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Mechanism of mahanine-induced apoptosis in human leukemia cells (HL-60)

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Abstract

Mahanine, a carbazole alkaloid occurs in the edible part of *Micromelum minutum*, *Murraya koenigii* and related species has been found to induce apoptosis in human myeloid cancer cell (HL-60). Concentration of 10 μM mahanine caused a complete inhibition of cell proliferation and the induction of apoptosis in a time dependent manner. Mahanine-induced cell death was characterized with the changes in nuclear morphology, DNA fragmentation, activation of caspase like activities, poly(ADP-ribose) polymerase cleavage, release of cytochrome *c* into cytosol and stimulation of reactive oxygen species generation. The cell death was completely prevented by a pancaspase inhibitor benzyloxycarbonyl-L-aspart-1-yl-[(2,6-dichlorobenzoyl)oxy]methane (Z-Asp-CH_2-DCB). Mahanine activated various caspases such as caspase-3, -6, -8 and -9 (like) activities but not caspase-1 like activity. More than 70% cell survival was observed in the presence of a caspase-3 inhibitor. In addition, co-treatment of cyclosporin A markedly increased the survival of mahanine-treated HL-60 cells. Flow cytometric analysis revealed that mahanine decreased the mitochondrial membrane potential of intact cells, and disrupted cell cycle progression by increasing the number of cells in sub-diploid region, concomitantly with the decrease of cells in diploid phases, particularly at late hours of apoptosis. The overall results suggest that mahanine down regulates cell survival factors by activation of caspase-3 through mitochondrial dependent pathway, and disrupts cell cycle progression.

Keywords: Mahanine; Apoptotic cell death; Cell cycle; Caspases; PARP

1. Introduction

Identifying mode of cell death has been given central importance in the study of various diseases, such as cancer and neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases) [1,2]. Cells undergo death by two major mechanisms: necrosis, in which primary damage to the metabolic or membrane integrity of the cell occurs, or apoptosis, which is an internal suicide program contained in all cells [3]. Programmed cell death (apoptosis) plays an important role in the regulation of tissue turnover in most normal and cancerous tissues.

The present trends in the management of cancer development include increasing awareness and chemoprevention that suggest using natural or synthetic substances to prevent initiational and promotional events associated with cancer development and this strategy has been considered to be the most direct way to counteract malignancy development [4]. Epidemiological studies suggest that dietary intake of fruits and vegetables, is effective against many diseases including cancer [5,6]. Mahanine (3,11-dihydro-3,5-dimethyl-3-(4-methyl-3-pentenyl)-pyrano[3,2-*a*]carbazol-9-ol), a carbazole alkaloid has been reported to be present in the edible parts of some plants such as *Micromelum minutum* and *Murraya koenigii*, that are consumed in some parts of Southeast Asia, particularly Thailand, and in South Asia [7,8]. The compound showed various bioactivities such as antimutagenicity against heterocyclic amines, antimicrobial activity against gram positives, anti-inflammatory effect, and appeared to be cytotoxic against several cancer cell lines [7–10] although the mechanism of action is elusive.

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It is becoming clear that caspases (cysteine aspartic acid-specific protease) play central roles in the execution of apoptosis [11,12]. They are found in cells as inactive precursors, being converted into active form dismantle key cellular structure by generating proteolytic torrent [13,14]. There are evidences that show caspase activation occurs in cell death pathway through the initiation of certain member of tumor necrosis factors [15]. However, more recently an alternative route to caspase activation involving mitochondria has emerged [16]. To investigate the possible mechanism(s) that involved in mahanine-induced apoptosis we examined the effect of this compound on cell morphology, DNA fragmentation, DNA ladder formation, activation of various caspases, release of cytochrome *c*, ROS production, mitochondrial membrane potential and cell cycle progression. In some experiments we used various inhibitors to conclude the observed results. In this study, HL-60 cell line was selected as a model system since it is broadly used in the study related to the evaluation of antiproliferative activity, differentiation, and apoptotic effect of potential active molecules.

This study revealed that mahanine actively induces apoptosis in HL-60 cells and reduces proliferation of HL-60 cells. The pathways that involved in the induction of apoptosis were activation pathways in the expression of caspase-3 activity related with the accumulation of cytochrome *c* in cytosol. Our data show that mahanine can induce ROS production in HL-60 cells and affects normal cell cycle progression.

2. Materials and methods

2.1. Reagents

Fluorogenic caspase substrates and specific caspase inhibitors were obtained from Peptide Institute Inc. Z-Asp-CH_2-DCB general caspase inhibitor, cyclosporin A (cs A), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbo-cyanine iodide (JC-1) were obtained from Wako Pure Chemicals. For Western blotting, anti-PARP (poly(ADP-ribose) polymerase) IgG was obtained from Santa Cruz Biotechnology, anti-cytochrome *c* IgG was obtained form R&D System, Inc. All other chemicals were purchased from Sigma unless otherwise mentioned.

2.2. Cell line

HL-60 cells (human myeloid leukemic cells, RCB0041 obtained form Riken Gene Bank) were maintained in RPMI 1640 medium (Gibco BRL Life Technologies) supplemented with 10% fetal bovine serum (FBS) and antibacterial antimycotic solution (Sigma). Cells were grown in a humidified incubator at 37° under 5% CO_2/air and used for assays during exponential phase of growth.

2.3. Cell proliferation activity assay

After 2 days of treatment with 10 μM mahanine the proliferation of HL-60 cells was measured by colorimetric method using alamar blue (Biosource International). Cell proliferation activity was also measured by counting viable cells using the trypan blue exclusion method.

2.4. Analysis of morphological changes and measurement of sub-diploid cells by flow cytometry

HL-60 cells treated with or without mahanine were fixed with 1% glutaraldehyde in PBS and the nuclei were stained with 1 mM bisbenzimide (Hoechst) 33258 (Wako Pure Chemical Industries) in PBS. Morphological changes were analyzed under a light or fluorescence microscope. Formation of sub-G0/G1 peak in mahanine-induced apoptosis was investigated by flow cytometry. For this, cells (1.25×10^6/5 mL) treated with or without mahanine were washed with PBS, and fixed in suspension in 70% ethanol on ice and then stored at −20° for more than 12 hr. After three washes with PBS fixed cells were incubated with 1 μg/mL RNAse at 37° for 30 min, and stained with propidium iodide (20 μg/mL) in the dark at room temperature for 20 min. The stained cells were analyzed on a FACSort flow cytometer (Becton Dickinson) for relative DNA content.

