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SHORT COMMUNICATION

Apoptosis-inducing and Antitumor Activity of Neolignans Isolated from *Magnolia officinalis* in HeLa Cancer Cells

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Two neolignans, 4′-methoxymagndialdehyde (1) and magnaldehyde B (2), were isolated from the stem bark of *Magnolia officinalis* (Magnoliaceae), evaluated for apoptosis-inducing effects in human cervical epitheloid carcinoma HeLa cells. The apoptosis-inducing activity of compounds 1 and 2 were assessed by DNA content using flow cytometric analysis. In the immunoblotting analysis, the treatment with 1 and 2 resulted in the cleavage of procaspase-8 and -3 and poly(ADP-ribose)polymerase into active forms. In addition, *in vivo*, the administration of 2 to Lewis lung carcinoma-inoculated mice evidenced a significant inhibition of tumor growth (volume) with reduction of 28.7% at concentration of 20 mg/kg, as compared with the control mice. These findings suggest that 2 can inhibit the proliferation of tumor cells, and might be an anti-tumoric agent. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: Magnolia officinalis; Magnoliaceae; neolignan; apoptosis; anti-tumor.

INTRODUCTION

Apoptosis is a programmed cell death in which tumor cells commit suicide, resulting in structural changes of the plasma membrane and DNA fragmentation in nuclei (Wyllie *et al*., 1980). Cysteine proteases called 'caspases' play an important role in apoptosis. A major event in the apoptotic process is the activation of caspases. Caspase family members are expressed as proenzymes, which are cleaved to be activated during the apoptotic process. The activation is caused by proteolysis of death substrates such as poly ADP-ribose polymerase (PARP), DNA-dependent protein kinase, and caspases themselves (Tewari *et al*., 1995). Inhibition of tumor cell growth and induction tumor cell apoptosis are ideal therapeutic strategies. Among a variety of pro-apoptotic agents, plant-derived compounds are characterized by their multiple mechanisms and low side effects (Darzynkiewicz *et al*., 2000; Pezzuto, 1997; Tiwari *et al*., 1999).

The stem bark of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae) has been used as a traditional medicine for the treatment of gastrointestinal disorders, anxiety, and allergic diseases including bronchial asthma in Korea, China, and Japan (Kuo *et al*., 2011). In recent studies, the major compounds, magnolol and honokiol, have been reported to exhibit a variety of biological effects, such as antiinflammation (Lin *et al*., 2007; Wang *et al*., 1995), antimicrobial (Chang *et al*., 1998; Syu *et al*., 2004), and antitumor (Ikeda and Nagase, 2002; Ikeda *et al*., 2003). Although, the minor compounds, 4′-methoxymagndialdehyde (**1**) and magnaldehyde B (**2**) (Fig. 1) are present in the stem bark of *M. officinalis* (Youn *et al*., 2007), there are no studies about their biological activity on cancer. Thus, as a part of our screening program to evaluate the chemopreventive effects of medicinal plants, we investigated compounds **1** and **2** isolated from *M. officinalis* on the induction of apoptosis in HeLa cells *in vitro*, and suppressive effects against the solid tumor growth in Lewis lung carcinoma (LLC)-inoculated mice *in vivo*.

MATERIALS AND METHODS

Plant material. The dried stem bark of *Magnolia officinalis* was purchased from a local market in Daejeon, Korea in June 2005, and was identified by Prof. KiHwan Bae, College of Pharmacy, Chungnam National University. A voucher specimen (CNU-768) was deposited at the herbarium of the College of Pharmacy, Chungnam National University.

Animal material. Male BDF1 mice, 20 ± 2 g body weight, were purchased from SLC, Inc. (Japan). They were fed with a commercial solid food (Samyang Yuji Co. Ltd., Seoul) and tap water. Eight mice were used in every group. The mice were housed at 23 ± 0.5 °C and 60%

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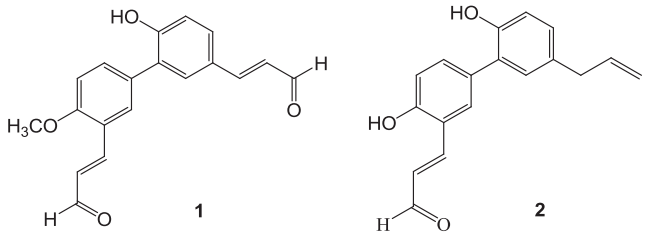

Figure 1. Chemical structures of **1** and **2** isolated from *M. officinalis*.

humidity in a 12 h light–dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals by Chungnam National University.

