

# Induction of caspase-dependent apoptosis in melanoma cells by the synthetic compound (*E*)-1-(3,4-dihydroxyphenethyl)-3-styrylurea

Ji-Hae Kim<sup>1</sup>, Young-Oh Jang<sup>2</sup>, Beom-Tae Kim<sup>1</sup>, Ki-Jun Hwang<sup>1</sup> & Jeong-Chae Lee<sup>1,3,\*</sup>

<sup>1</sup>Department of Bioactive Material Sciences and Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756,

<sup>2</sup>Korean Minjok Leadership Academy, Hoengseong 225-823, <sup>3</sup>Department of Orthodontics and Institute of Oral Biosciences, Brain Korea 21 program, Chonbuk National University, Jeonju 561-756, Korea

Recently, various phenolic acid phenethyl ureas (PAPUs) have been synthesized from phenolic acids by Curtius rearrangement for the development of more effective anti-oxidants. In this study, we examined the anti-tumor activity and cellular mechanism of the synthetic compound (*E*)-1-(3,4-dihydroxyphenethyl)-3-styrylurea (PAPU1) using melanoma B16/F10 and M-3 cells. Results showed that PAPU1 inhibited the cell proliferation and viability, but did not induce cytotoxic effects on primary cultured fibroblasts. PAPU1 induced apoptotic cell death rather than necrosis in melanoma cells, a result clearly proven by the shift of cells into sub-G<sub>1</sub> phase of the cell cycle and by the substantial increase in cells positively stained with TUNEL or Annexin V. Collectively, this study revealed that PAPU1 induced apoptosis in a caspase-dependent manner, suggesting a potential role as a cancer chemopreventive agent for melanoma cells. [BMB reports 2009; 42(12): 806-811]

## INTRODUCTION

Out of the numerous naturally occurring compounds used to treat disease through the reduction of oxidative stress, plant-derived phenolic compounds have been particularly identified. Accumulating evidence has shown that the anti-oxidant activities of certain fruits, vegetables and seeds are highly correlated with their total phenolic levels (1, 2). Phenolic compounds were also shown to possess anti-tumor and anti-inflammatory activities derived from their anti-oxidant properties (3-5).

Hydroxycinnamic acids are a major class of phenolic compounds contained in plants (6). Specifically, caffeic acid, ferulic acid and *p*-coumaric acid are found to produce various ben-

eficial effects, including anti-oxidation (7, 8), anti-inflammation (9), cancer chemoprevention (10) and anti-melanogenesis (6). Other findings suggest that phenolic acids containing aromatic hydroxyl groups have great potential for use in biological and pharmacological applications, a result derived from their anti-oxidant activities.

Considering that the anti-oxidant potential of phenolic compounds is closely correlated with anti-inflammation and anti-cancer activity, we focused on developing phenolic acid derivatives with stronger anti-oxidant activities than their corresponding phenolic acids. Recently, various phenolic acid phenethyl ureas (PAPUs) have been synthesized from phenolic acids containing one aromatic hydroxyl group using Curtius rearrangement. This study examined the degree to which each PAPU sample induces cell death in melanoma cells. In addition, the mechanisms by which these synthetic compounds cause cytotoxicity in cells were investigated by various cell-mediated assays capable of distinguishing the pattern of cell death, *i.e.* apoptosis or necrosis. Finally, we determined the cellular mechanism involved in PAPU-mediated apoptosis of melanoma cells.