2.5. Analysis of DNA fragmentation

DNA fragments in control and mahanine-treated cells were measured by terminal deoxynucleotidyl transferase-mediated fluorescent isothiocyanate deoxyuridine triphosphate (FITC-dUTP) nick end labeling (TUNEL) assay (APO-DIRECT, Phoneix Flow Systems Inc). The cells were fixed with 1% paraformaldehyde, followed by fixation with 70% ethanol. The DNA was subjected to an *in situ* tailing reaction in which residues of fluoroscent deoxyuridine triphosphate nucleotides (F-dUTP) were added catalytically to the 3′-OH sites of DNA strand breaks by terminal deoxynucleotidyl transferase (TdT). Cells were analyzed by flow cytometry on a FACSort (Beckton Dickinson). A concentration-dependent formation of fragmented DNA, in the presence of mahanine was determined spectroflurometrically by 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) method as described in a previous report [17]. The formation of high molecular weight and oligonucleosomal DNA fragments was examined by agarose gel electrophoresis with an Apoptosis Ladder Detection kit (Wako Pure Chemicals).

2.6. Assay for caspase-like activity

Cells were washed with ice cold PBS and lysed in lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP 40, 2 mM EDTA, 1 mM DTT, 1% phenylmethylsulfonyl

fluoride (PMSF), 10 μg/mL of each of leupeptin and aprotinin) for 30 min on ice followed by centrifugation at 15,000 *g* for 10 min. Twenty micro liter of protein solution containing 30 μg protein was mixed with 10 μL of 10 mM fluorogenic report substrate specific for each caspase such as 4-methyl-coumaryl-7-amide (YVAD-MCA) for caspase-1, Ac-DEVD-MCA for caspase-3, VEID-MCA for caspase-6, Ac-IETD-MCA for caspase-8, Ac-LEHD-MCA for caspase-9. The reaction mixture was mixed with 2970 μL of assay buffer (20 mM Hepes, pH 7.4, 0.1 M NaCl, 5 mM DTT, 0.1% NP 40) and incubated at 37° for 1 hr. Liberation of 7-amino-4-methyl-coumarin (AMC) was monitored using a spectrofluorometer with a pair of excitation/emission wavelength of 360/450 nm. For caspase inhibition assay following inhibitors were used. Benzyloxycarbonyl-L-aspart-1-yl-[(2,6-dichlorobenzoyl)oxy]methane Z-Asp-CH_2-DCB for interleukin-1-converting enzyme (ICE)-like protease (general caspase inhibitor), Ac-DEVD-CHO for caspase-3, Ac-LEHD-CHO for caspase-9. The inhibitors were diluted in DMSO and a 100 μg/mL (or 100 μM) of inhibitors were added to the culture 30 min to 15 hr before or during mahanine-treatment. Cell viability was measured using trypan blue exclusion method.

2.7. Western blot analysis of PARP cleavage and cytochrome c release

For PARP cleavage study cells were washed with ice-cold PBS containing protease inhibitors (1 mM PMSF and 0.5 mg/mL each of leupeptin and apoprotinin), and solubilized in a reducing loading buffer (62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.003% bromophenol blue, 5% 2-mercaptoethanol). Samples were sonicated on ice, and then incubated at 65° for 15 min. After centrifugation, an aliquot representing 1.5×10^5 cells was separated on a10% SDS–polyacrylamide. For cytochrome *c* analysis, cells were washed with ice cold PBS and resuspended in lysis buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM Hepes–NaOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, and protease inhibitors (100 μg/mL PMSF, 10 μg/mL leupeptin, 1 μg/mL chymostatin). After 20 min incubation on ice, cells were lysed by pushing them through a 22-gauge needle. The lysate was then centrifuged at 14,000 *g* for 15 min. The supernatants were removed and analyzed on 15% SDS–PAGE. After separation the proteins were transferred on to a polyvinylidene fluoride (PVDF) membrane (Amersham). The membrane was incubated with blocking solution (5% non-fat dry milk in TBS containing 0.1% Tween 20) overnight. They were then incubated with primary antibodies against PARP (1:500), cytochrome *c* (1: 2000) in Tris-buffered saline-Tween buffer. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:1250), and visualized by incubation of the membrane with the chemiluminescence Western blot kit (ECL, Amersham).

2.8. Flowcytometric analysis for mitochondrial membrane potential in HL-60 cells

HL-60 cells (1.5×10^6/5 mL) were cultured with either medium or medium containing the indicated concentrations of mahanine for up to 24 hr. At the end of the incubation cells were collected and washed twice with PBS, then stained with JC-1 (2 μg/mL) at room temperature in the dark for 15 min. After washing twice with PBS, FACS analysis of JC-1 fluoresecence was performed in a FACSort flow cytometer (Becton Dickinson) to determine the mitochondrial membrane potential in cells.

2.9. Treatment of cells with cs A

Cells were pretreated with cs A (dissolved in DMSO) at doses ranged from 0 to 10 μM for 30 min prior to the treatment of mahanine at 10 μM for 0–12 hr. Final concentration of DMSO never exceeded 0.2% level. Control cells were exposed to an equivalent concentration of DMSO. Cell viability was estimated by trypan blue exclusion method.

2.10. Spectrofluorometric determination of ROS

Cells (1.25×10^6/5 mL) were treated with 10 μM mahanine for up to 9 hr in RPMI 1640 (without phenol red) containing 5% serum. In some experiments *N*-acetyl-L-cysteine (NAC, 1–15 mM) and glutathione (GSH, 1–10 mM) were added 30 min before or during mahanine treatment. After three washes cells were resuspended in RPMI 1640 (without phenol red, with 5% FBS) containing 20 μM DCFH-DA (dichlorofluorescein diacetate). After 30 min of incubation at 37° cells were washed three times and resuspended in RPMI 1640 (without phenol red, with 5% FBS). The fluorescence intensity of the suspension was recorded using a RF-5300 PC Shimadzu Spectroflurometer with an excitation at 485 nm and an emission at 530 nm.

2.11. Analysis of data

All data are presented in this report as means ± SD of three separate experiments, and ANOVA with student Newman–Keuls post hoc comparison was used for statistical significance with $P \leq 0.05$. All the figures shown in this article were obtained from at least three independent experiments with a similar pattern.

3. Results

3.1. Effect of mahanine on cell proliferation, morphological changes, and cellular DNA of HL-60 cells

The doubling time of HL-60 was about 24 hr. To assess the effect of mahanine on cell proliferation alamar blue

reduction and trypan blue exclusion methods were used. Mahanine inhibited proliferation of HL-60 cell in a concentration- and time-dependent manner. Proliferation inhibition was observed as early as 8 hr, and a complete inhibition was detected when cells were treated with mahanine at a concentration higher than 10 μM for 72 hr. About 8.5 μM of mahanine inhibited 50% of cell growth after 48 hr. Morphological changes such as cell shrinkage, condensed and fragmented chromatin, associated with apoptotic cell death were observed as early as 4 hr under fluorescence microscopy using bisbenzamide dye (Hoechst) 33342 in the cells treated with 10 μM of mahanine but not in control cells (Fig. 1A). Figure 1B represents the cells treated with mahanine (10 μM) for 8 hr.