Extraction and isolation. The dried stem bark of *Magnolia officinalis* (5.5 kg) was extracted with methanol (MeOH) three times under reflux for 4 h. The MeOH solution was combined, filtered, and concentrated to yield a dry MeOH extract (640 g). The MeOH extract (640 g) was suspended in distilled water and fractionated with hexane, EtOAc, and BuOH to give hexane (180 g), EtOAc (270 g), and BuOH-soluble fractions (80 g). The EtOAc-soluble fraction was chromatographed over a silica gel column eluting with $CHCl_3$ – MeOH (100:1 to 2:1, v/v) to afford 12 fractions (E1 – E12). Fraction E3 was chromatographed on a silica gel column eluting with $CHCl_3$ – MeOH (50:1 to 5:1, v/v) to afford four subfractions (E3.1 – E3.4). Subfraction E3.2 was subjected to preparative HPLC [YMC-pack ODS-A, MeOH – H_2O (40:60)] to yield **1** (3 mg, t_R 90 min). Subfraction E3.3 was further purified with crystallization in MeOH to give **2** (500 mg).

Flow cytometric analysis. Cells were seeded onto 100-mm dishes and grown in RPMI 1640 supplemented with 10% FCS. After the cells had grown to subconfluence, they were rendered quiescent and challenged with 2% FCS. Then, after release using trypsin-EDTA, they were harvested at various times, washed twice with PBS/0.1% dextrose, and fixed in 70% ethanol at 4 °C. Nuclear DNA was stained with a reagent containing propidium iodine (50 μg/ml) and DNase-free RNase (2 U/ml) and measured using a fluorescence-activated cell sorter. The proportion of nuclei in each phase of the cell cycle was determined using established Cell FIT (Becton Dickinson, San Jose, CA) DNA analysis software.

Western blotting analysis. HeLa cells (5×10^5 cells/ml) were treated with tested compounds for 24 h at 37 °C. Cell lysates were prepared in 100 μl of lysis buffer (Sigma, U.S.A) containing a protease inhibitor cocktail (Roche, Germany). Insoluble material was removed by centrifugation at 14,000 rpm for 10 min. Then, the protein contents in the supernatant were measured using a Bio-Rad DC protein assay kit. The protein extracts (50 μg/well) were separated by SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad, U.S.A). The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) at 4 °C overnight and incubated with primary antibodies at room temperature for 1.5 h. The membranes were washed three times with TBS-T and blotted with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1.5 h, followed by washing three times in TBS-T. Immunoreactive proteins were visualized by an enhanced chemiluminescence procedure according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, U.S.A) and exposed to X-ray films. Protein contents were normalized by reprobing the same membrane with anti-β-actin antibody. For β-actin detection, previously used membranes were soaked in stripping buffer (Gene Bio-Application Ltd., Israel) at room temperature for 20 min.

Anti-tumor test *in vivo*. The anti-tumor activity was measured according to Teruhiro's method. $2 \times 2 \times 2$ mm^3 tumor fragments of LLC were transplated s.c. into the left axilla region of the BDF1 mice. Tested compound and adriamycin (a positive control) were dissolved in 2% acacia (gum arabic), and each solution was administered (ip) once a day for 14 consecutive days. The tumor volume (TV) was measured from 14^{th} to 24^{th} day and calculated according to the following formula: TV = L (mm) $\times$ W^2 (mm^2)/2, where L and W represent the length and the width of the tumor mass, respectively. The inhibition ratio of TV (IRTV) was calculated according to the formula: IRTV (%) = (Mean TV of control group – Mean TV of treated group)/Mean TV of control group $\times$ 100. The animals were sacrificed on the 24^{th} day, the tumor weight was counted, and the inhibition ratio of tumor weight (IRTW) was calculated with the same formula of IRTV.

Statistical analysis. All treatments were conducted in triplicate, and the results are presented as the mean ± standard deviation (S.D.). The statistical significance of all treatment effects was evaluated by Student's *t*-test with a probability limit for significance of $p < 0.05$, $p < 0.001$.

RESULTS AND DISCUSSION

The induction of apoptosis in cells treated with 4′-methoxymagndialdehyde (**1**, 5 and 10 μM) and magnaldehyde B (**2**, 10 and 30 μM) was confirmed by flow cytometric analysis of DNA content. Figure 2 illustrates the DNA content histograms of HeLa cells treated with **1** and **2** for 24 h. The sub-G1 DNA contents of compound-treated HeLa cells were increased in a dose-dependent manner.

The expression of initiator caspases and PARP was evaluated in compound-treated HeLa cells by western blotting. The caspase activation plays a central role in the induction of apoptosis. HeLa cells were treated with **1** (5 and 10 μM) and **2** (10 and 30 μM) for 24 h, and the activations of caspase-8 and -3 were detected (Fig. 3). On the contrary, activation of caspase-9 was not induced by **1** and **2**. In the immunoblotting experiment using the antibody against PARP, the proteolytic cleavage of PARP was used to assess the caspase-3 activation. PARP was cleaved from the intact form (116 kDa) into fragments (major fragment, 89 kDa) by treatment with compounds **1** (5 and 10 μM**)** and **2** (10 and 30 μM) for 24 h, and the major cleaved form of PARP was detected in cells treated with 10 μM compound **1**.