## RESULTS

### PAPU1 decreases the viability and DNA synthesis of melanoma cells in a dose-dependent manner

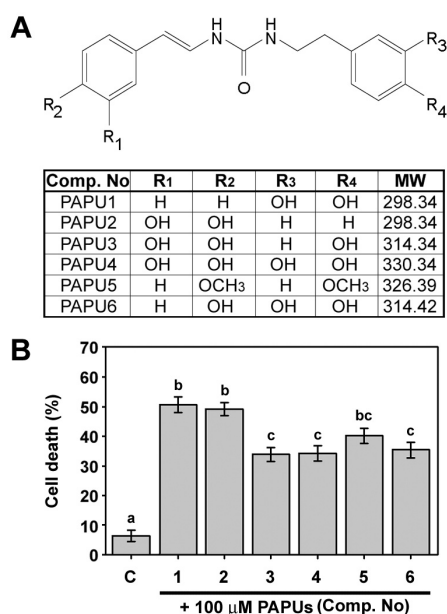
Since PAPU compounds have been observed as effective scavengers of DPPH radical, we initially selected six PAPU compounds thusly named PAPU1 to PAPU6 (Fig. 1A). Samples applied to cells exposed to hydrogen peroxide exhibited anti-oxidant activities in the following order: PAPU1 = PAPU2 > PAPU3, PAPU4, PAPU6 > PAPU5 (data not shown). Subsequent experiments using trypan blue staining showed that PAPU samples acted as anti-tumor agents in melanoma cells, and that PAPU1 and PAPU2 are the most effective forms (Fig. 1B). PAPU1, (*E*)-1-(3,4-dihydroxyphenethyl)-3-styrylurea, was therefore selected for use in the next experiments.

As shown in Fig. 2A, the addition of phenolic acids did not reduce cell viability, even at the highest concentration exam-

\*Corresponding author. Tel: 82-63-270-4049; Fax: 82-63-270-4049; E-mail: leejc88@chonbuk.ac.kr

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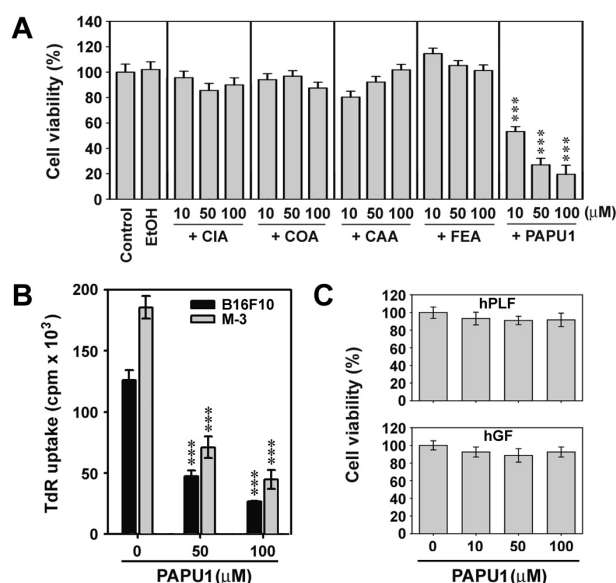


**Fig. 1.** Structures of PAPU samples and their cytotoxicity to B16F10 cells. (A) Structures of PAPUs synthesized by Curtius rearrangement by treating acylazide with phenethyl amines. (B) The cells were treated with 100  $\mu$ M of each PAPU sample for 48 h and processed for trypan blue staining assay. The superscripts represent significant differences ( $P < 0.05$ , Scheffe's test) among the experiments.

ined (100  $\mu$ M). However, treatment with PAPU1 markedly reduced the viability of B16F10 cells. Specifically, a 50  $\mu$ M treatment of PAPU1 reduced cell viability to  $23.1 \pm 4.5\%$  compared with untreated control. Similarly, PAPU1 caused a significant reduction in the viability of M-3 cells in a dose-dependent manner (data not shown). PAPU1 also inhibited DNA synthesis in both types of melanoma cells (Fig. 2B). To verify if PAPU1 exerts specific inhibition on the viability and proliferation of melanoma cells, we performed a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using primary cultured human gingival fibroblasts (hGF) and human periodontal ligament fibroblasts (hPLF) (Fig. 2C). The sample did not cause any loss of cell viability at the indicated concentrations.

### PAPU1 induces apoptotic cell death in melanoma cells

To understand the nature of PAPU1-mediated cytotoxicity in melanoma cells, PAPU1-exposed B16F10 and M-3 cells were subjected to various apoptosis detection assays. Changes in the number of B16F10 cells were initially characterized using flow cytometric analysis by permeabilizing and staining the cells with formaldehyde and propidium iodide (PI), respectively (Fig. 3A). In the absence of PAPU1, the proportion of cells in sub-G<sub>1</sub> phase of the cell cycle, also known as apoptotic cell death, was only 12.3% of the total cell population (Fig. 3D). However, the proportion of apoptotic cells was augmented upon treatment



