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Abstract

Purpose. Gypenosides (Gyp), found in *Gynostemma pentaphyllum* Makino, have been used as folk medicine for centuries and have exhibited diverse pharmacological effects, including antileukemia effects in vitro and in vivo. In the present study, Gyp were used to examine effects on cell viability, cell cycle, and induction of apoptosis in vitro. They were administered in the diet to mice injected with WEHI-3 cells in vivo. **Experimental design.** Effects of Gyp on WEHI-3 cells were determined by flow cytometric assay and Western blotting. **Results.** Gyp inhibited the growth of WEHI-3 cells. These effects were associated with the induction of G0/G1 arrest, morphological changes, DNA fragmentation, and increased sub-G1 phase. Gyp promoted the production of reactive oxygen species, increased Ca²⁺ levels, and induced the depolarization of the mitochondrial membrane potential. The effects of Gyp were dose and time dependent. Moreover, Gyp increased levels of the proapoptotic protein Bax, reduced levels of the antiapoptotic proteins Bcl-2, and stimulated release of cytochrome c, AIF (apoptosis-inducing factor), and Endo G (endonuclease G) from mitochondria. The levels of GADD153, GRP78, ATF6- α , and ATF4- α were increased by Gyp, resulting in ER (endoplasmic reticular) stress in WEHI-3 cells. Oral consumption of Gyp increased the survival rate of mice injected with WEHI-3 cells used as a mouse model of leukemia. **Conclusions.** Results of these experiments provide new information on understanding mechanisms of Gyp-induced effects on cell cycle arrest and apoptosis in vitro and in an in vivo animal model.

Keywords

gypenosides, antileukemia, WEHI-3 cells, in vitro, in vivo

Background and Introduction

Worldwide, leukemia is a common cause of death with about 3.7 individuals per 100 000 dying annually of leukemia in the United States.¹ In Taiwan, approximately 2.1 individuals per 100 000 die each year of leukemia based on 2008 reports from the Department of Health, Executive Yuan, ROC (Taiwan). Several different approaches have been used for treating leukemia patients, but overall success in treating this disease is not satisfactory.

Epidemiological studies and animal studies² have demonstrated that if people increase their consumption of plant-based diets, development of colon cancer is reduced.^{3,4} It is well known that herbal-based dietary supplements contain many phytochemicals, including flavonoids, which could act to induce cancer suppression.

The herb *Gynostemma pentaphyllum* (Thunb) Makino (*Cucurbitaceae*), which has been used in traditional Chinese medicine, has also been administered as a herbal tea

in randomly assigned type 2 diabetic patients, with results showing a prompt improvement in glycemia and insulin sensitivity.⁵ Approximately 90 dammarane-type saponin glycosides (called gypenosides [Gyp]) have been identified phytochemically from the plant by gas chromatography mass spectrometry.⁶ The leaves and stems of *G pentaphyllum* or the herb alone have been used as popular folk medicine for treatment of cardiovascular disease,⁷ hepatitis,⁸ hyperlipoproteinemia,^{9,10} and cancer¹¹ by the Chinese for centuries.

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Gyp have been reported to have anti-inflammatory, antioxidative,¹² antithrombotic,¹³ and anticancer effects.¹⁴⁻¹⁷ *N*-acetyltransferase activity and gene expression were altered by Gyp in human cervical cancer cells in vitro.¹⁸ Gyp induce apoptosis in human hepatoma cells¹⁹ and colon cancer cells through mitochondria-dependent pathways.²⁰ Recently, we also found that Gyp induced apoptosis in human tongue cancer SCC-4 cells involving endoplasmic reticular (ER) stress and mitochondria-dependent pathways.²¹

However, the mechanism of Gyp-induced apoptosis in leukemia in vitro and in vivo remains unclear, and the effects of Gyp on human leukemic cells have never been investigated. Therefore, in the present study, leukemia cells were selected for examining the effects of Gyp on WEHI-3 cells in vitro and in vivo. We show that Gyp induced apoptosis in WEHI-3 cells through mitochondria-dependent pathways and increased the survival rate of mice injected with WEHI-3 cells.

Materials and Methods

Preparation of Gypenosides

Gyp were kindly provided by Dr Jung-Chou Chen (Department of Chinese Medicine, China Medical University, Taichung, Taiwan). The plant material was purchased from Ankang Pharmaceutical Institute of the Beijing University, People's Republic of China. *G pentaphyllum* was extracted to generate the total Gyp: the material was subjected to reversed-phase short-column vacuum chromatography as previously described by Huang et al.²² In brief, an air-tight solid phase packed with silanized silica gel (thin-layer chromatography [TLC] grade, Merck [Darmstadt, Germany]) in a sintered glass column was primed under reduced pressure with stepwise mobile phases starting with 100% MeOH to MeOH-H₂O (10:90). The column was subsequently loaded with a sample in MeOH-H₂O (50:50) and eluted with a stepwise gradient mobile phase starting with MeOH-H₂O (10:90) to 100% MeOH under reduced pressure. The fraction eluted with 100% MeOH was at highest yield and was analyzed by TLC. TLC was analyzed on a Merck silica gel 60 F254 aluminum sheet with a mobile phase system, including chloroform/ethyl acetate/methanol/water (15:50:22:10).^{22,23}

Chemicals and Reagents

Dimethyl sulfoxide (DMSO), potassium phosphates, propidium iodide (PI), ribonuclease-A, thapsigargin (stimulating agent of ER stress), Tris-HCl, and Triton X-100 were obtained from Sigma Chemical Co (St Louis, MO). Tris-EDTA (ethylenediaminetetraacetic acid) buffer was purchased from Merck Co (Darmstadt, Germany). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), DiOC₆, and Indo 1/AM were obtained from Calbiochem (San Diego, CA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin,

and trypsin-EDTA were obtained from Gibco/Invitrogen Corp (Carlsbad, CA)

Part I: In Vitro Studies

Cell culture. The WEHI-3 (mouse BALB/c myelomonocytic leukemia, macrophage-like) cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in RPMI-1640 medium containing 10% FBS, 1% penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) and 2 mM L-glutamine in 75 cm² tissue culture flasks at 37°C under a humidified 5% CO₂ and 95% air atmosphere as described elsewhere.²⁴

Assessment of cell morphology and viability of WEHI-3 cells after exposure to Gyp. WEHI-3 cells (2×10^5 cells/well) were placed in 12-well plates and incubated at 37°C for 24 hours. Cells were then treated with 0, 60, 90, 120, 150, and 180 µg/mL Gyp for 24 and 48 hours or exposed to 150 µg/mL of Gyp for 6, 12, 24, 48, and 72 hours. DMSO (solvent) was used as a vehicle control. Cell morphology was determined using a phase-contrast microscope.²⁵ Cell viability was assayed using a flow cytometric protocol as previously described.^{24,25}

