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Propolin C from propolis induces apoptosis through activating caspases, Bid and cytochrome *c* release in human melanoma cells

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Abstract

We had demonstrated that two prenylflavanones, propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced apoptosis in human melanoma cells and significantly inhibited xanthine oxidase activity. Here, we have isolated a third compound called propolin C. The chemical structure of propolin C has been characterized by NMR and HRMS spectra, and was identical to nymphaeol-A. However, no biological activities of this compound have ever been reported. In the present study, propolin C effectively induced a cytotoxic effect on human melanoma cells, with an IC_{50} of about 8.5 μM. DNA flow cytometric analysis indicated that propolin C actively induced apoptosis in human melanoma cells and there is a marked loss of cells from the G2/M phase of the cell cycle. To address the mechanism of the apoptosis effect of propolin C, we evaluated the effect of propolin C on induction of apoptosis-related proteins in human melanoma cells. The levels of procaspase-8, Bid, procaspase-3, and poly(ADP-ribose) polymerase were decreased in dose- or time course-dependent manners. Moreover, propolin C was capable of releasing cytochrome *c* from mitochondria to cytosol. The findings suggest that propolin C may activate a mitochondria-mediated apoptosis pathway. On other hand, propolin C is a potential antioxidant agent and shows a strong capability to scavenge free radicals and inhibit on xanthine oxidase activity with IC_{50} of about 17.0 μM. In conclusion, the isolation and characterization of propolin C from bee propolis are described for the first time, and this compound is a powerful inducer of apoptosis in human melanoma cells.

Keywords: Propolin C; Nymphaeol-A; Taiwanese propolis; Apoptosis; Cytochrome *c*

1. Introduction

A natural product, propolis, is a resinous material gathered by honey bees from the buds and bark of certain trees and plants, and used in their hives [1]. It is a kind of crude medicine, long used as a folk remedy, chiefly in Europe [2]. Propolis contains various chemical components and exhibits a broad spectrum of biological activities, including antitumor [3], antioxidant [4], antibacterial [5], antiviral [6], antifungal [7], and anti-inflammatory activities [7]. More than 200 constituents have been identified from propolis, but its various biological activities have not yet been well understood [5]. Propolis contains wax, flavonoids, and phenolic compounds, such as cinnamic acids and their derivatives, as well as various aldehydes and ketones [8]. Caffeic acid phenethyl ester (CAPE) was one of the components isolated and identified to show antitumor and antioxidant properties [9].

Many natural products used in cancer chemotherapy, including taxol [10], adriamycin [11], VP16 [12], camptothecin [12], and carnosol [13], have apoptosis-inducing activity. We previously identified two novel prenylflavanones from Taiwanese propolis, flavonoid compounds with hydrated geranyl side chains, namely, propolin A and propolin B [14]. In this study, propolin C is isolated and characterized from Taiwanese propolis. The structure was elucidated mainly by NMR spectral evidence, and found to

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Abbreviations: XO, xanthine oxidase; PARP, poly(ADP-ribose) polymerase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MTT, (3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyl)tetrazolium bromide; A2058, human melanoma cells; B16F10, murine melanoma cells; MCF-7, human breast cancer cells; Hep.G2, human hepatoblastoma cells; Hep.3B, human hepatocellular carcinoma cells; HT-29, human colon adenocarcinoma cells.

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be identical with the reported prenylflavanones compound nymphaeol-A [15]. Propolin A and propolin B have hydrated geranyl side chains, but propolin C has an unhydrated geranyl side chains.

Flavonoids are found naturally in fruits and vegetables and have possessed chemoprevention or anticancer activity [16,17]. The anticancer activities of flavonoids are due to their inducing apoptosis [18]. Flavonoids shown to induce apoptosis in cancer cells include quercetin [19], bicalein [20], genistein [21], tangeritin [22], and sophoranone [23].

Apoptosis, the physiological mode of cell death, is related to the regulation of development and homeostasis. Its morphological characteristics, include plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies [24]. Many recent reports have indicated that many anticancer drugs or cancer chemopreventive agents act though the induction of apoptosis to prevent tumor promotion and progression. The process of apoptosis consists of different phases, including initiation, execution, and degradation [25], and is activated by two major pathways. One is the receptor-induced apoptosis that includes the TNF-R1, Fas (CD95), and TRAIL-receptor [26,27]. In the so-called ''extrinsic'' cell death pathway, a cytoplasmic death domain forms and trimerization of the receptor and recruits TRADD and FADD. The other major route leading to apoptosis is the ''intrinsic'' cell death pathway that is activation of proapoptotic factors from mitochondria, including cytochrome *c* and Apaf-1 [28,29].

We have demonstrated that propolin A and propolin B are cytotoxic to human melanoma cells, C6 glioma cells, HL-60 human leukemia cells, MCF-7 human breast cancer cells, and IMR32 neuroblastoma cells. Furthermore, both are potential antioxidant agents [14]. In the present study, propolin C was found to be more effective in inducing apoptosis than either propolin A or propolin B in human melanoma cells, and also more active in inhibiting xanthine oxidase (XO) activity. Furthermore, we found propolin C is more effective in inducing cytotoxicity than CAPE in six different cancer cell lines, and also that propolin C exhibits more potential inhibited XO activity than CAPE. However, no biological activities of this compound (nyphaeol-A) have ever been reported. These reasons prompted us to investigate and further to evaluate whether this compound had value as a chemotherapeutic drug.

2. Materials and methods

2.1. Cell culture and cytotoxicity assay

Human melanoma cells (A2058), murine melanoma cells (B16F10), human breast cancer cells (MCF-7), human hepatoblastoma cells (Hep.G2), human hepatocellular carcinoma cells (Hep.3B), and human colon adenocarcinoma cells (HT-29) were cultured in Dulbecoo's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin, and were maintained at 37° in a humidified atmosphere at 95% air and 5% CO_2. All cells (1×10^5) per well were cultured in 12-well plates and incubated for 14 hr were washed and different concentrations of propolin C (2.35–16.35 μM) were added to each well in triplicate for 24 hr. Treated cells were counted and cell viability was determined by a trypan blue exclusion or MTT assay.

2.2. Propolin C isolated from the Taiwanese propolis

Taiwanese propolis glue (245 g, mixture from hives collected near Bagwa Shan, Taiwan) was homogenized by stirring at 4° and washed thrice with 0.7 L of deionized water. The residue was extracted three times with 95% ethanol. The filtered ethanol extract was evaporated to dryness under reduced pressure to furnish a brown powder which was kept at −20° until further purification. The brown powder from the ethanol extract was dissolved in methanol and applied to a Sephadex LH-20 column (Amersham Pharmacia Biotech AB) using 95% ethanol as eluting solvent. All eluates, including fractions from the follow-up chromatographies, were assayed on human melanoma proliferation, and the active fractions were again chromatographed on Sephadex LH-20 column using 95% ethanol to elute. The active fractions were then subjected to silica gel column chromatography (Kiesel gel 60, E. Merck) using a solvent system of *n*-hexane–EtOAc. Purification of the most active fraction (*n*-hexane:EtOAc = 40:60) was carried out on a reversed-phase preparative HPLC. Fractions of retention times at 25.0 min. containing propolin C were collected. The conditions were: column-Luna Phenomenex (C18, 250 mm × 10 mm), solvent system: methanol:water (8:2); flow rate: 3 mL/min; detection: UV 280 nm. Propolin C was obtained as a slightly brown powder from methanol:water (8:2). FAB-MS (negative mode): *m/z* 423 ($[M - H]^-$), FAB-MS (positive mode): *m/z* 425.2 ($[M + H]^+$), HRFAB$^+$MS

Table 1
^{1}H NMR data of propolin C in acetone-d_6 and $CDCl_3$

Position	δ_H (J, Hz) in $CDCl_3$	δ_H in acetone-d_6
2β CH	5.12 (dd, 12.8, 2.8)	5.34
3β CH_2	2.60 (dd, 17.1, 2.8)	2.69
3α	2.93 (dd, 17.1, 12.8)	3.10
8 CH	5.85 (s)	6.02
5′ CH	6.73 (s)	6.84
6′ CH	6.68 (dd, 8.0, 1.4)	6.84
2′ CH	6.81 (br s)	7.00
1″ CH_2	3.15 (d, 6.9)	3.24
2″ CH	5.12[a]	5.23
4″ CH_3	1.66 (s)	1.67
5″ CH_2	1.85 (t, 6.9)	1.94
6″ CH_2	1.95 (t, 6.9)	2.05
7″ CH	4.96 (t)	5.07
9″ CH_3	1.46 (s)	1.55
10″ CH_3	1.53 (s)	1.60

Assignments were based on the HMQC and HMBC NMR data.
[a] Obscured.