Magnaldehyde B (**2**) was examined to evaluate the effectiveness on solid tumors *in vivo*. This compound was tested for its anti-tumor activity on BDF-1 mice

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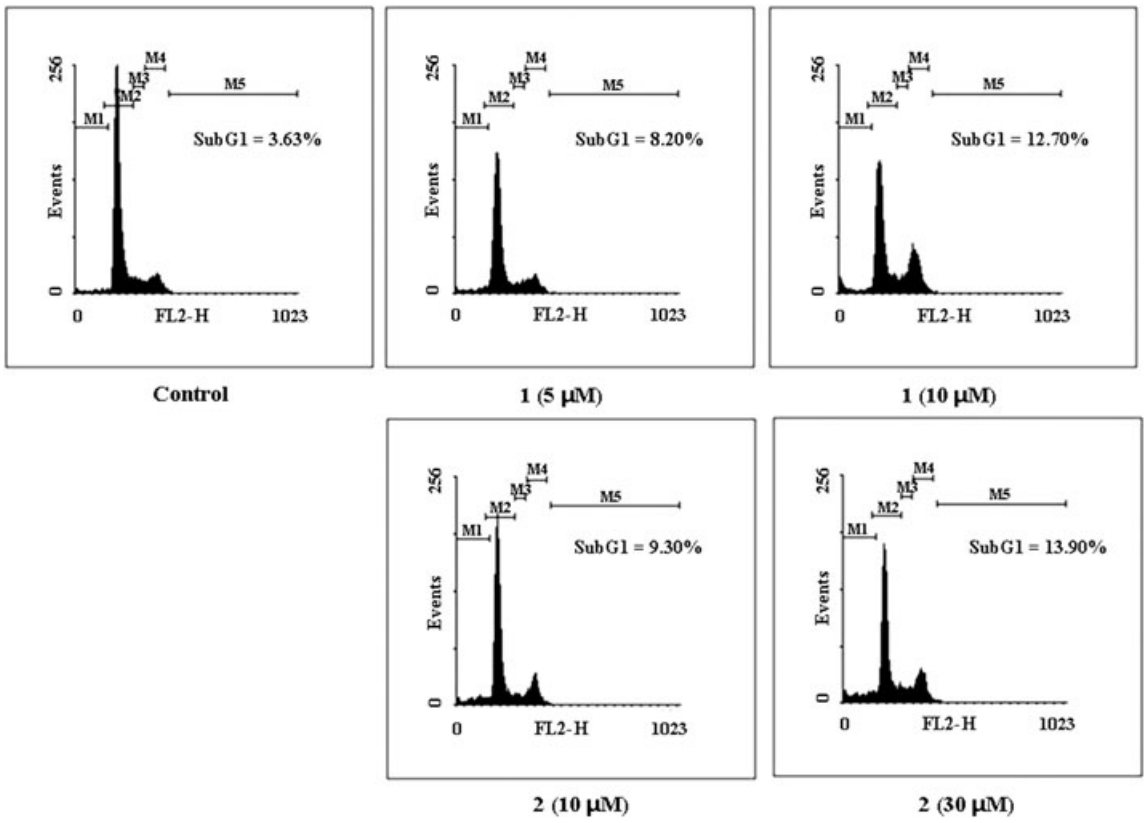

Figure 2. Flow cytometric analysis of the control and **1** and **2** treated HeLa cells. Cells treated with **1** (5 and 10 μM) and **2** (10 and 30 μM) or without **1** and **2** for 24 h were fixed and stained with propidium iodide, and the DNA content was quantified by flow cytometer.