Flow cytometry analysis of cell cycle distribution and sub-G1 group. Cells (2×10^5 cells/well) were seeded in 12-well plates and treated with Gyp (150 µg/mL) for 0, 6, 12, and 24 hours. Cells were harvested, washed, and fixed with ice-cold 70% ethanol and incubated in 800 µL phosphate-buffered saline (PBS), 100 µL RNase (1 mg/mL; Sigma, St Louis, MO), and 20 µL PI (2 mg/mL) for 30 minutes at 37°C in a dark room, followed by flow cytometric analysis using FACSCalibur (Becton Dickinson, NJ). The cell distribution of the G0/G1, S, and G2/M phases was assessed by ModFitLT software (Verity Software House Inc, Topsham, ME).^{24,25}

Comet assay for DNA damage. WEHI-3 cells (1×10^5 cells) were treated with Gyp (0, 80, 120, 160, and 200 µg/mL) for 24 hours and then cells were embedded onto 0.5% low-melting-point agar. The slide was then immersed in ice-cold cell lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), 1% *N*-laurylsarcosine, 1% Triton X-100, and 10% DMSO. Slides were then washed and digested with 2 units of endonuclease III (Endo III) per slide in the same buffer for 1 hour at 37°C. The slides were denatured with 0.3 M NaOH, 1 mM EDTA, pH 13.4 for 20 minutes, and electrophoresis was carried out at 25 V, 300 mA for 25 minutes. Cellular DNA was stained with PI (4 µg/mL) and examined under a fluorescence microscope. The dispersion of DNA from the nucleus of each cell was measured for the lengths or parameters of tail moments.^{21,26}

Detection of reactive oxygen species (ROS), Ca²⁺ levels, and mitochondrial membrane potential ($\Delta\Psi_m$) in WEHI-3 cells. WEHI-3 cells (2×10^5 WEHI cells/well) were seeded in 12-well plates and pretreated with or without *N*-acetylcysteine (NAC). Cells were then incubated with 150 µg/mL of Gyp or 1 µM of thapsigargin for 0, 1, 3, 6, 12, or 24 hours. The

cells were harvested and washed twice for determination of viability as described above or resuspended in 500 μL of DCFH-DA (10 μM), Indo 1/AM (3 $\mu\text{g}/\text{mL}$), or Flou-3/AM (2.5 $\mu\text{g}/\text{mL}$)—dyes contains fluorescence for staining of Ca^{2+} —then incubated at 37°C for 30 minutes to detect changes in ROS and Ca^{2+} release by flow cytometry as previously described.²⁷ The level of $\Delta\Psi_m$ was determined in WEHI-3 cells (2×10^5 cells/mL) treated with 150 $\mu\text{g}/\text{mL}$ Gyp for 0, 1, 6, and 12 hours. The cells were then harvested, washed twice, resuspended in 500 μL of DiOC₆ (1 $\mu\text{mol}/\text{L}$), and incubated at 37°C for 30 minutes and analyzed by flow cytometry.^{20,21}

Detection of caspase-8 and -3 activities in WEHI-3 cells by flow cytometry. WEHI-3 cells (2×10^5 cells/well) in 12-well plates were treated with 150 $\mu\text{g}/\text{mL}$ Gyp for 24 and 48 hours. The cells were harvested and washed twice for viability and apoptosis assays as described above or for determination of caspase-8 and -3 as previously described.²⁰ Cells were harvested by centrifugation, and 50 μL of a 10 μM substrate solution (PhiPhiLux-G₁D₁ for caspase-3 and CaspaLux 8-L₁D₂ for caspase-8) were then added to the cell pellet (1×10^5 cells per sample) and incubated at 37°C for 60 minutes in the dark before being washed twice with 1 mL of ice-cold PBS and resuspended in 1 mL fresh PBS as previously described.^{20,28} Activities of caspase-3 and caspase-8 were determined and analyzed according to the manufacturer's instructions.

Levels of proteins associated with cell cycle and apoptosis. Individual protein levels were determined by Western blotting analysis of cells treated with 150 $\mu\text{g}/\text{mL}$ Gyp for 0, 6, 12, 24, and 48 hours. Total protein was extracted by incubating cells in a lysis buffer (1 mL of lysis buffer [50 mM Tris-HCl, pH 7.5], 100 mM NaCl, 0.5% [v/v] Triton X-100, 1 mM EDTA, 2.5 mM sodium orthovanadate, 10 $\mu\text{g}/\text{mL}$ protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride (PMSF)). After removal of cell debris by centrifugation, the protein concentrations in the cell lysates were determined by Bradford assay according to the manufacturer's protocols.²⁹ SDS-PAGE was performed on 10% tricine gels with equal amounts (50 μg) of protein from each treatment loaded per lane. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in tris-buffered saline tween-20 (TBST) buffer for 1 hour and incubated for 18 hours at 4°C with monoclonal anti-p53, cyclin D, CDK2, CDK6, p21, p16, Bcl-2, t-Bid, Bax, Bcl-x_s, PARP, XIAP, caspase-9 (active), AIF (apoptosis-inducing factor), Endo G (endonuclease G), cytochrome *c*, GADD153, GRP78, ATF6- α , ATF4- α (act), caspase-12 (pro), Fas ligand, and Fas (CD95). β -Actin served as an internal control. After 3 washings with 0.1% Tween 20 in tris-buffered saline (TBS), membranes were incubated with secondary antibodies and conjugated with horseradish peroxidase at 1:2000 dilution for 1 hour at room temperature. All protein bands were visualized on X ray film (Kodak) using an enhanced chemiluminescence system.^{28,29} To observe the Gyp-induced ER stress, the levels

of 2 key markers of ER stress, which are the GRP78 and GADD153 proteins, were investigated after the addition of Gyp to the WEHI-3 cells and the protocol described above.

Confocal laser scanning microscopy. WEHI-3 cells (5×10^4 cells/well) were plated on 4-well chamber slides, treated with-out or with 150 $\mu\text{g}/\text{mL}$ Gyp for 24 hours and then fixed in 4% formaldehyde in PBS for 15 minutes, permeabilized with 0.3% Triton-X 100 in PBS for 1 hour with blocking of non-specific binding sites using 2% bovine serum albumin (BSA).³⁰ Fixed cells were then incubated with primary antibodies to AIF and Endo G (1:100 dilution; green fluorescence) overnight and then exposed to the secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG at 1:100 dilution], followed by DNA staining with PI (red fluorescence). Photomicrographs were obtained using a Leica TCS SP2 confocal spectral microscope.³⁰

Part II: In Vivo Studies

Male BALB/c mice and drug treatment. Male BALB/c mice 8 weeks of age (22–28 g body weight) were obtained from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan). A total of 40 mice were divided into 4 groups (10 mice per group). Group I was injected with WEHI-3 cells (1×10^5 cells/100 μL) and served as controls. Group II was injected with WEHI-3 cells (1×10^5 cells/100 μL) for treatment with 2 mg/kg of Gyp. Group III was injected with WEHI-3 cells (1×10^5 cells/100 μL) for treatment with 4 mg/kg of Gyp. Group IV mice were treated with Gyp (2 mg/kg) but not injected with cells. Gyp was dissolved in olive oil (Sigma-Aldrich Corp, St Louis, MO) and was administered orally for 2 weeks. At the end of treatment, all animals were killed by CO₂ inhalation.