Table 2
^{13}C NMR data of propolin C and nymphaeol-A[a]

Carbon No.	Propolin C in $CDCl_3$ δ_C	Propolin C in acetone-d_6 δ_C	Nymphaeol-A in acetone-d_6 δ_C	Carbon No.	Propolin C in $CDCl_3$ δ_C	Propolin C in acetone-d_6 δ_C	Nymphaeol-A in acetone-d_6 δ_C
2	78.9	79.9	79.5	1″	20.8	21.5	21.4
3	43.0	43.6	43.4	2″	122.0	123.5	123.2
4	197.2	197.3	196.5	3″	135.2	134.9	134.3
5	160.7	162.2	161.7	4″	15.8	16.2	16.1
6	102.3	109.0	108.6	5″	39.6	40.5	40.3
7	161.0	165.0	164.7	6″	26.5	27.4	27.2
8	94.6	95.3	95.0	7″	124.2	125.1	124.7
9	164.4	161.9	161.3	8″	131.0	131.6	131.0
10	108.6	103.0	102.5	9″	17.3	17.7	17.6
1′	130.3	131.6	130.9	10″	25.3	25.8	25.7
2′	115.0	114.6	114.2				
3′	144.6	145.1	145.4				
4′	145.0	146.4	145.8				
5′	113.2	115.9	115.7				
6′	118.2	119.1	118.6				

Assignments were based on the HMQC and HMBC NMR data.
[a] Data taken from ref. [15].

m/z 425.20 (calcd for $C_{25}H_{28}O_6$ 424.1992; ^1H and ^{13}C NMR data were illustrated in Tables 1 and 2).

2.3. Analysis of DNA fragmentation

Human melanoma cells in a 100-mm dish were treated with various concentrations of propolin C (2.35–16.35 μM) for 24 hr, trysinized, and collected with ice-cold PBS. After centrifugation (2000 *g*) for 10 min at 4°, cells were washed with PBS and recentrifuged at 13,200 *g* for 10 min at 4°. Cell pellets were resuspended in 0.1 mL of isolation buffer (10 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% SDS, 1 mg/mL proteinase K) and incubated at 12 hr at 50° and treated with RNase A (0.5 μg/mL) for another 4 hr at 37°. The lysate was centrifuged at 13,200 *g* for 10 min at 4° to separate the soluble fragmented DNA from the intact chromatin pellet. Fragmented DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, and analyzed by 1.8% agarose gel electrophoresis. Approximately 20 μg DNA was loaded in each well, visualized under UV light, and photographed.

2.4. Morphological analysis of apoptotic cells

Human melanoma cells on 60-mm culture dishes (5×10^5 per dish) were treated with propolin C at 11.75 μM for 24 hr. After treatments, cell were fixed with 4% formaldehyde for 30 min, washed with PBS, and then stained with 50 μg/mL propidium iodide (PI) in the presence of 25 μg/mL RNase A. The morphology of nuclear chromatin was defined by fluorescence of DNA-binding dye, PI, under a fluorescence microscope (Zeiss).

2.5. Analysis of the cell cycle

Human melanoma cells (1×10^6) in a 100-mm dish were treated with various concentrations (2.35–16.35 μM) for 24 hr or treated with propolin C (16.35 μM) for 0, 4, 8, 16, and 24 hr. Cells were trysinized and collected with ice-cold PBS. The cells were resupended in 200 μL PBS, and fixed in 800 μL of iced 100% ethanol at −20°. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase A), and incubated at 37° for 30 min. Then, 1 mL of PI solution (50 μg/mL) was added, and the mixture was allowed to stand on 4° for 30 min. Fluorescence emitted from the PI–DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson).

2.6. XO activity assay

The enzyme activity was measured spectrophotometrically by continuously measuring uric acid formation at 295 nm with xanthine as substrate. The XO assay consisted of a 500 μL reaction mixture containing 7.5 mM phosphate buffer, 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 38 μM EDTA (pH 7.0), 3 U/L XO, and 50 μM xanthine as substrate. The assay was started by addition of the enzyme to the reaction mixture without or with propolin C and CAPE. The assay mixture was incubated for 3 min at 37° and absorbancy readings were taken. The substrate concentration was less than 60 μM to avoid substrate inhibition. All data obtained for enzyme inhibition assays and plotting were preceded using the Microsoft Excel (Microsoft Office 2000).

2.7. Stable free radical scavenging capacity

The free radical scavenging capacity of propolin C was measured with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The DPPH radical has a deep violet color due to its unpaired electron and radical scavenging capability and

can be followed spectrophotometrically by absorbance loss at 517 nm when the pale yellow non-radical form is produced. A 0.5 mL aliquot of propolin C (1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μM, final concentration) dissolved in methanol was mixed with 0.5 mL 0.15 mM DPPH solution (in methanol) in a cuvette and the optical density change at 517 nm was followed for 1 min in a UV-Vis U-3210 spectrophotometer. The DPPH test was also performed with CAPE (1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μM, final concentration) as control.

2.8. Western blotting assay

Human melanoma cells on 100-mm culture dishes (1.5×10^6 per dish) were treated with propolin C at 2.35, 7.05, 11.75, and 16.35 μM for 24 hr or treated with propolin C at concentration of 16.35 for 0, 4, and 8 hr. After treatment, cells were collected and resuspended in 100 μL Gold lysis buffer. Equal amounts of proteins (20 μg), were mixed with 5 × sample buffer and resolved by 12% SDS–PAGE for β-actin, poly(ADP-ribose) polymerase (PARP), Bid, procaspase-3, procaspase-8, and cytochrome *c* detection. Proteins were electrotransferred to an immobilon membrane (PVDF; Millipore Corp.), and equivalent protein loading was verified by staining the membrane with reversible dye amido black (Sigma Chemical Co). This was followed by overnight blocking with a solution composed of 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 4% non-fat dry milk, and 0.1% sodium azide. Expression of β-actin, PARP, Bid, procaspase-3, procaspase-8, and cytochrome *c* was monitored by immunoblotting using a specific antibodies (1:500 of rabbit polyclonal antibodies to human PARP; Santa Cruz Biotechnology, Inc.), anti-caspase-3 antibody and anti-caspase-8 antibody (Pharmingen, Becton Dickinson), anti-Bid antibody (Santa Cruz Biotechnology, Inc.), anti-β-actin antibody (Cashmere Biotech), and cytochrome *c* antibody (Research Diagnostic Inc.). These proteins were detected by chemiluminescence (ECL, Amersham).

2.9. Cytochrome c release

Mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/mL phenylmethylsulfonylfluoride (PMSF), 8 μg/mL aprotinin, and 2 μg/mL leupeptin, pH 7.4). Cells were passed through a needle 10 times. Unlysed cells and nuclei were pelleted by centrifugation for 10 min at 750 *g*. The supernatant was then centrifuged at 100,000 *g* for 15 min. This pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was again centrifuged at 100,000 *g* for 1 hr. The supernatant from this final centrifugation step represents the cytosolic fraction.

2.10. Activity of caspase [36]

Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM PMSF, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 13,200 *g* for 20 min at 4°. Caspase activity in the supernatant was determined by a fluorogenic assay (Promega's CasACE™ Assay System). Briefly, 75 μg of total protein, as determined by bicinchoninic acid assay (Promega), was incubated with 50 μM substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) at 30° for 1 hr. The release of AMC was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (Hitachi F-4500).

3. Results

3.1. Purification and identification of propolin C

Propolin C (Fig. 1) was isolated through repeated chromatographies of the 95% ethanol extract of the propolis glue under the guidance of human melanoma proliferation. Final purification of the active fraction was achieved by HPLC on a reversed-phase column. The total content of the active component, propolin C, was roughly 4.5% of the propolis glue. Both ^1H and ^{13}C

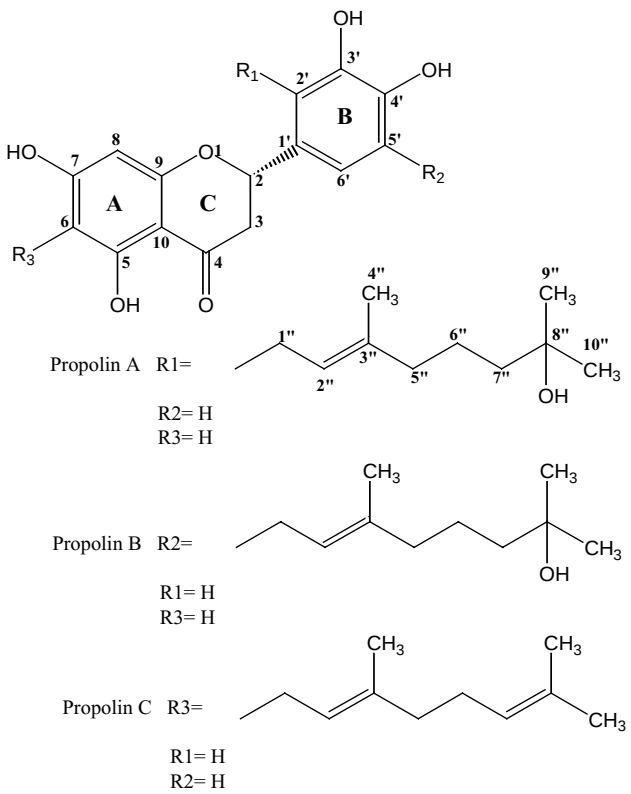

Fig. 1. Structures of propolin A, propolin B, and propolin C.