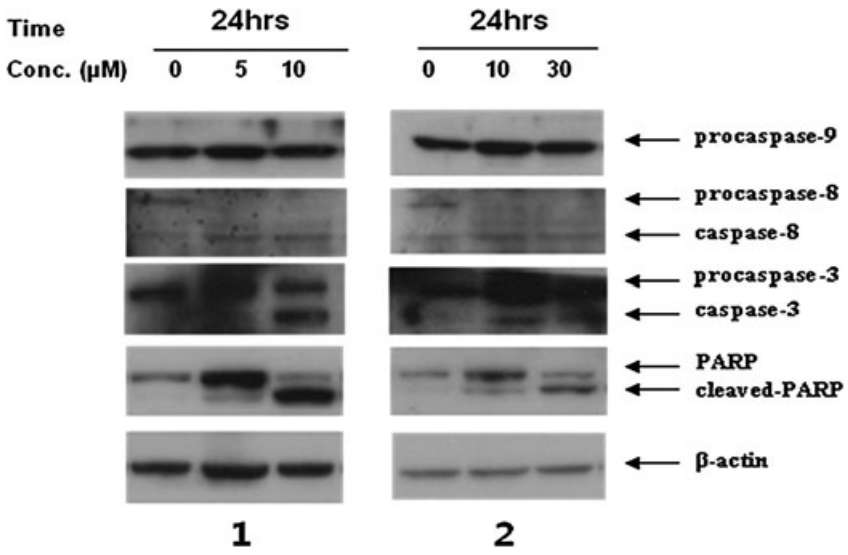

Figure 3. The induction of caspase activations and PARP degradation by **1** and **2** in HeLa cells. HeLa cells were treated with **1** (5 and 10 μM) and **2** (10 and 30 μM) for the 24 h, respectively. Each fraction was detected by Western blotting using specific antibody.

bearing LLC tumor cell by Teruhiro's method, which was intraperitoneally injected once a day with a daily dose of 50, 20, and 10 mg/kg for 14 consecutive days. Compound **2** reduced the TV with the IRTV values of 31.6%, 28.7%, 11.8% and the IRTW values of 20.4%, 18.0%, 8.8% at 50, 20, and 10 mg/kg, respectively (Table 1). No loss of body weight was observed at any concentration of compound after 24 days (data not shown). This result suggested that the compound **2** is effective at concentration more than 20 mg/kg. As a positive control, adriamycin at 20 mg/kg reduced the TV by 35.9% and the tumor weight by 23.4%.

We found that 4′-methoxymagndialdehyde (**1**) and magnaldehyde B (**2**) isolated from *M. officinalis* induced pro-apoptotic activity against HeLa cells via caspase activation. To confirm the apoptosis-inducing activity of compounds **1** and **2**, the flow cytometric analysis of compound-treated cells reveal that two compounds induced a significant accumulation of cells in the sub G1-phase of the cell cycle, suggesting that **1** and **2** blocked DNA replication and inhibited the cell cycle. Although compounds inhibited DNA replication in the cell cycle, caspase-activation and subsequent cleavage of PARP are regarded as necessary for the consequent

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Table 1. Antitumor activity of 2 in LLC bearing BDF1 mice

Group	Dose (mg/kg)	Tumor volume (mm^3)	IRTV (%)	Tumor weight (g)	IRTW (%)
Control	0	6980[a] ± 745	–	6.22[b] ± 0.52	–
MeOH extract[d]	50	5014[b] ± 652[c*]	28.1	5.28 ± 0.65[c*]	16.7
2	50	4770 ± 554[c*]	31.6	4.95 ± 0.16[c*]	20.4
	20	4972 ± 625[c*]	28.7	5.10 ± 0. 56[c*]	18.0
	10	5942 ± 712[c*]	11.8	5.67 ± 0. 36[c*]	8.8
Adriamycin	20	4470 ± 657[c*]	35.9	4.76 ± 0.56[c*]	23.4

[a]The value are the mean ± S.D. from five animals/group. [b]The tumor volume and weight were measured at 24[th] day after administered *i. p.* daily. [c]Statistical significance between the control and treatment groups was evaluated using Student's test. $*p < 0.05$. [d]Methanol extract from *M. officinalis*.

serial events of apoptosis (Lazebnik *et al.*, 1994). Our findings that the activations of caspase-8 and -3 were induced by treatment with compounds **1** and **2** in a dose-dependent manner imply that the death of HeLa cells was caused mainly by the apoptosis inducing activity. PARP, a substrate of caspase-3, is a target of caspase protease activity associated with apoptosis (Kaufmann *et al.*, 1993). During the apoptosis, PARP was also cleaved from its 116 kDa intact to 85 kDa fragments. These results demonstrate that compounds **1** and **2** induced the apoptosis via the activations of caspase-8 and -3. It was observed both compounds are effective in caspase activation and PARP degradation. Compound **2** showed *in vivo* antitumor in the mouse model. This *in vivo* effect implies that the anti-tumor effects may be attributable to the apoptotic process induced by treatment with **1** and **2**.

In summary, this study presented that lignans **1** and **2** isolated from *M. officinalis* effectively induced the apoptosis associated with the cell cycle arrest at the sub-G1 phase, activations of caspase-8 and -3, and degradation of PARP on human cervical epitheloid carcinoma HeLa cells although other pathways may have a role and that require further investigation. Our findings from *in vitro* and *in vivo* studies of **1** and **2** for anti-cancer effect suggest the potential applications in the treatment of human cancer.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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