**Fig. 2.** Inhibitory effects of PAPU1 on the viability and proliferation of melanoma cells. (A) B16F10 cells were treated with the indicated concentrations of phenolic acids or PAPU1 for 48 h and then processed for MTT assay. CIA, *e*-cinnamic acid; COA, *p*-coumaric acid; CAA, caffeic acid; FEA, ferulic acid. (B) B16F10 and M-3 melanoma cells were exposed to the indicated concentrations of PAPU1 for 24 h, then incubated in the presence of 1  $\mu$ Ci/ml [*methyl*-<sup>3</sup>H] thymidine deoxyribose for an additional 24 h. (C) hPLF and hGF were treated with various concentrations (10–100  $\mu$ M) of PAPU1 for 48 h, and their viabilities were analyzed by MTT assay. \*\*\* $P < 0.001$  vs. the untreated control values.

with PAPU1 in a dose-dependent manner. Following treatment with 10  $\mu$ M PAPU1 for 48 h, only 17% of cells were apoptotic, whereas more than 25% and 55% of the cells became apoptotic upon exposure to 50  $\mu$ M and 100  $\mu$ M PAPU1, respectively. Similar results were also found when M-3 cells were exposed to PAPU1 (data not shown).

The potential of PAPU1 to induce apoptosis in melanoma cells was also supported by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining. The majority of M-3 cells showed negative TUNEL staining in the absence of PAPU1 (Figs. 3B, 3E). However, when the cells were incubated with 100  $\mu$ M PAPU1 for 48 h, a large proportion showed intensive FITC-dUTP labeling. Similarly, exposure of B16F10 cells to PAPU1 increased the number of TUNEL positive cells (data not shown).

The results from Annexin V/PI double staining confirmed the potential of PAPU1 to induce apoptosis. Untreated control B16F10 cells showed either low or negative staining with both Annexin V and PI (Annexin V<sup>-</sup>/PI<sup>-</sup>), indicating non-apoptotic viable cells (Figs. 3C, 3F). Some cells were highly positive with Annexin V after exposure to PAPU1 for 48 h, but were almost entirely negative for PI uptake (Annexin V<sup>+</sup>/PI<sup>-</sup>). Similarly, M-3 cells were stained extensively with Annexin V

but not with PI (Annexin V<sup>+</sup>/PI<sup>-</sup>) after exposure to PAPU1 (data not shown). These results indicate that PAPU1 exerts its anti-tumor effects on melanoma cells by inducing apoptosis.

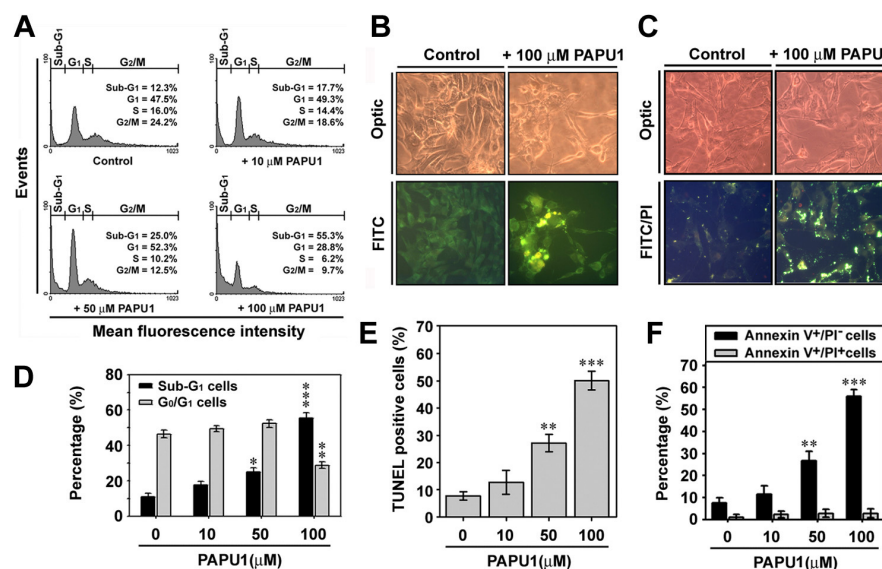
### PAPU1-induced apoptosis is mediated by a caspase-dependent pathway