Blood samples and immunofluorescence staining. Blood samples were collected (about 1 mL) from all mice at the end of the experiments. The blood was immediately treated with ammonium chloride for lysing of the red blood cells, followed by centrifugation for 15 minutes at 1500 rpm at 4°C. The isolated white blood cells were examined for cell markers, such as CD3 and CD19 by staining with anti-CD3-FITC and CD19-PE antibodies (BD Pharmingen Inc, San Diego, CA) and flow cytometry as previously described.^{24,25}

Spleen tissues samples and histopathology. Spleen samples were obtained, weighed individually, and used for histopathology.²⁴ Tissue samples from spleen were fixed in 4% formaldehyde and embedded in paraffin. Sections of 5 mm were stained with hematoxylin and eosin according to standard procedures.^{24,31}

Statistical Analysis

All data were expressed as mean \pm standard deviation of at least 3 separate experiments. Statistical calculations of the data were performed using an unpaired Student's *t* test. Statistical significance was set at $P < .05$.

Results

Effects of Gyp on Cell Viability, Morphology, Cell Cycle Arrest, Apoptosis, and DNA Damage in WEHI-3 Cells

WEHI-3 cells were exposed to different concentrations of Gyp for different time periods. Cells were then photographed and collected for PI staining for viability analysis. The results are presented in Figures 1A and 1B. It can be seen that Gyp decreased the number of viable cells in a time- and concentration-dependent manner when compared with control cells. Cells were morphologically altered by Gyp treatment (Figure 1C) and these effects are dose dependent. Figures 1D and 1E show cell cycle and sub-G1 analysis of WEHI-3 cells after exposure to various concentrations of Gyp. There was a decrease in the percentage of Gyp-treated cells in the S phase and an increase in the percentage of cells in G0/G1. Sub-G1 groups were observed in the cell cycle distribution, suggesting that Gyp induced apoptosis in WEHI-3 cells (Figure 1D). Increased concentrations of Gyp led to an increase in G0/G1 and sub-G1 phases in the WEHI-3 cells. Further supporting the Gyp-induced apoptosis in WEHI-3 cells was the finding that longer exposure to Gyp produced more apoptotic cells (Figure 1E). Gyp-induced DNA damage was confirmed by using the comet assay, and the results are seen in Figure 1F. Higher concentrations of Gyp led to more damaged cells being stained (Figure 1F).

Gyp Alters ROS Production, Ca^{2+} Levels, $\Delta\Psi_m$, and Caspase Activity in WEHI-3 Cells

Figure 2A shows that Gyp induced ROS production quite early, and this was time dependent, but after 6 hours of treatment, there was actually a reduction in ROS levels. Gyp increased Ca^{2+} levels in WEHI-3 cells, and this effect was time dependent (Figure 2B). Mitochondrial depolarization (levels of $\Delta\Psi_m$) was increased in a time-dependent manner (Figure 2C). Data in Figure 2D and 2E indicated that Gyp stimulated activity of caspase-3 and -8. After pretreatment with NAC and then treatment with or without Gyp, the level of ROS production was measured (presented in Figure 2F), and it indicated that NAC pretreatment can lead to an increase in the production of ROS.

Effects of Gyp on Levels of Proteins Associated With Cell Cycle and Apoptosis in WEHI-3 Cells

Individual protein levels determined by Western analysis are shown in Figure 3 (A: p53, Cyclin D, CDK2, CDK6, p21, and p16; B: Bcl-2, t-Bid, Bax, and Bcl-xs; C: PARP, XIAP, and active caspase-9; D: AIF, Endo G, and cytochrome *c*; E: GADD153, GRP78, ATF6- α [pro], ATF6- α [act], ATF4- α

[act], and caspase-12 [pro]; F: Fas ligand, Fas, and t-Bid). Levels of cyclin D, CDK2, CDK4 (Figure 3A), which are involved in G0/G1 arrest, Bcl-2 (Figure 3B), PARP and XIAP (Figure 3C), and caspase-12 (pro; Figure 3E), which are involved in apoptosis, were decreased. In contrast, levels of p53, p21, and p26 (Figure 3A), which are involved in G0/G1, and t-Bid, Bax, and Bcl-x1 (Figure 3B); active caspase-9 (Figure 3C), AIF, Endo G, and cytochrome *c* (Figure 3D); GADD153, GRP78, ATF6- α , and ATF4- α (Figure 3E); and Fas ligand, Fas, and t-Bid (Figure 3F), which are all involved in apoptosis, were increased. Gyp also increased levels of Bax, cytochrome *c*, caspase-9 (act), caspase-3 activation, AIF, and Endo G but decreased the levels of Bcl-2 and Bcl-xs, which may have contributed to apoptosis. It was also observed that results from confocal laser microscopy indicated that AIF (Figure 4A) and Endo G (Figure 4B) both are released from mitochondria and translocated to the nucleus.

Effects of Gyp on Survival Rate, Body and Liver Weights, and Cell Markers of White Blood Cells From BALB/c Mice

Mice were injected with WEHI-3 cells (1×10^5 cells/100 μ L) for 2 weeks and treated with Gyp (2 and 4 mg/kg) for 2 weeks. Gyp increased the survival rate of WEHI-3 leukemia mice, and this effect was dose dependent (Figure 5A). Gyp (2 mg/kg) promoted body weight but decreased the spleen weight of WEHI-3 leukemia mice (Figure 5B). The levels of CD3 and CD19 surface markers of white blood cells were increased in Gyp-treated mice (Figure 5C and 5D). We also observed that the spleens either disclosed markedly decreased number of neoplastic cells, or the cells were difficult to detect in the red pulp. The number of megakaryocytes increased in the Gyp-treated mice (Figure 5E).

