Methyl protodioscin induces G2/M cell cycle arrest and apoptosis in A549 human lung cancer cells

Yang Bai, Xiao-Yuan Qu[1], Jun-Qiang Yin[2], Liangcai Wu[2], Hong Jiang[1], Han-Wu Long[1], Qiang Jia[1]

Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, [1]Institute of Biology, Guizhou Academy of Sciences, Guiyang, [2]First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

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ABSTRACT

Background: Methyl protodioscin (MPD) is a furostanol bisglycoside with antitumor properties. It has been shown to reduce proliferation, cause cell cycle arrest. **Objective:** The present study elucidates the mechanism underlying MPD's apoptotic effects, using the A549 human lung cancer cell line. **Materials and Methods:** The human pulmonary adenocarcinoma cell line A549 was obtained from the Cell Bank of the Animal Experiment Center, North School Region, Sun Yat-Sen University. All of the cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), penicillin (10,000 U/l), and streptomycin (100 mg/l) at 37°C in a 5% CO_2 humidified atmosphere. The induction of apoptosis was observed in flow cytometry and fluorescent staining experiments. **Results:** MPD showed growth inhibitory effects in A549 cells in a dose- and time-dependent manner. The significant G2/M cell cycle arrest and apoptotic effect were also seen in A549 cells treated with MPD. MPD-induced apoptosis was accompanied by a significant reduction of mitochondrial membrane potential, release of mitochondrial cytochrome c to cytosol, activation of caspase-3, downregulation of Bcl-2, p-Bad, and upregulation of Bax. **Conclusion:** Our results show that the induction of apoptosis by MPD involves multiple molecular pathways and strongly suggest that Bcl-2 family proteins signaling pathways. In addition, mitochondrial membrane potential, mitochondrial cytochrome c and caspase-3 were also closely associated with MPD-induced apoptotic process in human A549 cells.

Key words: A549, apoptosis, Bcl-2 family, Methyl protodioscin

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INTRODUCTION

Lung cancer is now the most common cause of cancer-related deaths, which accounts for more than one million worldwide annual deaths.[1] It is one of the most malignant types of human cancer and about 70% of patients die as a result of cancer metastasis. Approximately 80% of people diagnosed with lung cancer have non-small cell lung cancer, such as adenocarcinoma, which remains an aggressive cancer with poor prognosis.[2]

Multimodal therapy remains the norm for patients with locally advanced disease, and patients with advanced metastatic disease benefit from palliative chemotherapy. Additional therapeutic agents should be evaluated to improve the survival of lung cancer patients.

Dioscoreaceae saponins comprise a diverse class of plant glycosides, which have many biological activities, which include two kinds of saponins (spirostanol saponins and furostanol saponins). Previous studies have demonstrated that methyl protodioscin (MPD) has notable anti-tumor activities. It has been shown to reduce proliferation, cause cell cycle arrest in the G_2/M-phase and apoptosis[3,4] in a concentration range of 2.5-20 μM in *in vitro* experiments with various cell lines. Its activities are mediated by the induction of apoptosis and cell differentiation; the regulation of various genes and proteins is also involved in the process.

However, the antitumor activities of MPD has been previously tested by the National Cancer Institute's (NCI) anti-cancer drug discovery screen,[5] which is an *in vitro* disease-oriented screening system with a panel of 60 human cancer cell lines.[6]

Its activities are mediated by the induction of apoptosis and cell differentiation, for which the regulation of various genes and proteins are also involved. However, little is known about its effects on human pulmonary adenocarcinoma cell line (A549). In this study, we evaluated the effects of MPD on cell proliferation, cell cycle arrest,

Address for correspondence:
Dr. Qiang Jia, Institute of Biology, Guizhou Academy of Sciences, Guiyang - 550 009, China. E-mail: ytjq0629_cn@sina.com

and apoptosis in human A549 cells. The results showed that MPD inhibited proliferation via blocking cell cycle progression at G2/M phase and subsequently progressing into apoptosis. The mechanism of apoptosis was also elucidated by analyzing the regulation of apoptotic-related proteins. The aim of this study was to determine the effects of this agent on human pulmonary adenocarcinoma cell *in vitro*.

