Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 19 (2008) 166–174
Journal of Nutritional Biochemistry

# Cell death signal by glycine- and proline-rich plant glycoprotein is transferred from cytochrome *c* and nuclear factor kappa B to caspase 3 in Hep3B cells☆

Sei-Jung Lee[a,b], Kye-Taek Lim[a,*]

[a]*Molecular Biochemistry Laboratory, Institute of Biotechnology, Chonnam National University, Yongbong-Dong 500-757, South Korea*
[b]*Center for the Control of Animal Hazards using Biotechnology (BK 21), Chonnam National University, Yongbong-Dong 500-757, South Korea*

Received 17 October 2006; received in revised form 1 December 2006; accepted 8 February 2007

**Abstract**

This study was carried out to investigate the apoptotic effects of glycine- and proline-rich glycoprotein [*Solanum nigrum* Linne (SNL) glycoprotein, 150-kDa] isolated from SNL, which has been used as an antipyretic and anticancer agent in Korean herbal medicine. We found that SNL glycoprotein has obviously cytotoxic and apoptotic effects at 80 μg/ml of SNL glycoprotein for 4 h in Hep3B cells (hepatocellular carcinoma cells). In mitochondria-mediated apoptosis pathway, SNL glycoprotein has abilities to stimulate release of mitochondrial cytochrome *c*, activations of caspase-9 and caspase-3, cleavage of poly(ADP-ribose)polymerase and production of intracellular reactive oxygen species in Hep3B cells. In nuclear factor-kappa B (NF-κB)-mediated apoptosis pathway, the results showed that SNL glycoprotein dose-dependently blocked DNA binding activity of NF-κB, activity of inducible nitric oxide synthase (iNOS) and production of inducible nitric oxide (NO). Interestingly, pyrrolidine dithiocarbamate (for NF-κB inhibitor) and *N*ω-nitro-L-arginine methylester hydrochloride (for NO inhibitor) effectively stimulated the caspase-3 activation and induced apoptosis in Hep3B cells. These results indicate that SNL glycoprotein transfers its cell death signal from cytochrome *c* to caspase 3 by inhibiting NF-κB and iNOS activation in Hep3B cells. Here, we speculate that SNL glycoprotein is one of the chemotherapeutic agents to modulate mitochondria-mediated apoptosis signals in Hep3B cells.


*Keywords:* SNL glycoprotein; Hep3B cells; Caspase-3; Nuclear factor-kappa B; Reactive oxygen species

## 1. Introduction

Hepatocellular carcinoma is one of the leading causes of cancer deaths in both men and women in Korea. Since multiple molecular steps that collectively bring about this disease are known, its chemotherapy has been focused on the regulations of biochemical targets such as Wnt/β-catenin signaling mediators, reactive oxygen species (ROS), transcription and epigenetic factors, angiogenetic proteins and key molecules in cell proliferation and apoptosis using chemotherapeutic agents [1–3]. However, the problem is that the great majority of chemical compounds, which have been identified as specific agents for killing cancer cells, are also toxic to normal cells [4]. In addition, many hepatocellular carcinoma cells are either resistant to chemotherapy, or they develop resistance during the course of therapy [5]. Hence, the discovery and identification of new safe drugs, without severe side effects, have become an important goal of research in the biomedical sciences.

Apoptosis, or programmed cell death, is the result of a highly complex cascade of cellular events that result in chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing and cell shrinkage. Apoptosis is signaled through several death receptors and ligands stimulated by apoptotic mediators, which are placed mainly in the cell surface, the cytosol, the mitochondria and the nucleus [6]. Especially, the mitochondria play a central role in apoptosis occurrence resulting from many chemical or chemotherapeutic agent [7]. The mitochondria-mediated apoptosis pathway is dependent on the release of cytochrome *c* from the mitochondria to the cytosol. This process, in turn, activates the downstream caspases, which are cysteine-dependent enzymes [8]. Among these caspases,

☆ This study was financially supported by research fund of Chonnam National University in 2005.
* Corresponding author. Tel.: +82 62 530 2115; fax: +82 62 530 0485.
*E-mail addresses:* ktlim@chonnam.ac.kr, kyetaeklim@hotmail.com (K.-T. Lim).

0955-2863/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2007.02.006

caspase-3 has been implicated in the execution phase of apoptosis and has been shown to cleave over 100 substrates, including poly(ADP-ribose)polymerase (PARP), that respond to DNA strand breaks and eventually lead to apoptosis [9]. Moreover, it is also well known that the generation of intracellular ROS plays an important role in mitochondrial apoptosis occurrence through the disruption of redox homeostasis [10]. The generated ROS can directly or indirectly cause the loss of mitochondrial membrane potential by activating mitochondrial permeability transition and induce apoptosis by releasing apoptotic protein including cytochrome *c* [11]. In these contexts, we postulate that modulation of mitochondrial apoptotic mediators and intracellular ROS production by chemotherapeutic agents is one of cancer therapeutic strategies to eliminate cancer cells [12].

Nuclear factor kappa B (NF-κB) is an ubiquitously expressed Rel oncogene family (e.g., Rel A/p65, p50, p52, c-Rel, v-Rel and Rel B) involved in the regulation of variety of biological processes, such as immune response, inflammatory response, cell adhesion, growth control and cell death, depending on cell type and stimulus. It has been reported that NF-κB can exist as either heterodimeric or homodimeric complex, but it is classically composed predominantly of p50 and Rel A/p65 subunits [13]. In a highly simplified scenario of NF-κB activation by an appropriate stimulus, I-κB becomes phosphorylated, and this results in its rapid ubiquitination and subsequent proteolysis by the 26s proteasome. Proteasome-dependent degradation of I-κB causes the translocation of NF-κB to the nucleus, where it initiates gene expression [14]. NF-κB has also been implicated in the inhibition of apoptotic cellular pathways. In other words, NF-κB is constitutively expressed in many tumor cell lines, including hepatocellular carcinoma cells [15].

Nitric oxide (NO), which is formed from $Ca^{2+}$-independent inducible NO synthase (iNOS), could also be regulated by NF-κB in physiological and pathological conditions [16]. Such NO production has been implicated in carcinogenesis and tumor promotion, including angiogenesis and multidrug resistance in hepatocellular carcinoma [17,18]. Therefore, we postulate that any kind of agent, which is able to block iNOS expression resulting from NF-κB activation, will act as a promising anti-tumor promoter in hepatocellular carcinoma.