NMR (in $CDCl_3$, Tables 1 and 2) displayed absorptions of characteristic of the prenylflavanone type, very similar to those of propolin A and propolin B, the two more polar components, isolated from the same source (3 and 1%, respectively) [14]. The HRMS of a pure sample of propolin C indicated a $[M]^+$ of 424 with the molecular composition as $C_{25}H_{28}O_6$. Obviously, a water molecule was lost from the geranyl side chain. Moreover, the geranyl substitution is not on the C-ring anymore, judging from the coupling patterns of the two aromatic rings (H 5.85, s; 6.76, d, $J = 8.0$ Hz; 6.68, dd, $J = 8.0$, 1.4; and 6.81, br s). In other words, the side chain is either at C6 or at C8 of ring A. A known compound, nymphaeol-A, isolated from *Hernandia nymphaefolia*, happened to have the same geranyl group at C6 [15]. Upon comparison of the ^{13}C NMR of propolin C in d_6-acetone with the reported data of nymphaeol-A, the identity of both compounds could be recognized. However, the bioactivities of nymphaeol-A have never been reported, and its chemical shifts of proton were only partially assigned. In Tables 1 and 2, a complete assignments of each proton and carbon of propolin C in both *d*-chloroform and d_6-acetone were compiled on the basis of two-dimensional NMR (HMQC, HMBC, NOESY) performed. The cytotoxicity and free radical scavenging capability of propolin C showed much more potency than those of either propolin A or propolin B [14] as further demonstrated in the following.

3.2. Inhibition of cell growth by propolin C

The cytotoxicity effect of propolin C was first evaluated in the human melanoma cells. As shown in Fig. 2a, treatment with propolin C concentrations of 2.35–16.35 μM caused a dose-dependant decrease of cell viability. The IC_{50} values of propolin C on the human melanoma cells was 8.5 μM. The growth-inhibiting activity of propolin C was much stronger than that of either propolin A or propolin B that had been described previously. The IC_{50} values of propolin A and propolin B were 13.6 and 16.0 μM, respectively. Figure 2b shows the time courses of the induction of cytotoxic effect in human melanoma cells by propolin C at a concentration of 16.35 μM. Treatment of human melanoma cells with 16.35 μM propolin C for 12 hr induced cytotoxic effects in approximately 62% of the cells. Treatment of the human melanoma cells with 2.35–11.75 μM propolin C for 24 hr induced cell morphological change as shown in Fig. 2c. Morphological features, such as condensation of chromatin of the nucleus and cell shrinkage, were seen in propolin C-treated cells. It appears that the cells morphologic changes may be *via* induced apoptosis as described below.

3.3. Comparison of propolis major compound CAPE with propolin C on various cancer cell viabilities

The cytotoxic effects of CAPE and propolin C on six different cancer cell lines were investigated. All cancer cell lines were treated with various concentrations (2.35–16.35 μM) of propolin C and CAPE. After 24 hr of treatment, the number of live cells was determined by means of MTT assay. As shown in Table 3, the viability of MCF-7 cells was less than 6% after exposure to propolin C (11.75 μM) for 24 hr, but, the same concentration of CAPE induced no marked cytotoxic effect. Four different cancer cell lines were sensitized by treatment with propolin C, including A2058, B16F10, Hep.3B, and MCF-7. However, both Hep.G2 and HT-29 were more resistant to propolin C than the other four lines. Furthermore, B16F10 were more sensitized to CAPE than the other five cancer cell lines, but, all the data indicated that propolin C was markedly more active than CAPE in inhibiting the growth of cancer cells.

3.4. Effect of propolin C on DNA fragmentation and ladder formation

We next questioned whether propolin C induced apoptosis in human melanoma cells. After treatment of human melanoma cells with various concentrations of propolin C for 24 hr, the genomic DNA from cells was subjected to agarose gel electrophoresis. The DNA fragmentation ladder significantly increased (Fig. 3a). The induction of

Table 3
Cytotoxic activity of propolin C and CAPE in various human and murine cancer cell lines

Cancer cell lines	Cell viability (%)								
	Propolin C concentrations (μM)					CAPE concentrations (μM)			
	0	2.35	7.05	11.75	16.35	2.35	7.05	11.75	16.35
A2058	100	85 ± 5	62 ± 4	18 ± 2	4 ± 0.5	98 ± 6	95 ± 5	92 ± 4	82 ± 4.5
B16F10	100	98 ± 6	49 ± 3	12 ± 2	2 ± 0.5	99 ± 7	75 ± 3.5	60 ± 3	42 ± 2.5
Hep.G_2	100	99 ± 6	95 ± 4	92 ± 3	85 ± 4	98 ± 3	94 ± 3.5	92 ± 5	91 ± 5
Hep.3B	100	98 ± 3	80 ± 4	57 ± 3	6 ± 0.5	97 ± 3	95 ± 6	92 ± 4	85 ± 3
MCF-7	100	95 ± 2	35 ± 3	6 ± 2	1 ± 0.2	97 ± 3	96 ± 2.5	90 ± 4	82 ± 3
HT-29	100	95 ± 4	82 ± 3	71 ± 4	53 ± 2	97 ± 3.5	95 ± 4	92 ± 4	90 ± 3

Cells were treated with propolin C or CAPE for 24 h. Each cell viability value is the mean of three repeated experimesnts by MTT assay.

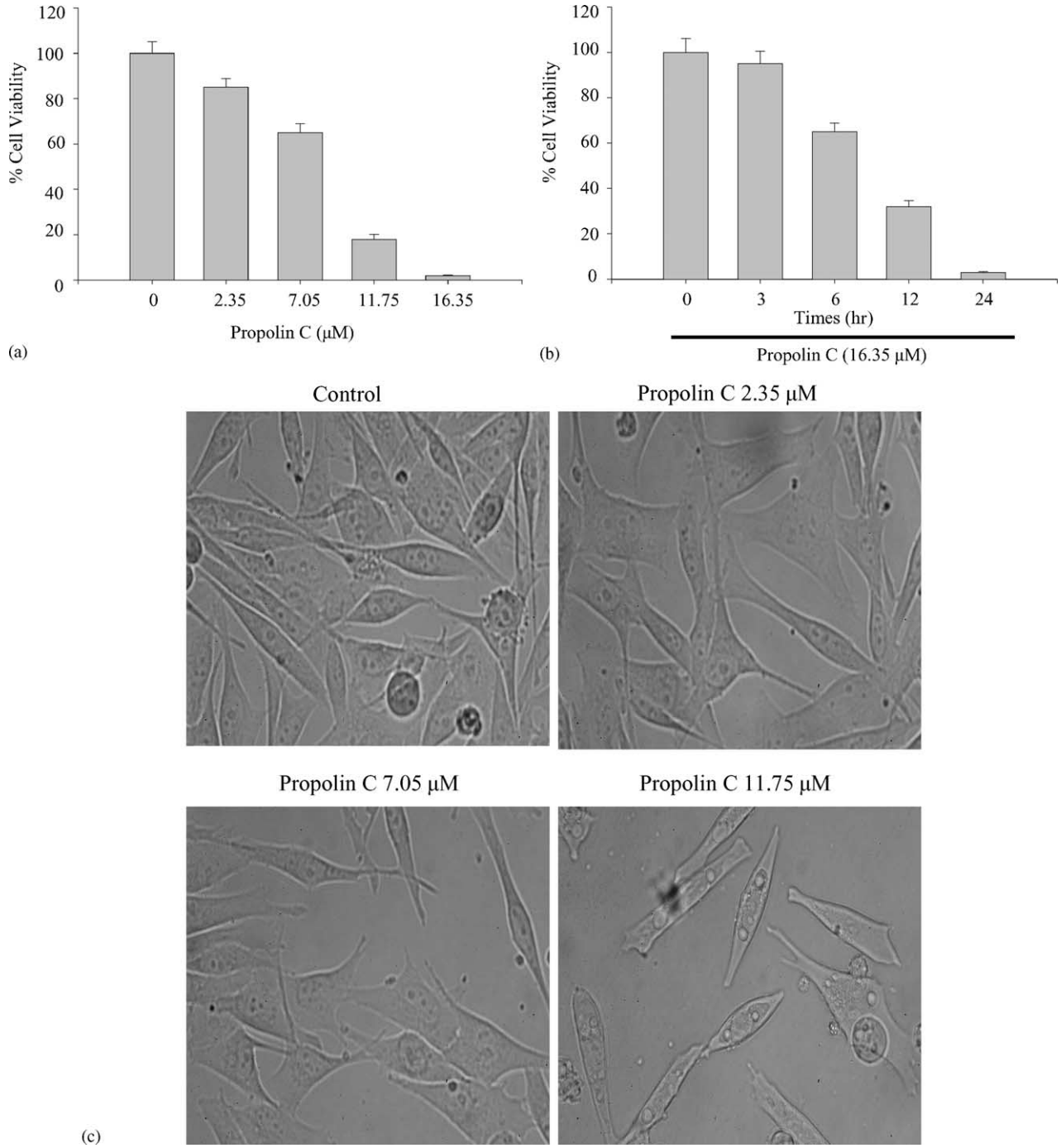

Fig. 2. Effect of propolin C on the viability of human melanoma cells. (a) The cells were treated with various concentrations (2.35–16.35 μM) of propolin C for 24 hr and (b) various time courses at concentration of 16.35 μM for 0, 3, 6, 12, and 24 hr and subsequent cell viability was measured by a trypan blue exclusion assay. (c) Phase-contrast micrographs of human melanoma cells treated with various concentrations of propolin C (2.35–11.75 μM). Results are from one experiment representation of three similar experiments. Magnification 400×. The data are presented in terms of proportional viability (%) by comparing the propolin C-treated group with the untreated cells, the viability of which was assumed to be 100%. Results represent the mean ± SE of three independent experiments.