Discussion

Gyp have been used as traditional Chinese herbal medicine for hundreds of years, but its functional effects and molecular signaling pathways are not understood. Furthermore, our previous studies had shown that Gyp (1) inhibited *N*-acetyltransferase activity and gene expression in human cervical cancer Ca Ski cells,¹⁸ (2) induced apoptosis in human colon cancer cells via mitochondrial- and caspase-3-dependent pathways,²⁰ and (3) induced apoptosis in human tongue cancer cells via ER stress.²¹ There is no report on the effects of Gyp on leukemia cells. To address that deficiency, we examined the effects of Gyp on leukemia cells both in vitro and in vivo. Gyp induced G0/G1 arrest and apoptosis in murine leukemia WEHI-3 cells, and Gyp affected WEHI-3 cells in vivo, which provides a useful model system to characterize the cytotoxic and/or apoptotic effects of Gyp.

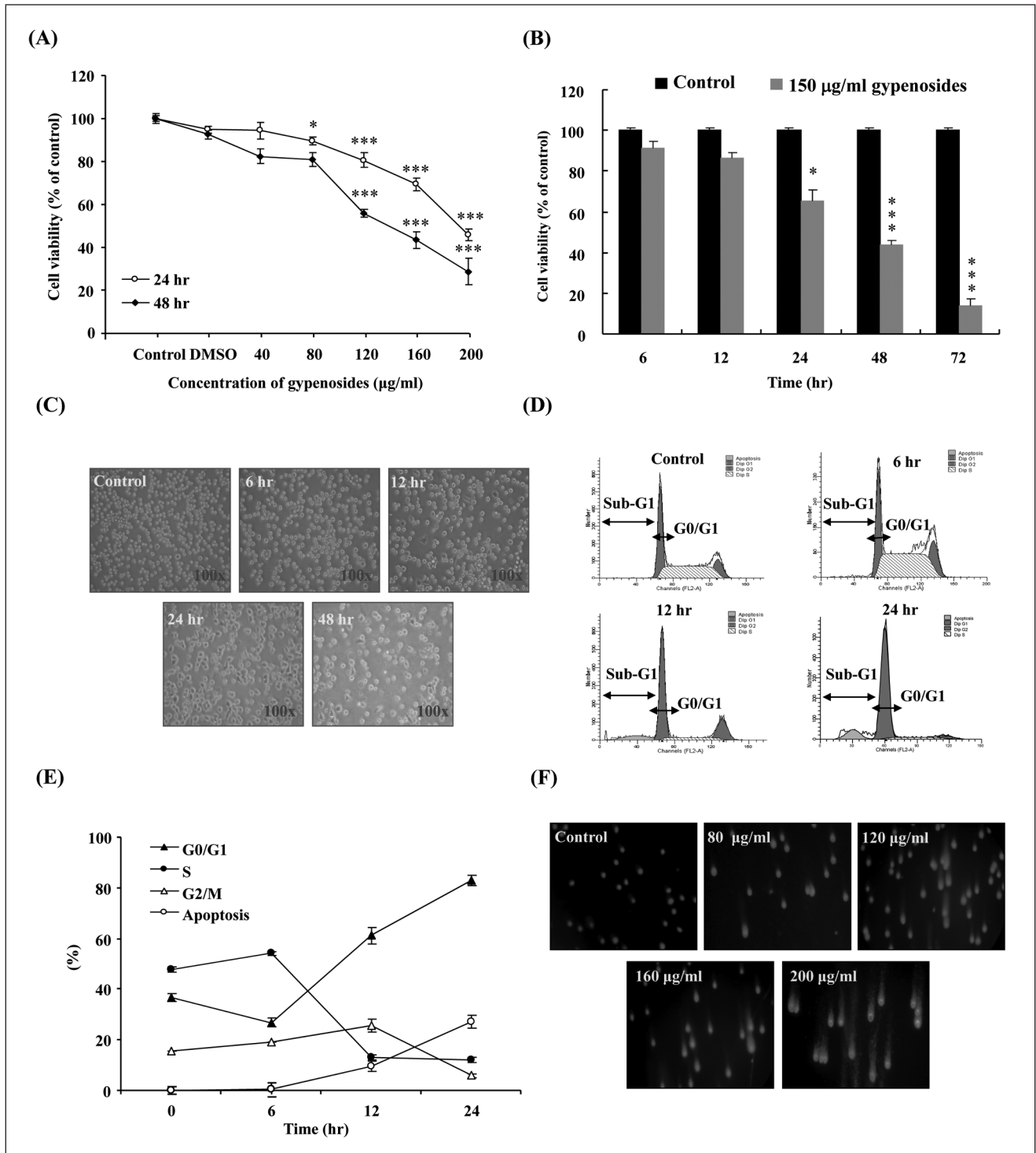


Figure 1. Gyp effects on percentage of viable cells, cell morphology, cell cycle arrest, apoptosis, and DNA damage in WEHI-3 cells. Cells were cultured in RPMI-1640 + 10% fetal bovine serum with various concentrations of Gyp for 24 and 48 hours (A) or were treated with 150 µg/mL Gyp for 6, 12, 24, 48, and 72 hours (B) for percentages of viable cells. The cells were examined and photographed by phase-contrast microscopy (200×) for morphological changes (C); cell cycle distribution (D), apoptosis (E), and DNA damage (F) were determined by flow cytometry and comet assay, as described in the Materials and Methods section. Each point is mean ± standard deviation of 3 experiments

Abbreviations: Gyp, gypenosides; DMSO, dimethyl sulfoxide.

*P < .05. ***P < .001.