Numerous antitumor polysaccharide-protein complexes (glycoprotein) have been discovered from mushrooms, fungi, yeasts, algae, lichens and plants. The research for a novel glycoprotein with antitumor properties originated as a result of shortcomings of existing cancer chemotherapy and radiotherapy, although the mechanism of antitumor action of the glycoprotein is not fully understood [19]. Recently, we found a glycoprotein with an approximate molecular mass of 150 kDa, isolated from *Solanum nigrum* Linne (SNL), which is made up of carbohydrate content (69.74%) and protein content (30.26%) and contains more than 50% hydrophobic amino acids such as glycine and proline [20]. This active substance, designated as SNL glycoprotein, has pharmacological activities such as antimicrobial against JA221 and XL1-blue [21] and apoptotic activity in colon and breast cancer cells [20,22,23]. In addition, the specific anticancer active site of SNL glycoprotein is postulated to be located in the protein part [22]. However, it has been still undefined whether SNL glycoprotein activates mitochondria-mediated apoptosis pathways by modulating of NF-κB activities and intracellular ROS production in hepatocellular carcinoma cells.

Therefore, we investigated whether the SNL glycoprotein regulates mitochondrial apoptotic signal mediators and whether or not it consequently induces apoptosis through suppressions of NF-κB and iNOS activities in Hep3B cells.

## 2. Materials and methods

### 2.1. Chemicals

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). Bisbenzimide (H33342, B2261), penicillin G (H0474), streptomycin (H0447), *N*ω-nitro-L-arginine methylester hydrochloride (L-NAME, N5751), neutral red (N7005), phenol solution (P4682), phenylmethanesulfonyl fluoride (P7626), pyrrolidine dithiocarbamate (PDTC, P8765), ribonuclease A (R4875), silica gel (S4883) and trypsin (T4549) were obtained from Sigma (St. Louis, MO, USA). Ac-LETD-CHO (218776) and Ac-DEVD-CHO (235420) were purchased from Calbiochem (Darmstadt, Germany). Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest quality available.

### 2.2. Preparation of SNL glycoprotein

Fruit of SNL was collected from Naju, located in the Chonnam province, Korea, in October 2004. SNL was cut into small pieces and soaked in distilled water for several months in a dark basement. The extract was filtered through Whatman filter paper (no. 2) and concentrated using a rotary evaporator (B465; Bunchi, Flawil, Switzerland). The resulting solution was dried with a freeze-dryer (SFDS06; Sam won, Seoul, Korea). Five grams of dried-crude extract was dissolved in distilled water. The dried powder 2.0 g (40% of the initial amounts) was dissolved again with distilled water. The solution was precipitated with 80% ammonium sulfate and then dialyzed with a dialysis membrane (Spectra/por, MWCO 6000–8000, San Diego, CA, USA) against 20 mM Tris-Cl (pH 7.4) at 4°C overnight. After dialysis, the solution was centrifuged at 3000*g* for 15 min at 25°C using Microcon concentrators (MWCO 100,000) according to the manufacturer's protocol (Amicon Inc, MA, USA), and the supernatant was dried with a freeze-dryer and stored at −70°C. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was per-

formed with the sample of protein (50 mg/ml) containing 0.1% SDS, using a 10% polyacrylamide mini-gel and a Mini-PROTEIN II electrophoresis cell (Bio-Rad, Hercules, CA, USA) at 110 V, 30 mA for 2.5 h. The gels were stained with Schiff's reagent for the glycoprotein [24]. After staining, the SNL glycoprotein (150 kDa) was eluted with an electroeluter (Mini Whole Gel Eluter; Bio-Rad), and the SNL glycoprotein solution was further dried with a freeze-dryer and stored at −70°C. The final amount of SNL glycoprotein was 4.0 mg (0.08%) from the initial SNL sample. After verification of high purity (approximately more than 95%) of glycoprotein, we analyzed that SNL glycoprotein consists of carbohydrate content (69.74%) and protein content (30.26%), as described previously [22]. In addition, we analyzed that the protein in SNL glycoprotein mainly contains glycine (37.92%) and proline (20.30%) using Pico-Tag method (Waters, Milford) [25,26]. Here, it should be noted that 80 μg/ml of SNL glycoprotein correspond to 532 μM based on calculation of molarity with molecular weight 150 kDa.

### 2.3. Cell cultures

Hep3B cells, the human hepatocellular carcinoma cells, were obtained from the Korean Cell Line Bank (Seoul, Korea). The Hep3B cells were incubated in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% $CO_2$ atmosphere, respectively. The medium was renewed two times per week. The number of cells ($1\times10^6$ cells/ml) was divided into 35-mm culture dishes or 96-well flat bottom plates. The final volumes were adjusted to 2 ml per dish on the 35-mm culture dishes and 100 μl per well on the 96-well flat bottom plates.

### 2.4. Cytotoxicity of SNL glycoprotein

The cellular cytotoxicity induced by SNL glycoprotein was measured using neutral red assay [27]. Cells were treated either with or without SNL glycoprotein and then the cells were incubated in 100 μl of new medium containing 10 mg/ml neutral red for 90 min at 37°C. After the complete removal of the medium, each well was washed three times with 100 μl of phosphate buffered saline (PBS). One hundred microliters of 50% ethanol containing 50 mM sodium citrate (pH 4.2) was added into each well on the 96-well multiple plates. After 20 min, the absorbance was measured at 510 nm using a SpectraCount (Packard Instrument, Downers Grove, IL, USA) ELISA reader.

### 2.5. Western blot analysis

The whole cell and mitochondrial extracts for immunoblotting of caspase-3, caspase-9, PARP, iNOS and cytochrome *c* were made from Hep3B cells [20]. These sample proteins were separated on a 12% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini-PROTEIN II electrophoresis cell (Bio-Rad). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h at room temperature in TBS-T solution [10 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% (v/v) Tween-20] containing 5% (w/v) nonfat dry milk. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibody [1:3000; caspase-3, caspase-9, PARP, iNOS and cytochrome *c*, Santa Cruz Biotechnology, Santa Cruz, CA, USA] in TBS-T solution containing 5% nonfat dry milk. After washing with TBS-T, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat antirabbit IgG (1:10,000; Santa Cruz Biotechnology) in TBS-T containing 5% nonfat dry milk. The protein bands were visualized by incubation with nitrobluetetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical).