MATERIALS AND METHODS

Drugs and reagents

MPD got from the Graduate School at Shenzhen, Tsinghua University, was purified from the rhizome of Dioscorea collettii var. hypoglauca[7,8] with purity higher than 97% based on reversed-phase HPLC analysis. Working concentrations were then prepared by diluting stock solutions in culture medium immediately before use. MTT, Hoechst 33258, and SB216763 were purchased from Sigma (St. Louis, MO, USA). The DNAzol reagent was purchased from Invitrogen Life Technologies (Paisley, Scotland, UK). Antibodies to Bax, Bak, and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of analytical reagent quality.

Cell culture

The human pulmonary adenocarcinoma cell line A549 was obtained from the Cell Bank of the Animal Experiment Center, North School Region, Sun Yat-Sen University. All of the cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), penicillin (10,000 U/l), and streptomycin (100 mg/l) at 37°C in a 5% CO_2 humidified atmosphere.

MTT assay

The effects of MPD on cell proliferation and the viability of A549 cells were assessed using the MTT assay. A549 cells were harvested and seeded in 24-well plates at 4.0×10^4 cells/well in a final volume of 500 μl. After 24 hours of incubation, drugs were added to duplicate plates at appropriate concentrations. After 48 hours, MTT dye (5 mg/ml) was added to each well. DMSO (150 μl) was added to each well and vortexed at low speed for 10 minutes to fully dissolve the blue crystals. The concentrations required to inhibit cell growth by 50% (IC_{50}) were calculated from the cytotoxicity curves (Bliss's software; Bliss Co., CA, USA), at least 3 independent experiments.

Fluorescent staining

Initially, 2×10^5 cells were seeded on a 6-well plate and treated with the indicated concentrations of cinobufagin for 48 hours. The apoptotic morphology was subsequently studied by staining the cells with Hoechst 33258. Cells displaying condensed, fragmented DNA were considered apoptotic.

Cell cycle analysis by flow cytometry

A549 cells were treated with 100 nM cinobufagin for 12 hours, washed with PBS, and fixed with 70% ice-cold ethanol at 4°C overnight. Then, the cells were resuspended in a staining solution containing 1% Triton-× 100, 0.1 mg/ml RNase, and 6 μg/ml PI. The cell suspensions were subsequently incubated at 37°C for 30 minutes in the dark and analyzed on a fluorescence-activated cell sorter (FACS-calibur, Becton Dickinson, San Jose, CA, USA).

Assay of mitochondrial membrane potential

After incorporation of fluorescent probe, the cells were incubated up to 4 hours with or without 20 μM MPD. Initially, 1×10^6 A549 cells/ml was incubated with 10 μM rhodamine 123 for 10 minutes at 37°C. At the end of incubation, the cells were washed twice with PBS, harvested by centrifugation, and then resuspended in 1.5 ml PBS. The fluorescent intensity of each cell suspensions was measured at a wavelength 530 nm in a Perkin Elmer L15B fluorescence spectrophotometer. The fluorescence intensity was used as an arbitrary unit representing the mitochondrial transmembrane potential.

Assay of caspase-3 activity

After the treatment with indicated agents, A549 cells were harvested and washed with PBS by centrifugation at 750 × g for 5 minutes at 4°C. The cell pellets were resuspended in lysis buffer (caspase colorimetric assay kits; Bivision, Inc.) and left on ice for 30 minutes. The lysates were centrifuged at 10,000 × g for 10 minutes and the supernatant (20 μl) was collected for caspase-3 activity assay in the lysis buffer containing DEVD-pNA, a specific substrate to caspase-3. The concentration of pNA, as the product from enzymatic converting of DEVD-pNA by caspase-3, was measured at 405 nm and used as an indicative of caspase-3 activity.

Mitochondrial cytochrome c release

A549 cells were seeded in 2 ml fresh medium at an initial density of 1×10^6 cells/ml and incubated up to 4 hours with or without 20 μM MPD. After the incubation, the cells were harvested by centrifugation and washed twice with PBS. The cells were suspended in 200 μl lysis buffer (195 mM mannitol; 65 mM sucrose; 2 mM HEPES, pH 7.4; 0.05 mM EGTA; 0.01 mM MgCl2; 0.5 mg/ml BSA) and lysed by the addition of 0.01% digitonin. The cytosolic fraction was obtained from 10,000 × g centrifugation for 10 minutes and was collected for cytochrome c assay in 1 × RD5P calibrator diluent (cytochrome c Immunoassay Kit; R and D Systems, MN, USA).