apoptosis was further confirmed in fluorescence photomicrographs of human melanoma cells stained with PI after treatment with 11.75 μM propolin C for 24 hr. The morphological features of apoptosis, such as condensation of chromatin and fragmentation of the nucleus into discrete masses scattered throughout the cytosol, were seen in propolin C-treated cells (Fig. 3b).

3.5. Relation between cell cycle and DNA fragmentation after propolin C

To investigate the induction of a sub-G1 cell population, the DNA content of human melanoma cells treated with propolin C at various concentrations was analyzed by flow cytometry as shown in Fig. 4a. The percentages

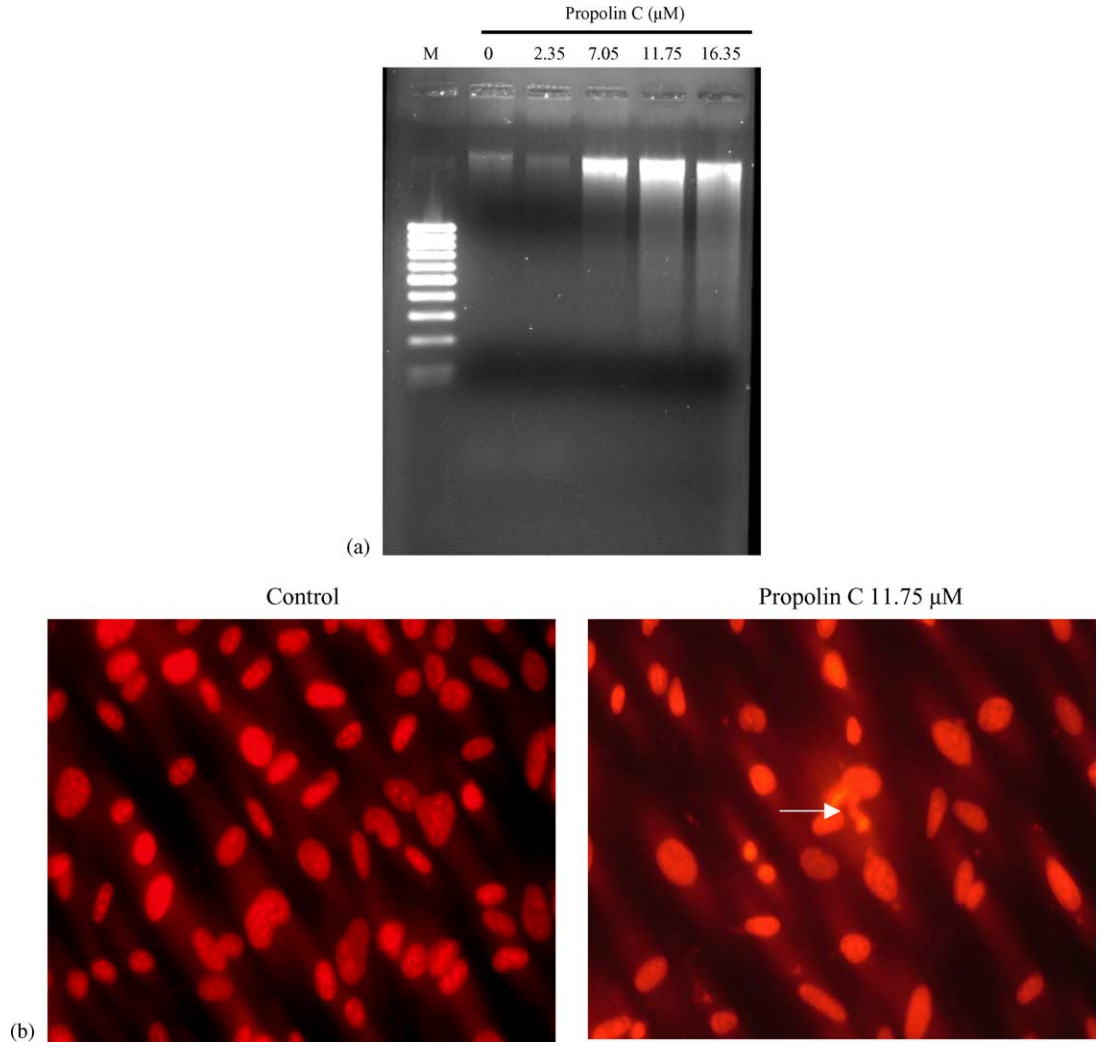

Fig. 3. Propolin C-induced DNA fragmentation in human melanoma cells. (a) DNA fragmentation by propolin C in human melanoma cells. Human melanoma cells were treated with various concentrations (2.35–16.35 μM) for 24 hr. Genomic DNA was isolated and analyzed by 1.8% agarose gel electrophoresis. (b) Effect of propolin C on the morphology of nuclear chromatin. The untreated and propolin C (11.75 μM)-treated human melanoma cells for 24 hr were fixed and stained as described in Section 2. Morphological changes of nuclear chromatin were then viewed under a fluorescence microscope. The control cells showed round and homogeneous nuclei; propolin C-treated cells showed condensed and fragmented nuclei. Results are from one experiment representative of three similar experiments. Magnification 400×.

of apoptotic human melanoma cells observed after 0, 2.35, 7.05, 11.75, and 16.35 μM of propolin C (left column in Fig. 4a) for 24 hr were as follows: 1.70, 0.72, 4.16, 25.24, and 52.72%. A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA, was detected. However, we next questioned whether cell damage might be attributable to the cell cycle program or might have become arrested at any cell cycle phases by propolin C-induced apoptosis in human melanoma cells. Our data in Fig. 4a right and left columns demonstrate that propolin C was markedly loss of cells from the G2/M phase of the cell cycle (from 22.4 to 8.2%). Figure 4b (sub-G1) and 4c (DNA content) shows the time courses of the induction of apoptosis effect in human melanoma cells by propolin C at a concentration of 16.35 μM. Treatment of human melanoma cells with 16.35 μM propolin C for 16 hr induced apoptosis effects in approximately 48.57% of sub-G1 (sub-2N) DNA peak. These results show that propolin C at a concentration of 11.75 μM or treated with propolin C at concentration 16.35 μM for 16 hr was efficient to induce apoptosis in human melanoma cells, and there is a marked loss of cells from the G2/M phase of the cell cycle. Our data indicate that propolin C had cytotoxic effects on human melanoma cells, and the cytotoxic effects may be through apoptosis induction.

3.6. *Propolin C-induced apoptosis via activated caspase-dependent pathway*

Initiator caspases (including 8, 9, 10, and 12) are closely coupled to proapoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including 3, 6, and 7), which in turn cleave

cytoskeletal and nuclear proteins, such as PARP and lamin A, and finally induce apoptosis. Proapoptotic stimuli, including the FasL, TNF-α, DNA damage, and ER stress. We first evaluate whether caspase-dependent signal pathways were involved in the apoptotic cell death induced by propolin C in human melanoma cells. Treatment of human melanoma cells with 16.35 μM for 0, 4, and 8 hr resulted in dramatic decreases in PARP

