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Mechanisms of Apoptotic Effects Induced by Resveratrol, Dibenzoylmethane, and Their Analogues on Human Lung Carcinoma Cells

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While lung cancer accounts for approximately 20% of cancer diagnoses, it is the leading cause of tumor-related deaths. The apoptotic effects of 3,5,4′-trihydroxystilbene (resveratrol), dibenzoylmethane (DBM), and their analogues on human lung cancer cells are generally unclear. The aims of this study were to evaluate the apoptotic effects and molecular mechanisms of resveratrol, DBM, and their analogues on human lung cancer cells. The results of the MTT and lactate dehydrogenase (LDH) leakage assays indicated that resveratrol, 3,5,4′-trimethoxy-*trans*-stilbene (MR-3), and 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB) could inhibit cell population growth and induce cell injury in A549 and CH27 cell lines. Resveratrol and HMDB could induce apoptotic cell death in the A549 and CH27 cell lines. Moreover, cellular growth of the A549 and CH27 cell lines might be inhibited by MR-3 through induction of apoptosis and regulation of the cell cycle. The A549 and CH27 cell lines treated with resveratrol, MR-3, and HMDB showed a time-dependent reduction of mitochondrial membrane potential, and the Bax/Bcl-2 ratio increased gradually with a higher concentration of polyphenols. The resveratrol-, MR-3-, and HMDB-induced apoptosis in the A549 and CH27 cell lines were controlled through activation of caspase-9 and caspase-3 and subsequent cleavage of PARP. In conclusion, we have demonstrated that resveratrol, DBM, and their analogues could be effective candidates for chemoprevention of lung cancer and HMDB might have the strongest ability for inducing apoptosis.

KEYWORDS: Apoptosis; Bcl-2; caspase; dibenzoylmethane; human lung carcinoma; PARP; resveratrol

INTRODUCTION

Polyphenols, such as resveratrol (3,5,4′-trihydroxystilbene) and dibenzoylmethane (DBM) (**Figure 1**), are common antioxidants found in various plants. Their pharmacological and physiological activities have been studied extensively in many fields, including cardioprotection (*1*), inhibition of platelet aggregation, anti-inflammation, and antioxidant, vasorelaxant, and anticarcinogenic capacities (*2*, *3*). Resveratrol, an isoflavone present in grapes, berries, pine nuts, and peanuts, has been reported to suppress growth of various cancer cells by regulating NF-κB and AP-1 activities (*4*). It can also induce apoptosis through mitochondrial pathways in human pancreatic cancer cells (*5*) and in 7,12-dimethyl-benz[α]anthracene (DMBA)-induced mouse skin tumors (*6*). DBM is a flavonoid phytochemical that is a minor constituent of the root extract of licorice (*Glycyrrhiza glabra* in the family Leguminosae). Dietary administration of DBM can inhibit DMBA-induced mammary tumor growth in SENCAR (sensitive to carcinogenesis) mice (*7*). Treatment with DBM can cause cell cycle arrest of prostate cancer cells in the G_1 and S phases (*8*). The highly potent inhibitory effects of resveratrol and DBM toward tumorigenesis suggest that they are efficient cancer chemoprevention agents. However, the bioavailability of such phytochemicals is restricted by their metabolic rate in the liver. There are various natural resveratrol and DBM analogues existing in plants. For example, isorhapontigenin and 3,5,4′-trimethoxy-*trans*-stilbene (MR-3) are analogues of resveratrol that are isolated from *Belamcanda chinensis* and *Pterobolium hexapetallum* (*9*), respectively. The effects of resveratrol and its methoxy derivative on human cancer cell lines have been tested by Pan et al. (*10*). The methoxy derivative markedly exhibited an inhibitory effect toward COLO 205 cell growth. This result indicated that the difference in bioactivity of the methoxy derivative is related to the presence and positionality of methoxy groups on the basic resveratrol chemical structure. 1-(2-Hydroxyphenyl)-3-phenyl-1,3-propanedione (HDB) and 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB) are identical in structure to DBM and similar to curcumin in that they possess a β-diketone (1,3-diketone), except that they possess a hydroxyl group and methyl group on the aromatic rings, respectively.

