Biochemical Pharmacology, Vol. 60, pp. 677–685, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00362-2

Induction of Apoptosis by a Novel Intestinal Metabolite of Ginseng Saponin via Cytochrome *c*-Mediated Activation of Caspase-3 Protease

Sang-Jun Lee, Won-Gil Ko,* Jeong-Hee Kim,† Jong-Hwan Sung,‡ Sang-Joo Lee,‡ Chang-Kiu Moon§ and Byung-Hoon Lee*‖*

*College of Pharmacy and Medicinal Resources Research Center, Wonkwang University, Iksan, Chonbuk 570-749; †Department of Biochemistry, College of Dentistry, Kyung-Hee University, Seoul 130-701; ‡Laboratory of Natural Products Research, Central Research Institute, IL HWA Co. Ltd., Guri, Kyonggi-do 471-030; and §Department of Hygienic Pharmacy, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

ABSTRACT. Ginseng saponins exert various important pharmacological effects with regard to the control of many diseases including cancer. The novel intestinal bacterial metabolites of ginseng protopanaxadiol saponins have recently been found and isolated after the oral administration of ginseng extract in human and rats. 20-*O*-(β-D-Glucopyranosyl)-20(*S*)-protopanaxadiol (IH-901) formed from ginsenosides Rb1, Rb2, and Rc is of particular interest in cancer chemoprevention and treatment. We investigated the effects of IH-901 on the human myeloid leukemia cell line HL-60 in terms of inhibition of proliferation and induction of apoptosis. IH-901 showed a significant cytotoxic activity in HL-60 cells (IC_{50} = 24.3 μM) following a 96-hr incubation. Treatment of HL-60 cells with IH-901 resulted in the formation of internucleosomal DNA fragments. The dose- and time-dependent induction of apoptosis by IH-901 was demonstrated in sandwich enzyme immunoassay and the results were confirmed by flow cytometric analysis. Morphological examination of IH-901-treated samples showed cells with chromatin condensation, cell shrinkage, and nuclear fragmentation, all typical characteristics of apoptotic cells. The treatment of HL-60 cells with IH-901 caused activation of caspase-3 protease and subsequent proteolytic cleavage of poly(ADP-ribose) polymerase. IH-901 did not affect the expression of antiapoptotic protein Bcl-2 but did cause a release of mitochondrial cytochrome *c* into cytosol. In conclusion, our results demonstrate that IH-901 dramatically suppresses HL-60 cell growth by inducing programed cell death through activation of caspase-3 protease, which occurs via mitochondrial cytochrome *c* release independently of Bcl-2 modulation. These results may provide a pivotal mechanism for the use of IH-901 in the prevention and treatment of leukemia. BIOCHEM PHARMACOL **60**;5:677–685, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; HL-60 Cells; ginseng saponin metabolite (IH-901); cytochrome *c*; caspase-3 protease; poly(ADP-ribose) polymerase

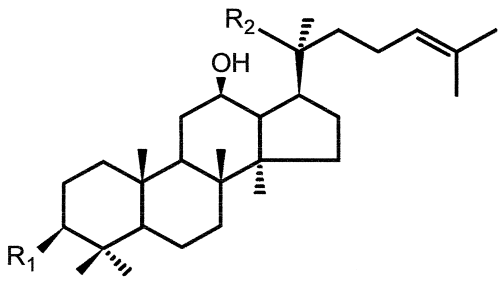
Panax ginseng C.A. Meyer, belonging to Araliaceae, has been reported to possess diverse biological and pharmacological activities on the central nervous system, cardiovascular system, endocrine system, and immune function, that are mainly attributed to its triterpenoid saponin components. A number of saponins have so far been isolated from ginseng and its congeners and have been extensively investigated with regard to their possible antitumor activity. Protopanaxadiol saponins such as ginsenoside Rh2 and Rg3, for example, have been found to inhibit tumor cell proliferation and tumor growth [1, 2], induce differentiation and apoptosis [3, 4], and inhibit tumor cell invasion and metastasis [5, 6] in *in vitro* as well as *in vivo* systems.

The minor constituents of ginseng have been receiving increasing attention because of their unique biological properties. Recently, the ginseng saponin metabolites formed by intestinal bacteria were identified after oral administration of ginseng extract in human and rats [7]. One of the major metabolites detected in urine and blood after the administration of ginseng total saponins to rats is 20-*O*-(β-D-Glucopyranosyl)-20(*S*)-protopanaxadiol (IH-901¶; Fig. 1). It has been demonstrated that the *in vivo* antimetastatic effect by the treatment of ginsenosides is mediated by the metabolite [8]. IH-901 is known to be non-toxic, to inhibit glucose uptake by tumor cells [9], and to reverse multidrug resistance in tumor cells [10]. Moreover, we found that IH-901 possesses chemopreventive

‖ Corresponding author: Professor Byung-Hoon Lee, Hygienic Pharmacy & Molecular Toxicology, Wonkwang University, College of Pharmacy, Iksan, Chonbuk 570-749, Korea. Tel. +82 653 850 6806; FAX +82 653 850 7309; E-mail: bhlee@wonnms.wonkwang.ac.kr

Received 14 September 1999; accepted 13 March 2000.

¶ *Abbreviations:* IH-901, 20-*O*-(β-D-glucopyranosyl)-20(*S*)-protopanaxadiol; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; CDDP, cisplatin; and BrdU, 5-bromo-2′-deoxyuridine.



	R_1	R_2
IH-901	OH	O-Glc
Rb1	O-Glc(2→1)Glc	O-Glc(6→1)Glc

FIG. 1. Structural formula of the novel ginseng saponin metabolite 20-O-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901). Glc: β-D-glucopyranose.

potential against chemical carcinogens, which showed antigenotoxic and anticlastogenic activity induced by benzo[*a*]pyrene [11].

Apoptosis is a selective process of physiological cell deletion that plays an important role in the balance between cell replication and cell death. Since it has recently been suggested that cancer chemotherapeutic as well as chemopreventive agents exert part of their pharmacological effects by triggering apoptotic cell death or cell cycle transition, the induction of apoptosis in tumor cells has become a predictor of tumor treatment response [12, 13]. Conversely, several tumor promoters have also been shown to inhibit apoptosis [14].