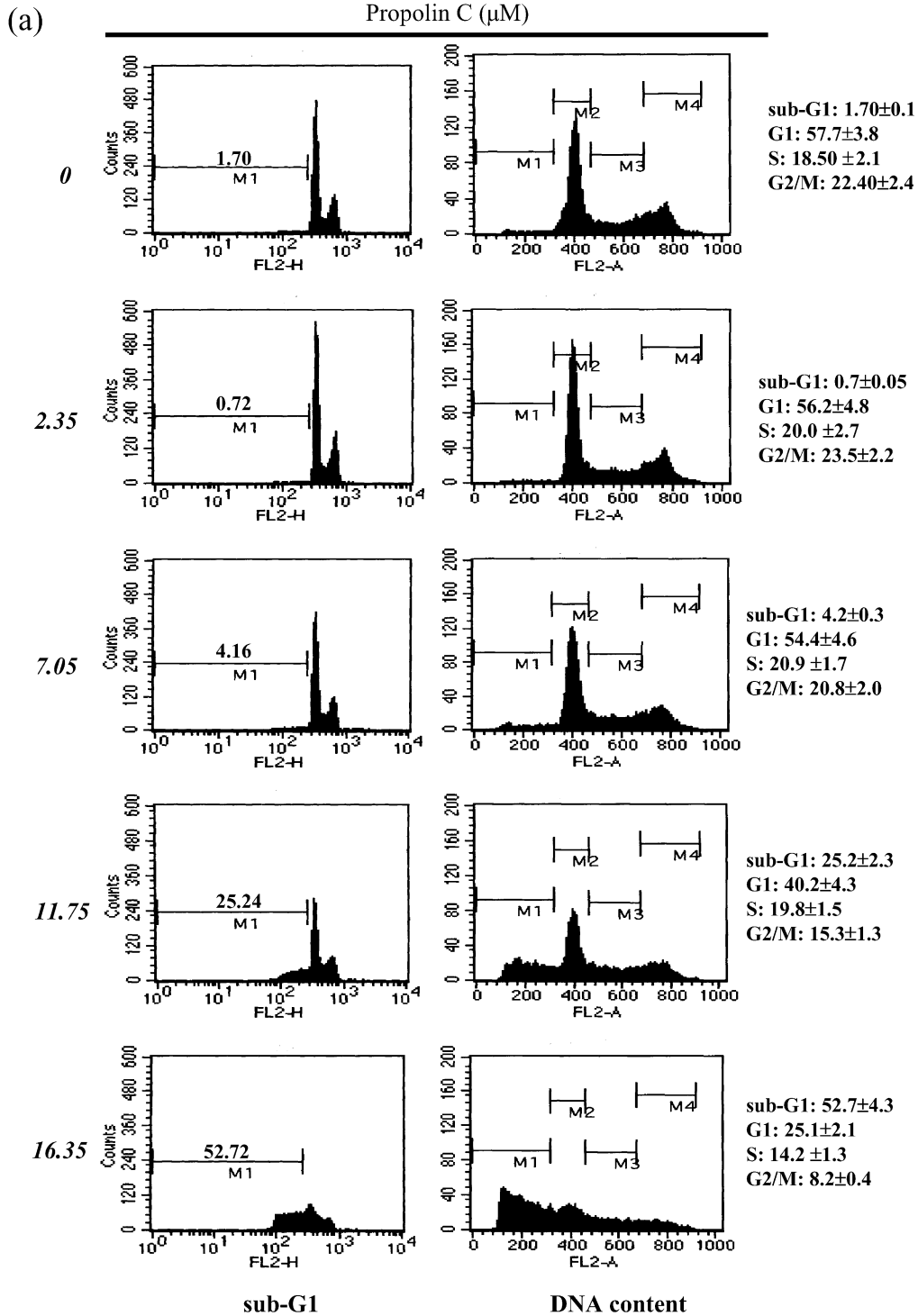

Fig. 4. Propolin C-induced apoptosis in human melanoma cells. (a) Effect of propolin C on the cellular sub-G1 and DNA content. The cells were treated with various concentrations (2.35–16.35 μM) of propolin C for 24 hr and stained with PI as described in Section 2. Following flow cytometric analysis, cellular DNA profile was further analyzed by Cell Quest software. Data represent the percentage of cell counts and display a hypoploid DNA (sub-G1) population. (b) Effect of propolin C treated with concentration of 16.36 μM for 0, 4, 8, 16, and 24 hr on the human melanoma cells. Data represent the percentage of cell counts and display a sub-G1. (c) Effect of propolin C on the time course manner for cell cycle distribution of human melanoma cells. Cell cycle distribution as assessed by flow cytometry. Results are from one experiment representative of three similar experiments.

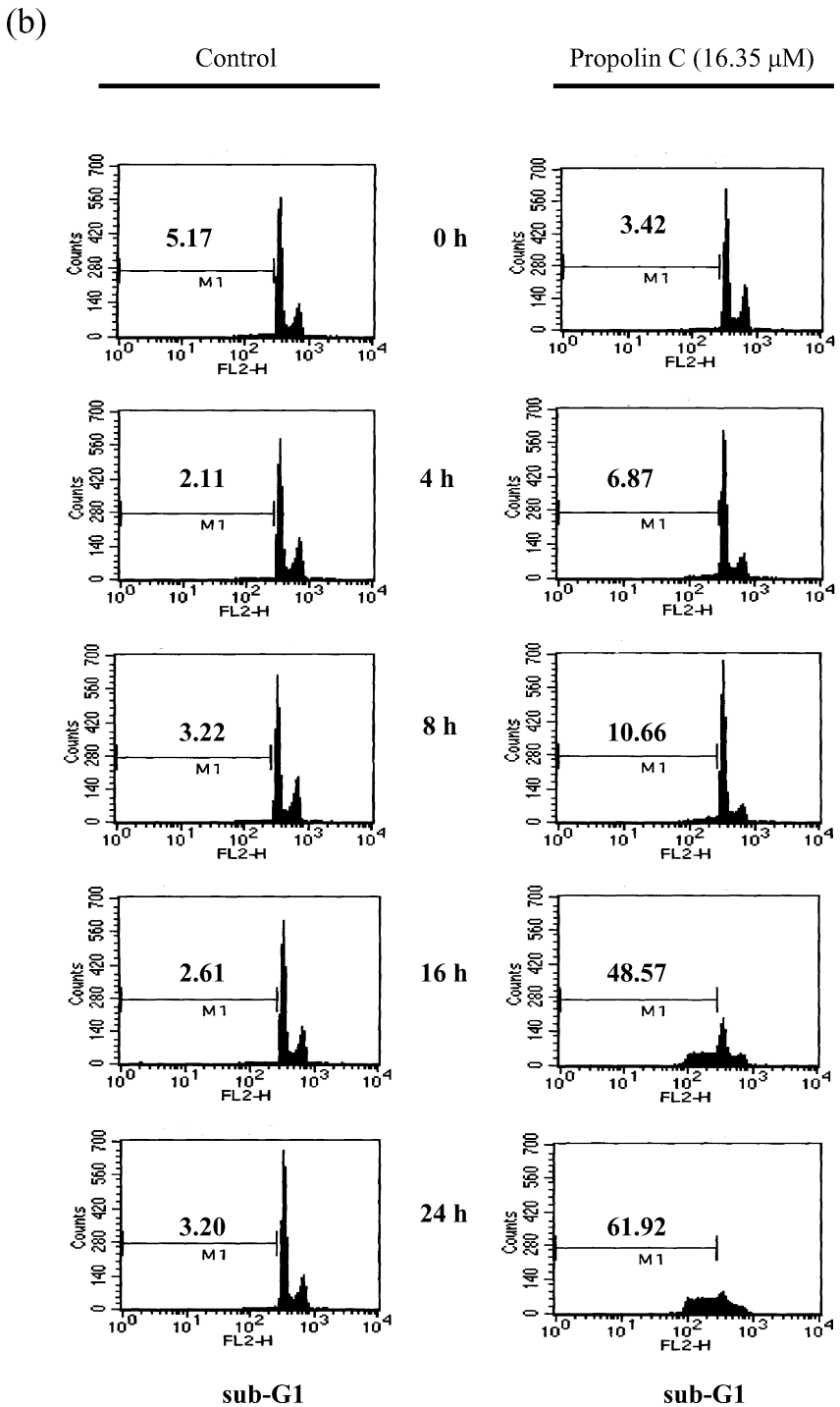

Fig. 4. (*Continued*)

and procaspase-3 protein levels (Fig. 5a). Next, we further evaluate whether caspase-8 might be activated during the induction of apoptosis by propolin C, because caspase-8 plays an important role in the mitochondria dependent or independent pathway in apoptosis. Our data indicate PARP, procaspase-8, procaspas-3, and Bid protein expressions were markedly decreased by treated with propolin C at concentration of 11.75–16.75 μM. (Fig. 5b).