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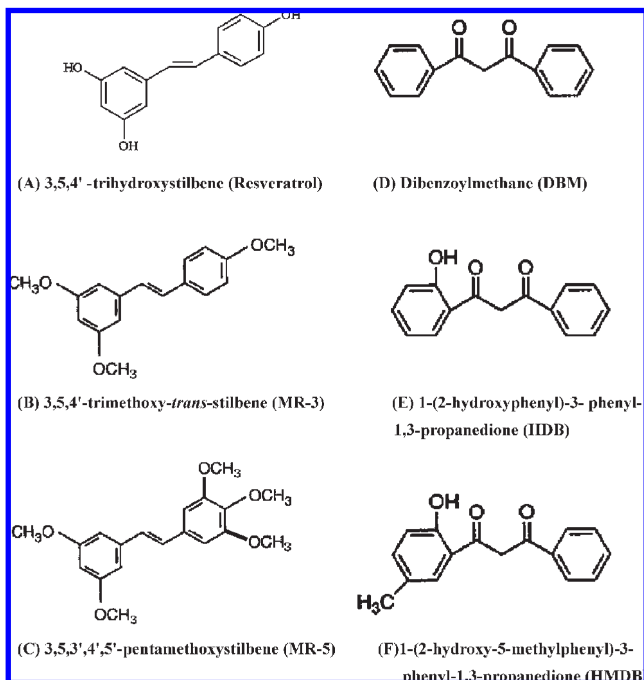

Figure 1. Chemical structures of resveratrol, DBM, and their analogues.

Lung cancer is the leading cause of approximately 20% of tumor-related deaths. The major cause of cancer formation is the stimulation of cells by internal or external factors, which leads to mutations in one or multiple genes. These mutations usually disrupt the balance of proliferation and death in normal cells. The cell cycle might become uncontrolled, and the rapid proliferation of cells finally leads to benign or malignant tumor formation (*11*). Cells could respond to DNA damage by arresting the cell cycle and facilitating DNA repair or by undergoing cell suicide. Cell apoptosis is a process that plays an important role during development and homeostasis of multicellular organisms because it facilitates the destruction of undesired cells. It is associated with many characteristic morphological changes, including membrane blebbing, formation of apoptotic bodies, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (*12*). Two principal pathways are known to be involved in initiating apoptosis. One of these depends upon the participation of pro- and anti-apoptotic Bcl-2 family members to regulate the mitochondrial pathway (*13*). The other pathway is stimulated by the interaction of a death receptor and its ligand at the plasma membrane. There are numerous proteins involved in programmed cell death, and one of these is the caspase family of proteins. Caspases are cysteine proteases that are activated during the execution of apoptosis (*14*). There are at least two major mechanisms, involving caspase-8 or caspase-9, by which a caspase cascade results in the activation of caspase-3 (*15*, *16*).

Chemotherapy is a common clinical cancer therapy that inhibits cell proliferation by stopping the cell cycle or by inducing apoptosis in cancer cells. However, the effective doses of chemotherapeutic agents often cause serious side effects for patients with a variety of cancers. Thus, it becomes important to search for potent chemotherapeutic agents that allow for the reduction of treatment dosage to minimize side effects. It has been demonstrated that the methylated flavonoids could increase cytotoxicity in tumor cells, such as leukemic cells and hepatocarcinomas. Additionally, the pharmacological effect of phytochemicals may vary with the modification of different groups on it. Although the induction of apoptosis and the suppression of cell growth by resveratrol and DBM have been observed in various cancer cell lines (*17*, *18*), the apoptotic effects of resveratrol, DBM, and their analogues on human lung cancer cells remain unclear. In this study, human lung adenocarcinoma A549 and lung squamous carcinoma CH27 cell lines were employed as *in vitro* lung cancer model systems to investigate the apoptotic potential and the underlying mechanisms of resveratrol, DBM, and their methylated or hydroxylated analogues.