### 2.6. Measurement of intracellular reactive oxygen species (ROS)

The intracellular ROS production was measured by using nonfluorescent 2',7'-dichlorofluorescein (DCFH-DA). This compound is deacetylated by intracellular esterases to the

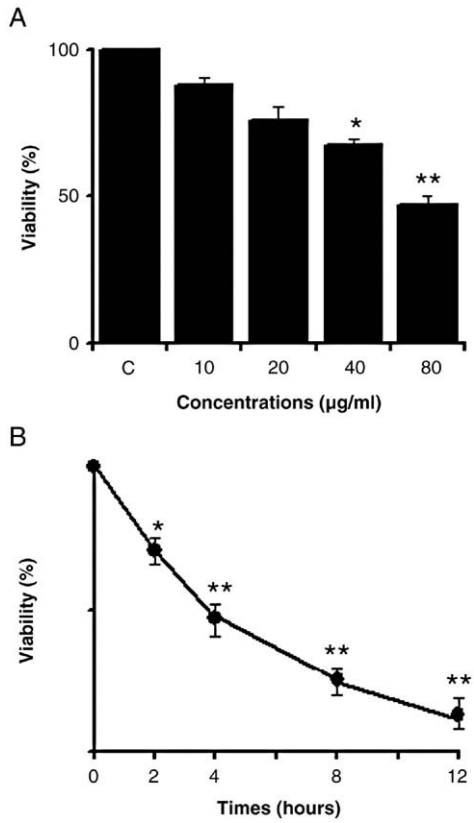


Fig. 1. Cytotoxicity of SNL glycoprotein. Hep3B cells were treated with various concentrations of SNL glycoprotein (10, 20, 40 and 80 μg/ml) alone for 4 h (A) or 80 μg/ml of SNL glycoprotein alone for various times (2, 4, 8 and 12 h) (B) at 37°C in an atmosphere containing 5% $CO_2$. Cellular cytotoxicities were measured by neutral red assay as described in Materials and methods. The values in the neutral red assay are relative percentages, compared to the control, which was treated with culture medium. Each bar represents the mean±S.D. of triplicate experiments ($n=3$). Asterisks represent a significant difference between the SNL glycoprotein treatments and the control (*$P<.05$ and **$P<.01$, respectively).

nonfluorescent DCFH, which is oxidized to the fluorescent compound DCF by ROS [28]. For the measurement of ROS production, Hep3B cells were preincubated with 10 μM DCFH-DA for 30 min at 37°C, and then the cells were washed twice with PBS to remove the excess DCFH-DA. After that, the cells were treated with or without SNL glycoprotein at different concentrations for 4 h. Finally, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluoresencent microplate reader (Dual Scanning SPECTRAmax, Molecular Devices, Sunnyvale, CA, USA). The values were calculated as a relative of the DCF fluorescence intensities, compared to the control.

### 2.7. Electrophoretic mobility shift assay

Nuclear extracts for electrophoretic mobility shift assay (EMSA) of NF-κB were isolated from the Hep3B cells as described previously [22]. The amount of protein in the supernatant was measured by the Lowry method [29] and stored at −70°C prior to use. On the other hand, to make the double-stranded oligonucleotide, each strand of NF-κB oligonucleotide was annealed by heating at 37°C for 30 min. Then, it was labeled with [α-$^{32}$P]dCTP (0.25 mCi, Amersham Pharmacia Biotech, Buckinghamshire, UK) by Klenow polymerase and purified on a QIAquick Nucleotide Removal Kit according to the manufacturer's protocol (LRS Laboratory, QIAGEN Distributor, Seoul, Korea). The following NF-κB oligonucleotide sequences were used for probing: 5′-AAG GTC CAG GCC AGG GAA AGT CCC GGA GCA CAG G-3′. The DNA-protein binding reaction was performed by incubation of the NF-κB probes and 10 μg of nuclear protein extracts and 0.5 μg/ml poly dI/dC (Sigma Chemical) in a binding buffer [0.2 M DTT, 20 mg/ml bovine serum albumin, buffer C (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40), buffer D (20% ficoll 400, 100 mM HEPES, 300 mM KCl)] at room temperature for 1–2 h. The DNA-protein complexes were resolved by applying 4% nondenaturing polyacryl-