Western blot analysis

Briefly, the cells were lysed and harvested by scraping with a protein extraction reagent (Pierce Biotechnology, Rockford, USA). Protein concentrations were determined

using the Bradford assay. For each sample, 20 μg of protein extract was separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies for 12 hours at 4°C, washed three times in Tris-buffered saline with Tween-20 (TBST) for 5 minutes each, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The protein bands were visualized on X-ray film using an enhanced chemiluminescence detection system.

Data analysis

All data were derived from at least 3 independent experiments and results are expressed as mean ± standard error. Differences were assessed using the Student's *t*-test or the Kruskal-Wallis test. $P < 0.05$ was deemed statistically significant.

RESULTS

Effects of MPD on the viability of the A549 cell lines

To investigate the effect of MPD on A549 cell proliferation, the cells were treated for 48 hours in medium containing varying concentrations of MPD up to 20 μM. Cells were counted by MTT studies. In the present study, MPD showed potent cytotoxic effect in A549 cells in a dose-dependent manner, as expressed as percentage of cell survival [Figure 1]. The survival rate of human A549 cells treated with 20 μM MPD started to decrease at first 6 hours of treatment and sharply dropped after 24 hours of incubation. Thus, 20 μM of MPD was selected to monitor the changes in molecular events for the subsequent experiments.

MPD-induced apoptosis of A549 cells

To characterize MPD-induced cell death, several hallmarks of apoptosis were examined, namely, nuclear chromatin condensation and fragmentation of DNA by Hoechst 33258. As shown in Figure 2, in contrast to control cells, cells exposed to 20 μM MPD had nuclei with chromatin condensation and fragmentation. During morphological examination, the results revealed that MPD-treated cells showed typical apoptotic morphological changes, such as cell shrinkage, nuclear fragmentation, and apoptotic body formation.

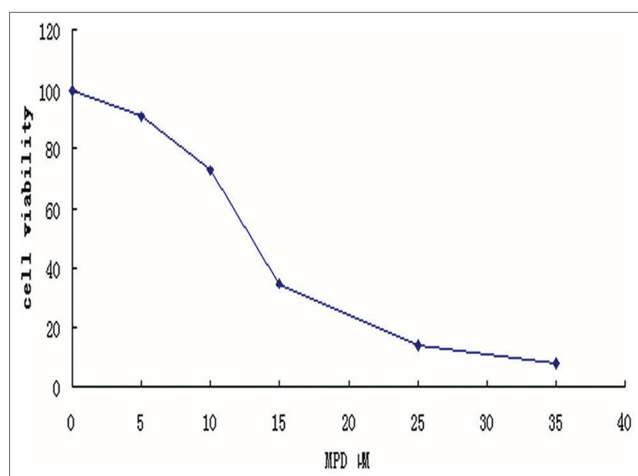

Figure 1: Cytotoxicity of MPD on human A549 cells. A549 cells were incubated with 0, 5, 10, 15, 25, 35 μM of MPD for 48 hours

Effect of MPD on A549 cells cycle distribution

In order to quantify the kinetics of events both on apoptosis and on cell cycle phases, we performed a flow cytometric analysis. We cultured A549 cells for various time lengths with 20 μM MPD and analyzed DNA content by flow cytometry. As shown in Figure 3, MPD induced a significant cell population in G2/M phase following 48 hours of treatment with different concentrations induced a time-dependent accumulation in A549 cells, and then the cells underwent apoptosis.

Effects of MPD on apoptosis-related proteins

We also investigated the expression of several proteins that are involved in MPD-induced apoptosis. The expression of several members of Bcl-2 family protein was examined by western blot analysis. Exposure of A549 cells to MPD in various concentrations resulted in a marked decrease of Bcl-2 protein expression, but a drastic increase in the expression of Bax and Bak proteins [Figure 4].

Changes of mitochondrial membrane potential and release of cytochrome c from mitochondria

As shown in Figure 5, MPD induced a time-dependent mitochondrial transmembrane depolarization, represented as the decrease of mitochondrial membrane potential. Concomitantly, a MPD-induced time-dependent, cytochrome c release was also observed in A549 cells, which represents as significant increase of cytosolic cytochrome c concentration. In this study, the cytochrome c release and mitochondrial membrane potential were analyzed spectrophotometrically. These data suggest that loss of mitochondrial membrane potential may be required for MPD-induced cytochrome c release into cytosol, that later triggered the cleavage and activation of mitochondrial downstream caspases and onset of apoptosis.