3.7. Propolin C-induced apoptosis via activated mitochondrial-dependent pathway

However, the apoptotic processes that follows two signaling pathways, namely, the mitochondrial-independent activation of caspase-3 and the mitochondrial-dependent apoptosis due to cleavage of Bid by caspase-8, the formation of apoptosomes, and activation of caspase-9 and the downstream caspases. We next determined the Bid protein level

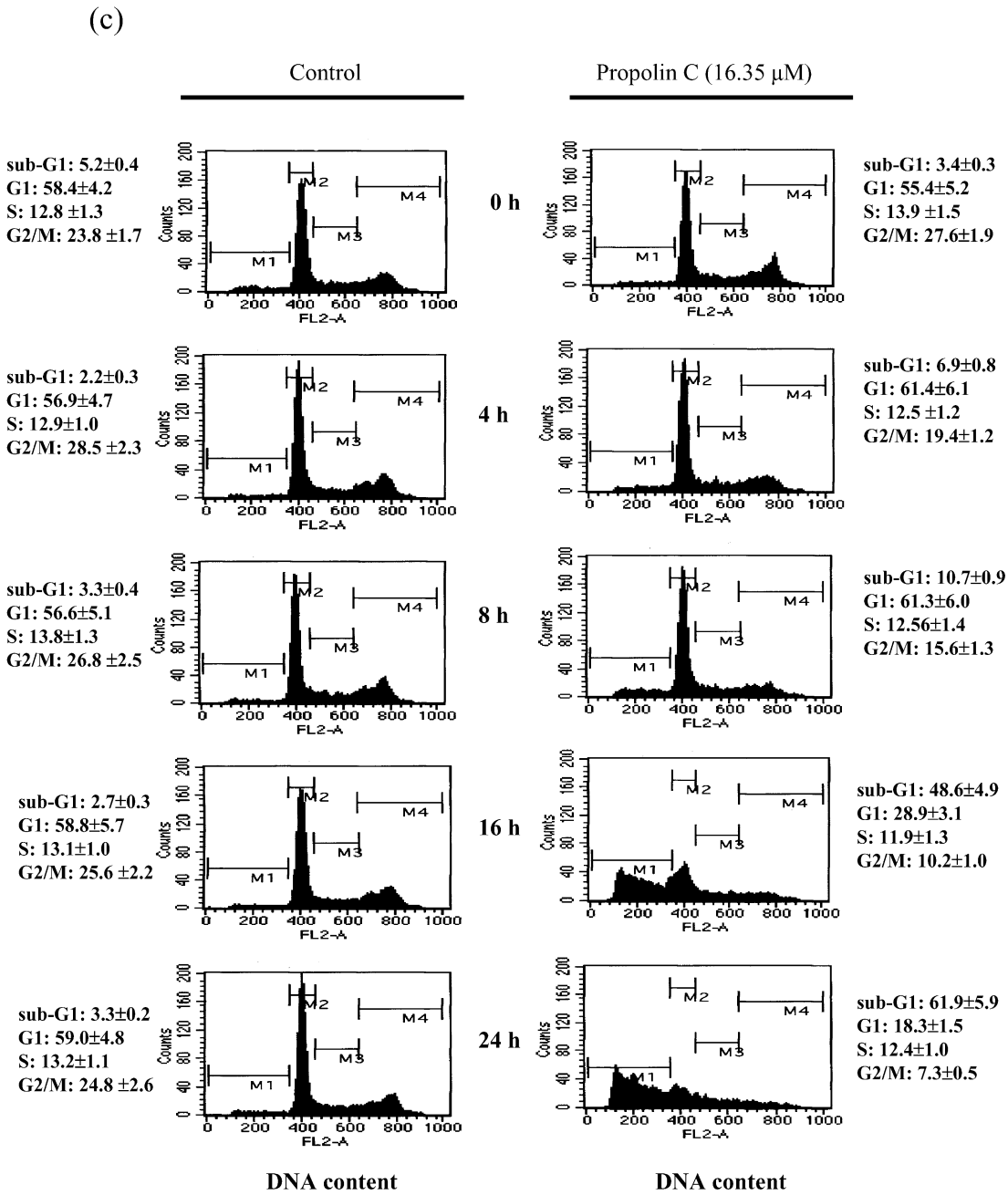

Fig. 4. (*Continued*).

after propolin C treatment (0, 4, and 8 hr) in human melanoma cells. The results indicate this level decreased markedly at 8 hr after treatment with propolin C (Fig. 5c). To address the question of whether propolin C induces cytochrome *c* release from mitochondria, a cytoplasm fraction prepared from human melanoma cells was incubated in the presence (7.05, 11.75, and 16.35 μM) or absence of propolin C for 24 hr. Our data indicated that the cytoplasm fraction markedly increased the cytochrome *c* protein level in a dose-dependent manner (Fig. 5d). All results suggest conclusively that the propolin C-induced apoptosis of human melanoma cells was *via* mitochondrial-dependent pathway, as were the activation of caspase-8, Bid, and caspase-3.

3.8. Caspase-3 activation of human melanoma cells by propolin C

To further elucidate the mechanisms of propolin C-induced apoptosis, we think of the possible involvement of caspases. Activation of caspase-3 plays an important role in the induction of apoptosis by various stimuli. Upon treatment of human melanoma cells with 16.35 μM propolin C, the activity of a caspase-3-like enzyme increased significantly within 4 hr after the start of treatment (Fig. 6). The results further obtained in these experiments suggested that caspase-3 involved in propolin C-induced apoptosis of human melanoma cells.

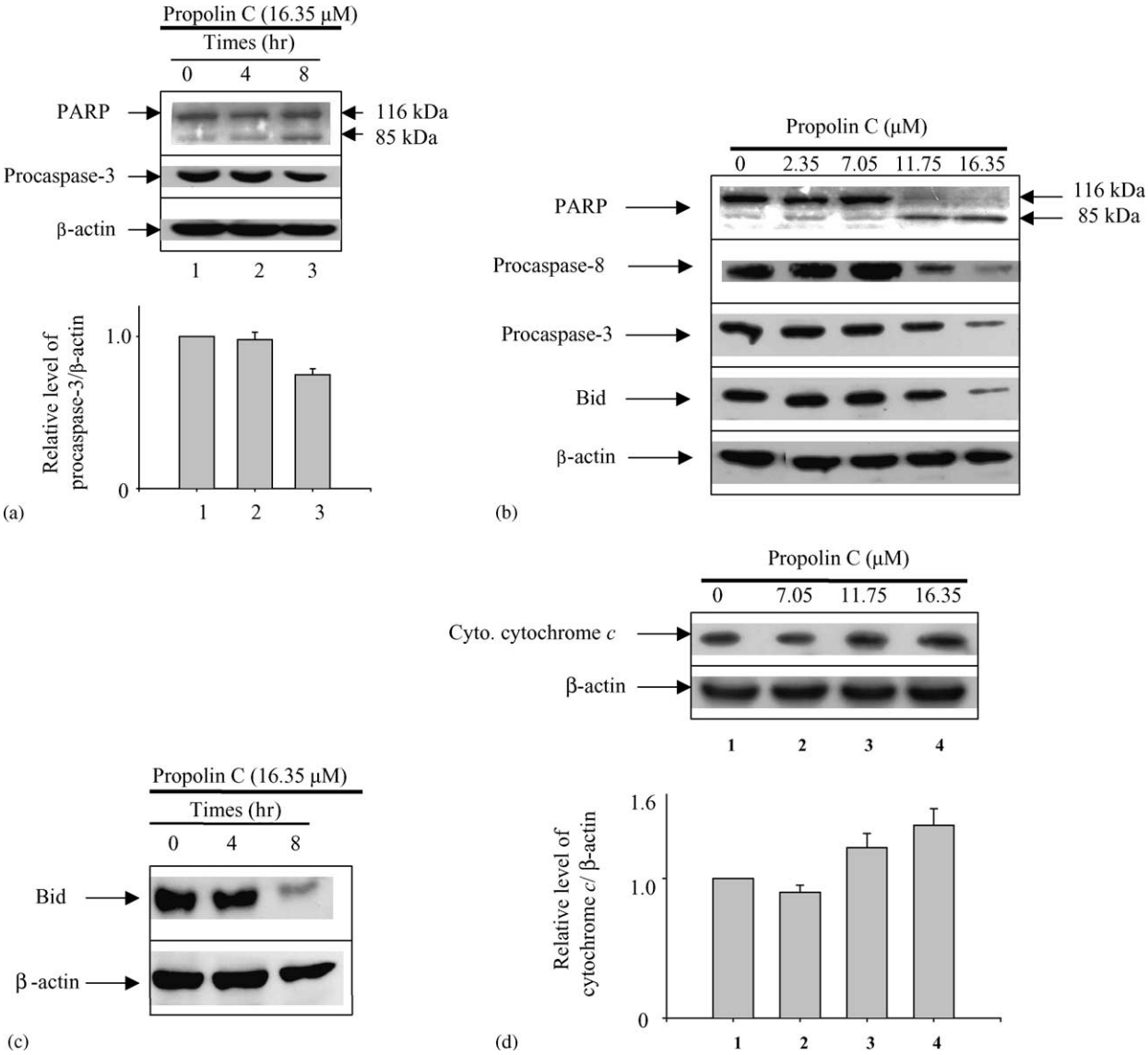

Fig. 5. Induction of apoptosis *via* caspases-dependent pathway, and activation of caspase-8, Bid, caspase-3, and PARP. Human melanoma cells were incubated with 2.35, 7.05, 11.75, and 16.35 μM propolin C for 24 hr or treated with propolin C for 0, 4, and 8 hr. Cell lysates were prepared, and subjected to SDS–PAGE. (a) Procaspase-3 and PARP were determined by immunoblotting using specific antibodies. (b) PARP, procaspase-8, procaspase-3, and Bid were determined by immunoblotting using specific antibodies. (c) Human melanoma cells were incubated with propolin C at 0, 4, and 8 hr at concentrations of 16.35 μM. Cell lysates were prepared, subjected to SDS–PAGE, and Bid determined by immunoblotting using specific antibodies. (d) Release of cytochrome *c* from mitochondria was dose dependent. Human melanoma cells were treated with 7.05, 11.75, and 16.35 μM propolin C for 24 hr, and cytochrome *c* in the cytosolic fraction was detected by western blotting analysis with antibodies against cytochrome *c*. The results are representative of those from three separate experiments.