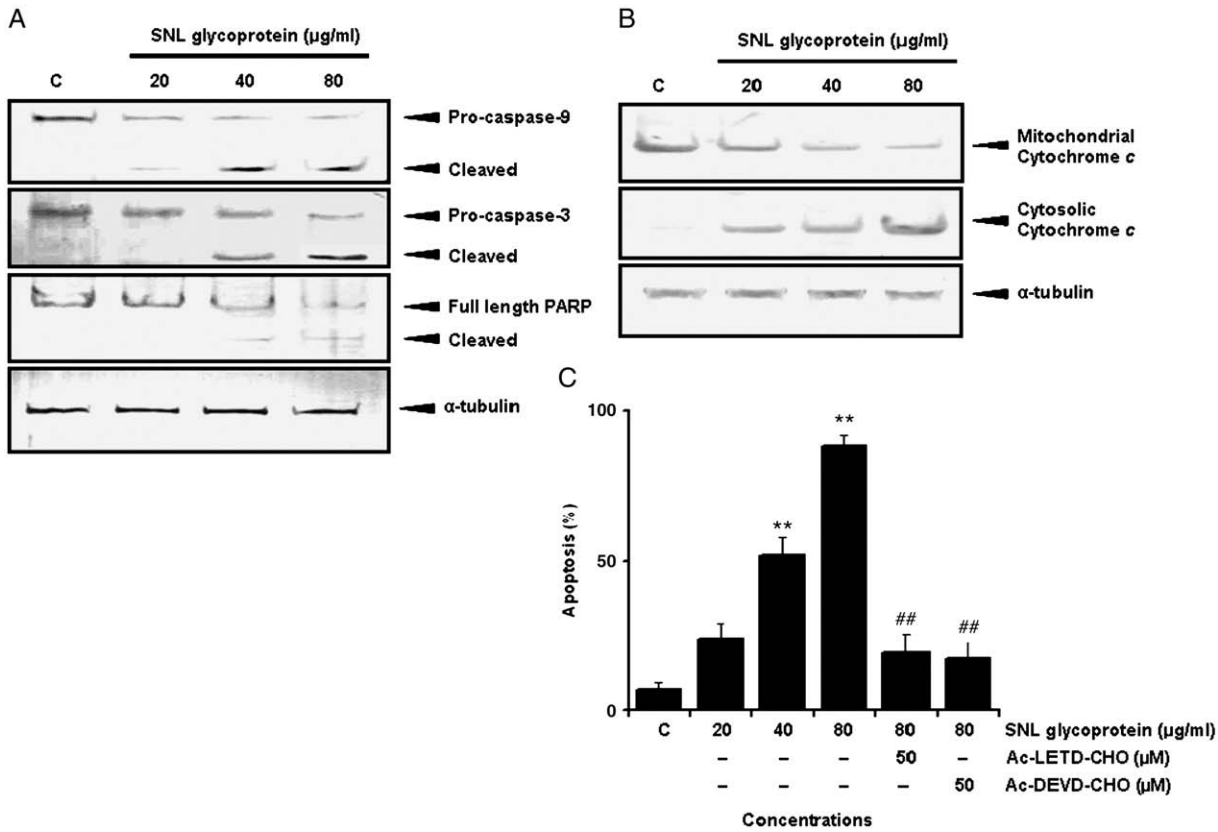


Fig. 2. Effect of SNL glycoprotein on mitochondria-mediated apoptosis pathway. Hep3B cells were treated with various concentrations of SNL glycoprotein (20, 40 and 80 μg/ml) alone for 4 h. Whole cell extracts for immunoblotting of caspase-9, caspase-3 and PARP proteins (A), and cytosolic and mitochondrial extracts for immunoblotting cytochrome *c* protein (B) were made from Hep3B cells, as described in Materials and methods. Detection of cytochrome *c*, caspase-9, caspase-3 and PARP proteins was performed by Western blotting using an anti-cytochrome *c*, anti-caspase-9, anti-caspase-3 and anti-PARP polyclonal antibodies. Lane 1, control; Lane 2, SNL glycoprotein (20 μg/ml); Lane 3, SNL glycoprotein (40 μg/ml); Lane 4, SNL glycoprotein (80 μg/ml). α-Tubulin was used as an internal control. (C) The apoptotic inducing effect of SNL glycoprotein was also evaluated by H33342/ethidium bromide staining. Cells were pretreated with either 50 μM inhibitors of caspase-9 (Ac-LETD-CHO) or caspase-3 (Ac-DEVD-CHO) for 1 h and then treated with 80 μg/ml SNL glycoprotein for another 4 h. Results were expressed as a means ± S.D. of triplicate experiments ($n = 3$). Asterisks represent a significant difference between the SNL glycoprotein treatments and the control (**$P < .01$). Pound signs represent a significant difference between the SNL glycoprotein (80 μg/ml) and caspase inhibitor treatments ($^{\#\#}P < .01$).

amide gel in 0.5 × tris-borate EDTA buffer (45 mM Tris-borate, 1 mM EDTA). Electrophoresis was carried out at 200 V for 3 h in a cold room. Gels were then dried on 3M blotting paper (Whatman) and exposed to X-ray film at −70°C overnight.

### 2.8. Nitric oxide and apoptosis assays

Nitric oxide production was measured according to the method of Green et al [30], as described previously [22]. Apoptotic effects of SNL glycoprotein were confirmed by DNA fragmentation and H33342/ethidium bromide staining assays as described previously [20].

### 2.9. Statistical analysis

All experiments were done 3 times in triplicate ($n=3$), and the results were expressed as means±S.D. A one-way analysis of variance and the Duncan test were used for multiple comparisons (SPSS program, ver 10.0).

## 3. Results

### 3.1. Cytotoxicity of SNL glycoprotein

When Hep3B cells were exposed to various concentrations of SNL glycoprotein and for suitable incubation times, the cellular cytotoxicities were significantly increased (Fig. 1). In dose-dependent manner, the viability values were 88, 76, 68 and 47% at 10, 20, 40 and 80 μg/ml SNL glycoprotein for 4 h, respectively (Fig. 1A). In time-dependent manner, the viability values were 71, 47, 25 and 13% for 2, 4, 8 and 12 h at 80 μg/ml SNL glycoprotein, respectively (Fig. 1B). From the results of cytotoxicity, which approximately showed $IC_{50}$, the experimental condition (80 μg/ml SNL glycoprotein, 4 h) was chosen in this experiment to study the apoptotic signal pathway.

### 3.2. Effect of SNL glycoprotein on apoptotic related proteins

When the cells were exposed to SNL glycoprotein (20–80 μg/ml) for 4 h, caspase-9, caspase-3 and PARP proteins in whole-cell extract fractions were cleaved in a dose-dependent manner (Fig. 2A). After treatment of 40 and 80 μg/ml of SNL glycoprotein for 4 h, the cleaved forms of the pro-caspase-9, pro-caspase-3 and PARP proteins could be detected, compared to the control. In cytochrome *c* protein, intensities of its bands were decreased after treatment with SNL glycoprotein in the mitochondria, whereas its intensities were gradually increased in the cytosol in a dose-dependent manner (Fig. 2B, lanes 2–4). To confirm whether or not the caspase activations are essential to the SNL glycoprotein-induced apoptosis pathway, we performed H33342/ethidium bromide assay for nuclear staining after treatments with Ac-LETD-CHO and Ac-DEVD-CHO as inhibitors of caspase-9 and caspase-3, respectively (Fig. 2C). The results showed that the percentages of apoptotic cells were increased by 17%, 45% and 81% at 20, 40 and 80 μg/ml SNL glycoprotein for 4 h, compared to the control. However, when the cells were pretreated with Ac-LETD-CHO (50 μM) and Ac-DEVD-CHO (50 μM) for 1 h, then exposed to 80 μg/ml of SNL glycoprotein for another 4 h, the percentages of SNL glycoprotein-induced apoptotic cells were reduced by 69% and 71%, compared to the treatment with 80 μg/ml SNL glycoprotein alone. Here, the activity of caspase-8 showed that there is significantly no difference between the control and treatments with SNL glycoprotein in Hep3B cells (data not shown).