Fig. 4A shows that the PAPU1-induced cytotoxicity of M-3 cells was significantly inhibited by the pancaspase inhibitor z-VAD-fmk (20 μM). However, any inhibitors of mitogen-activated protein kinases (MAPK) did not protect cells from PAPU1-mediated cytotoxicity. PAPU1 treatment seems to promote caspase-dependent apoptosis, as evidenced by the in-

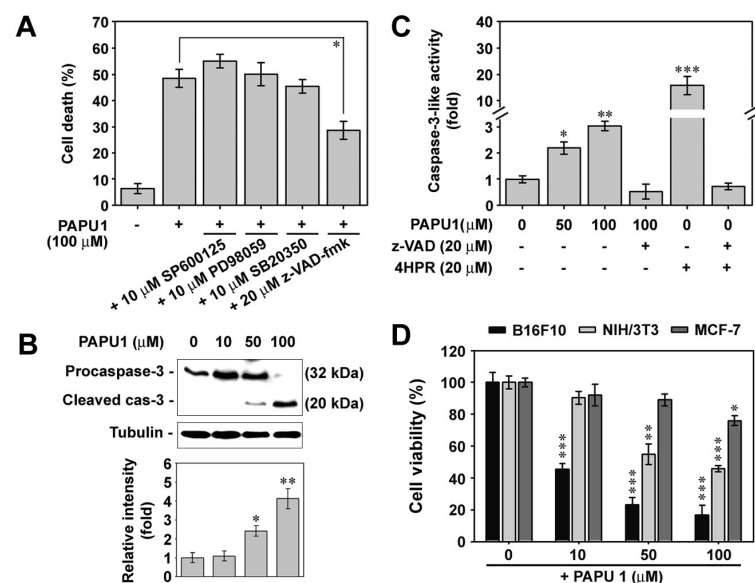
duction of a cleaved caspase-3 band and the attendant reduction of its inactive form (Fig. 4B). This was further supported by an observable increase in the activity of caspase-3 after cells were treated with PAPU1, an event completely inhibited by z-VAD fmk (Fig. 4C). Fig. 4D reveals that PAPU1-mediated cytotoxicity was more specific to melanoma cells than other cells such as NIH/3T3 fibroblasts and MCF-7 human breast cancer cells.

### DISCUSSION

The present study evaluated the hypothesis that potent anti-ox-



**Fig. 3.** PAPU1 induces apoptosis in melanoma cells. (A) Flow cytometric analyses of PAPU1-exposed B16F10 cells for 48 h after PI staining. The figures show a representative staining profile for 10,000 cells per experiment. (B) TUNEL staining of PAPU1-exposed M-3 melanoma cells for 48 h. Photographs were taken by fluorescence microscopy at 400× (Axioskop 2, Carl Zeiss). (C) Annexin V/PI double staining of B16F10 cells after exposure to 100 μM PAPU1 for 48 h. The figures in A, B and C show a representative result from three experiments. (D) The percentages of each cell cycle population after PI staining were calculated using the WinMDI 2.9 program. Sub-G<sub>1</sub> cells refer to apoptotic cells. The number of cells stained positively for TUNEL (E) and the percentages of Annexin V/PI-stained cells (F) were counted using fluorescence microscopy. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 vs. the untreated control values.



**Fig. 4.** PAPU1 induces caspase-dependent apoptosis in melanoma cells. (A) M-3 melanoma cells were exposed to 100 μM PAPU1 for 48 h in the presence of MAPK inhibitors or pancaspase inhibitor and then processed for trypan blue staining. \*P < 0.05 vs. the experiments. Cells were also treated with increasing doses (0-100 μM) of PAPU1 for 24 h, and the protein extracts were prepared for analysis of procaspase-3 cleavage by western blotting (B), or for determination of caspase-3 activity (C). N-(4-hydroxyphenyl) retinamide (4HPR, 20 μM) was used as a caspase-dependent positive control. (D) Various types of other cells were treated with PAPU1, and were tested for viability by MTT assay after 48 h of exposure. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 vs. the untreated control values.

idants possess high degrees of anti-tumor activities. Among PAPU samples, PAPU1 exhibited the highest anti-oxidant efficiency as well as the highest potential to induce cell death in melanoma cells. PAPU1 also reduced the viability of melanoma cells without inflicting any cytotoxic effects on normal cells. Altogether, these effects are thought to be related to the anti-proliferative potential of PAPU1 in that DNA synthesis was efficiently inhibited in the cells. Therefore, we postulate that the synthetic compound PAPU1 possesses anti-tumor activity that is associated with its status as an anti-oxidant.