Determination of the involvement of caspase-3 activation

In order to verify the requirement for caspases on MPD-induced apoptosis in A549, we used the cell permeable caspase inhibitors (z-DEVD-fmk). A549 cells were pretreated with 20 μM caspase-3 inhibitors for 2 hours, and then induced to undergo apoptosis by treatment with MPD. The results clearly showed that administration of caspase-3 inhibitor alone did not affect the cell viability and

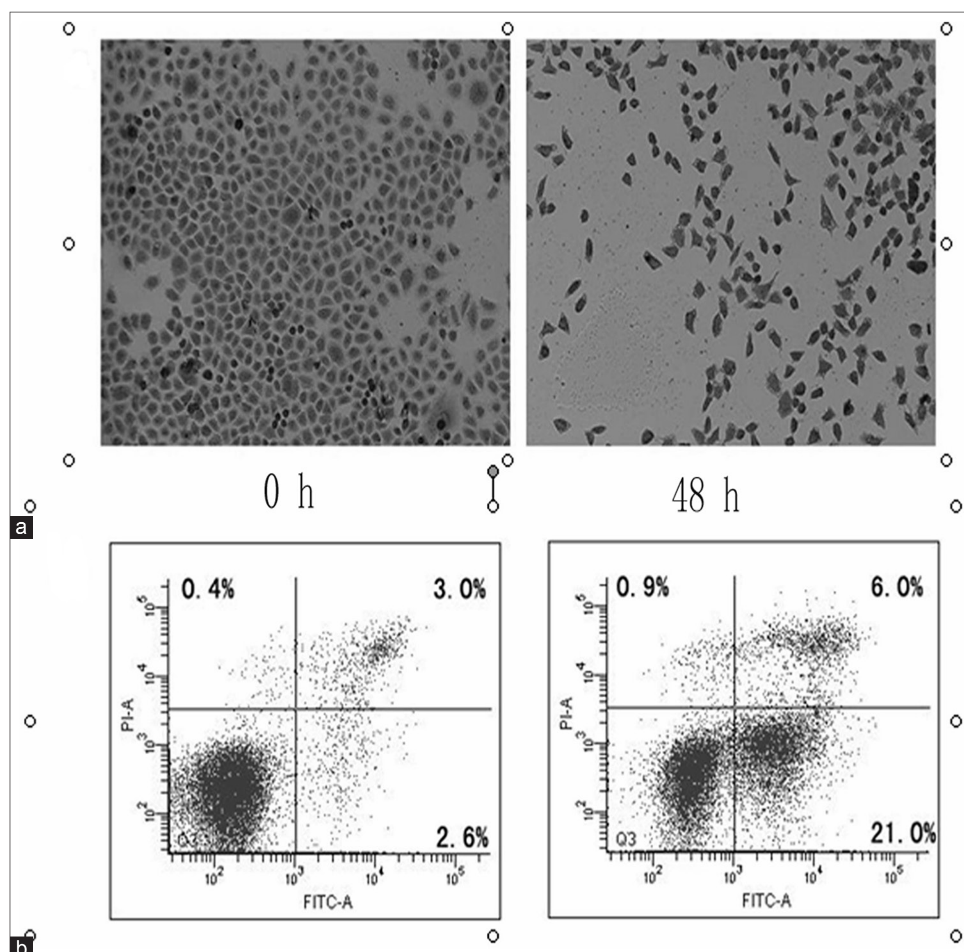

Figure 2: Apoptosis-inducing effects of MPD in A549 cells by flow cytometry. Cells were incubated with MPD for 48 hours. (a) Cells were incubated with 0, 20 μM MPD for 48 hours and then Hoechst staining was performed to detect the morphology change. Each picture was taken at a magnification of ×100, early apoptotic cells (0 h), and late apoptotic and dead cells (48 hours). (b) Annexin V/PI staining assay were used to detect the apoptosis rate

caspase-3 activity, but significantly inhibited MPD-induced cell death and caspase-3 activation in A549 cells [Figure 5].