MATERIALS AND METHODS

Materials and Reagents. MR-3 and 3,5,3′,4′,5′-pentamethoxystilbene (MR-5) were synthesized and purified through column chromatography, and a purity greater than 99% was verified by high-performance liquid chromatography (HPLC) (*19*). DBM, HDB, and HMDB were purchased from Aldrich Chemical Co. (Milwaukee, WI). The purities of the chemicals used in this study, DBM, HDB, and HMDB, were 98, 98, and 97%, respectively. All chemicals were dissolved in dimethylsulfoxide (DMSO). RPMI 1640 medium was purchased from Invitrogen, Inc. (Grand Island, NY), and fetal bovine serum (FBS) was acquired from HyClone, Inc. (Logan, UT). Resveratrol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylsulfoxide (DMSO) was obtained from Tedia Co. (Fairfield, OH). The annexin V-FITC assay kit was purchased from AbD Serotec Ltd. (Kidlington, Oxford, U.K.). The lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Takara Biomedicals, Inc. (Otsu, Shiga, Japan). The mitochondrial permeability transition detection kit (MitoPT) was purchased from Immunochemistry Technologies (Kidlington, Oxford, U.K.). The mitochondria fractionation kit and the caspase-9 fluorometric assay kit were obtained from BioVision, Inc. (Mountain View, CA). The caspase-3 activity detection kit was purchased from Upstate (Lake Placid, NY). Antibodies for Bcl-2, Bax, caspase-3, PARP, and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell Culture. Human lung adenocarcinoma A549 cells (BCRC 60074) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Human lung squamous carcinoma CH27 cells were provided by Dr. Hsu of Taichung Veterans General Hospital. Cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 2.0 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Cell Viability Assay. Cell viability was determined using the MTT assay. Cells were seeded into 96-well plates at a concentration of 1×10^4 cells/well in RPMI 1640 medium. After 24 h of incubation, the cells were treated with various concentrations (0, 1, 5, 10, 25, 50, and 100 μM) of the chemicals and returned to the incubator. Control cells were treated with 0.1% DMSO alone. The dye solution [10 μL; 5 mg of dye/1 mL of phosphate-buffered saline (PBS)] was added to each well for an additional 2 h of incubation at 37 °C. After the addition of DMSO (100 μL/well), the reaction solution was incubated for 30 min in the dark. The absorbances at 570 and 630 nm (reference) were recorded using a FLUOstar galaxy plate reader (BMG LabTechologies, GmbH, Offenburg, Germany). The percent viability of the treated cells was calculated as follows:

$$(A_{570\ \mathrm{nm}} - A_{630\ \mathrm{nm}})_{\mathrm{sample}}/(A_{570\ \mathrm{nm}} - A_{630\ \mathrm{nm}})_{\mathrm{control}} \times 100$$

LDH Leakage Assay. LDH activity was examined using a commercial kit. Cells in 96-well plates (1×10^4 cells/well) were incubated with various concentrations (0, 1, 5, 10, 25, 50, and 100 μM) of each chemical for 24 h and then analyzed for LDH leakage into the culture medium. Total LDH leakage was determined after the cells were thoroughly disrupted by sonication. The absorbance at 492 nm was recorded using a FLUOstar galaxy plate reader. The percentage of LDH leakage was expressed as follows:

$$[(\text{activity in the medium})/(\text{activity in the medium} + \text{activity of the cells})] \times 100$$

Cell Apoptosis Analysis by PI Staining. Cells were treated with various concentrations (0, 10, 25, 50, and 100 μM) of chemical for 24 h. The cells were then harvested with a trypsin−ethylenediaminetetraacetic

acid (EDTA) (TE) solution (0.05% trypsin and 0.02% EDTA in PBS), washed twice with PBS, and fixed in 80% ethanol for 30 min at 4 °C. Fixation was followed by incubation with RNase (100 μg/mL) for 30 min at 37 °C. The cells were then stained with PI (40 μg/mL) for 15 min at room temperature in the dark and subjected to a flow cytometric analysis of DNA content using a FACScan flow cytometer (Becton−Dickinson Immunocytometery Systems, San Jose, CA). Approximately 1×10^4 counts were made for each sample. The percentage of cells undergoing apoptosis was calculated using CELL Quest software.