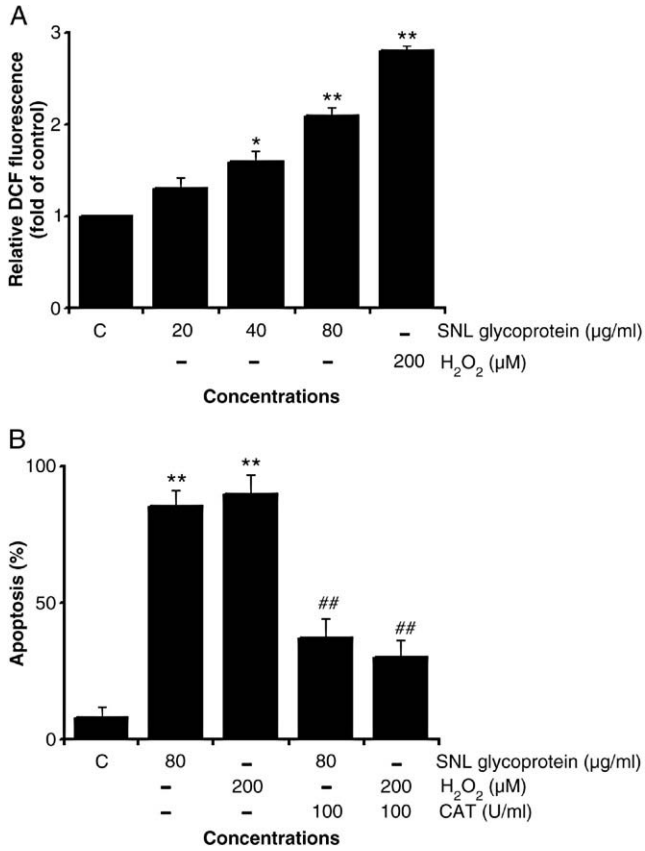


Fig. 3. Effects of SNL glycoprotein on intracellular ROS production. (A) Hep3B cells were treated with various concentrations of SNL glycoprotein (20, 40 and 80 μg/ml) alone or $H_2O_2$ (200 μM) alone for 2 h. The intracellular ROS production was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescent microplate reader. The values were calculated as a relative of the DCF fluorescence intensities, compared to the control. $H_2O_2$ (200 μM) was used as a positive control for ROS generation. Each bar represents the mean±S.D. of triplicate experiments ($n=3$). Asterisk represents a significant difference between the control and SNL glycoprotein treatment (40 μg/ml) (*$P<.05$). Double asterisk represents a significant difference between the control and SNL glycoprotein treatment (80 μg/ml) and the control and $H_2O_2$ treatment (**$P<.01$). (B) Cells were pretreated with CAT (100 U/ml) for 1 h and then treated with SNL glycoprotein (80 μg/ml) or $H_2O_2$ (200 μM) for another 4 h. The apoptotic inducing effect of SNL glycoprotein or $H_2O_2$ was evaluated by H33342/ethidium bromide staining. Each bar represents the mean±S.D. of triplicate experiments ($n=3$). Double asterisks represents a significant difference between the SNL glycoprotein treatments and the control and the $H_2O_2$ treatment and the control (**$P<.01$). Pound signs represent a significant difference between the SNL glycoprotein treatment and CAT treatment in the presence of SNL glycoprotein, and $H_2O_2$ treatment and CAT treatment in the presence of $H_2O_2$ ($^{\#\#}P<.01$).

### 3.3. Effect of SNL glycoprotein on intracellular ROS production

In the intracellular ROS production by SNL glycoprotein, the levels of intracellular ROS were increased by 1.3-, 1.6- and 2.1-fold at 20, 40 and 80 μg/ml, compared to the control (Fig. 3A). After exposure to the 200 μM $H_2O_2$ alone, which was used as a positive control, the levels of intracellular ROS significantly were increased by 2.8 fold, compared to the control, indicating that SNL glycoprotein might induce the apoptosis via a ROS-dependent mechanism in Hep3B cells. To support the evidence that SNL glycoprotein-induced apoptosis is dependent of ROS generation, we investigated whether free radical scavengers [catalase (CAT)] can protect against SNL glycoprotein-induced apoptosis, using a nuclei staining assay (Fig. 3B). The results showed that the percentages of apoptotic cells were increased by 79% and 82% after treatment with SNL glycoprotein (80 μg/ml) or $H_2O_2$ (200 μM) for 4 h, compared to the control, whereas pretreatment with CAT (100 U/ml) for 1 h in these conditions resulted in the diminishment of percentage of apoptotic cells by 53% and 60% in SNL glycoprotein- or $H_2O_2$-induced Hep3B cells, compared to SNL glycoprotein or $H_2O_2$ alone, respectively.

### 3.4. Inhibitory effects of SNL glycoprotein on NF-κB and iNOS activities

Results in Fig. 4A and B showed that SNL glycoprotein inhibit the DNA-binding activity of NF-κB and iNOS activity in Hep3B cells. When the cells were treated with various concentrations of SNL glycoprotein (20–80 μg/ml) for 4 h, the DNA-binding activity of NF-κB complex in nuclear extract fractions and iNOS activity in whole cell fractions were diminished in a dose-dependent manner, compared to control. In addition, the level of inducible NO production was significantly decreased by 17 μM after treatment with 80 μg/ml of SNL glycoprotein for 4 h in Hep3B cells, compared to control, respectively (Fig. 4C). Because the inhibition of NF-κB activity induced by SNL glycoprotein is upstream event in iNOS activity, we also compared the inhibitory effect of SNL glycoprotein on the iNOS activity and inducible NO production with NF-κB inhibitor, PDTC (Fig. 4B and C). The result showed that the iNOS activity markedly is abolished, lowering the production of inducible NO by 19 μM after treatment with 100 μM PDTC for 4 h, compared to control, in Hep3B cells.

### 3.5. Apoptotic effect of SNL glycoprotein on caspase-3 activity

We also examined the apoptotic effect of SNL glycoprotein, PDTC (NF-κB inhibitor) and L-NAME (NO inhibitor) on caspase-3 activation in Hep3B cells (Fig. 5A). As expected, when the cells were treated with 80 μg/ml SNL glycoprotein, 100 μM PDTC or 100 μM L-NAME for 4 h, the cleaved forms of the caspase-3 were definitely observed. The apoptotic activity of SNL glycoprotein, PDTC and L-NAME was also confirmed by nuclei staining and DNA fragmentation assays. After the addition of 80 μg/ml SNL glycoprotein, 100 μM PDTC or 100 μM L-NAME for 4 h, the percentages of apoptotic cells were increased by 74, 77 and 72 (Fig. 5B), and the nucleosomal DNA fragmentations (apoptotic DNA frag-