DISCUSSION

The present study showed that MPD could exert a strong growth inhibitory activity against human pulmonary adenocarcinoma cancer A549 cells, through increasing the mitochondrial membrane permeability by downregulation of Bcl-2 and upregulation of p53, Bax, and Bak, then triggering the cytochrome c release from mitochondria into cytosol and activation of caspase-3.

Apoptosis plays an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells. Currently, natural and dietary agents being able to induce cancer cells apoptosis grasped many researchers' eyes to seek for new anti-cancer drugs. MPD has been shown to reduce proliferation, cause cell cycle arrest in the G_2/M-phase, and apoptosis[3,4] in a concentration range of 2.5-20 μM in *in vitro* experiments with various cell lines. MPD has been previously tested by the NCI's anti-cancer drug discovery screen,[8] which is an *in vitro* disease-oriented screening system with a panel of 60 human cancer cell lines.[6] However, little is known about its effects on human osteosarcoma cells.

In this study, we demonstrated that MPD treatment of A549 cells resulted in significant cell growth inhibition G2/M phase arrest and apoptosis in a time- and dose-dependent manner. A549 cells treated with MPD exhibited characteristic morphological features of apoptosis, such as membrane shrinkage, chromosomal condensation, and increases of caspase-3 activity. These data showed that MPD induced A549 cells apoptosis in caspase-dependent apoptotic pathway.

Exposure to MPD, progressive decrease of the mitochondrial membrane potential, and release of cytochrome c into the cytosol were also observed in A549 cells. It has been noticed