Since IH-901 offers potential for use in the prevention and treatment of cancer, we investigate in this report the regulation of growth and apoptosis of human myeloid leukemia cells by IH-901. The aim of the present study was to examine whether IH-901 was able to induce apoptosis in the human myeloid leukemia cell line HL-60, a valid model system for testing antileukemic or general antitumoral compounds [15]. We also investigated the mechanisms underlying apoptosis induction, particularly focusing on the expression of oncoprotein Bcl-2, release of mitochondrial cytochrome *c* into cytosol, the activation of caspase-3 protease, and subsequent cleavage of poly(ADP-ribose) polymerase (PARP). Our findings indicate that IH-901 induces apoptosis in HL-60 cells through activation of caspase-3 protease, which occurs via mitochondrial cytochrome *c* release independently of Bcl-2 modulation.

MATERIALS AND METHODS

Materials and Cell Culture

IH-901 was biosynthesized by incubating ginseng saponins and intestinal bacteria according to Hasegawa *et al.* [7]. IH-901 was freshly dissolved in DMSO, the final concentration of which was not exceeded by 0.2%. The human myeloid leukemia HL-60 cell line (American Type Culture Collection) was maintained in the logarithmic phase of growth in RPMI-1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 2 mM L-glutamine (Sigma Chemical Co.) at 37° in a 5% CO_2–95% air humidified incubator.

Cell Proliferation Analysis

HL-60 cells were seeded at 1×10^4 cells/well in a 96-well plate and treated with the appropriate concentrations of IH-901 or vehicle as described in the figure legends. The general viability of cultured cells was determined by either trypan blue exclusion or by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan [16].

DNA Fragmentation Analysis

HL-60 cells grown at a density of 2×10^6 cells/mL were exposed to IH-901 for different time periods and concentrations as described in the figure legends. Cells were washed with ice-cold PBS (pH 7.4), centrifuged, and resuspended in 10 mM Tris–HCl/1 mM EDTA buffer (TE buffer; pH 7.5) to a concentration of 5×10^7 cells/mL. Cells were lysed (0.5% SDS, 25 mM Tris–HCl, and 5 mM EDTA; pH 7.5) and incubated with 1 mg/mL of proteinase K (GIBCO BRL) at 50° for 3 hr. After extraction with phenol–chloroform (1:1) and chloroform, DNA was precipitated with 3 M sodium acetate (pH 5.2) and absolute ethanol, washed, dried, and resuspended with TE buffer. DNase-free RNase A (200 μg/mL) was added and incubated at 37° for 30 min and at 65° for 5 min. The DNA was electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining.

Quantification of Apoptosis by Sandwich Enzyme Immunoassay

Quantification of apoptosis of HL-60 cells induced by IH-901 was performed with mouse monoclonal antibody directed against DNA and BrdU using a cellular DNA fragmentation kit (Boehringer Mannheim). Briefly, cells were labeled with BrdU to a final concentration of 10 μM for 18 hr. Following treatment of the cells with various concentrations of IH-901 for appropriate periods, cells were lysed and centrifuged at $250 \times g$ for 10 min. Aliquots of the supernatant were removed and assayed for DNA fragmentation as recommended by the supplier.

Flow Cytometric Analysis

Aliquots of 1×10^6 cells/mL exposed to IH-901 for 12 hr were centrifuged at $800 \times g$ for 5 min. The pellets were mixed with 1:1 (v/v) mixture of PBS and 0.2 M Na_2HPO_4–0.1 M citric acid (pH 7.5) and fixed with ice-cold ethanol at 4° for 1 hr. The cells were washed twice with PBS and resuspended in 1 mL of a staining solution containing 10

μg/mL of PI (Sigma Chemical Co.) and 100 μg/mL of DNase-free RNase A. The cell suspensions were incubated at room temperature for 1 hr and 20,000 cells were analyzed on a fluorescence-activated cell sorter (FACScalibur) flow cytometer (Becton Dickinson).

Morphological Analysis

Logarithmically growing HL-60 cells were treated with IH-901, washed with PBS, and placed onto sterile microscope slides using a cytospin. Cells were fixed with ethanol and stained with Giemsa or PI solution (50 μg/mL of PI, 0.1% Nonidet P-40, and 100 μg/mL of RNase A). The morphology of the cells was examined using the Leitz phase-contrast microscope or Olympus Fluoview laser scanning confocal microscope.

Preparation of Cytosolic Extracts for Cytochrome c Analysis

HL-60 cells were washed with ice-cold PBS and resuspended in ice-cold lysis buffer (20 mM HEPES–KOH, pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of pepstatin A, and 10 μg/mL of leupeptin) containing 250 mM sucrose. After incubation on ice for 20 min, cells were homogenized with 20 strokes of prechilled Dounce homogenizer, and the homogenates were centrifuged at 1000 × *g* for 10 min at 4° and at 10,000 × *g* for 15 min at 4°. The supernatants were subjected to the next centrifugation at 100,000 × *g* for 1 hr at 4° and stored at −80° for the analysis of cytochrome *c*.

Western Blot Analysis

HL-60 cells were treated with IH-901 as described in the figure legends. At indicated times, cells were washed with PBS and lysed (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM $MgCl_2$, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, and 10 μg/mL of aprotinin). Cell lysates were centrifuged and the protein content was determined. After SDS–polyacrylamide gel electrophoresis, proteins were transferred onto immobilon nitrocellulose membrane (Millipore) at 150 mA for 3 hr at 4°. Blots were probed with mouse monoclonal anti-human anti-Bcl-2 (Boehringer Mannheim), anti-caspase-3 (Transduction Laboratory), anti-PARP (PharMingen), and anti-cytochrome *c* antibody (PharMingen).

Immune complexes were detected using anti-mouse peroxidase-conjugated secondary immunoglobulin G antibody (Boehringer Mannheim) and were visualized by ECL (electrochemiluminescence) Western Blotting Detection Reagents (Amersham).