Annexin V-FITC/PI Double Staining Assay. Annexin V-FITC/PI double staining of the cells was performed with the Annexin V-FITC kit (ANNEX100F, SEROTEC, U.K.). Cells (1×10^6 cells/6 cm dish) were treated with various concentrations (0, 10, 25, 50, and 100 μM) of each chemical for 24 h at 37 °C. The cells were collected by centrifugation and stained for 10 min at room temperature with Annexin V-FITC and PI in a Ca^{2+}-enriched binding buffer (Annexin V-FITC kit). The cells were then analyzed by the FACScan flow cytometer. Annexin V-FITC and PI emissions were detected using emission filters of 525 and 575 nm, respectively. The Annexin V-FITC−/PI− population was regarded as normal, while Annexin V-FITC+/PI−, Annexin V-FITC+/PI+, and Annexin V-FITC−/PI+ populations were taken as measurements of early apoptotic, late apoptotic, and necrotic cells, respectively. Approximately 1×10^4 counts were made for each sample.

Mitochondrial Membrane Potential ($\Delta\Psi$m) Analysis. The mitochondrial membrane potential was determined using the MitoPT 100 Test kit (Immunochemistry Technologies, Bloomington, MN). Cells were seeded into a 12-well plate. After 24 h of incubation, the cells were treated with 50 μM of each chemical for 3, 6, 9, 12, and 18 h. The passage of cells included rinsing cells once with PBS in a 12-well plate, harvesting the cells with 0.1 mL of TE solution, adding 1 mL of fresh culture medium, and thoroughly dispersing the cells. Aliquots of these cell suspensions (1×10^6 in 1 mL of culture medium) were placed in an eppendorf and centrifuged at 1000 rpm for 5 min. The harvested cells were then incubated with JC-1 (a dye, 10 μg/mL) for 15 min at 37 °C in a humidified 5% CO_2 incubator, followed by washing with 1× assay buffer from the MitoPT 100 Test kit. Cells were resuspended in an adequate volume of the same solution, and they were analyzed using the FLUOstar galaxy fluorescence plate reader, with an excitation wavelength of 485 nm and an emission wavelength of 520 nm for red fluorescence.

Measurement of Caspase-9 Activity. The activity of caspase-9 was measured using a caspase-9 fluorometric assay kit. Briefly, cells were collected after treatment with 50 μM of the chemical for various time intervals. Then, the cells were washed with PBS and lysed in lysis buffer [1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris−HCl (pH 8), 2 mM dithiothione, 10 μg/mL pepstatin A, 2 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin] for 20 min at 4 °C. Lysis was followed by centrifugation (10000*g*) for 30 min. The cytosolic extract (50 μg of total protein) was mixed with the LEHD-AFC fluorogenic substrate (1 mM) in 50 μL of reaction buffer and incubated at 37 °C for 2 h. Fluorescence, using an excitation wavelength of 400 nm and an emission wavelength of 505 nm, was measured with a FLUOstar galaxy fluorescence plate reader.

Measurement of Caspase-3 Activity. The activity of caspase-3 was performed using a commercial kit. Briefly, cytosolic cellular extracts were obtained following the protocol from the caspase-9 activity assays, and they were mixed with a fluorometric substrate (50 μM) in 200 μL of modified RIPA buffer. Fluorescence, using an excitation wavelength of 380 nm and an emission wavelength of 460 nm, was measured after 5 min with a FLUOstar galaxy fluorescence plate reader.