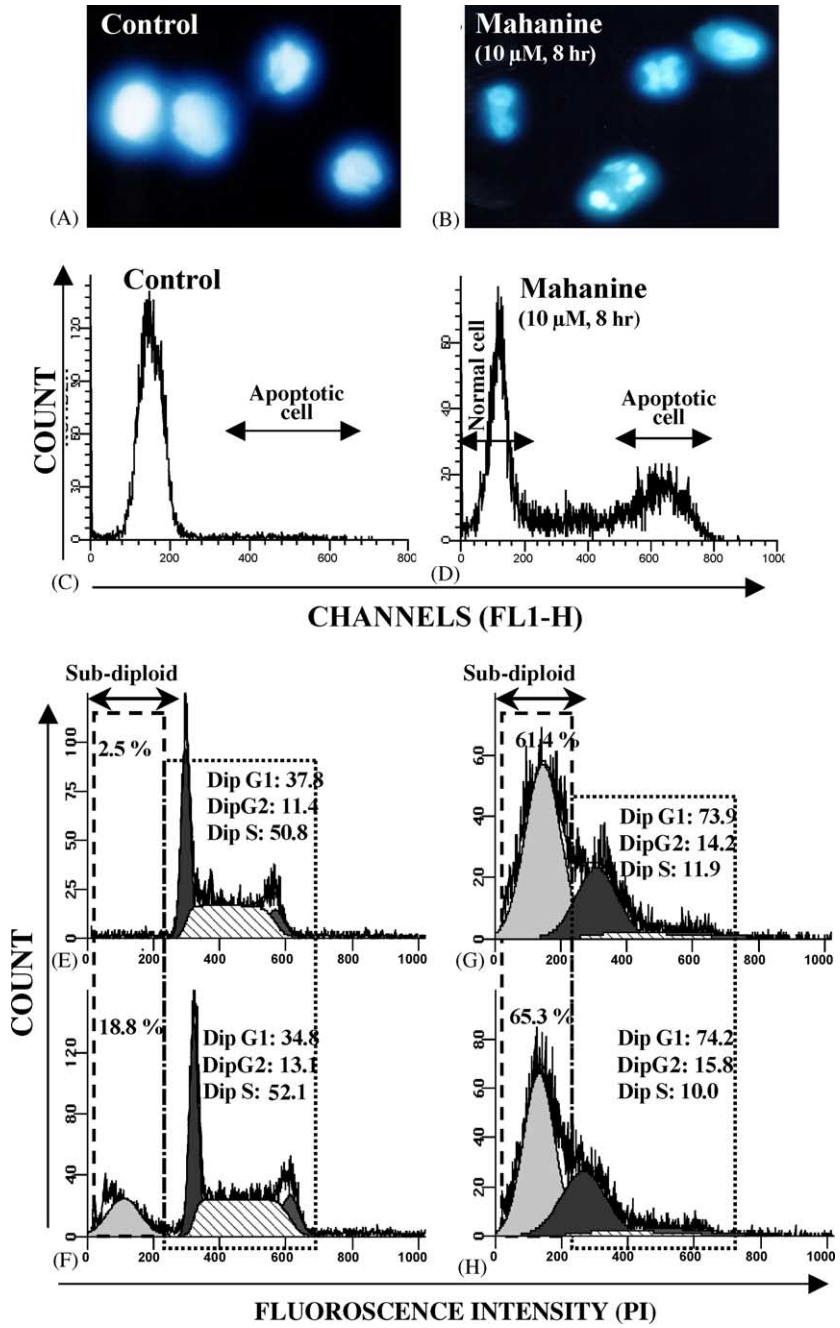

Fig. 1. Effect mahanine on cell morphology, DNA fragmentation and DNA distribution in HL-60 cells. Panels A and B: HL-60 cells were incubated with either media (control, panel A) or with 10 μM mahanine for 8 hr. Cells were stained with bisbenzimide (Hoechst) 33258 and examined under a fluorescent microscope. Panels C and D: cells were analyzed by TUNEL reaction using FACS. The vertical and horizontal axes represent cell number and FL1-H, respectively. Cells shifted to higher FL1-H represent TUNEL positive (apoptosis) cells (panel D). Panels E–H: cells were fixed and stained with PI as described under Section 2. Number of cells in different phases was determined by flow cytometry. Panel E represents control cells, panels F, G, and H represent cells when treated with 10 μM mahanine for 6, 24, and 48 hr, respectively. Results are representative from three separate experiments.

TUNEL assay, based on flow cytometry was used to detect the DNA breaks and total cellular DNA in cells exposed to mahanine. A substantial increase in the fluorescence of labeled DNA breaks in mahanine (10 μM) treated cells was observed at 8 hr compared to the control cells (Fig. 1C and D), an indicative of the occurrence of apoptosis.

Cell cycle analysis by flow cytometry was used to quantitatively estimate the number of cells in each phase of cell cycle. For this DNA distribution in HL-60 cells after treatment with mahanine (10 μM) for 6, 24 and 48 hr was analyzed. The untreated cells (Fig. 1E) showed a typical distribution in dip G1, dip S, and dip G2 phases on flow cytometry. After mahanine treatment, number of cells in sub-diploid area increased by 18% at 6 hr and more than 60% at 24 hr. The data also showed that transition of cells from diploid regions occurs with the accumulation of cells in dip G1 region, particularly at late hours.

3.2. Effect of mahanine on DNA fragmentation and DNA laddering

DNA fragmentation is a typical feature of apoptosis [18]. The extent of DNA fragmentation induced by mahanine was quantified by a method that involved separation of low molecular weight DNA by centrifugation (27,000 *g*) and staining with DAPI. HL-60 cells treated with mahanine at a concentration over 5 μM for 10 hr resulted in remarkable increase in DNA fragmentation (Fig. 2A). Kinetic analysis of DNA fragmentation revealed that formation of fragmented DNA in HL-60 cells became apparent at 6–12 hr after incubation with 10 μM mahanine (Fig. 2B). A time dependent occurrence of cellular DNA fragmentation was also studied by gel electrophoresis, and a ladder like pattern, typical of DNA cleavage between nucleosomes was visible during 6–12 hr after incubation with 10 μM mahanine (Fig. 2C).

3.3. Effect of Z-Asp-CH_2-DCB on mahanine-induced DNA fragmentation

Z-Asp-CH_2-DCB, a pan caspase inhibitor was used to investigate the possible role of caspases on mahanine-induced apoptosis. DNA fragmentation induced by mahanine was effectively suppressed when cells were pretreated with 100 μg/mL of Z-Asp-CH_2-DCB as analyzed using agarose gel electrophoresis and DAPI method (Fig. 3). The results indicate that activation of caspases were crucial for mahanine-induced apoptosis in HL-60 cells.