3.9. Inhibition of XO by propolin C and CAPE

XO is the major source of reactive oxygen species (ROS). Normal loads of ROS are removed by the enzyme superoxide dismutase (SOD), catalase, and glutathione peroxidase. Excessive amounts of ROS increase the oxidative stress in the body. This accelerates membrane damage, DNA base oxidation, DNA strand breaks, and chromosome aberrations, most of which are involved in the cacinogenesis process. XO activity can be determined because it forms uric acid, which has a maximum absorption in OD = 295 nm, providing the basis for a spectrophtometric assay. The effects of two polyphenols on XO activity were tested, and the dose–response curves and IC_{50} values for these two compounds are shown in Fig. 7. The IC_{50} values of propolin C and CAPE [30] were 17 and 44 μM, respectively. In the system, the IC_{50} value for the well-known XO inhibitor allopurinol is 0.7 μM (data not shown). The results suggest that the chemical structures of propolin C are closely related to hypoxanthine, xanthine, and uric acid, and may act as substrate analogs for XO.

3.10. Free radicals scavenging by propolin C and CAPE

The radical scavenging abilities of propolins C and CAPE were tested with DPPH, and the results are pre-

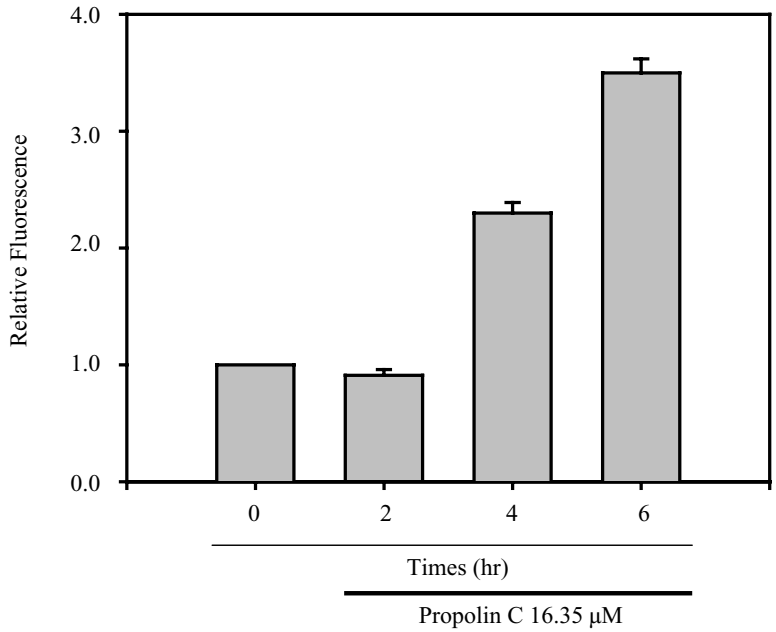

Fig. 6. Analysis of the caspase-3-like activity induced by propolin C. Human melanoma cells were treated with 16.35 μM propolin C for 0, 2, 4, and 6 hr and caspase-3-like activity in cell extracts was measured with a specific fluorogenic substrate. Results are representative of those from three similar experiments.

sented in Fig. 8. The data indicate IC_{50} values of both propolin C and CAPE were approximately 7.5 and 8.5 μM, respectively. These results suggest that propolins C is a potential antioxidant agent.

4. Discussion

In the present study, we have demonstrated that propolin C potently inhibits the proliferation of human melanoma cells through inducing a cytotoxic effect and triggering apoptosis. Propolin C exhibited a dose- and time-dependent inhibition of cellular proliferation in the human melanoma cells. Our present data demonstrate that propolin C induces more cytotoxic effect than CAPE at the same concentrations. We have identified two novel prenylflavanones from Taiwanese propolis, flavonoid compounds with hydrated geranyl side chains, namely, propolin A and propolin B [14]. Both propolin A and propolin B have hydrated geranyl side chains on the ring B attached at C2 and C5, respectively, but propolin C has an unhydrated geranyl side chain on the ring A attached at C6 (Fig. 1). We have demonstrated that both propolin A and propolin B induce apoptosis in different cancer cell lines and are potential antioxidant agents.

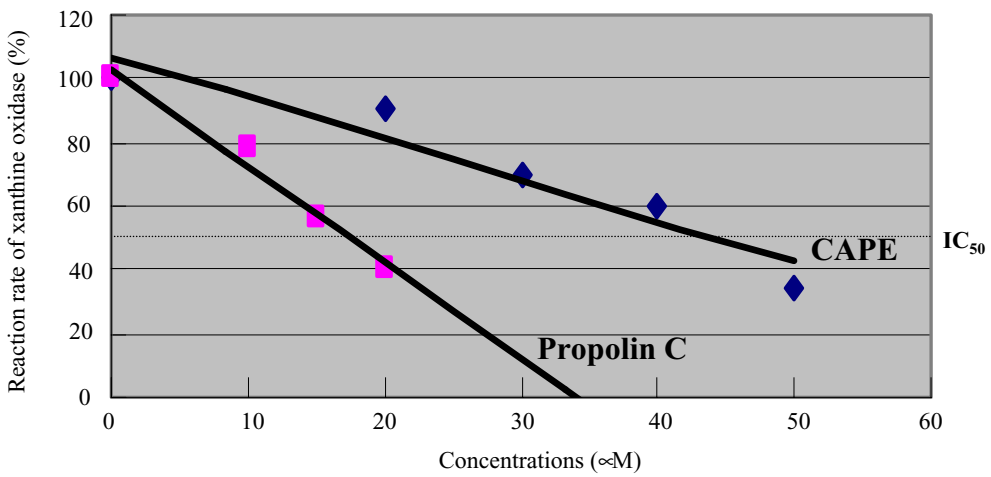

Fig. 7. Inhibition of xanthine oxidase activity by propolin C. Inhibition of xanthine oxidase by propolin C, CAPE, and allopurinol. The formation of uric acid was determined spectrophotometrically at 295 nm as described in Section 2.

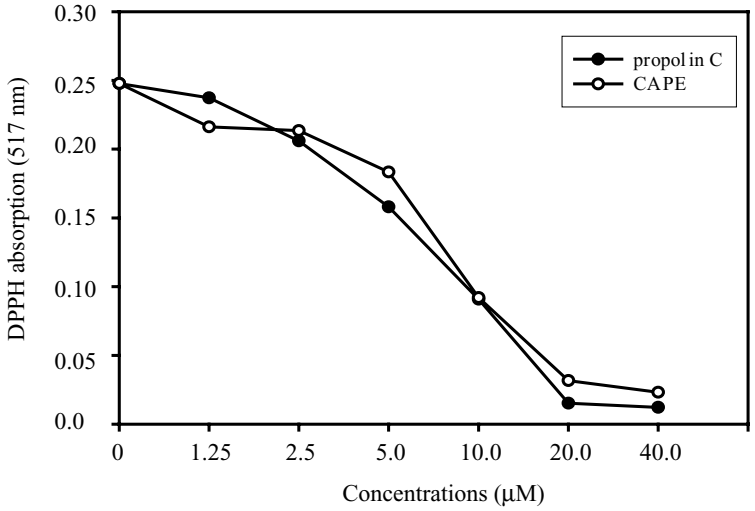

Fig. 8. Free radicals scavenging activity by propolin C. Antioxidant activity of propolin C and CAPE in DPPH test as described in Section 2. Results are from one experiment representative of three similar experiments.

^{1}H NMR and ^{13}C NMR data indicate that propolin C is a flavonoid compound with a geranyl side chain and identical to known compound nymphaeol-A from *H. nymphaefolia* [15]. However, the physiological activity of nymphaeol-A has never been reported. This prompted us to study the anticancer activity of propolin C. Another closely related compound called sophoranone, extracted from a traditional Chinese medicine *shan dou gen*, induced apoptosis in human leukemia U937 cells [23]. Hyperforin was isolated from St. John's wort (*Hypericum perforatum*), and inhibition of tumor cell growth by it has been reported [31]. Hyperforin is an acylphloroglucinol-type compound that consists of a phloroglucinol skeleton substituted with four lipophilic isopren chains. Many reports have indicated that geranylgeranylacetone [32] or Vitamin K_2 with four isoprene units [33], significantly inhibits cell growth and induces the differentiation of leukemia cells.