Western Blot Analysis. Cells (1×10^7 cells/10 cm dish) were treated with chemicals for various time intervals at 37 °C. The cells were collected and lysed in an ice-cold lysis buffer. Total proteins (50−60 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 12% polyacrylamide gels and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blot was subsequently blocked with 5% skim milk in PBST [0.05% (v/v) Tween-20 in PBS at pH 7.2] for 1 h and probed with each specific antibody (1:5000) overnight at 4 °C. Detection was performed with an appropriate

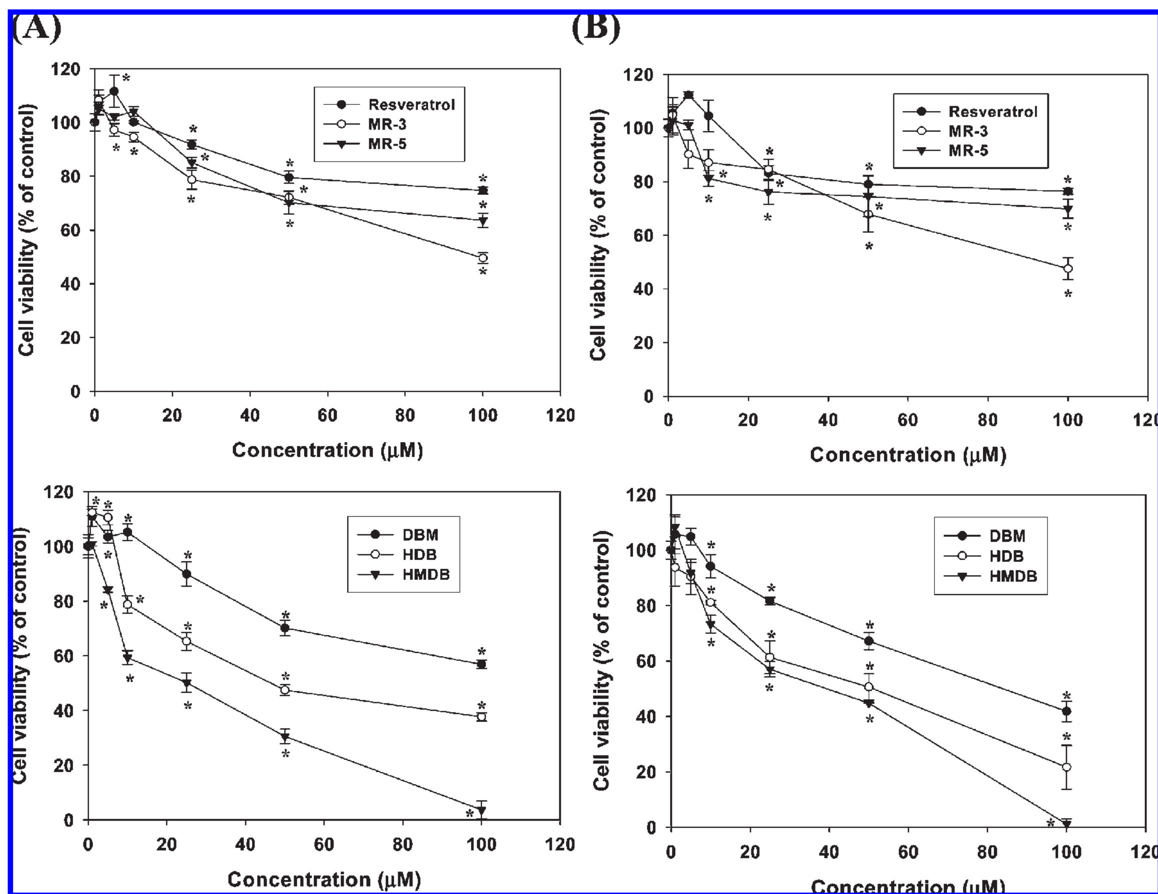

Figure 2. Effects of resveratrol, DBM, and their analogues on the cell viability of (A) A549 and (B) CH27 cells, using the MTT assay. Cells were treated with resveratrol, DBM, or their analogues at various concentrations (0, 1, 5, 10, 25, 50, and 100 μM) for 24 h. (*) $p < 0.05$ compared to the control.

peroxidase-conjugated secondary antibody (1:5000) at room temperature for 1 h. Intensive washing with PBS was performed after each incubation. After the final PBS wash, the signal was detected using the enhanced chemiluminescence (ECL) detection system and Kodak BioMax Light Film (Rochester, NY). The relative expression of proteins was quantified densitometrically using LabWorks 4.5 software, and calculations were performed on the basis of the β-actin reference bands.