RESULTS

Antiproliferative Effects of IH-901

Inhibition of cell proliferation was observed following treatment of HL-60 cells with IH-901. IH-901 significantly decreased cell proliferation in a dose-dependent way (Fig. 2A). The concentration required for 50% inhibition of growth (IC_{50}) at 96 hr was 24.3 μM. In contrast, ginsenoside Rb1, a metabolic precursor of IH-901, did not alter the proliferation of HL-60 cells (Fig. 2B). For the comparison of the antiproliferative effects of IH-901 on other cancer cells, the cytotoxicity of IH-901 on PC-14, HepG2, and MKN-45 cells was determined. Figure 2, C–E shows that the IC_{50}s of IH-901 on these cells ranged from 24.3 to 56.6 μM.

Induction of Apoptosis by IH-901

Morphological analysis of Giemsa-stained cells indicated that they had undergone gross morphological changes (Fig. 3, A–C). After a 4.5-hr exposure to IH-901 at 30 μM, the cells showed typical apoptotic changes, including cell shrinkage, chromatin condensation, and loss of normal nuclear architecture. At a concentration of 50 μM, the disruption of cell membrane integrity was more prominent. Confocal microscopic observation after PI staining showed that a relevant number of cells treated with IH-901 acquired apoptotic features, as evident from nuclear fragmentation (Fig. 3, D and E). To elucidate these observations more definitively, we investigated more thoroughly the possibility that IH-901 induces apoptosis.

Two different methods were employed to examine DNA fragmentation in HL-60 cells induced by IH-901: detection of DNA ladder on agarose gels and sandwich enzyme immunoassay. As evident by the formation of internucleosomal DNA fragments compared with the vehicle-treated group, the induction of apoptosis by IH-901 was observed at 50 μM after 8- and 12-hr exposure (Fig. 4A) or at 40 and 50 μM after 12 hr (Fig. 4B). The effect of IH-901 on DNA fragmentation was comparable to that of CDDP (Fig. 4C). To quantify the degree of DNA fragmentation more rigorously, the sandwich enzyme immunoassay was performed using a cellular DNA fragmentation kit as described in Materials and Methods. After exposure to 12.5 μM IH-901 for 12 hr, a 3.7-fold increase in DNA fragmentation was observed. The induction of DNA fragmentation in HL-60 cells by IH-901 was concentration-and time-dependent (Fig. 4, D and E).

DNA histograms generated from the analysis of the total cell proliferation of HL-60 cells treated with IH-901 for 12 hr are shown in Fig. 5, A1–A4. Cells with sub-G1 DNA contents were scored as apoptotic based on the previous study [17]. At a concentration of 50 μM IH-901, 78% of cells presented an apoptotic/hypodiploid peak. We observed 13 and 9% of total cells in the apoptotic region at 35 and 12.5 μM, respectively (Fig. 5, B and C).