The results of the comparative analysis of polyphenolic compounds with melanoma cells suggest that many flavonoids possess anti-proliferative potential, and that the anti-tumor activities of flavonoids vary depending on chemical structure (11). Most flavonic molecules with only one or two OH groups do not exhibit strong anti-proliferative activity (12). Furthermore, flavonoids with 4~6 OH groups act as powerful anti-oxidants, whereas those with fewer OH groups lacked substantial anti-oxidant potential (12, 13). These reports seem to imply that the number rather than the position of OH groups is the important factor for the anti-proliferative potential of phenolic compounds. Namely this could mean that anti-tumor potentials of polyphenolic anti-oxidants depends on an adequate content of OH groups according to the number of aromatic ring. From this point of view, we suggest that two OH groups than more or less OH groups are required for the strong anti-oxidant and anti-tumor potentials of PAPU compounds. However, it is not excluded that the position of OH groups in phenolic compounds could possibly influence the chemical properties of the molecules. Such an effect would be due to OH groups in the *ortho*-position of the B ring as well as those associated with a 2,3-double bond combined with a 4-keto group in the C-ring, as they are known to be important structural determinants for the anti-oxidant activity of flavonols (14).

Another mechanism that likely contributes to the anti-tumor activity of phenolic anti-oxidants is the induction of apoptosis. Apoptosis physiologically regulates the cell number of developing organs and organisms. In many types of tumor cells, the selective induction of apoptotic cell death is considered the most ideal approach for cancer treatment. Some hallmarks of apoptosis are cellular shrinkage, nuclear condensation and DNA fragmentation (15-17). The current study demonstrates that PAPU1 induces apoptotic death of melanoma cells in a dose-dependent manner without arresting cells in the G<sub>1</sub> and/or G<sub>2</sub>/M phases of the cell cycle. This conclusion is clearly supported by the migration of cells into sub-G<sub>1</sub> phase, as well as by the appearance cells stained positively with TUNEL or Annexin V after PAPU1 treatment. In addition, only inhibitors of pancaspase and not of MAPK suppressed PAPU1-mediated cytotoxicity in melanoma cells. Moreover, a cleaved form of caspase-3 was present with an increased level of activity in PAPU1-treated melanoma cells. From these results, we suggest that the PAPU-mediated inhibition of viability and proliferation

of melanoma cells is related to the direct induction of apoptosis by executive caspases.

We also found that melanoma cells are more sensitive to PAPU1-induced cell death than any other cell type examined. Therefore, it is likely that the cellular response to PAPU1 varies according to cell type and the physiological conditions present. More detailed experiments are needed to verify the mechanism through which PAPU1 exerts specific effects on melanoma cells.

Many biochemical and clinical studies have suggested that anti-oxidants are capable of treating disease mediated by oxidative stress. Specifically, phenolic acids can be absorbed through the intestine by an active transport system whereupon they can prevent oxidative stress (18, 19). In addition, compounds containing one or more aromatic hydroxyl groups replace  $\alpha$ -tocopherol as a chain-breaking anti-oxidant and restore reduced glutathione as a protective agent in cells (15). The results in this report show that PAPU1 containing two OH groups exerts anti-tumor effects that are stronger than commonly generated phenolic acids, which only contain one OH group. These findings suggest that the synthetic compound PAPU1 can be used as an anti-cancer agent for the treatment of melanoma. However, more detailed *in vivo* studies on PAPU1 are needed in order to elucidate the mechanisms responsible for the anti-tumor effects on melanoma cells.

## MATERIALS AND METHODS

### Chemicals and plastics

Unless otherwise specified, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and all plastics were Falcon Labware obtained from Becton-Dickinson (Franklin Lakes, NJ, USA). PAPU samples, synthesized and identified in the Research Center of Bioactive Materials at Chonbuk National University, were dissolved in absolute ethanol prior to use, and the final ethanol concentration did not exceed 0.5% (v/v) in any experiment.