Protein Content Determination. The protein content was determined by the Bradford method (*20*), with bovine serum albumin as a standard.

Statistical Analysis. Statistical analysis was performed using SAS software. Analysis of variance was performed using analysis of variation (ANOVA) procedures. Data are presented as mean ± standard deviation (SD) for three independent measurements. Differences between variants were analyzed by Duncan's multiple range tests for unpaired data. Values of $p < 0.05$ (*) or $p < 0.01$ (**) were regarded as statistically significant.

RESULTS

Effect of Resveratrol, DBM, and Their Analogues on the Viability of Human Lung Carcinoma Cells. The viability of A549 (**Figure 2A**) and CH27 (**Figure 2B**) cells treated for 24 h with 0−100 μM resveratrol, DBM, and their analogues (structures shown in **Figure 1**) was determined using the MTT assay. It was observed that the viability of both cell types was gradually decreased with increasing concentrations of the polyphenols, above 10 μM. Among their analogues, MR-3 and HMDB showed the strongest inhibitory effect on the cell population growth of both cell types. The IC_{50} values of MR-3 on A549 and CH27 cells were 98 and 92 μM, respectively, and for HMDB, the IC_{50} values on A549 and CH27 cells were 25 and 39 μM, respectively.

Cytotoxicity of Resveratrol, DBM, and Their Analogues on Human Lung Carcinoma Cells. To further evaluate the influence of resveratrol, DBM, and their analogues on cell injury of A549 and CH27 cell lines, a quantitative analysis of LDH activity was used to determine the percentage of dead cells. As shown in **Figure 3**, while being treated with 0−100 μM of resveratrol, DBM, and their analogues, the LDH leakage of cells increases significantly ($p < 0.05$) compared to the control. This result demonstrates that these compounds exhibited cytotoxicity against A549 and CH27 cell lines in a dose-dependent manner. For this particular experiment, MR-3 and HMDB showed the highest cytotoxicity toward both cell lines.

Effect of Resveratrol, DBM, and Their Analogues on the Induction of Apoptosis. The A549 and CH27 cells were treated for 24 h with various concentrations of resveratrol, DBM, and their analogues. The amount of sub-G1 DNA was analyzed by flow cytometry and quantified by the CELL Quest software. The results demonstrate that treatment with these compounds increases the number of apoptotic cells in a dose-dependent manner (**Figure 4**). Furthermore, HMDB induced the strongest apoptosis in A549 and CH27 cells, while resveratrol, among its analogues, had the most robust apoptotic induction in CH27 cells. Interestingly, the percentage of CH27 cells undergoing apoptosis did not increase significantly while being treated with 10−100 μM MR-3. To further evaluate the modes of cell death (apoptosis or necrosis) induced by these compounds, A549 and CH27 cells were treated with different concentrations of resveratrol, MR-3, and HMDB for 24 h and stained with Annexin V-FITC and PI. The cells were further analyzed by flow cytometry. As shown in **Table 1**, the treatment of resveratrol, MR-3, and HMDB decreases the number of normal cells in a dose-dependent manner. In contrast,