3.4. Effect of mahanine on caspase-like activity

Because of the inhibitory effect of Z-Asp-CH_2-DCB on the fragmentation of DNA in mahanine-induced apoptosis, we examined the effect of mahanine on the activation of caspase-1, -3-, -6-, -8- and -9-like activities in HL-60 cells. For this purpose, we incubated cytosolic extract of control cells and mahanine-treated cells with specific caspase substrates for each caspase. As shown in Fig. 4, mahanine treatment resulted in time dependent increase in the proteolytic activity of various caspases in HL-60 cells by comparing the activity of each caspases in the control cells. A significant increase in the activity of caspase-3 and -6 was observed since 8 hr of mahanine treatment. Activity of caspase-8 and -9 slowly increased until 12 hr of mahanine treatment. However, a sharp increase in the activity of caspase-3 and -6 was observed when the activity of caspase-8 and -9 significantly increased. The activity of caspase-1 was almost unaffected by mahanine treatment.

3.5. Effects of Z-Asp-CH_2-DCB, Ac-DEVD-CHO and Ac-LEHD-CHO on mahanine-induced cell death

The effect of a pan caspase inhibitor (Z-Asp-CH_2-DCB), and two specific caspase inhibitors, Ac-DEVD-CHO for

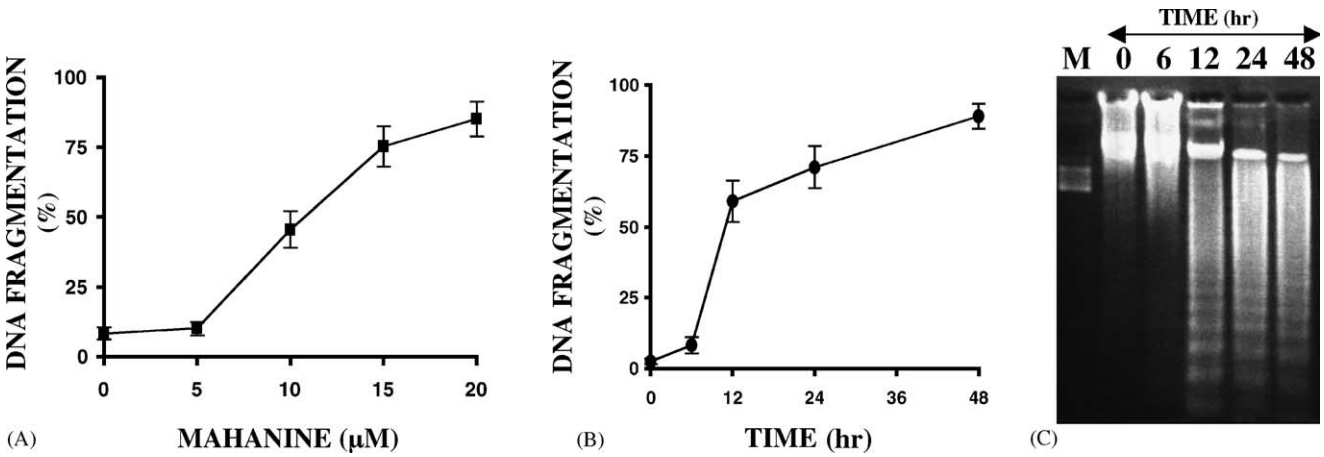

Fig. 2. Mahanine-induced dose and time-dependent DNA fragmentation and DNA laddering in HL-60 cells. (A) Cells were incubated with mahanine as indicated for 10 hr and DNA fragmentation was estimated by DAPI method. (B and C) Concentration-dependent DNA fragmentation at 10 hr measured by DAPI method or visualized by gel electrophoresis. Data are means ± SD derived from three separate experiments or representative from three separate experiments.

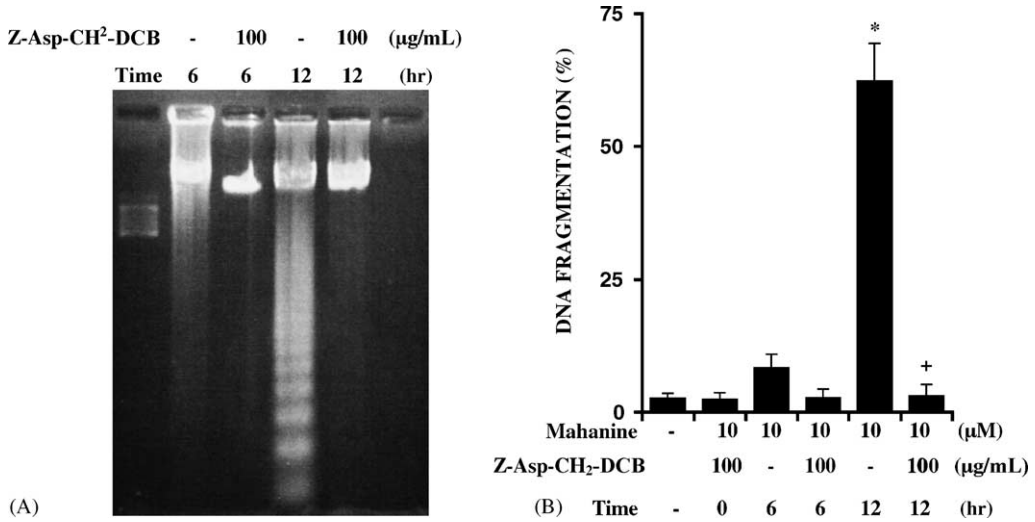

Fig. 3. Effect of a pan caspase inhibitor on mahanine-induced DNA fragmentation. Cells were pretreated with or without pan caspase inhibitor (Z-Asp-CH_2-DCB) for 30 min, and then incubated with 10 μM of mahanine for 6 and 12 hr. DNA fragmentation was visualized by agarose gel electrophoresis (A, representative from three separate experiments) or estimated by DAPI method (B, data are means ± SD derived from three separate experiments, $^{*}P \leq 0.05$ vs. control, $^{+}P \leq 0.05$ vs. mahanine alone).

caspase-3 and Ac-LEHD-CHO for caspase-9 on mahanine-induced apoptosis was investigated. Z-Asp-CH_2-DCB completely prevented mahanine-induced cell death. Among the specific caspase inhibitors, Ac-DEVD-CHO was found to be most effective in preventing cell viability loses (Fig. 5). The cell survival reduced to 30% at 12 hr after 10 μM of mahanine treatment was increased up to 74% when the cells were pretreated with a caspase-3 inhibitor (100 μM) however at the same condition a caspase-9 inhibitor increased cell viability up to 40%.