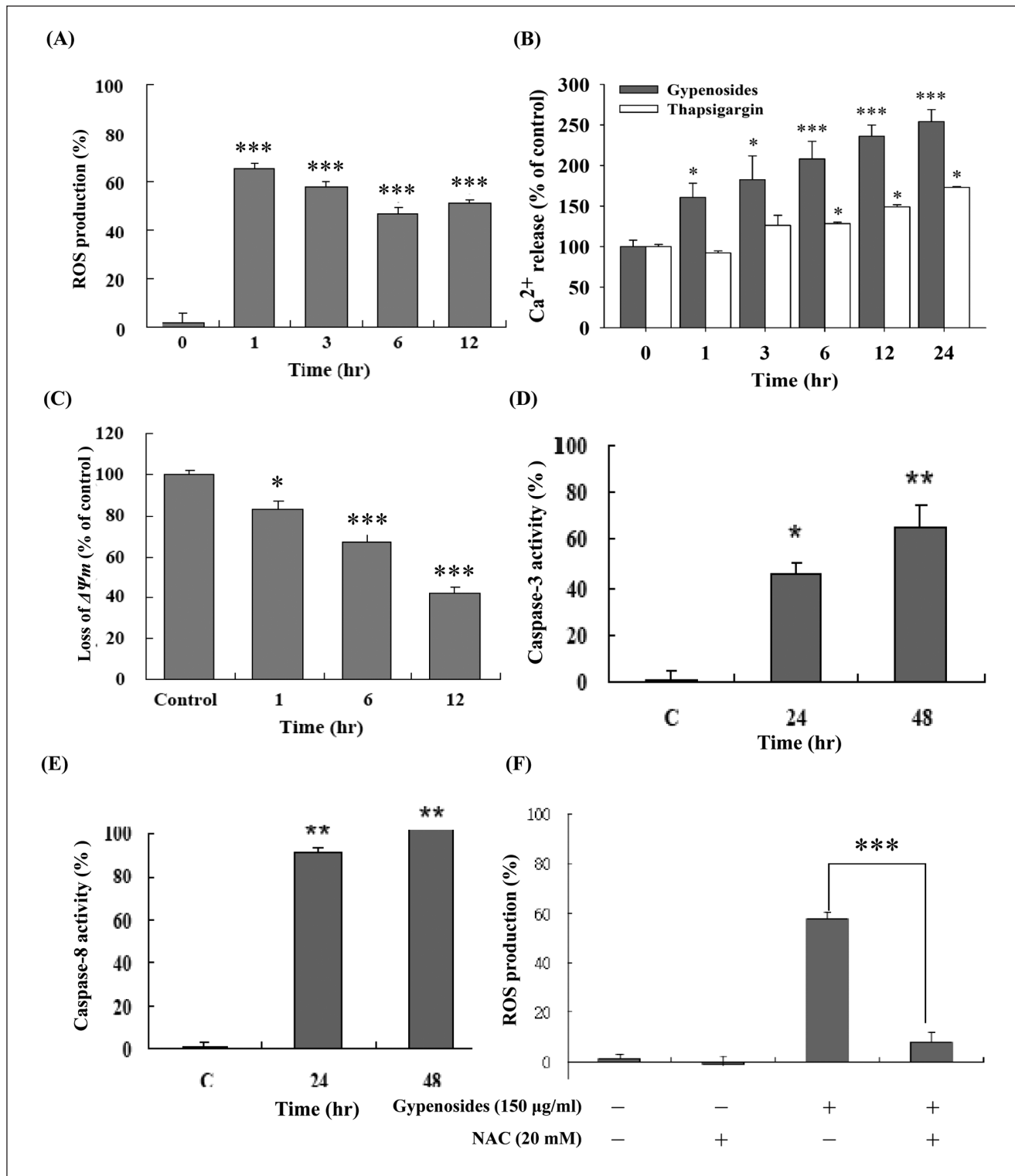


Figure 2. Gypenosides (Gyp) affected the production of reactive oxygen species (ROS) and the levels of Ca²⁺ and mitochondria membrane potential ($\Delta\Psi_m$) and caspase activities in WEHI-3 cells. Cells were treated with 150 μ g/mL Gyp for 0, 1, 3, 6, 12, or 24 hours before being collected; they were stained by DCFH-DA and the ROS levels determined (A), stained by Indo 1/AM and the Ca²⁺ levels determined (B), and stained with DiOC6 and the $\Delta\Psi_m$ levels determined (C) as described in Materials and Methods. Cells were pretreated with or without *N*-acetylcysteine (NAC) and then were treated with 150 μ g/mL Gyp for 24 hours. The cells were harvested and washed twice for activities of caspase-3 (D), caspase-8 (E), and ROS levels (F) as described in Materials and Methods ***P* < .01. ****P* < .001.