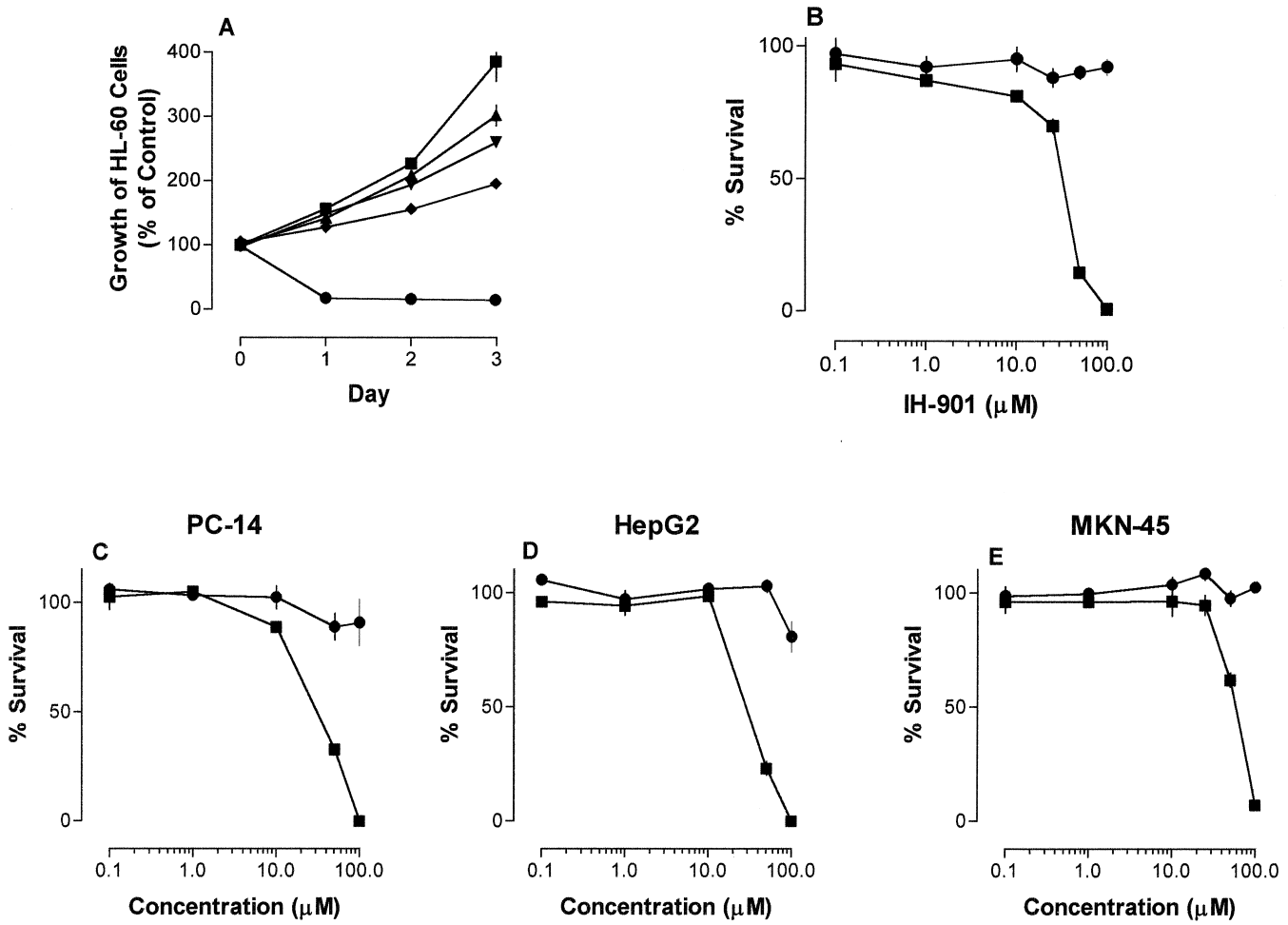


FIG. 2. Antiproliferative effect of IH-901 in various cancer cell lines. HL-60 cells were incubated with IH-901 and the time–course (A: ■, control; ▲, 10 μM; ▼, 20 μM; ◆, 30 μM; ●, 50 μM) and the dose dependency after 96 hr of treatment (B: ●, Rb1; ■, IH-901) were determined. PC-14, HepG2, and MKN-45 cells were also tested for cytotoxicity by the same method as with the HL-60 cells (C–E: ●, Rb1; ■, IH-901). Each point represents the mean and standard error from three experiments.

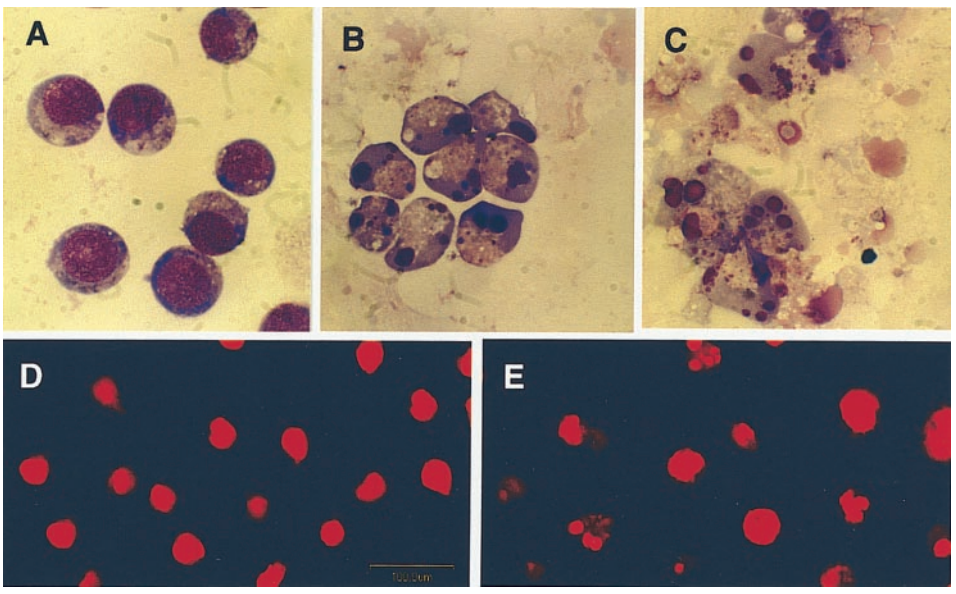


FIG. 3. Morphology of HL-60 cells was examined after exposure to IH-901 using a light microscope after Giemsa staining (A, vehicle; B, 30 μM IH-901 for 4.5 hr; C, 50 μM IH-901 for 2 hr) and a confocal microscope after PI staining (D, vehicle; E, 50 μM IH-901 for 9 hr).