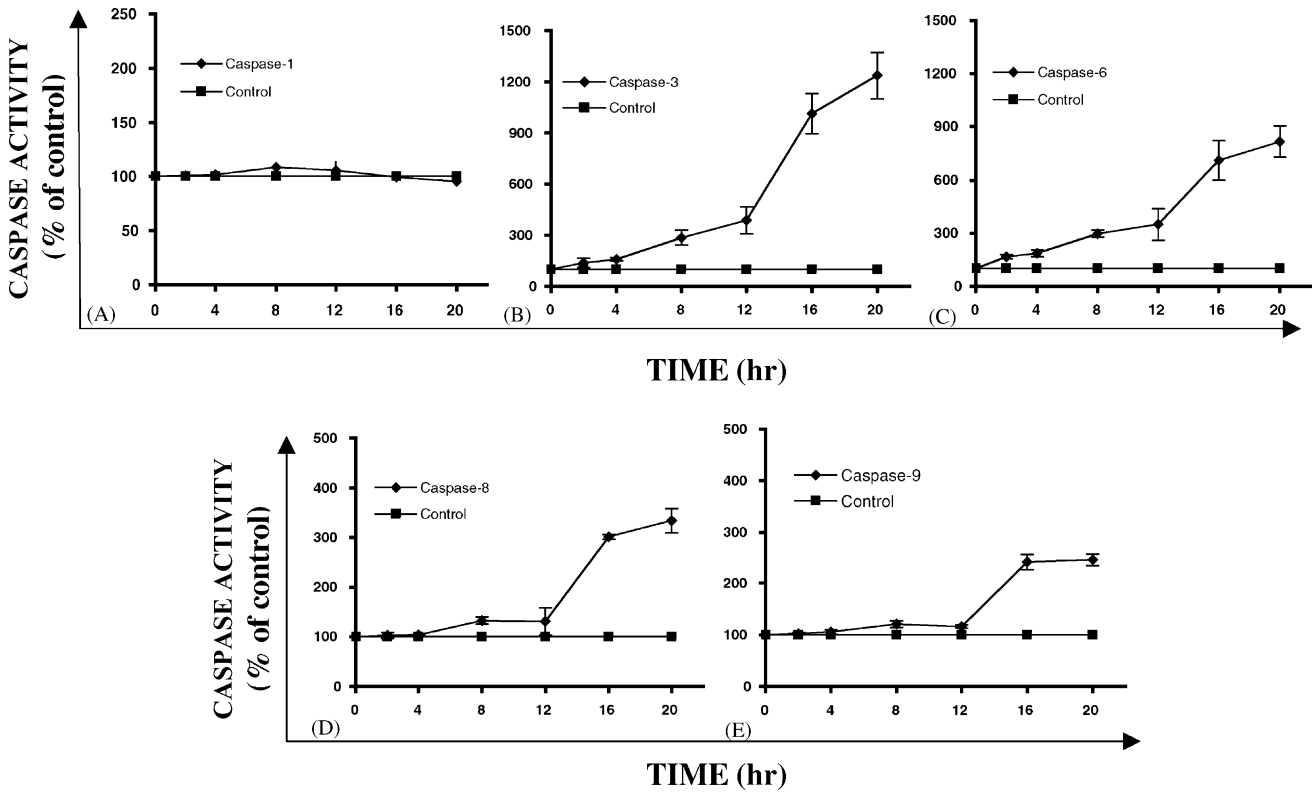

Fig. 4. Time course activation of various caspase-like enzymes in mahanine-treated HL-60 cells. HL-60 cells were incubated with 10 μM of mahanine for various time periods (0, 2, 4, 8, 12, 16, and 20 or 24 hr). Cytosolic fraction of cells was analyzed for changes in the activity of caspase-1 (A), caspase-3 (B), caspase-6 (C), caspase-8 (D), and caspase-9 (E) were determined as described in the Section 2. Data are means ± SD, N = 3. $^{*}P \leq 0.05$ vs. control.

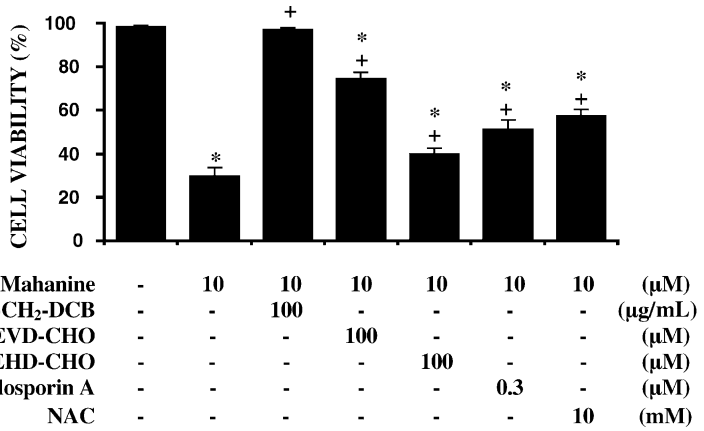

Fig. 5. Inhibition of mahanine mediated proliferation inhibition by caspase inhibitors and cs A. HL-60 cells were pretreated with or without Z-Asp-CH_2-DCB (pan caspase inhibitor), Ac-DEVD-CHO (caspase-3 inhibitor), Ac-LEHD-CHO (caspase-9 inhibitor), cs A and NAC for 30 min and exposed to 10 μM mahanine for 10 hr. Cell viability was measured by trypan blue exclusion method. Data are means ± SD, N = 3. $^{*}P \leq 0.05$ vs. control, $^{+}P \leq 0.05$ vs. mahanine alone.

3.6. Effect of mahanie on the cleavage of PARP

An additional evidence of caspase-3 activation was the cleavage of PARP as investigated by Western blot analysis using a mouse monoclonal antibody. Incubation of cells with mahanine resulted in the formation of 85 and 31 kDa protein fragments compared to the intact protein (116 kDa) in a time-dependent manner. Figure 6A shows the cleavage of PARP in HL-60 cells started at 4 hr after treatment with mahanine (10 μM) and became clearer when caspase-3 activation increased significantly.