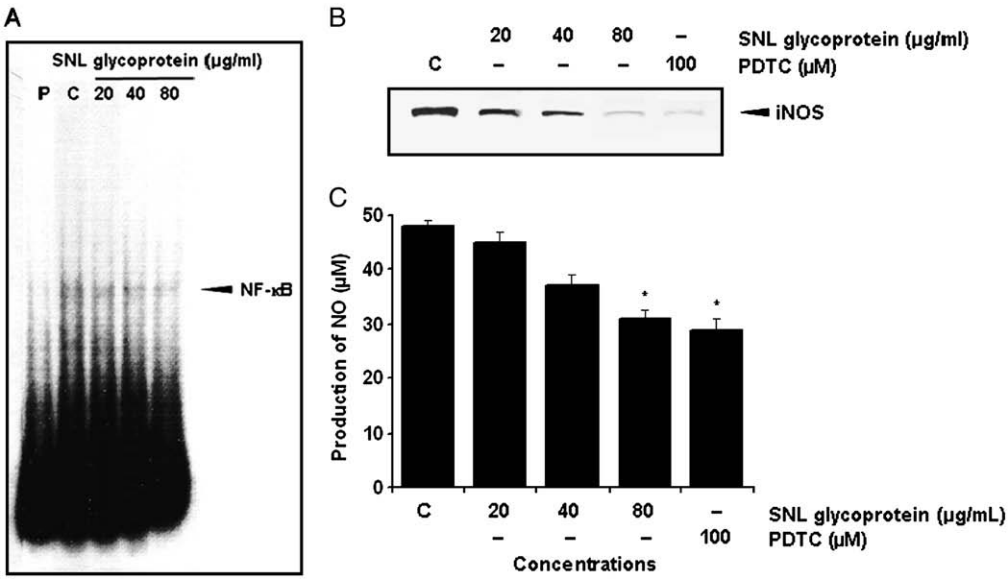


Fig. 4. Inhibitory effects of SNL glycoprotein on NF-κB and iNOS activities. Hep3B cells were treated with various concentrations of SNL glycoprotein (20, 40 and 80 μg/ml) for 4 h. (A) Detection of NF-κB DNA binding activity was performed by EMSA using the NF-κB oligonucleotide. Lane 1, free probe alone (no nuclear extracts); Lane 2, control; Lane 3, SNL glycoprotein (20 μg/ml); Lane 4, SNL glycoprotein (40 μg/ml); Lane 5, SNL glycoprotein (80 μg/ml). (B) Cells were treated with various concentrations of SNL glycoprotein (20, 40 and 80 μg/ml) or 100 μM of PDTC alone for 4 h. Detection of iNOS activation was performed by Western blotting using an anti-iNOS polyclonal antibody. (C) For measurements of NO production, the supernatants of culture medium were used as described in Materials and methods. The concentration of NO was assessed using a standard curve of $NaNO_2$.

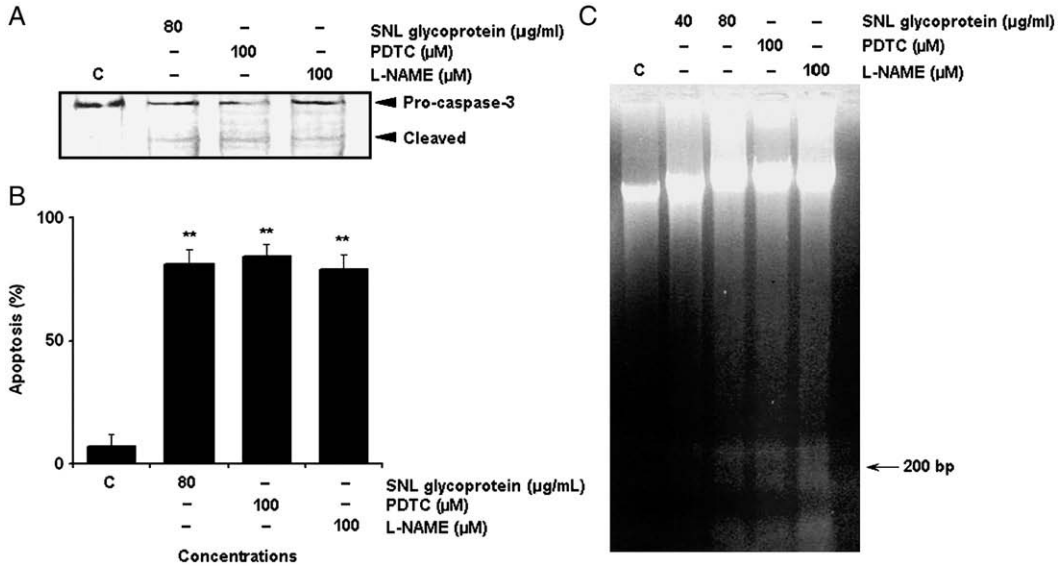


Fig. 5. Apoptotic effect of SNL glycoprotein on caspase-3 activity. Hep3B cells were exposed to SNL glycoprotein (80 μg/ml), PDTC (100 μM) or L-NAME (100 μM) for 4 h, respectively. (A) Detection of caspase-3 activation was performed by Western blotting using an anti-caspase-3 polyclonal antibody. (B) The apoptotic inducing effect of SNL glycoprotein (80 μg/ml), PDTC (100 μM) or L-NAME (100 μM) was evaluated by H33342/ethidium bromide staining. Each bar represents the mean ± S.D. of triplicate experiments ($n = 3$). Double asterisk represents a significant difference between the treatments and the control (**$P < .01$). (C) The apoptotic DNA fragments induced by treatment with SNL glycoprotein (80 μg/ml), PDTC (100 μM) or L-NAME (100 μM) were detected using DNA fragmentation assay. Electrophoresis was carried out on a 2% agarose gel. Lane 1, control; Lane 2, SNL glycoprotein (40 μg/ml); Lane 3, SNL glycoprotein (80 μg/ml); Lane 4, PDTC (100 μM); Lane 5, L-NAME (100 μM).

ments) of 180–200 base pairs could be detected (Fig. 5C, lanes 3–5), compared to the control.

## 4. Discussion

Many glycoproteins isolated from plants have been investigated with a view to their application as host defense potentiators or biological response modifiers for inhibiting tumor growth. In the present study, we investigated whether glycine and proline-rich SNL glycoprotein, which mostly consists of carbohydrate content (69.74%) and protein content (30.26%), can stimulate the activities of mitochondria-mediated apoptotic signals (cytochrome *c*, caspases, PARP) and inhibit the activities of NF-κB and iNOS in Hep3B cells (hepatocellular carcinoma cells). First, the result in neutral red assay indicated that the SNL glycoprotein has strong cytotoxic ability, because it showed approximately $IC_{50}$ at 80 μg/ml for 4 h (Fig. 1). These findings are consistent with the observations that glycine-rich peptide and proline-rich peptide such as cecropin, defensin, drosoin and diptericin have antimicrobial, chemopreventive and chemotherapeutic activities [31–33]. From these results, we speculated that SNL glycoprotein has a potential activity of apoptosis in Hep3B cells.