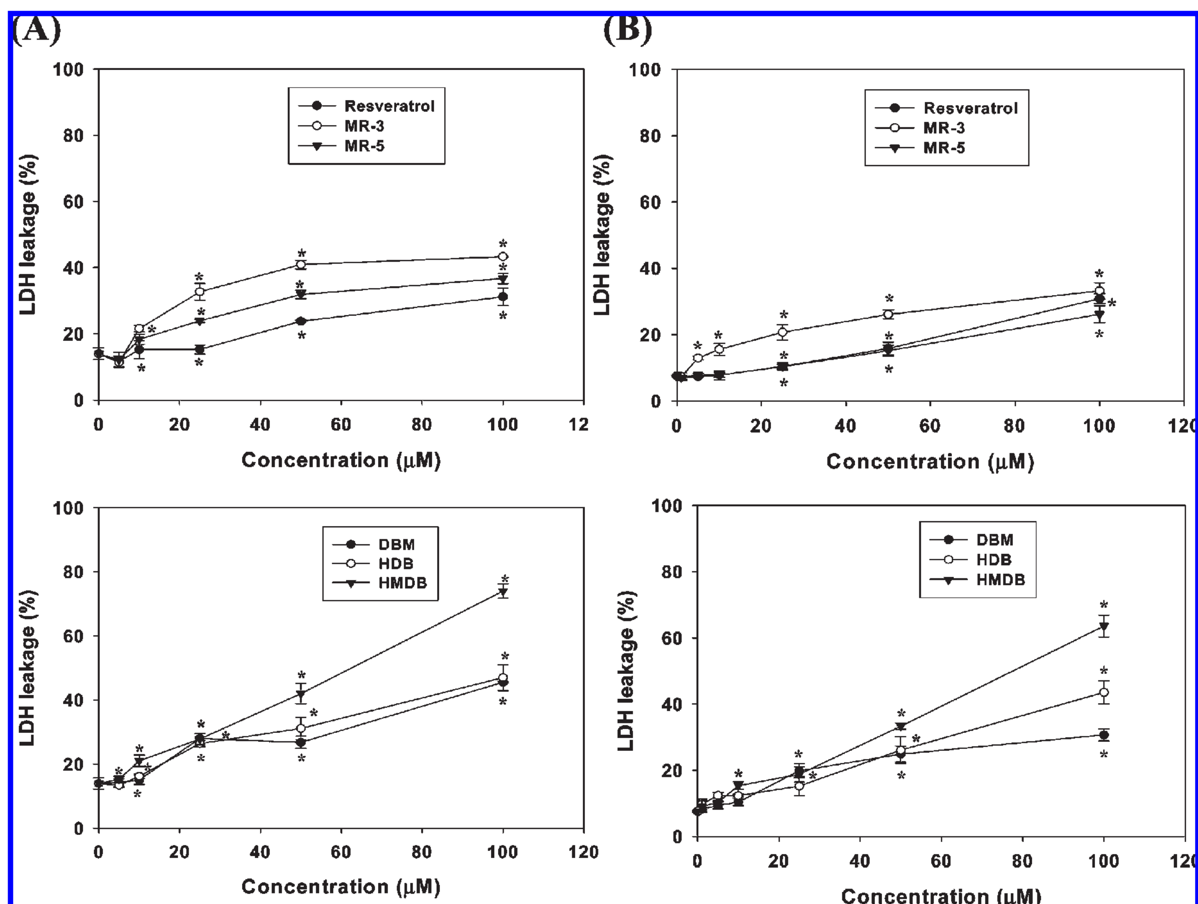

Figure 3. Analysis of cytotoxic effects of resveratrol, DBM, and their analogues on (A) A549 and (B) CH27 cells using the LDH leakage assay. Cells were treated with resveratrol, DBM, or their analogues at various concentrations (0, 5, 10, 25, 50, and 100 μM) for 24 h. (*) $p < 0.05$ compared to the control.

the numbers of apoptotic cells, including both early and late apoptotic cells, were increased dose-dependently. Treatment of A549 cells with HMDB at a concentration of 50 μM yielded 28.2% apoptotic (early and late stages) cells and 2.2% necrotic cells. However, the percentages of apoptotic (early and late stages) and necrotic A549 cells changed to 19.1 and 54.7%, respectively, as the concentration of HMDB increased to 100 μM. These data reveal that 100 μM HMDB might cause serious cellular injury and increase necrotic cell death. In addition, the percentages of CH27 cells undergoing apoptosis after treatment with various concentrations of MR-3 did not increase significantly compared to the results obtained with resveratrol. These data indicated that MR-3 might inhibit CH27 cell population growth through another mechanism besides the induction of apoptosis.