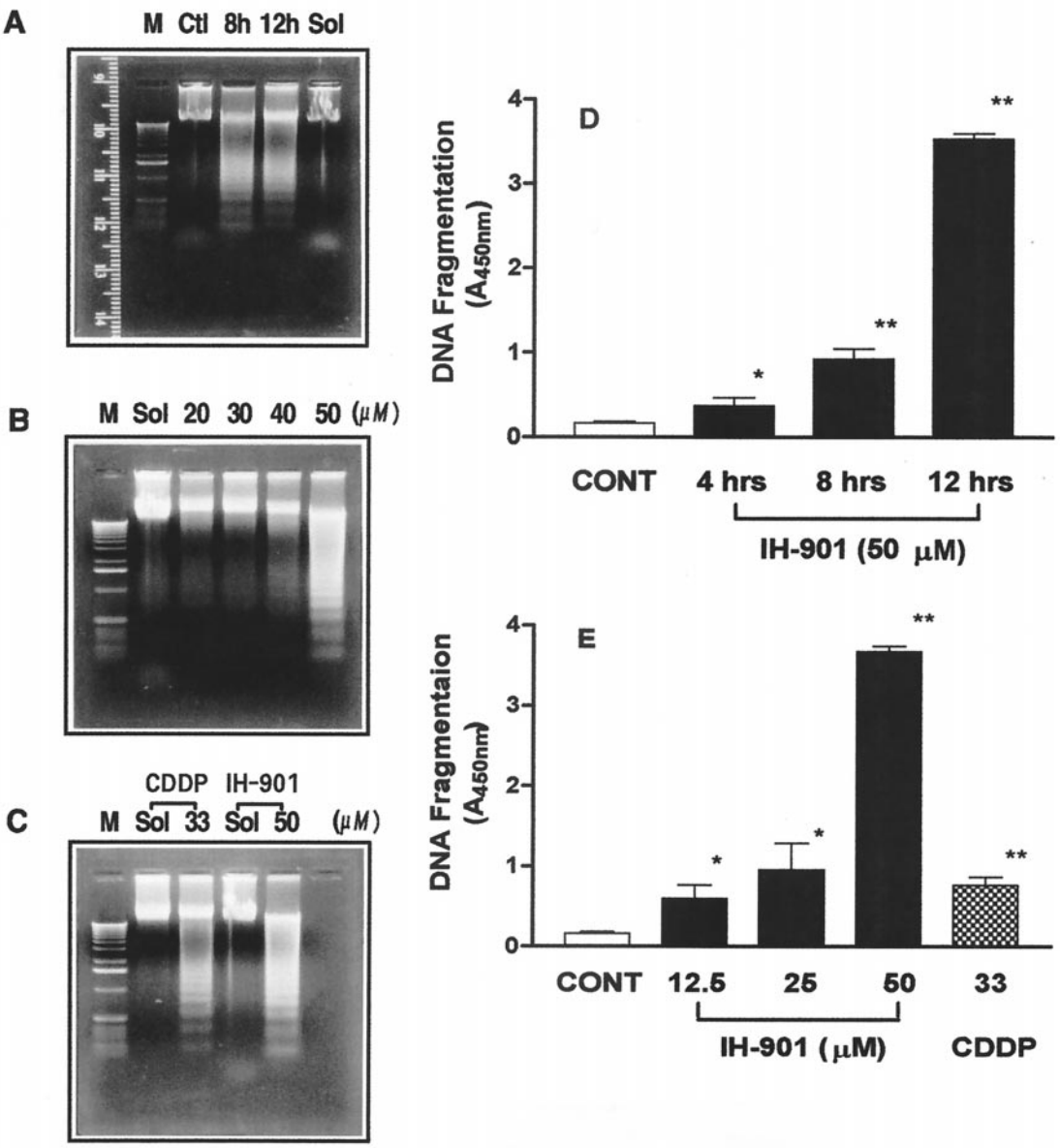


FIG. 4. DNA fragmentation after exposure to IH-901. HL-60 cells were exposed to 50 μM IH-901 for the indicated period (A), exposed to the various concentrations of IH-901 for 12 hr (B), or exposed to CDDP and IH-901 for comparison (C) (M, marker; Ctl, control; Sol, solvent control). Genomic DNA was extracted as described in Materials and Methods, electrophoresed in a 1.5% agarose gel, and visualized with ethidium bromide staining. For the quantitative determination of DNA fragmentation, HL-60 cells were labeled with BrdU, exposed to IH-901 (50 μM) for appropriate periods (D) or to the indicated concentrations of IH-901 for 12 hr (E). Each bar represents the mean ± SEM of 3 different experiments. CDDP was used as a positive control at a concentration of 33 μM. (*$P < 0.05$; **$P < 0.01$).

Western Blot Analysis

To begin to address the mechanism by which IH-901 causes apoptosis, we first examined whether caspase-3 protease is involved in the cell death response. IH-901 induced the proteolytic processing of caspase-3 as early as 1 hr after treatment. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis. Treatment of HL-60 cells with 50 μM IH-901 caused a time-dependent proteolytic cleavage of PARP, with accumulation of the 85 kDa and the concomitant disappearance of the full-size 116 kDa molecule (Fig. 6). Recent evidence indicates that apoptosis involves a disruption of mitochondrial membrane integrity that is decisive for the cell death process. We next evaluated the effects of IH-901 on the release of mitochondrial cytochrome *c* into the cytosol and tested whether Bcl-2, a fundamental death antagonist protein known to inhibit mitochondrial megachannel opening, is affected. Western blot analysis, shown in Fig. 6, revealed that treatment of HL-60 cells with 50 μM IH-901 induced the release of mitochondrial cytochrome *c* into cytosol. The