### Cell cultures and PAPU treatment

Mouse and human melanoma cells, B16F10 and M-3 respectively, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics (100 IU/ml penicillin G and 100  $\mu$ g/ml streptomycin). Primary cultures of hGF and hPLF were also used as control cells (20, 21). This study was approved by the Review Board of Chonbuk National University Hospital (CNUH).

Just prior to PAPU treatment, all cultures were replaced with medium supplemented with a low concentration (1%) of FBS. This replacement did not induce cell growth arrest during the experimental period. The cells were processed at various times after 10 to 100  $\mu$ M PAPU treatment for the analyses of DNA synthesis, cytotoxicity and apoptosis.

### Measurement of cell viability and DNA synthesis

This study used MTT to evaluate the viability of cells exposed to PAPU samples (22). The level of DNA synthesis in cells after treatment with PAPU samples was measured by the addition of 1  $\mu$ Ci of [*methyl*-<sup>3</sup>H] Thymidine deoxyribose (TdR) (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) to each well during the last 24 h period prior to cell harvesting (Inotech Inc., Switzerland). The levels of incorporated tritium were measured using a liquid scintillation counter (Packard Instrument Co.).

### Apoptosis detection

TUNEL assay was performed using a TUNEL Apoptosis Detection Kit (#17-141; Millipore, Temecula, CA, USA) according to manufacturer's instructions. The cells were washed with PBS and observed by fluorescence microscopy (Axioskop 2, Carl Zeiss, Germany).

The level of PAPU-induced DNA fragmentation was determined by flow cytometric analysis after PI staining. Initially, the suspension ( $2 \times 10^6$  cells) of PAPU-treated cells was fixed with 80% ethanol at 4°C for 24 h, and then incubated overnight at 4°C with 1 ml of PI staining mixture (250  $\mu$ l of PBS, 250  $\mu$ l of 1 mg/ml RNase in 1.12% sodium citrate, and 500  $\mu$ l of 50  $\mu$ g/ml PI in 1.12% sodium citrate). After staining, 10,000 cells were analyzed using the FACS Calibur<sup>®</sup> system (Becton Dickinson, San Jose, CA, USA).

In order to confirm the patterns of PAPU-mediated cell death, sample-treated cells were examined via double-staining with FITC-labeled Annexin V and PI without cell fixation (23). Briefly, cells grown in cover slips were exposed to 100  $\mu$ M PAPU for 48 h and then washed twice with PBS and with once Annexin V binding buffer. The cells were stained using an Annexin V-FITC Fluorescence Microscopy Kit (51-8074KC; BD Biosciences, USA) at room temperature for 15 min. After washing once with PBS, the cells were incubated in HEPES buffer containing 1  $\mu$ g/ml PI at room temperature for 10 min and then observed by fluorescence microscopy (Axioskop 2, Carl Zeiss).

### Measurement of caspase-3 activity

Cells were collected and resuspended in lysis buffer 24 h after exposure to PAPU. To measure caspase-3-like activity, the lysates (100  $\mu$ g) were incubated with 100  $\mu$ M of the synthetic substrate Ac-DEVD-AMC at 37°C for 2 h in reaction buffer (1 ml) containing 100 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS and 10 mM dithiothreitol (DTT). Fluorescence intensities were measured using a microplate spectrophotometer (Bio-Tek Ins., Vermont, USA).

### Western blot analysis

Protein lysates were prepared in lysis buffer as described elsewhere (21), and each protein (30  $\mu$ g) sample were separated by 12% SDS-PAGE and blotted onto PVDF membranes. The

blots were probed with primary and secondary antibodies and then developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) immediately before exposure to X-ray film (Eastman-Kodak, Rochester, NY, USA). Polyclonal antibody specific to caspase-3 (sc-7148) and monoclonal antibody specific to  $\alpha$ -tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and BD Bioscience Pharmingen (San Diego, CA, USA), respectively.

### Statistical analyses

Unless otherwise specified, all data were expressed as a mean  $\pm$  standard deviation (SD) of triplicate experiments. A one-way ANOVA using SPSS ver. 16.0 software was used for multiple comparisons. A value of  $P < 0.05$  was considered statistically significant.

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