To provide evidence to support a possible mechanism of the apoptotic effect of SNL glycoprotein, we investigated the effects of SNL glycoprotein on the activities of mitochondria-mediated apoptosis mediators in Hep3B cells (Fig. 2). Our results in this study indicate that SNL glycoprotein has apoptotic effects by stimulating the activities of cytochrome *c*, caspase-9, caspase-3 and PARP proteins in a dose-dependent manner. In mammalian cells, two major apoptosis pathways have been defined as the death receptor-mediated (extrinsic) apoptosis pathway and the mitochondria-mediated (intrinsic) apoptosis pathway. In contrast to the extrinsic apoptosis pathway, it has been reported that the mitochondria-mediated apoptosis pathway is activated by various cellular stress stimuli such as radiation, oxidant agent and other chemicals (eg, drugs) and dependent on the release of cytochrome *c* from the mitochondria [8]. Following the efflux of mitochondrial cytochrome *c* to cytosol, the pro-caspase-9 is cleaved and activated by formation of apoptosome and then causes caspase-3 activations and PARP cleavage and finally results in apoptosis [9]. Although we have not determined the interaction between SNL glycoprotein and death ligands/death receptors, our results suggest that SNL glycoprotein activates mitochondria-mediated apoptosis pathway by stimulating the release of mitochondrial cytochrome *c* and the cleavages of pro-caspase-9, pro-caspase-3 and PARP proteins in Hep3B cells.

Reactive oxygen species (ROS) are constantly generated and eliminated in the biological system and play important roles in a variety of normal biochemical functions and abnormal pathological processes [10]. In present study, high level of intracellular ROS production was observed in SNL glycoprotein-treated Hep3B cells (Fig. 3). Accumulating evidence has indicated that there are interrelationships between intracellular ROS production and apoptotic factors in the mitochondrial apoptotic pathway. Namely, the overproduction of ROS caused by chemotherapeutic agent can directly stimulate the mitochondrial permeability

transition and subsequently results in the loss of mitochondrial membrane potential. These changes in mitochondrial membrane structure allow the release of cytochrome *c*, which binds to Apaf-1, forming an apoptosome in cytosol that consequently lead to the caspases cascade by activating caspase-9 and caspase-3 [11]. To know the production of intracellular ROS is essential for SNL glycoprotein-induced apoptosis, we further investigated whether free radical scavenger (CAT) can protect against SNL glycoprotein-induced apoptosis. Our results in this study show that CAT blocks SNL glycoprotein-induced apoptosis in Hep3B cells, suggesting that SNL glycoprotein stimulates the mitochondria-mediated apoptosis pathway via ROS-dependent mechanism in Hep3B cells. Interestingly, the results in this study showed that the apoptotic stimulation of SNL glycoprotein (80 μg/ml) is similar to $H_2O_2$ (200 μM).

In terms of biochemical therapeutic avenues for hepatocellular carcinoma, it is also important to understand the transcriptional activity of NF-κB on the SNL glycoprotein-induced apoptosis pathway because the inhibition of the NF-κB activity by an agent are closely related to its anticancer, antiresistance and apoptosis activities in hepatocellular carcinoma [2,15]. In this experiment, the DNA binding activity of NF-κB complex was markedly inhibited by treatment with SNL glycoprotein in a dose-dependent manner (Fig. 4A). Here it should be noted that the antiapoptotic actions of NF-κB could be mediated by preventing caspase-3 activation [34]. This means that the caspase-3 activation through suppression of NF-κB might be involved in SNL glycoprotein-induced apoptosis pathways in Hep3B cells.

We further investigated whether SNL glycoprotein can regulate the activity of iNOS as a mediator between NF-κB and caspase-3 (Fig. 4B). Our results in present study showed that the activity of iNOS is weakened by the addition of 80 μg/ml SNL glycoprotein in Hep3B cells. Moreover, the production of inducible NO is significantly reduced (Fig. 4C). Although the activity of iNOS in hepatocellular carcinoma is still a controversial issue, numerous studies have shown that functional roles of iNOS and inducible NO in tumor progression represent tumor-cell proliferation, survival, migration and resistance [16–18]. From this point of view, our results suggest that SNL glycoprotein is a potent inhibitor of iNOS activity. We also speculate that the reason for the low activity of iNOS after treatment with SNL glycoprotein might be resulted from modulation of upstream signal, such as NF-κB. As expected, the treatment with 100 μM PDTC alone apparently abolished the iNOS activity and inducible NO production, indicating that the iNOS activity correlated with the activation of NF-κB in Hep3B cells.

To elucidate the mechanism of apoptotic signals involving NF-κB, iNOS, caspase-3 by SNL glycoprotein, we further investigated whether the inhibitors of NF-κB and iNOS can regulate the caspase-3 activation and the apoptosis in Hep3B cells (Fig. 5). Interestingly, the cleaved forms of caspase-3 and increased apoptotic DNA fragments were observed after treatments with PDTC (100 μM) or L-NAME (100 μM), as well as SNL glycoprotein (80 μg/ml), suggesting that the apoptotic activity of SNL glycoprotein to block the NF-κB and iNOS activities is associated with its ability to stimulate the caspase-3 activation in Hep3B cells. In the relationship between NO production and caspase-3 activation, it was recently reported that the inhibition of apoptosis by NO production is to be a consequence through the suppression of caspase enzymatic activity because NO can directly inhibit caspase activity through *S*-nitrosylation of the active cysteine conserved in all caspases, leading to tumorigenesis [35]. Thus, the low activation of iNOS after treatment with SNL glycoprotein may stimulate caspases activities through inhibition of *S*-nitrosylation. Moreover, recent study also showed that suppression of NO stimulates several other events associated with caspase activation, including release of cytochrome *c* from mitochondria, decrease in mitochondrial transmembrane potential and cleavage of PARP in carcinoma cells [36]. From our results, we assume that SNL glycoprotein may also inhibit the activation of NF-κB, which leads to suppression of iNOS activity and inducible NO production and then activates the caspase-3-mediated apoptosis pathway in Hep3B cells. The results in the recent report showed that the inhibitions of NF-κB and iNOS activities relate to stimulate caspase cascade signaling through Fas/TNF-R1 death receptor activation [37]. Since SNL glycoprotein has high molecular weights (150 kD) and high polarity, it is not possible to penetrate into the cytoplasm through the cell membrane. In this context, we assumed that SNL glycoprotein as ligand binds to the receptor of the cell death (Fas/TNF-R1) to build complex, and then the results from complex building stimulates the apoptotic intracellular factors in the cytoplasm. Finally, the activated signal induced by SNL glycoprotein stimulates caspase-3 induction via suppression of NF-κB and iNOS in the Hep3B cells.

In conclusion, these results in this study pointed out that SNL glycoprotein induces apoptosis deduced from activation of mitochondria-mediated apoptosis pathway (cytochrome *c*, caspase-9, caspase-3 and PARP), production of intracellular ROS and inactivation NF-κB-mediated signals (NF-κB and iNOS) in Hep3B cells. Therefore, we speculate that SNL glycoprotein can be used as a chemotherapeutic agent for hepatocellular carcinoma. However, further research must be carried out to elucidate the mechanisms of apoptotic signals involving mitogen-activated protein kinase, and the expression of apoptotic mRNA at the molecular biological level, such as Bax, p53 and Bcl-2 expressions.