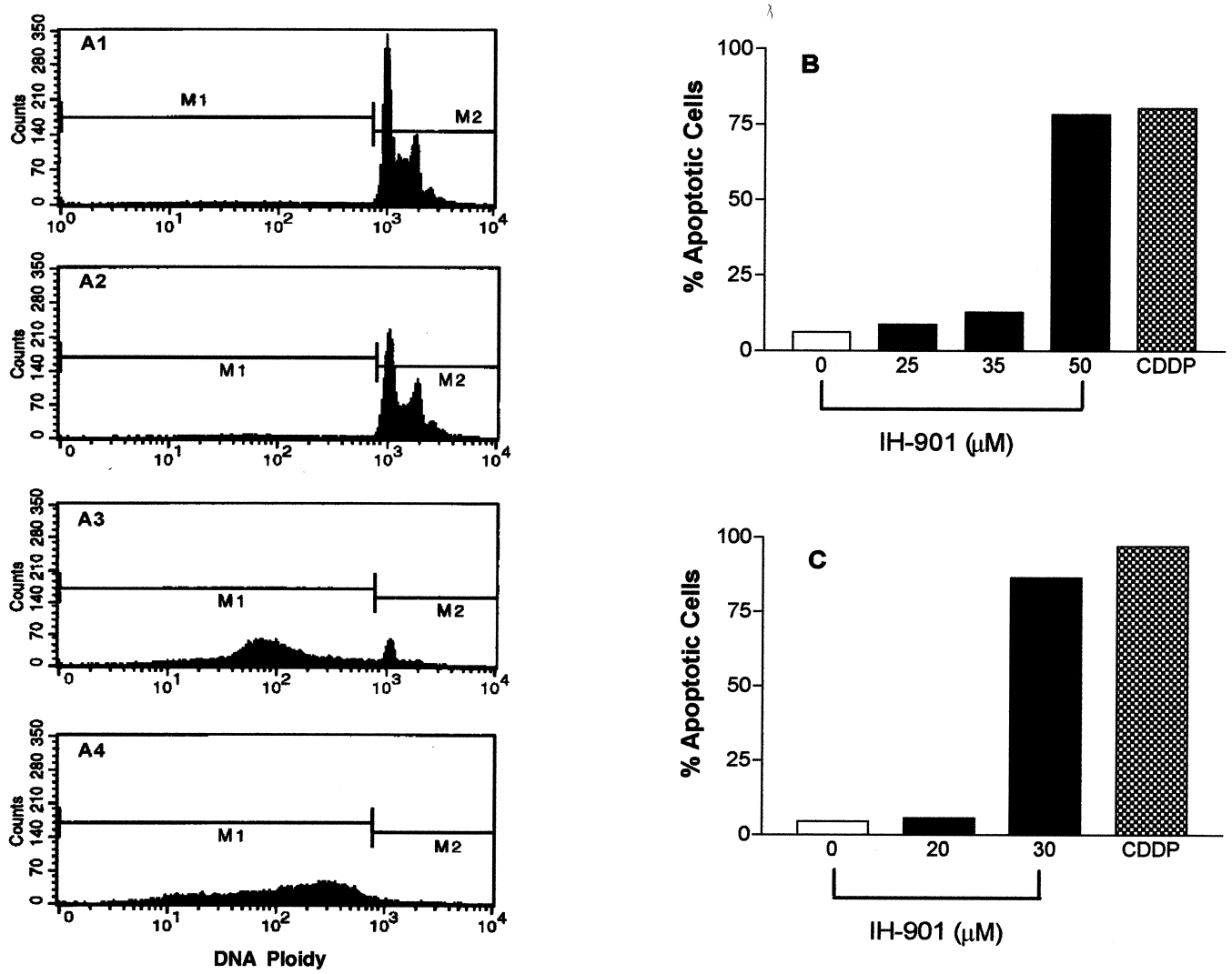


FIG. 5. Flow cytometric analysis of apoptosis induced by the treatment of vehicle only (A1), 20 μM (A2), and 30 μM (A3) IH-901 and 33 μM CDDP (A4) for 24 hr. Percent of apoptotic cells (M1) is shown after treatment with IH-901 at the indicated concentrations for 12 hr (B) and 24 hr (C).

treatment of IH-901 failed to modify the level of Bcl-2. Taken together, these results allow us to conclude that the mitochondria play an important role in the activation of caspase-3, proteolytic cleavage of PARP, and induction of apoptosis triggered by IH-901 in HL-60 cells.

DISCUSSION

The study presented here demonstrates that IH-901, a novel ginseng saponin metabolite formed by intestinal bacteria, inhibits proliferation and induces apoptosis in human myeloid leukemia HL-60 cells. Apoptosis was judged by three criteria: morphology of cells, detection of DNA fragmentation, and subdiploid DNA content by flow cytometry. Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated or activated in response to specific stimuli or various forms of cell injury. In cancer biology, it is now evident that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self-destruction. Therefore, it would be advantageous to tip the balance in favor of apoptosis over mitosis in cancer chemotherapy and prevention.

IH-901 is a novel ginseng saponin metabolite formed by intestinal bacteria after oral administration of ginseng extract in human and rats [7]. The intestinal bacteria *Prevotella oris*, which is responsible for the hydrolysis of ginsenoside Rb1 to IH-901, was found in 79% of the human fecal specimens. Since IH-901 is one of the metabolites detected in blood after oral administration of mice with ginsenoside Rb1, it has been speculated that IH-901 is most likely the major form of protopanaxadiol saponin absorbed from the intestine [18]. The hypothesis that IH-901 may be the active metabolite responsible for the anticarcinogenic effects of ginseng saponins has prompted several groups to investigate the effects of IH-901 in more detail. Wakabayashi *et al.* [19] reported that the antimetastatic effects of ginseng saponins are mediated by this metabolite.

In the present study, we show for the first time that

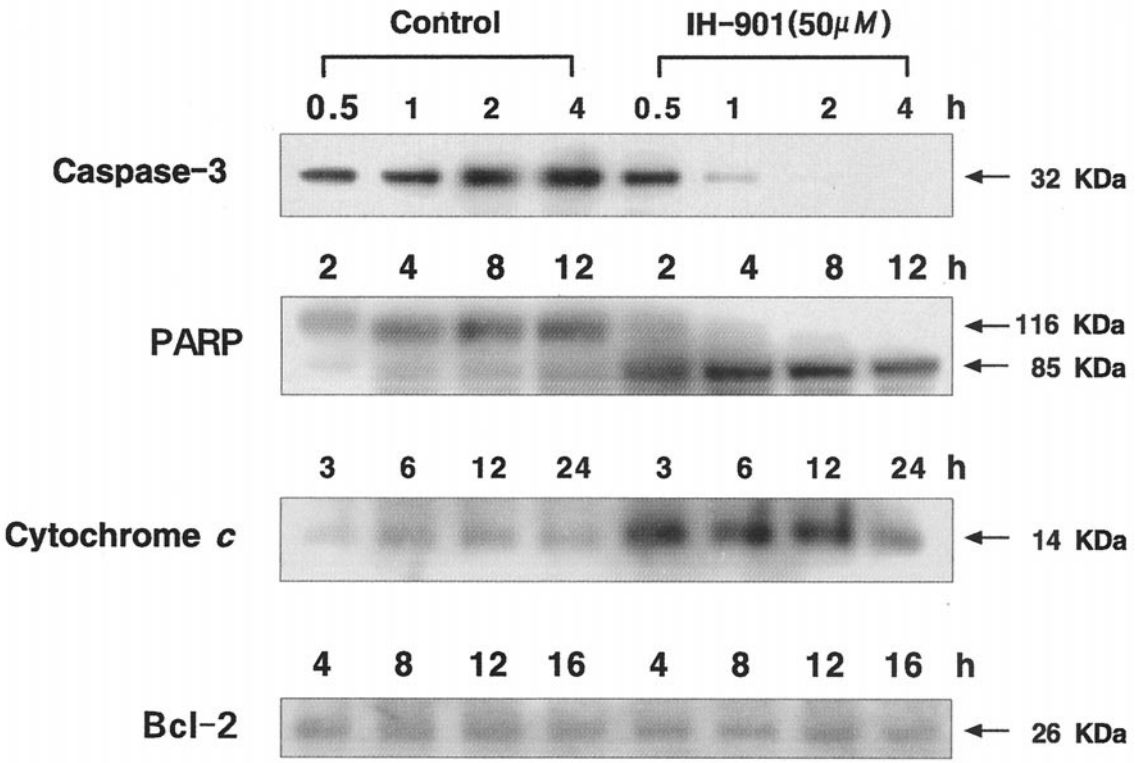


FIG. 6. Effect of IH-901 on caspase-3, PARP cytochrome c, and Bcl-2 proteins. Exponentially growing HL-60 cells were treated with 50 μM IH-901 for the indicated periods. Cells were washed with PBS, lysed, and Western blot analysis performed as described in Materials and Methods. Proteins were visualized by ECL (electrochemiluminescence) Western Blotting Detection Reagent.