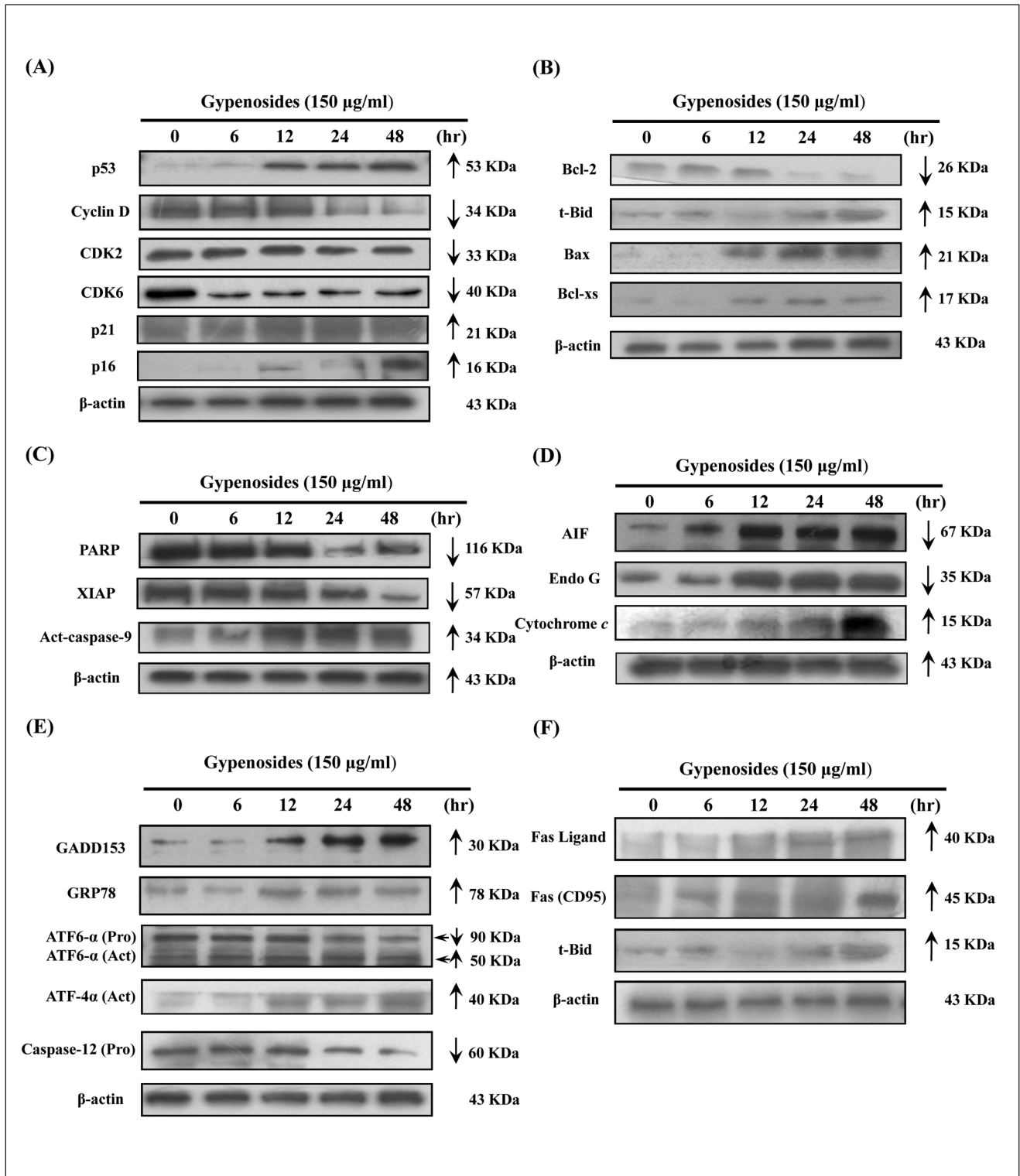


Figure 3. Representative Western blotting showing changes in the levels of associated proteins in G0/G1 arrest and apoptosis of WEHI-3 cells after exposure to Gyp. The WEHI-3 cells were treated with Gyp at 150 µg/mL for various periods of time (0, 6, 12, 24, and 48 hours) before the total proteins were prepared and determined as described in Materials and Methods. The levels of associated protein expressions (A: p53, Cyclin D, CDK2, CDK6, p21, and p16; B: Bcl-2, t-Bid, Bax, and Bcl-xl; C: PARP, XIAP, and active-caspase-9; D: AIF, Endo G and cytochrome c; E: GADD153, GRP78, ATF6-α [pro], ATF6-α [act], ATF4-α [act], and caspase-12 [pro]; F: Fas ligand, Fas, and t-Bid) were estimated by Western blotting, as described in Materials and Methods. Abbreviations: Gyp, gypenosides; AIF, apoptosis-inducing factor; Endo G, endonuclease G.

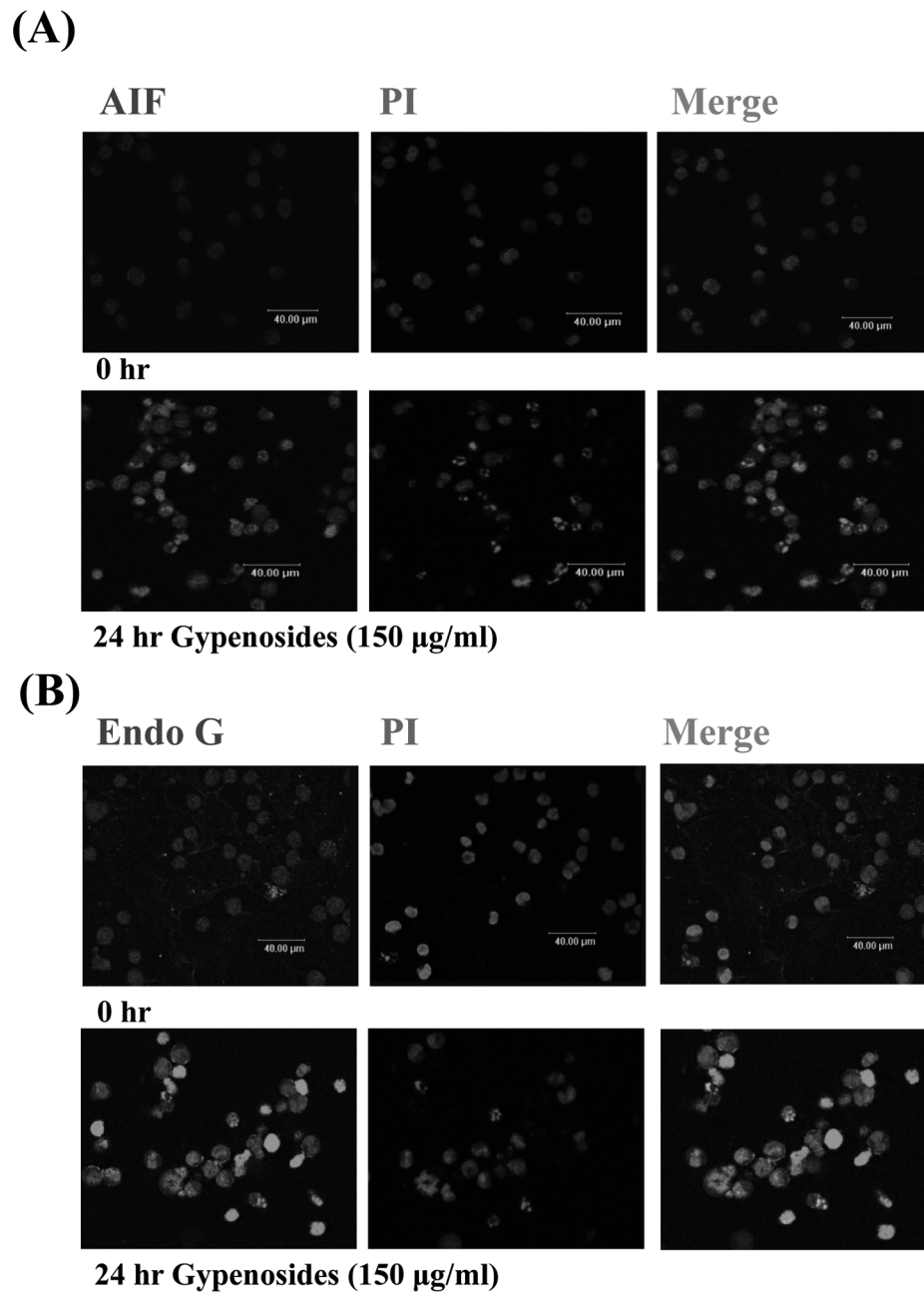


Figure 4. Gyp affected AIF and Endo-G distribution in WEHI-3 cells. Cells were incubated with Gyp at 150 µg/mL for 24 hours and then were fixed and stained with primary antibodies to AIF (A) and Endo G (B) before FITC-labeled secondary antibodies were used (green fluorescence); the proteins were detected by a confocal laser microscopic system. The nuclei were stained by PI (red fluorescence). Areas of colocalization between AIF and Endo G expressions, and cytoplasm and nuclei in the merged panels are yellow (scale bar, 40 µm)

Abbreviations: Gyp, gypenosides; AIF, apoptosis-inducing factor; Endo G, endonuclease G; PI, propidium iodide.