3.7. Mahanine induced releases of cytochrome c in cytosol

To unravel the role of mitochondria in the system of mahanine-induced apoptosis, we tested the effect of mahanine on cytochrome *c* release by evaluating the cytochrome

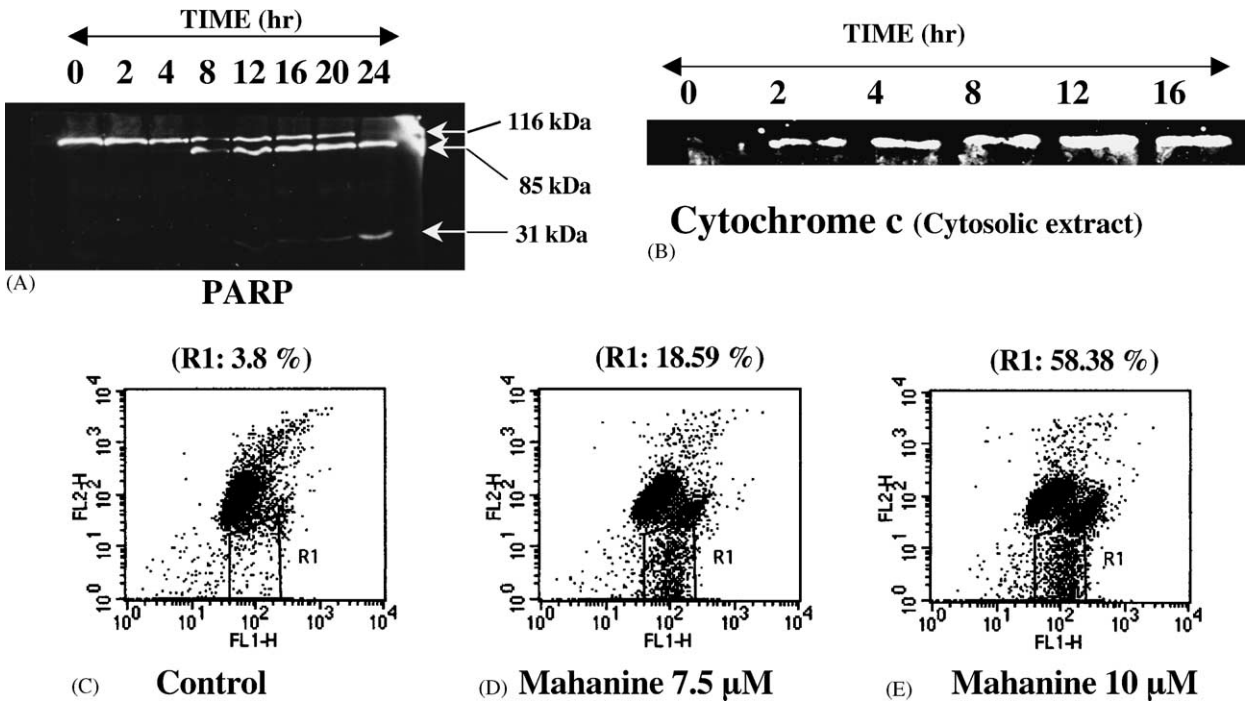

Fig. 6. Time-dependent cleavage of PARP, cytosolic accumulation of cytochrome *c*, and concentration-dependent depolarization of mitochondrial membrane in mahanine-induced apoptosis. (A and B) The cells were treated with 10 μM mahanine for the indicated periods. Total cell lysates were prepared and analyzed by immunoblotting for PARP analysis as described under Section 2 (A). Cytosolic fraction (about 30 μg) was analyzed to detect release of cytochrome *c* in cytosol by immunoblotting with an anti-cytochrome *c* antibody (B). (C–E) Cells were incubated with media (C, control) or mahanine (B and C) as indicated for 12 hr, then intensity of JC-1 fluorescence was analyzed as described under Section 2. Similar results were obtained in three separate experiments.

c content in cytosol of HL-60 cells treated with 10 μM mahanine through 0–20 hr. As shown in Fig. 6B, progressive accumulation of cytochrome *c* in cytosolic compartment is demonstrated. The release of cytochrome *c* was observed as early as 2 hr of mahanine treatment and peaked at 12 hr. The release of cytochrome *c* preceded the activation of caspase-3 and -6, PARP cleavage thus suggesting that it might be important in the execution of mahanine-induced apoptosis.

3.8. Effect of cs A on mahanine-induced cell death

To reveal the potential role for cytochrome *c* in mahanine-induced apoptosis in HL-60 cells, we examined the effect of cs A, a mitochondrial membrane permeability transition (MPT) inhibitor [19] on mahanine-induced cell death. As shown in Fig. 5, cs A (0.3 μM) pretreatment inhibited mahanine-induced cell death, which suggested mitochondrial swelling or mitochondrial membrane permeabilization plays an important role in mahanine-induced apoptosis.

3.9. Mahanine depolarizes mitochondrial membrane in HL-60 cells

Gradual increase of cytosolic cytochrome *c* in mahanine-treated cells and the inhibitory effect of cs A on mahanine-induced cell death increased the possibility of depolarization of mitochondrial membrane in HL-60 cells in the presence of mahanine. To confirm this, we used JC-1 that selectively enters into mitochondria and depending on the membrane potential this molecule is able to form J-aggregates that stains red. However, in the cells with mitochondrial membrane becomes more polarized monomeric JC-1 stains cytosol green. HL-60 cells treated with mahanine for 12 hr showed decreased intensity in the fluroscence of JC-1 aggregates (Fig. 6C–E) in a dose dependent manner which was concomitant with the fragmentation of nucleosomal DNA. Lower intensity in the fluorescence of JC-1 aggregates was also evident when cells were treated with mahanine in a time-dependent manner. The results suggested that mahanine depolarizes mitochondrial membrane, and the event is crucial in the release of cytochrome *c* into cytosol.

3.10. Mahanine-induced ROS generation and cytotoxicity in HL-60 cells

The probe DCHF-DA was used to monitor the generation of ROS in mahanine-treated HL-60 cells. DCFH-DA is converted by ROS to the highly fluorescent 2′7′-dichlorofluorescine (DCF), which can easily be monitored by a spectrofluorometer. Figure 7 shows the significant increase in fluorescent intensity (DCF formation) when HL-60 cells were treated with 10 μM of mahanine. After 6 hr of incubation, mahanine increased ROS generation almost

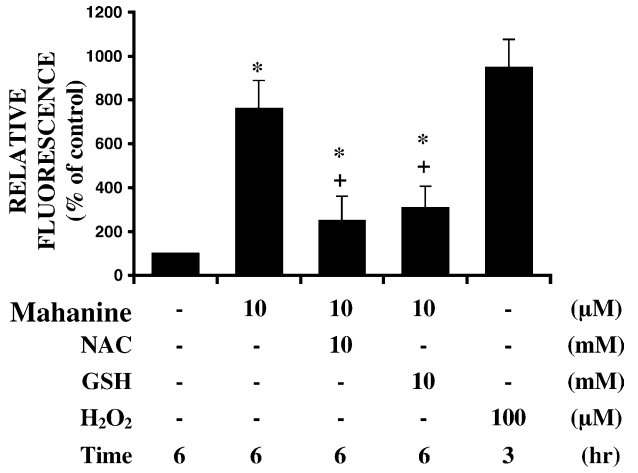

Fig. 7. ROS production in mahanine-induced cells. HL-60 cells were pretreated or not with NAC and GSH (10 mM) for 30 min and further incubated with or without mahanine (10 μM) for indicated times 6 and 12 hr before incubation with DCFH-DA for 30 min. For each time point fluorescence intensity was recorded by spectrofluorometry. Results were expressed as the relative fluorescence intensity with respect to untreated cells. A positive control, H_2O_2-treated cells was used to monitor the level of ROS. Data are means ± SD derived from three separate experiments. $^{*}P \leq 0.05$ vs. control, $^{+}P \leq 0.05$ vs. mahanine alone.