Our data indicate that propolin C had cytotoxic effects on human melanoma cells, and the effect may be due to its induction of apoptosis. Apoptosis can be evaluated by three techniques: DNA laddering, fluorescence microscopy, and flow cytometry. When human melanoma cells were grown in the presence of propolin C and cells were isolated and analyzed electrophoretically for nuclear DNA degradation as shown in Fig. 3a, the results indicated that propolin C markedly promoted DNA fragmentation at a concentration of 11.75 μM. In DNA content analysis, low molecular weight DNA is lost from apoptotic cells, resulting in a peak at the sub-G1 position. Human melanoma cells were grown in the presence of various concentrations of propolin C (2.35–16.35 μM) for 24 hr or treated with propolin C (16.35 μM) for 0, 4, 8, 16, and 24 hr, and cells were analyzed by flow cytometry. Results indicated that propolin C efficiently induced apoptosis in human melanoma cells, and there is a clear loss of cells from the G2/M phase of the cell cycle as shown in Fig. 4a and c. We further investigated the molecular mechanism of the propolin C-induced apoptosis pathway. Our data indicated that propolin C can induce the activation of caspase-8, Bid, caspase-3, and PARP, finally producing DNA fragmentation (Fig. 5). On whether propolin C induced apoptosis through the mitochondrial-dependent pathway, our data indicated that it can increase cytochrome *c* release in cytoplasm (Fig. 5d).

A high level of XO may cause gout and is responsible for oxidative damage to living tissues. It catalyzes the oxidation of hypoxanthine and xanthine to uric acid-yielded superoxide radical and raises the oxidation level in the organism. Our previous studies have indicated that propolin A and propolin B inhibit XO activity. The data in this report demonstrate that propolin C and CAPE might inhibit XO activity with IC_{50} values at a concentration of 17 and 44 μM, respectively (Fig. 7). The results suggest that the chemical structures of propolin C are closely related to hypoxanthine, xanthine, and uric acid and may act as substrate analogs for XO.

The mechanism of apoptosis induction by propolin C appears *via* caspase pathways. Recently, many reports have suggested that the caspase family plays an important role in apoptosis [34,35]. The caspases are unique in their requirement for an Asp residue at the cleavage site in their substrates. Recently, from the14 known members of the caspase family of proteases, caspase-3 has been suggested to be a key part in the apoptotic machinery. Caspase-3 has been indicated to be activated in apoptotic cells, and it cleaves several cellular proteins, including PARP protein, and DFF-45 protein. The cleavage of these vital proteins is a hallmark of apoptosis. The Fas/Fas ligand (FasL) death pathway is an important mediator of apoptosis. Deregulation of this pathway is reportedly involved in the immune escape of breast cancer and the resistance to anticancer drugs. In the present study, our results indicate that the mitochondrial-dependent pathway of cell death signaling is involved in propolin C-induced apoptosis in human melanoma cells.

In summary, we propose that propolin C-induced apoptosis may be *via* the activation of caspase-8, Bid, and the induction of cytochrome *c* release from mitochondria to the cytosol, activating of caspase-3, leading to cleavage of PARP, causing DNA fragmentation, and finally the execution of apoptosis.

Acknowledgments

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References

[1] Burdock GA. Review of the biological properties and toxicity of bee propolis. Food Chem Toxicol 1998;36:347–63.
[2] Ghisalberti EL. Propolis: a review. Bee World 1979;60:59–84.
[3] Gribel N, Pashinskii VG. Antitumor properties of honey. Vopr Onkol 1984;36:704–9.
[4] Krol W, Czuba Z, Scheller S, Gabrys J, Grabiec S, Shani J. Antioxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminal. Biochem Int 1990;21:593–7.
[5] Bankova V, Christov R, Kujumgiev A, Marcucci MC, Popov S. Chemical composition and antibacterial activity of Brazilian propolis. Z Nat Sect C Biosci 1995;50:167–72.
[6] Amoros M, Sauvager F, Girre L, Cormier M. In vitro antiviral activity of propolis. Apidologie 1992;23:231–40.
[7] Dobrowolski JW, Vohora SB, Sharma K, Shah SA, Naqvi SA, Dandiya PC. Antibacterial, antifungal, antiamoebic, anti-inflammatory and antipyretic studies on propolis bee products. J Ethnopharm 1991;35:77–82.
[8] Greenaway W, May J, Scaysbrook T, Whatley FR. Identification by gas chromatograph-mass spectrometry of 150 compounds in propolis. Z Naturforsch 1991;46C:111–21.
[9] Sud'ina GF, Mirzoeva OK, Pushkareva MA, Korshunova GA, Sumbatyan NV, Varfolomeev SD. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. FEBS Lett 1993;329:21–4.
[10] Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME, Huang Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia 1993;7:563–8.
[11] Friesen C, Herr I, Krammer PH, Debatin KM. Involvement of the CD95 (Apo-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 1996;2:574–7.
[12] Kaufmann SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 1989;49:5870–8.
[13] Dorrie J, Sapala K, Zunino SJ. Carnosol-induced apoptosis and downregulation of Bcl-2 in B-lineage leukemia cells. Cancer Lett 2001;170:33–9.
[14] Chen CN, Wu CL, Shy HS, Lin JK. Cytotoxic prenylflavanones from Taiwanese propolis. J Nat Prod 2003;66:503–6.
[15] Yakushijin K, Shibayama K, Murata H, Furukawa H. New prenylflavanones from *Hernandia nymphaefolia* (Presl) kubitzki. Heterocycles 1980;14:397–401.
[16] Stavric B. Role of chemopreventers in human diet. Clin Biochem 1994;27:319–32.
[17] Matsukawa Y, Marui N, Sakai T, Satomi Y, Yoshida M, Matsumoto K, Nishino H, Aoike A. Genistein arrests cell cycle progression at G2-M. Cancer Res 1993;53:1328–31.
[18] Yanagihara K, Ito A, Toge T, Numoto M. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. Cancer Res 1993;53:5815–21.
[19] Wei Y-Q, Zhao X, Kariya Y, Fukata K, Teshigawara K, Uchida A. Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res 1994;54:4952–7.
[20] Matsuzaki Y, Kurokawa N, Terai S, Matsumura Y, Kobayashi N, Okita K. Cell death induced by bicalein in human hepatocellular carcinoma cell lines. Jpn J Cancer Res 1996;87:170–7.
[21] Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynklewiez Z. The cell cycle-related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res 1993; 53:3186–92.
[22] Hirano T, Abe K, Gotoh M, Oka K. Citrus flavone tangeretin inhibits leukemia HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. Br J Cancer 1995; 72:1380–8.
[23] Sachiko K, Noriko T, Tetsuya K, Man X, Judong C, Yutaka M, Toshihiro A, Shigeo N, Yoshiteru I, Kazuyasu N. Sophoranone, extracted from a traditional Chinese medicine *shan dou gen*, induces apoptosis in human leukemia U937 cells via formation of reactive oxygen species and opening of mitochondrial permeability transition pores. Int J Cancer 2002;99:879–90.
[24] Wyllie AH. Apoptosis: an overview. Br Med Bull 1997;53:451–65.
[25] Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 1997;3:614–20.
[26] Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:305–8.
[27] Martinez-Lorenzo MJ, Alava MA, Gamen S, Kim KJ, Chuntharapai A, Pineiro A, Naval J, Anel A. Involvement of APO2 ligand/TRAIL in activation-induced death of Jurkat and human peripheral blood T cells. Eur J Immunol 1998;28:2714–25.
[28] Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312–6.
[29] Reed JC. Cytochrome *c*: can't live with it-can't live without it. Cell 1997;91:559–62.
[30] Grunberger D, Banerjee R, Eisinger K, Oltz EM, Efros L, Caldwell M, Estevez V, Nakanishi K. Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. Experientia 1988;44:230–2.
[31] Christoph MS, Vladimir K, Birgit S-H, Astrid K, Judit K, Christian CT, Bernhard G, Thomas K, Christoph B. Inhibition of tumor cell growth by hyperforin, a novel anticancer durg from St. John's wort that acts by induction of apoptosis. Oncogene 2002;21:1242–50.
[32] Sakai I, Tanaka T, Osawa S, Hashimoto S, Nakaya K. Geranylgeranylacetone used as an antiulcer agent is a potent inducer of differentiation of various human myeloid leukemia cell lines. Biochem Biophys Res Commun 1993;191:873–9.
[33] Sakai I, Yoda M, Hida T, Osawa S, Nakajo S, Nakaya K. Novel role of Vitamin K_2 a potent inducer of differentiation of various human myeloid leukemia cell lines. Biochem Biophys Res Commun 1994; 205:1305–10.
[34] Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326:1–16.
[35] Chen YL, Wang JY, Chen SA, Yang BC. Granulocytes mediates the Fas-L-associate apoptosis during lung metastasis of melanoma that determines the metastatic behaviour. Br J Cancer 2002;87: 359–65.
[36] Pan MH, Lin JH, Lin-Shiau SY, Lin JK. Induction of apoptosis by pento-*O*-galloyl-β-D-glucose through activation of caspase-3 in human leukemia HL-60 cells. Eur J Pharmacol 1999;381:171–83.