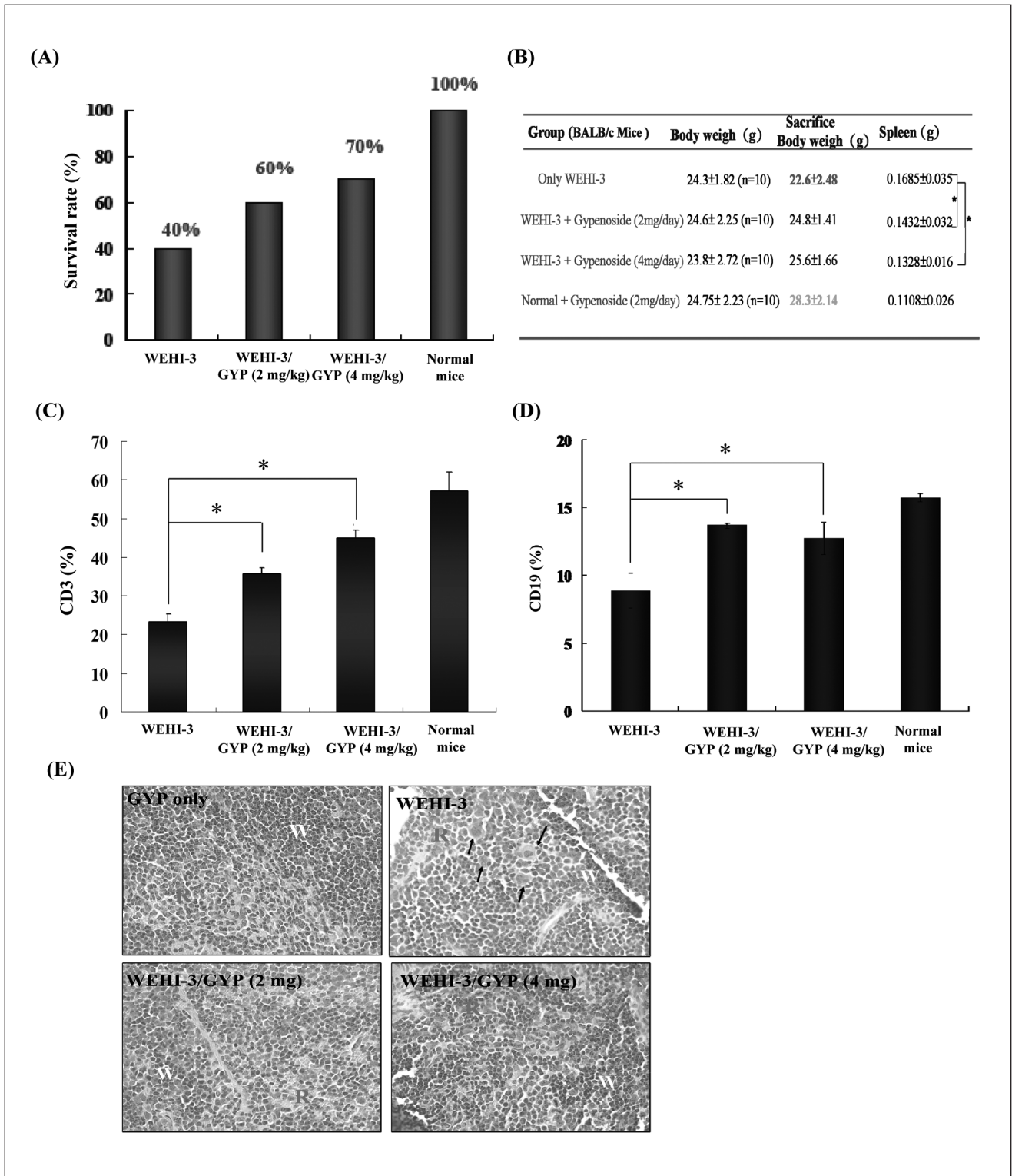


Figure 5. Gypenosides (Gyp) affected the survival rate, the body and liver weights, and the cell markers of white blood cells from BALB/c mice. The animals were injected with WEHI-3 cells (1×10^5 cells/100 μ L) for 2 weeks and treated with or without Gyp (2 and 4 mg) for 2 weeks. Then, the percentage of survival mice were counted (A), the body and livers were individually weighed (B), and spleen tissues were histopathologically examined (E). The animals were injected with WEHI-3 cells for 2 weeks and treated with or without Gyp for 2 weeks. Blood was collected from individual animals and was analyzed for cell markers (C and D) by flow cytometry as described in Materials and Methods (each point is the mean \pm standard deviation of 3 experiments)

*P < .05.

Our results indicated that Gyp induced morphological changes and cytotoxic effects, including apoptosis, in WEHI-3 cells, which were dose and time dependent. This is in agreement with our earlier report on SCC-4 cells.²¹ The results from flow cytometric assays indicated that Gyp increased the number of cells in the G0/G1 phase but reduced cells in the S and G2 phases. Gyp also inhibited cell cycle progression of WEHI-3 cells by blocking the transition from the G1 to the S phase. It was reported that cyclin D1, which is expressed in G1 cells, binds to the cyclin-dependent kinases CDK4 and CDK6 causing activation of those 2 kinases.³²⁻³⁴ The analysis of DNA content versus light scatter of the Gyp-treated WEHI-3 cells also showed that apoptosis followed Gyp-induced G0/G1 phase arrest based on the sub-G1 group occurrence. It was reported that cells going from the G1 to the S phase are regulated by cdk2, which is associated with cyclin E.^{35,36} Subsequent upregulation of p53 has been found to be sufficient to activate protein kinase c-mediated p53 gene transcription, followed by the induction of G1 phase arrest, and promote cellular repair mechanisms in model cells.^{37,38} In the present study, the results indicated that Gyp induced growth inhibition mainly via regulation of p53, p21, and p16 in WEHI-3 cells associated with inhibition of CDK2.

Gyp induced apoptosis, and this conclusion is based on several lines of evidence. Changes in WEHI-3 cells' morphology, DNA damage, DNA fragmentation, and the appearance of the sub-G1 group are consistent with apoptosis. Gyp decreased the amounts of Bcl-2 and Bcl-xs proteins but increased the amounts of Bax protein. It is well known that the ratio of antiapoptotic protein Bcl-2 to proapoptotic protein Bax is associated with the sensitivity or resistance of a cell to apoptotic stimuli.³⁹ Gyp-treated WEHI-3 cells exhibited upregulation of p53 and downregulation of the Bcl-2 protein levels. Flow cytometric assay also showed that Gyp promoted ROS and Ca²⁺ production, and decreased levels of $\Delta\Psi_m$ were accompanied by cytochrome *c*, AIF, and Endo G release, which was also confirmed by confocal laser microscope examination. These effects showed that AIF and Endo G migrated from mitochondria into nuclei.

Elevated levels of GADD153 and GRP78 are hallmarks of ER stress,⁴⁰ and some of the agents affecting ER function have been recognized as inducers of GADD153. For example, thapsigargin depleted ER calcium stores, tunicamycin blocked protein glycosylation, and dithiothreitol disrupted disulfide bond formation.⁴⁰ Therefore, the induction of GADD153 is highly responsive to ER stress. Gyp induced GADD153 and GRP78 expressions may be associated with the release of Ca²⁺ and a decrease in a mitochondrial depolarization, which was positively associated with apoptosis in WEHI-3 cells.