five times compared with the signals generated by control cells (Fig. 7). Whether ROS generation has any role in the events involved in the mahanine-induced apoptosis, we examined two antioxidants (NAC, GSH) for their capacity to interfere with the cell death process. For this purpose, HL-60 cells (1×10^5 cells/mL) were grown in culture medium for 24 hr, and then incubated with antioxidants at doses from 1 to 15 mM for 30 min. After pretreatment, mahanine (10 μM) was added to the medium and cell viability was measured using trypan blue exclusion method. Among the antioxidants, NAC was very effective in suppressing cell death induced by mahanine at concentrations used in other experiments (Fig. 5).

4. Discussion

There are reports that indicate mahanine possess a wide variety of physiological activities [7,8,20]. It has also been shown that the vegetable species *M. minutum*, *M. koenigii*, *M. zeylanicum*, and *M. euchrestiolia* have significant pharmacological values, the edible parts of which contain many cabazole alkaloids [7–10,20,21] including mahanine. Very recently, identifying active components from foods with apoptosis inducing activity against cancer cell lines has been emphasized as apoptosis is considered as a primary mechanism for chemoprevention of cancer. Thus, the studies devoting to assess their mechanism(s) of action on cancer cells appear of remarkable importance.

In this investigation we have characterized the effects of mahanine on the proliferation of human promyelocytic cell

lines namely HL-60 cells. Primary results demonstrated that mahanine inhibited growth of HL-60 cells in a concentration at range 6–10 μM and the values probably be expected *in vivo* due to mahanine content (4% w/w) in the leaves of *M. minutum* [7].

Further analysis demonstrated that cultured HL-60 cells treated with mahanine exhibited morphological features of apoptosis such as membrane shrinkage, and chromosomal condensation (Fig. 1B), what is supported by additional findings. A landmark of cellular self-destruction by apoptosis is the degradation of higher order chromatin structure of DNA into fragments of 50–300 kbp. These DNA fragments result in the appearance of ladder like pattern when analyzed by agarose gel electrophoresis. The presence of fragmented DNA in mahanine-treated cells was first confirmed using TUNEL assay (Fig. 1C and D). Then concentration- and time-dependent formation of fragmented DNA was determined, or visualized by means of DAPI method and agarose gel electrophoresis (Fig. 2). We observed that morphological changes appeared in nuclear chromatin were earlier than the formation of fragmented DNA.

Mahanine-induced DNA fragmentation was completely prevented by the pretreatment of cells with Z-Asp-CH_2-DCB (100 μg/mL) a cell permeable inhibitor of cysteine proteases (Fig. 3) as determined by agarose gel electrophoresis and DAPI method. The cells pretreated with Z-Asp-CH_2-DCB showed no change in cellular morphology when treated with mahanine. The results primarily confirmed the involvement of caspases in mahanine-induced cell death. There are several caspases that play important role in the regulation of apoptosis. They are broadly grouped into initiator or effector caspases, due to the role they play in apoptosis inducing system. The cellular level of these caspases greatly varied depending on the inducers and type of cell lines. Here it is worth mentioning that activation of caspases is a hallmark of promoting apoptosis in response to death inducing signals originated form cell surface receptors, mitochondria or endoplasmic reticulum [21–23]. In particular, activation of caspase-3 plays the central role in the initiation of apoptosis [24,25]. This enzyme has a substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves poly(-ADP-ribose) polymerase (PARP). Our study revealed that mahanine induced the elevation of some caspases including caspase-3, -6, -8 and -9 but not caspase-1 as shown in Fig. 4. During investigation, from 2 to 20 hr, caspase-3 activation was dominant and reflected in the cleavage of PARP, a well-known caspase-3 substrate. Treatment of HL-60 cells with 10 μM mahanine-induced proteolytic cleavage of PARP (116 kDa) with the accumulation of 85 and 26 kDa cleaved products. PARP cleavage was apparent at 4 hr after 10 μM mahanine treatment with similar time course analysis of evaluating caspase-3 like activity. In mahanine-induced apoptosis, involvement of caspase-3 was further confirmed when Ac-DEVD-CHO (100 μM), a specific inhibitor of caspase-3 prevented cell death as more than 70% cells remained viable after 10 hr of mahanine (10 μM) treatment with the co-treatment of the caspase-3 inhibitor (Fig. 5). It has been shown that activation of caspase-3 and -6, the major effector caspases, requires the activation of initiator caspases such as caspase-8 or -9 in response to the proapoptotic signals [22]. Until recently it was considered that caspase activation was largely due to the engagement of certain members of tumor necrosis factor with death receptors on cell surface. Caspase-8 is believed to be activated through this pathway [26]. In contrast, involvement of mitochondria has been found in caspase-9 activation [16]. There are also reports that emphasized on the presence of cytochrome *c* for activation of caspase-3. Liu *et al.* successfully proved that cytochrome *c* depleted cellular extract did not activate caspase-3 unless exogenous cytochrome *c* was added in the cellular extract [27]. However, we cannot rule out the involvement of caspase cascades in mahanine-induced caspase-3 activation. And the event is very clear, particularly after 8 hr of mahanine treatment when a rapid increase in the activity of effector caspases, caspase-3 and -6 was observed with the increased level in the activity of caspase-9 and/or caspase-8. Our results suggest us to conclude that cytosolic accumulation of cytochrome *c* and caspase cascades are the important factors in the activation of caspase-3 or -6 in mahanine-induced apoptosis. Increase of cell viability in the presence of cs A and caspase-9 inhibitor supports this. It is considered that depolarization of the mitochondrial membrane potential play important role in the release of cytochrome *c* into cytosol in many apoptosis inducing system. To verify the event in mahanine-induced apoptosis we used JC-1, and lower intensity in the fluroscence of JC-1 aggregates confirmed the loss of mitochondrial membrane potential in mahanine-treated HL-60 cells.