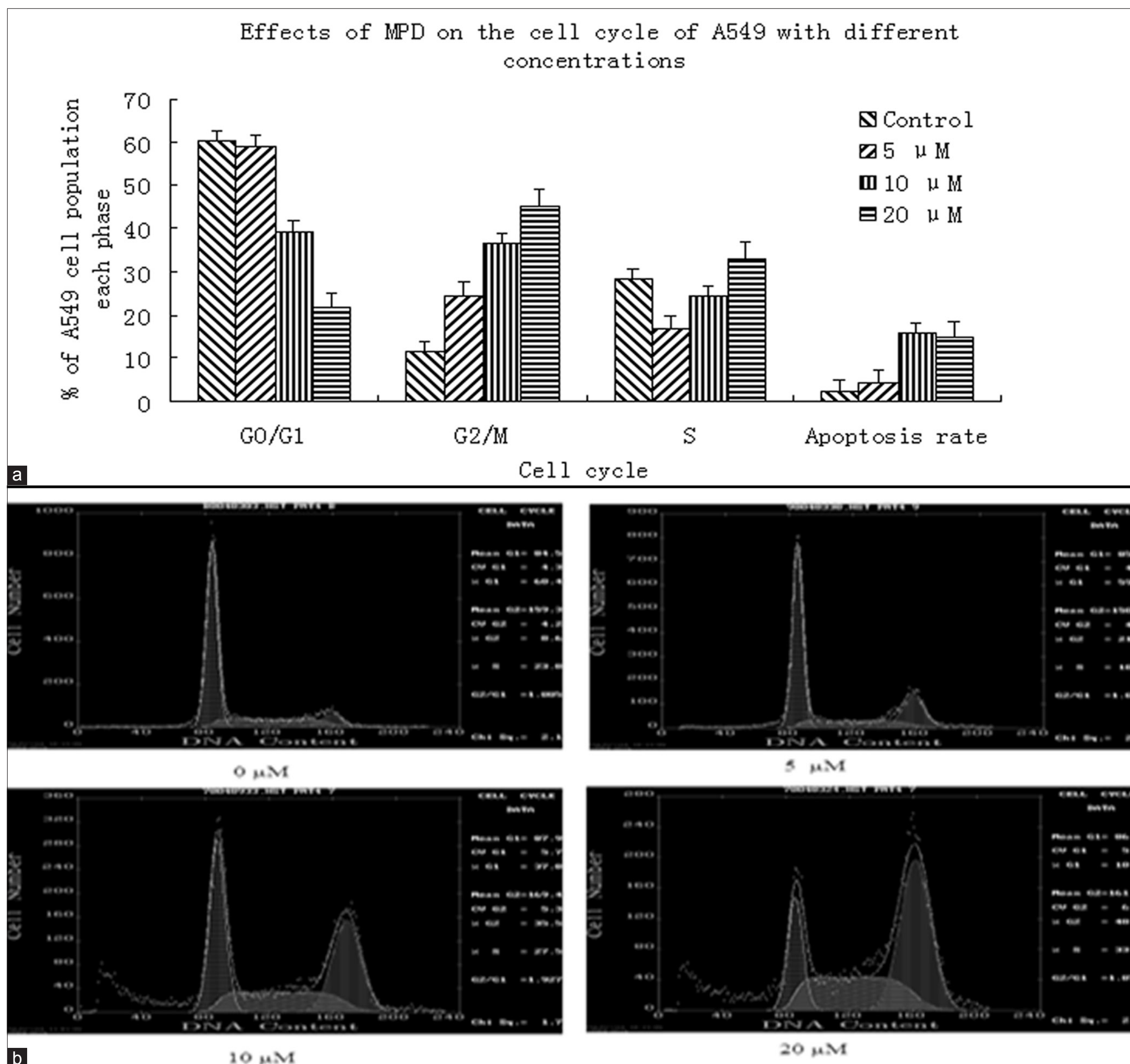

Figure 3: A549 cells were treated with 0, 5, 10, or 20 μM of bostrycin for 48 hours. (a) G2/M arrested effects of MPD. Cells were treated with 0, 5, 10, and 20 μM MPD for 48 hours. B: Cells were harvested and subjected to analyze the DNA content by flow cytometry. Data were presented as mean ± S.D. from five independent experiments. *$P < 0.05$; compared with control; (b) shows representative flow cytometry plots

in many *in vitro* systems that apoptosis was associated with a loss of mitochondrial membrane potential, which may correspond to the opening of an outer membrane pore. Thus, this event has been suggested to be responsible for cytochrome c release into cytosol from mitochondria.[9] In this study, the cytosolic cytochrome c accumulation in MPD-induced human A549 cells is probably the consequence of the loss of mitochondrial membrane potential, which finally leads to the cell death. Although many studies suggested that caspase-3 can also be activated through the mitochondria-dependent signaling proteins by releasing cytochrome c from its intermembrane space into the cytoplasm,[10] it is not clear how cytosolic cytochrome c accumulation and caspase activation are achieved upon MPD treatment.

Bcl-2 is a member of a family of genes that regulates the apoptosis threshold. The family includes members, which act as pro-apoptotic protein (such as Bax and Bak) or anti-apoptotic proteins (such as Bcl-2)[9] (Uthalakath and Strasser, 2002). The interactions and relative frequency of occurrence of these proteins appear to modulate the propensity of a cell to undergo apoptotic cell death.[11] Mitochondrial-mediated apoptosis is facilitated by members

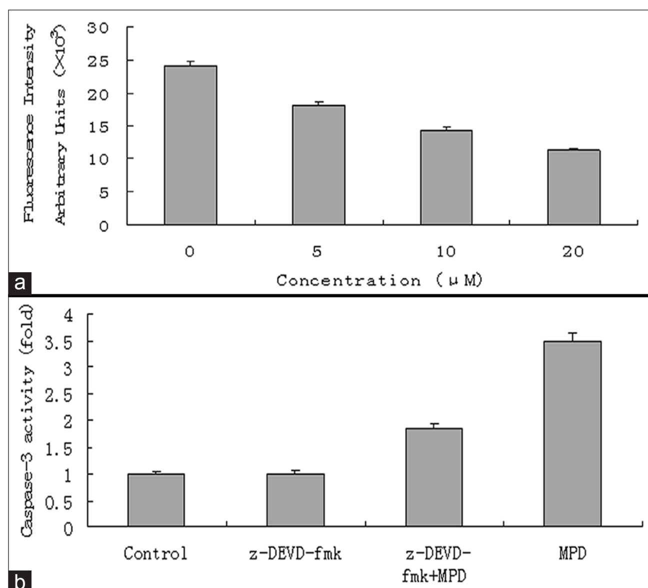

Figure 4: Expression of cytochrome C, p53, and Bcl-2 family proteins in MPD-treated A549 cells. A549 cells were treated with MPD for 48 hours. After the treatment, cell lysates were extracted, and the levels of Bcl-2 family proteins were analyzed by western blot analysis