Gyp-induced GADD153, GRP78, ATF6- α , ATF4- α , and caspase-12 expressions may be associated with the release of Ca²⁺ and a decrease in mitochondrial depolarization, which

was positively associated with apoptosis in WEHI-3 cells. WEHI-3 cells that were pretreated with catalase and then followed by Gyp treatment or pretreated with BAPTA (a chelator of Ca²⁺) and then treated with Gyp showed a reduction in GADD153 levels and caspase-3 activation (data not shown). Catalase and BAPTA, however, did not completely block the GADD153 level.

Gyp induced apoptosis in mice leukemia WEHI-3 cells *in vitro*. We expanded on those results by determining if Gyp can affect WEHI-3 cells *in vivo*. WEHI-3 cells were injected into mice as a model of leukemia. This model has been used for monitoring agents affecting leukemia *in vivo*. Our results showed that dietary Gyp promoted immune response and increased the survival rate of mice injected with WEHI-3 cells *in vivo*.

In the present study, we found that Gyp acted via mechanisms similar to that observed in previous studies on human tongue cancer SCC-4 cells to inhibit the growth of WEHI-3 cells.²¹ Mechanisms through which Gyp inhibits cell viability are (a) induction of apoptosis (Figure 3); (b) induction of G0/G1 arrest (Figure 2), and repression of G0/G1 and apoptosis-associated protein levels and expression; and (c) enhancement of AIF and Endo G expression (Figure 6). Furthermore, we found that oral intake of Gyp could reduce the leukemia burden in a leukemia mouse model. Interestingly, we also found that oral intake of Gyp for a longer period can promote the survival rate of leukemia mice. A portion of Gyp may elude esterase digestion in the gastrointestinal tract. The undigested Gyp could accumulate in blood and exhibit antitumor activity after cessation of Gyp administration. Nevertheless, our study supports the hypothesis that oral intake of Gyp reduces the WEHI-3 leukemia burden *in vivo*. Further studies that are needed to determine whether Gyp has any effect on metastasis or angiogenesis will be accomplished in the future.

Conclusion

In conclusion, the present results showed that Gyp induced ER stress; promoted ROS and Ca²⁺ production; changed the ratio of Bax/Bcl-2; caused a mitochondrial depolarization (the decrease of $\Delta\Psi_m$); led to cytochrome *c*, AIF, and Endo G release from mitochondria; and activated caspase-8 and -3 resulting in apoptosis. A model of the proposed effects of Gyp can be seen in Figure 6. The results of the leukemia mouse model indicated that Gyp promoted the survival of mice. Taken together, these findings provide new insights (Figure 6) into the possible pathways and functions *in vitro* and *in vivo* of Gyp.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

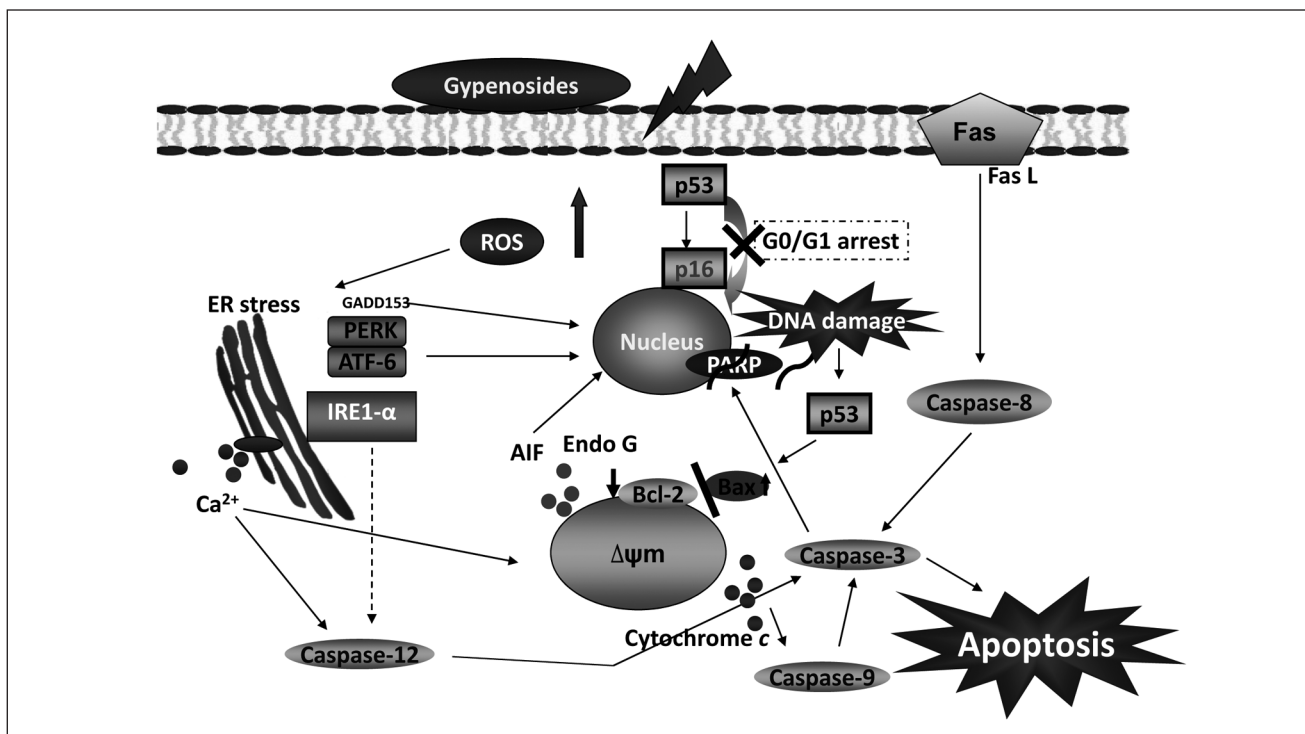


Figure 6. The proposed signaling pathways of Gyp-induced G0/G1 arrest and apoptosis in WEHI-3 cells: Gyp-induced G0/G1 phase arrest and apoptosis in WEHI-3 cells are through the Fas–FasL interaction; G0/G1 phase arrest is through p53, p21, and p16, and induction of apoptosis is through mitochondria-dependent and mitochondria-independent pathways. The mitochondria-dependent pathway is a result of the changes of the Bax/Bcl-2 ratio and involves cytochrome c release and activations of caspase-9 and -3. The mitochondria-independent pathway is involved in caspase-8 then caspase-3 activation or through the release of AIF from mitochondria for induction apoptosis

Abbreviations: ROS, reactive oxygen species; ER, endoplasmic reticular; Gyp, gypenosides; AIF, apoptosis-inducing factor; Endo G, endonuclease G; $\Delta\Psi_m$, mitochondrial membrane potential.

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