It is becoming increasingly clear that mitochondria play critical roles in the process that lead to cell death. Elevated level of oxidative stress might be the result of mitochondrial dysfunction and has been considered as a mediator of apoptosis [28,29]. Release of cytochrome *c* into cytosol in mahanine-treated HL-60 cells has increased our speculation of mitochondrial dysfunction. Therefore, we speculated that intracellular generation of ROS could also be an important factor in mahanine-induced cell suicide process. To verify this, we performed experiments to confirm the level of oxidative stress and the effects of antioxidant on mahanine-induced oxidative stress as well as cell death. HL-60 cells exposed to mahanine (10 μM) showed a significant elevation of ROS signals as measured using oxidation-sensitive fluorescent probe, DCFH-DA. Pretreatment with antioxidants, NAC, GSH, which are also used as free radical scavenger [30,31] caused a significant suppression in DCF fluorescence in mahanine-treated cells (Fig. 7). We also found that NAC exposure of HL-60 cells produced increased viability against mahanine and the result is in

agreement with the reports where antioxidants like NAC, GSH, Vit C have been shown to suppress apoptotic cell death [32] even though the mechanisms of action are not very clear.

Induction of apoptosis and/or cell proliferation inhibition is highly correlated with the activation of a variety of intracellular signaling pathways to arrest the cell cycle in the G1, S, or G2 phase. In malignant tumors cell population in G1 phase appear less frequent (<70%) than in normal tissue (>90%). The damages that cause G1-check point arrest are believed to be irreversible process, and the cells ultimately undergo apoptosis. Our data showed that mahanine causes the increase of cells in sub-diploid regions in a time-dependent manner, with the decrease of cells in diploid regions (G1, G2 and S-phase). Thus, it can be concluded that mahanine has the ability to induce apoptosis in proliferating and resting cells. However, the cells in diploid regions seem to be accumulated at G1 phase before shifting to sub-diploid region, as the percentage of cells at diploid-G1 region greatly increased with the decrease of cells in S and G2 phases. Here it will be worth mentioning that many anti-cancer drugs such as 6-mercaptopurin, thioguanin, prednisolone, tyrphostins, adriamycin [33–35] exert their anticancer effect by inhibiting cell cycle or inducing apoptosis, even though their discovery did not depend on their activities in apoptosis or cell cycle. Thus, it would be interesting to examine whether the mahanine-induced apoptosis and cell cycle perturbation can play any role in anticancer activity *in vivo*.

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References

[1] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456–62.
[2] Nicholson DW. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. Nat Biotechnol 1996;14:297–301.
[3] Majno G, Joris I. Apoptosis, oncosis, and necrosis: an overview of cell death. Am J Pathol 1995;146:3–15.
[4] Greenwald P, Kelloff G, Burch-Whitman C, Kramer BS. Chemoprevention. Cancer J Clin 1995;45:31–49.
[5] Potter JD, Steinmetz K. Vegetables, fruit and phytoestrogens as preventive agents. IARC Sci Publ 1996;139:61–90.
[6] Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc 1996;96:27–39.
[7] Nakahara K, Trakoontivakorn G, Alzoreky NS, Ono H, Onishi-Kameyama M, Yoshida M. Antimutagenicity of some edible Thai plants, and a bioactive carbazole alkaloid, mahanine, isolated from *Micromelum minutum*. J Agric Food Chem 2002;50:4796–802.
[8] Ramsewak RS, Nair MG, Strasburg GM, DeWitt DL, Nitiss JL. Biologically active carbazole alkaloids from *Murraya koenigii*. J Agric Food Chem 1999;47:444–7.
[9] Bowen IH, Perera KPWC. Alkaloids, coumarins and flavonoids of *Micromelum zeylanicum*. Phytochemistry 1982;21:433–7.
[10] Wu TS. Murrayamine-A, -B, -C (+) mahanine, carbazole alkaloids from *Murraya euchrestifolia*. Phytochemistry 1991;30:1048–51.
[11] Cohen GM. Caspases: the executioners of apoptosis (Pt 1). Biochem J 1997;326:1–16.
[12] Los M, Stroh C, Janicke RU, Engels IH, Schulze-Osthoff K. Caspases: more than just killers? Trends Immunol 2001;22:31–4.
[13] Martin SJ, Green DR. Protease activation during apoptosis: death by a thousand cuts? Cell 1995;82:349–52.
[14] Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 1999;68:383–424.
[15] Krammer PH. CD95s deadly mission in the immune system. Nature 2000;407:789–95.
[16] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.
[17] Roy MK, Kuwabara Y, Hara K, Watanabe Y, Tamai Y. Peptides from the N-terminal end of bovine lactoferrin induce apoptosis in human leukemic (HL-60) cells. J Dairy Sci 2002;85:2065–74.
[18] Cohen GM, Sun XM, Fearnhead H, MacFarlane M, Brown DG, Snowden RT, Dinsdale D. Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. J Immunol 1994;153:507–16.
[19] Pritchard DE, Singh J, Carlisle DL, Patierno SR. Cyclosporin A inhibits chromium(VI)-induced apoptosis and mitochondrial cytochrome *c* release and restores clonogenic survival in CHO cells. Carcinogenesis 2000;21:2027–233.
[20] Tachibana Y, Kikuzaki H, Lajis NH, Nakatani N. Antioxidative activity of carbazoles from *Murraya koenigii* leaves. J Agric Food Chem 2001;49:5589–94.
[21] Kong YC, Ng KH, But PP, Li Q, Yu SX, Zhang HT, Cheng KF, Soejarto DD, Kan WS, Waterman PG. Sources of the anti-implantation alkaloid yuehchukene in the genus *Murraya*. J Ethnopharmacol 1986;15:195–200.
[22] Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. FASEB J 1996;10:587–97.
[23] Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269–90.
[24] Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. Proc Natl Acad Sci USA 1999;96:10964–7.
[25] 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, Miller DK. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995;376:37–43.
[26] Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305–8.
[27] Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 1996;86:147–57.
[28] Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. J Biol Chem 2001;276:4588–96.
[29] Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, Robinson JP. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem 2003;278:8516–25.

[30] Kong AN, Yu R, Lei W, Mandlekar S, Tan TH, Ucker DS. Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis: the role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. Restor Neurol Neurosci 1998;12:63–70.

[31] Lappas M, Permezel M, Rice GE. *N*-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes *in vitro*. J Clin Endocrinol Metab 2003;88:1723–9.

[32] Li N, Ragheb K, Lawler G, Sturgis J, Rajwa BJ, Melendez JA, Robinson JP. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem 2003;278:8516–25.

[33] Tsurusawa M, Saeki K, Fujimoto T. Differential induction of apoptosis on human lymphoblastic leukemia Nalm-6 and Molt-4 cells by various antitumor drugs. Int J Hematol 1997;66:79–88.

[34] Palumbo GA, Yarom N, Gazit A, Sandalon Z, Baniyash M, Kleinberger-Doron N, Levitzki A, Ben-Yehuda. The tryphostin AG17 induces apoptosis and inhibition of cdk2 activity in a lymphoma cell line that overexpresses bcl-2. Cancer Res 1997; 57:2434–9.

[35] Siu WY, Yam CH, Poon RY. G1 vs. G2 cell cycle arrest after adriamycin-induced damage in mouse Swiss3T3 cells. FEBS Lett 1999;461:299–305.