## References

[1] Roberts LR, Gores GJ. Hepatocellular carcinoma: molecular pathways and new therapeutic targets. Semin Liver Dis 2005;25:212–25.
[2] Arsura M, Cavin LG. Nuclear factor-kappaB and liver carcinogenesis. Cancer Lett 2005;229:157–69.
[3] Kountouras J, Zavos C, Chatzopoulos D. Apoptotic and anti-angiogenic strategies in liver and gastrointestinal malignancies. J Surg Oncol 2005;90:249–59.

[4] Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. Mushrooms, tumors, and immunity. Proc Soc Exp Biol Med 1999; 221:281–93.
[5] Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med 2002;53:615–27.
[6] Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 1993;75:241–51.
[7] Robertson JD, Orrenius S. Role of mitochondria in toxic cell death. Toxicology 2002;181-182:491–6.
[8] Yin XM. Bid, a critical mediator for apoptosis induced by the activation of Fas/TNF-R1 death receptors in hepatocytes. J Mol Med 2000;78:203–11.
[9] Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell 1997;91:443–6.
[10] Brunelle JK, Chandel NS. Oxygen deprivation induced cell death: an update. Apoptosis 2002;7:475–82.
[11] Zou H, Li Y, Liu X, Wang X. An APAF-1. cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 1999;274:11549–56.
[12] Sun SY, Hail N, Lotan R. Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 2004;96:662–72.
[13] Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 1996;274:782–4.
[14] Karin M. How NF-kappa B is activated: the role of the Ikappa B kinase (IKK) complex. Oncogene 1990;18:6867–74.
[15] Foo SY, Nolan GP. NF-kappa B to the rescue: RELs, apoptosis and cellular transformation. Trends Genet 1999;15:229–35.
[16] Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell 1994;78:915–8.
[17] Fantappie O, Masini E, Sardi I, Raimondi L, Bani D, Solazzo M, et al. The MDR phenotype is associated with the expression of COX-2 and iNOS in a human hepatocellular carcinoma cell line. Hepatology 2002;35:843–52.
[18] Lasagna N, Fantappie O, Solazzo M, Morbidelli L, Marchetti S, Cipriani G, et al. Hepatocyte growth factor and inducible nitric oxide synthase are involved in multidrug resistance-induced angiogenesis in hepatocellular carcinoma cell lines. Cancer Res 2006;66:2673–82.
[19] Ooi VE, Liu F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Cur Med Chem 2000;7:715–29.
[20] Lee SJ, Ko JH, Lim KT. Glycine- and proline-rich glycoprotein isolated from *Solanum nigrum* Linne activates caspase-3 through cytochrome *c* in HT-29 cells. Oncol Rep 2005;14:789–96.
[21] Lim KT, Heo KS, Son YO. Antioxidative and antimicrobial effects of glycoprotein isolated from *Solanum nigrum* Linne. Food Sci Biotechnol 2002;11:484–9.
[22] Lee SJ, Oh PS, Ko JH, Lim K, Lim KT. A 150-kDa glycoprotein isolated from *Solanum nigrum* L. has cytotoxic and apoptotic effects by inhibiting the effects of protein kinase C alpha, nuclear factor-kappa B and inducible nitric oxide in HCT-116 cells. Cancer Chemother Pharmacol 2004;54:562–72.
[23] Heo KS, Lim KT. Glycoprotein isolated from *Solanum nigrum* L. modulates the apoptotic-related signals in 12-*O*-tetradecanoylphorbol 13-acetate-stimulated MCF-7 cells. J Med Food 2005;8:69–77.
[24] Neville DM, Glossmann H. Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. Methods Enzymol 1974; 32:92–102.
[25] Tarr GE. Manual Edman sequencing system. In: Shively JE, editor. Methods of protein microcharacterization. New Jersey: Humana Press; 1986. p. 155–94.
[26] Liu TY, Chang YH. Hydrolysis of proteins with *p*-toluenesulfonic acid. Determination of tryptophan. J Biol Chem 1971;246:2842–8.
[27] Wadsworth TL, Koop DR. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. Biochem Pharmacol 1999;57:941–9.
[28] Royall JA, Ischiropoulos H. Evaluation of 2′ ,7′ -dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular $H_2O_2$ in cultured endothelial cells. Arch Biochem Biophys 1993;302:348–55.
[29] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin-phenol reagent. J Biol Chem 1951;193:265–75.
[30] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126:131–8.
[31] Johnstone SA, Gelmon K, Mayer LD, Hancock RE, Bally MB. In vitro characterization of the anticancer activity of membrane-active cationic peptides. I. Peptide-mediated cytotoxicity and peptide-enhanced cytotoxic activity of doxorubicin against wild-type and P-glycoprotein over-expressing tumor cell lines. Anticancer Drug Des 2000;15:151–60.
[32] Zhang WM, Lai ZS, He MR, Xu G, Huang W, Zhou DY. Effects of the antibacterial peptide cecropins from Chinese oak silkworm, *Antheraea pernyi* on 1, 2-dimethylhydrazine-induced colon carcinogenesis in rats. Di Yi Jun Yi Da Xue Xue Bao 2003;23:1066–8.
[33] Otvos Jr L. Antibacterial peptides isolated from insects. J Pept Sci 2000;6:497–511.
[34] Miyajima A, Nakashima J, Yoshioka K, Tachibana M, Tazaki H, Murai M. Role of reactive oxygen species in *cis*-dichlorodiammine-platinum-induced cytotoxicity on bladder cancer cells. Br J Cancer 1997;76:206–10.
[35] Hug H, Strand S, Grambihler A, Galle J, Hack V, Stremmel W, et al. Reactive oxygen intermediates are involved in the induction of CD95 ligand mRNA expression by cytostatic drugs in hepatoma cells. J Biol Chem 1997;272:28191–3.
[36] Lee YJ, Lee KH, Kim HR, Jessup JM, Seol DW, Kim TH, et al. Sodium nitroprusside enhances TRAIL-induced apoptosis via a mitochondria-dependent pathway in human colorectal carcinoma CX-1 cells. Oncogene 2001;20:1476–85.
[37] Hatano E, Bennett BL, Manning AM, Qian T, Lemasters JJ, Brenner DA. NF-kappaB stimulates inducible nitric oxide synthase to protect mouse hepatocytes from TNF-alpha- and Fas-mediated apoptosis. Gastroenterology 2001;120:1251–62.