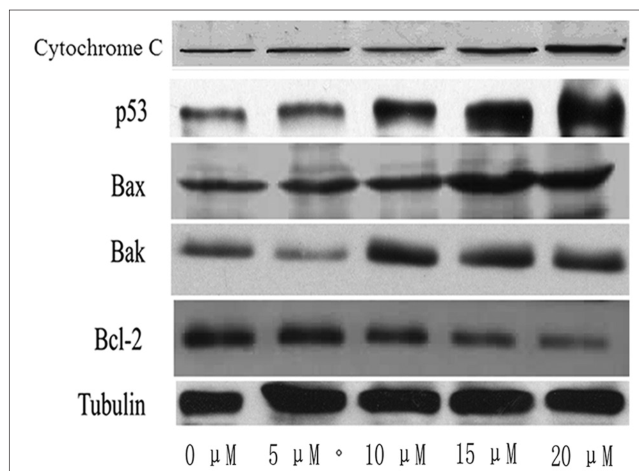

Figure 5: Inhibition of caspase 3 activity and attenuation of MPD-induced cell death by z-DEVD-fmk. A549 cells were treated with 20 μM z-DEVD-fmk 2 hours prior to 48 hours of 20 μM MPD treatment. After incubation, caspase-3 activity (a) After MPD treatment, caspase-3 activity was detected by a colorimetric assay. $^*P < 0.05$ vs. control (b) Effect of z-VAD-fmk on the MPD-induced caspase-3 activation. A549 cells were pretreated with/without z-VAD-fmk (20 μM) for 2 hours and then incubated with MPD (20 μM) for 48 hours. $^*P < 0.05$ compared with the respective MPD and z-DEVD-fmk free control and $^{**}P < 0.001$ comparison between the absence and presence of z-DEVD-fmk in the same MPD treatment group

of the Bcl-2 homology family of proteins.[12,13] The reduction of mitochondrial membrane potential leads to the release of intermembrane proteins, such as cytochrome c and apoptosis-inducing factors, into the cytosol and induces apoptosis.[14] Several studies have shown that overexpression of Bcl-2 prevents the mitochondrial release of cytochrome c, thereby inhibiting the activation of caspases cascade and apoptosis.[15,16] The proapoptotic molecules, Bax and Bak, occur in the cytosol in the absence of apoptotic stimuli.[17,18] During apoptosis, Bax undergoes homodimerization, translocation to the mitochondrial membrane triggering an apoptotic cascade leading to cell death.[19] Moreover, expression of Bax is capable of directly initiating apoptosis.[20] In the present study, MPD-induced apoptosis in A549 cells was accompanied by upregulation of Bax and Bak and downregulation of Bcl-2. Other studies have demonstrated that Bcl-2, Bax, and Bak can act as channel proteins within the mitochondrial membrane.[21,22] It is conceivable that the channel property of Bax and Bak may control the mitochondrial permeability transition and other early mitochondrial perturbation. Thus, Bax and Bak may facilitate the passage of some important proteins, such as cytochrome c or other apoptosis-inducing factors that trigger the activation of caspase cascade and apoptosis. Previous reports have also documented that the ratio of pro- and anti-apoptotic proteins determines, at least in part, the susceptibility of cells to a death signal.[23,24] Our results showed that expression of Bcl-2 family proteins, Bcl-2, Bax, and Bak, can be differently regulated by MPD, suggesting that the MPD-induced apoptosis is controlled by a balanced expression between those apoptosis-inducing and apoptosis-suppressing molecules. Thus, enforced dimerization of Bax and Bak may result in altering permeability, triggering mitochondrial cytochrome c release into cytosol, activating caspase-3 cascade, and eventually promoting cell death. These results suggested that the molecular events of chemical-induced apoptosis may depend on the kind of tested cell types or actions of different chemicals.

In conclusion, the present study demonstrates that MPD induces apoptosis in A549 cells. The treatment of A549 cells with MPD activates one cell death pathway. The pathway regulates the mitochondrial membrane permeability by downregulation of Bcl-2 and upregulation of p53, Bax, and Bak, triggering the cytochrome c release from mitochondria into cytosol. And subsequently induces the caspase-3 activation, and then cleaves specific substrates. Overall, our results showed that the MPD-induced apoptosis involves multiple molecular pathways and very likely Bcl-2 family proteins signaling pathways. In addition, caspase-3 was also closely associated with MPD-induced apoptotic process in human A549 cells. MPD is a promising candidate as an anti-tumor agent on A549 cells.

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