IH-901 inhibits the growth of human myeloid leukemia cells and induces apoptosis, which is associated with proteolytic degradation of PARP via activation of caspase-3. The only naturally occurring ginsenoside reported to date to induce apoptosis is Rh2 on SK-HEP-1 human hepatocarcinoma cells [4], B16 mouse melanoma cells, and Meth-A mouse sarcoma cells [20]. The ginsenoside Rb1, the metabolic precursor of IH-901, had no effects on the proliferation and apoptosis of human hepatocellular carcinoma and cholangiocarcinoma cells [21], which is inconsistent with the results of our present study. Studies on HL-60 cells showed that IH-901 possesses a similar antiproliferative effect on this cell line.

Apoptosis is an active process that ultimately leads to the activation of endonuclease and cleavage of DNA into fragments of about 180–200 base pairs [22]. The observed breakage of cellular DNA by IH-901 detected by agarose gel electrophoresis shows the characteristics of apoptotic cell death. To confirm and quantify DNA fragmentation in HL-60 cells, we performed a sandwich enzyme immunoassay. The assay uses two mouse monoclonal antibodies directed against DNA and BrdU that detect BrdU-labeled DNA fragments in the cytoplasm and the medium fraction of the culture. The double-antibody sandwich ELISA is extremely sensitive in some cases about 500 times more so than the detection of apoptotic DNA ladder by agarose electrophoresis [23]. In our experiment, 3.7- and 2.3-fold increases in DNA fragmentation were already seen at concentrations of 12.5 μM for 12-hr and 50 μM for 4-hr treatment with IH-901, respectively. Besides biochemical indicators of DNA fragmentation, IH-901 induces morphological changes that are characteristic of apoptosis, such as chromatin condensation and fragmentation as well as cell shrinkage, as demonstrated by light and confocal microscopy. Also, cells with sub-G1 DNA content were detected by FACS analysis, another indicator of apoptosis [24]. These cytofluorimetric characteristics showed a very similar time and concentration dependence to biochemical assays of DNA fragmentation.

Several mechanisms apparently regulate apoptosis, such as induction of a p53-dependent pathway after DNA-damaging agents, modulation by the Bcl-2 family of proteins, and activation of effectors including the interleukin-converting enzyme (ICE) family of proteases and endonucleases [25, 26]. For example, the induction of apoptosis by up-regulation of the p53 protein is reported to be very closely related to the chemopreventive effects of selenodiglutathione [27]. Flavopiridol, a cyclin-dependent kinase inhibitor, and ginsenoside Rh2 induce apoptosis by decreasing Bcl-2 or by activating caspase-3 and subsequently PARP [4, 28]. The mechanism by which IH-901 shows an antiproliferative and apoptogenic effect remains largely unknown.

In many cancer cell lines, Bcl-2 overexpression or c-Myc down-regulation may contribute to the therapeutic efficacy of cytotoxic drugs [29]. Although it does not seem to be

necessary for induction of apoptosis in HL-60 cells by IH-901, the cleavage of caspase-3 appears to correlate with IH-901-induced apoptosis in this cell line. Caspase-3 is a cysteine protease that exists as an inactive zymogen in cells, and is activated by sequential proteolytic events that cleave the 32-kDa precursor at aspartic acid residues to generate an active heterodimer of 20- and 12-kDa subunits [30]. It has been demonstrated that Apaf-1, cytochrome *c*, and caspase-9 participate in the activation of caspase-3. *In vitro* depletion of caspase-9 from cytosolic fractions resulted in the failure of caspase-3 activation [31]. These data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* released from the mitochondria. Evidence indicates that caspase-3 is both necessary and sufficient to trigger apoptosis, as has been demonstrated in caspase-3 knockout mice [32], in various strategies for inducing apoptosis, including Fas activation or exposure to ionizing radiation [33], and in the inhibition of apoptosis by specific tetrapeptide aldehyde [30]. Cytochrome *c* normally resides in the mitochondrial intermembrane space, where it serves as a transducer of electrons in the respiratory chain. Several antitumor drugs with diverse intracellular targets have been demonstrated to cause the mitochondrial release and cytosolic accumulation of cytochrome *c* and activation of caspase-3 [34, 35]. Although the mechanism is not yet fully understood, the release of cytochrome *c* is considered to be a very important event for the induction of apoptosis by IH-901.

In conclusion, IH-901, a novel ginseng saponin metabolite, inhibits proliferation and induces apoptosis of human HL-60 cells. IH-901 exposure resulted in activation of caspase-3 through release of cytochrome *c* from the mitochondria. The more precise signaling pathway by which IH-901 triggers caspase-3 activation, cytochrome *c* release, and the other apoptotic phenomena described here remain to be identified. Nevertheless, our data should contribute to the development of IH-901 or related drugs as potential cancer chemotherapeutic or chemopreventive agents.

This work was supported by grants from the Good Health R&D Project (HMP-96-D-5-1047), the Ministry of Health and Welfare, and the Medicinal Resources Research Center at Wonkwang University, which is supported by KOSEF and the Chonbuk provincial government.

