₂⁰° + 32.7 (c = 1.00, MeOH); IR (KBr) νmax cm⁻¹: 3384, 2930, 1715, 1455, 1384, 1029, 630, 470, 420; HR-ESI-MS: 509.3847 [M+H⁺]⁺ (calculated for C₃₀H₅₂O₆: 509.3842); ¹H-NMR and ¹³C-NMR: see Table I.

### Biological material

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Pan-caspase inhibitor (z-VAD-fmk) was purchased from Enzyme Systems (Sacramento, CA, U.S.A.). Polyclonal antibodies against p53 and phospho-p53 were obtained from Oncogene Research Products (Boston, MA, U.S.A.). Polyclonal antibodies against caspase-3 and β-actin and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). TACSTM2 TDT-DAB *In Situ* Apoptosis Detection Kit was obtained from Trevigen (Gaithersburg, MD, U.S.A.).

### Cell culture

HepG2 cells were purchased from American Type Culture Collection (#HTB-8065, ATCC, Manassas, VA, U.S.A.). The cells were cultured in RPMI-1640 medium (GIBCO, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS) (Shengma Yuanheng, Beijing, China), 100 mg/L streptomycin, 100 IU/ml penicillin, and 0.03% L-glutamine, and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

### Cytotoxicity assay

Compounds 1-4 were resolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.05% throughout the cell culture period and did not exert any detectable effect on cell growth or cell death. The HepG2 cells were incubated at 1×10⁵ cells/well in 96-well plates. The cells were incubated with the four compounds at various concentrations for 36 h. Cell growth was measured by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The percentage of cell growth inhibition was calculated as follows:

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\text{Cell growth inhibition} = \frac{A_{570 \text{ (control)}} - A_{570 \text{ (compound)}}}{A_{570 \text{ (control)}}} \times 100
\]

### Cell cycle distribution assay

HepG2 cells (1×10⁶) were harvested and washed once in cold phosphatase-buffered saline (PBS). The cells were fixed in 70% ethanol and washed in cold PBS again. Then the cells were suspended in 1 mL propidium iodide (PI) solution [50 μg/mL PI, 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton-X]. Cell samples were incubated at 4°C in the dark for at least 15 min and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

### Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

The TUNEL assay was used for detection of DNA strand breaks. The detection was performed according to the instructions of the TACS™ II TDT-DAB *In Situ* Apoptosis Detection Kit. Briefly, the cells were rinsed once with PBS and fixed in 3.7% buffered formaldehyde at room temperature for 10 min. The fixed cells were pretreated with 10% H₂O₂, and end-labeling was performed with the TdT labeling