References

1. Kikuchi Y, Sasa H, Kita T, Hirata J, Tode T and Nagata I, Inhibition of human ovarian cancer cell proliferation *in vitro* by ginsenoside Rh2 and adjuvant effects to cisplatin *in vivo*. *Anticancer Drugs* **2:** 63–67, 1991.
2. Tode T, Kikuchi Y, Kita T, Hirata J, Imaizumi E and Nagata I, Inhibitory effects by oral administration of ginsenoside Rh2 on the growth of human ovarian cancer cells in nude mice. *J Cancer Res Clin Oncol* **120:** 24–26, 1993.
3. Lee YN, Lee HY, Chung HY, Kim SI, Lee SK, Park BC and Kim KW, *In vitro* induction of differentiation by ginsenosides in F9 teratocarcinoma cells. *Eur J Cancer* **32A:** 1420–1428, 1996.
4. Park JA, Lee KY, Oh YJ, Kim KW and Lee SK, Activation of caspase-3 protease via a Bcl-2-insensitive pathway during the process of ginsenoside Rh2-induced apoptosis. *Cancer Lett* **121:** 73–81, 1997.
5. Shinkai K, Akedo H, Mukai M, Imamura F, Isoai A, Kobayashi M and Kitagawa I, Inhibition of *in vitro* tumor cell invasion by ginsenoside Rg3. *Jpn J Cancer Res* **87:** 357–362, 1996.
6. Iishi H, Tatsuta M, Baba M, Uehara H, Nakaizumi A, Shinkai K, Akedo H, Funai H, Ishiguro S and Kitagawa I, Inhibition by ginsenoside Rg3 of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in Wistar rats. *Clin Exp Metastasis* **15:** 603–611, 1997.
7. Hasegawa H, Sung JH, Matsumiya S and Uchiyama M, Main ginseng saponin metabolites formed by intestinal bacteria. *Planta Med* **62:** 453–457, 1996.
8. Hasegawa H, Sung JH and Huh JD, Ginseng intestinal bacterial metabolite IH901 as a new antimetastatic agent. *Arch Pharm Res* **20:** 539–544, 1997.
9. Hasegawa H, Matsumiya S, Uchiyama M, Kurokawa T, Inouye Y, Kasai R, Ishibashi S and Yamasaki K, Inhibitory effect of some triterpenoid saponins on glucose transport in tumor cells and its application to *in vitro* cytotoxic and antiviral activities. *Planta Med* **60:** 240–243, 1995.
10. Hasegawa H, Sung JH, Matsumiya S, Uchiyama M, Inouye Y, Kasai R and Yamasaki K, Reversal of daunomycin and vinblastine resistance in multidrug-resistant P388 leukemia *in vitro* through enhanced cytotoxicity by triterpenoids. *Planta Med* **61:** 409–413, 1995.
11. Lee BH, Lee SJ, Hur JH, Lee S, Sung JH, Huh JD and Moon CK, *In vitro* antigenotoxic activity of novel ginseng saponin metabolites formed by intestinal bacteria. *Planta Med* **64:** 500–503, 1998.
12. Kim YS, Jin SH, Lee YH, Kim SI and Park JD, Ginsenoside Rh2 induces apoptosis independently of Bcl-2, Bcl-xL, or Bax in C6Bu-1 cells. *Arch Pharm Res* **22:** 448–453, 1999.
13. Ahmad N, Feyes DK, Nieminen AL, Agarwal R and Mukhtar H, Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* **89:** 1881–1886, 1997.
14. Wright SC, Zhong J and Larrick JW, Inhibition of apoptosis as a mechanism of tumor promotion. *FASEB J* **8:** 654–660, 1994.
15. Suh N, Luyengi L, Fong HH, Kinghorn AD and Pezzuto JM, Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. *Anticancer Res* **15:** 233–239, 1995.
16. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J and Boyd M, Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* **83:** 757–766, 1991.
17. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C, A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139:** 271–279, 1991.
18. Hasegawa H, Sung JH and Benno Y, Role of human intestinal *Prevotella oris* in hydrolyzing ginseng saponins. *Planta Med* **63:** 436–440, 1997.
19. Wakabayashi C, Hasegawa H, Murata J and Saiki I, *In vivo* antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. *Oncol Res* **9:** 411–417, 1997.
20. Ota T, Maeda M, Odashima S, Ninomiya-Tsuji J and Tatsuka M, G1 phase-specific suppression of the Cdk2 activity by ginsenoside Rh2 in cultured murine cells. *Life Sci* **60:** PL39–PL44, 1997.
21. Yano H, Mizoguchi A, Fukuda K, Haramaki M, Ogasawara S,

Momosaki S and Kojiro M, The herbal medicine Sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0/G1 phase. *Cancer Res* **54:** 448–454, 1994.
22. Kerr JF, Winterford CM and Harmon BV, Apoptosis: Its significance in cancer and cancer therapy. *Cancer* **73:** 2013–2026, 1994.
23. Salgame P, Varadhachary AS, Primiano LL, Fincke JE, Muller S and Monestier M, An ELISA for detection of apoptosis. *Nucleic Acids Res* **25:** 680–681, 1997.
24. Cejna M, Fritsch G, Printz D, Schulte-Hermann R and Bursch W, Kinetics of apoptosis and secondary necrosis in cultured rat thymocytes and S49 mouse lymphoma and CEM human leukemia cells. *Biochem Cell Biol* **72:** 677–685, 1994.
25. Rao L and White E, Bcl-2 and ICE family of apoptotic regulators: Making a connection. *Curr Opin Genet Dev* **7:** 52–58, 1997.
26. Chinnaiyan AM, Orth K, O'Rourke K, Duan H, Poirier GG and Dixit VM, Molecular ordering of the cell death pathway. *J Biol Chem* **271:** 4573–4576, 1996.
27. Lanfear J, Fleming J, Wu L, Webster G and Harrison PR, The selenium metabolite selenodiglutathione induces p53 and apoptosis: Relevance to the chemopreventive effects of selenium? *Carcinogenesis* **15:** 1387–1392, 1994.
28. König A, Schwartz GK, Mohammad RM, Al-Katib A and Gabrilove JL, The novel cyclin-dependent kinase inhibitor flavopiridol downregulates Bcl-2 and induces growth arrest and apoptosis in chronic B-cell leukemia lines. *Blood* **90:** 4307–4312, 1997.
29. Banker DE, Groudine M, Norwood T and Appelbaum FR, Measurement of spontaneous and therapeutic agent-induced apoptosis with BCL-2 protein expression in acute myeloid leukemia. *Blood* **89:** 243–255, 1997.
30. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL and Miller DK, Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376:** 37–43, 1995.
31. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X, Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91:** 479–489, 1997.
32. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P and Flavell RA, Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384:** 368–372, 1996.
33. Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry NA, Yamin TT and Nicholson DW, CPP32/apopain is a key interleukin 1β converting enzyme-like protease involved in Fas-mediated apoptosis. *J Biol Chem* **271:** 1841–1844, 1996.
34. Fang G, Chang BS, Kim CN, Perkins C, Thompson CB and Bhalla K, "Loop" domain is necessary for taxol-induced cytosolic accumulation of cytochrome *c* and apoptosis. *Cancer Res* **58:** 3202–3208, 1998.
35. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP and Wang X, Prevention of apoptosis by Bcl-2: Release of cytochrome *c* from mitochondria blocked. *Science* **275:** 1129–1132, 1997.