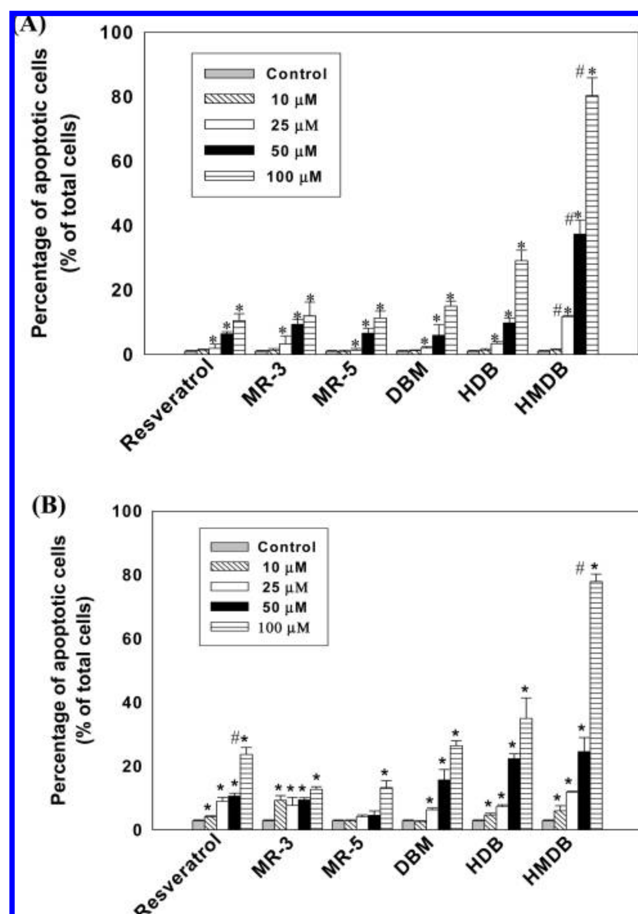

Figure 4. Apoptotic induction by resveratrol, DBM, and their analogues in (A) A549 and (B) CH27 cells. The cells were analyzed by flow cytometry after PI staining. The percentages of apoptotic cells were calculated with the CELL Quest software. (*) $p < 0.05$ compared to the control. (#) $p < 0.05$ compared to their analogues at the same dose.

Collapse of Mitochondrial Membrane Potential (ΔΨm) by Resveratrol, MR-3, and HMDB. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. The loss of mitochondrial membrane potential is a hallmark for apoptosis. In other words, the electrochemical gradient across the mitochondrial membrane collapses during the apoptotic process. While treating A549 and CH27 cells with resveratrol, MR-3, and HMDB at a concentration of 50 μM for 6−18 h, a significant ($p < 0.05$) reduction of red fluorescence intensity was observed (**Figure 5**). This decrease in fluorescence intensity indicated that the early damage of the cells was due to the collapse of the mitochondrial membrane potential, which might subsequently activate the intrinsic pathway of apoptosis.

Effect of Resveratrol, MR-3, and HMDB on the Protein Expression of Bcl-2 and Bax. The collapse of the mitochondrial membrane potential occurs through the formation of pores on the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Bax and Bcl-2 are proteins that belong to the Bcl-2 family, and they serve as pro- and anti-apoptotic effectors, respectively, to govern mitochondrial outer membrane permeabilization (MOMP). The imbalance of anti- and pro-apoptotic protein expression is one of the major mechanisms underlying the ultimate fate of cells during the apoptotic process. In comparison to the control, a 6 h treatment of A549 cells with 50 μM resveratrol, MR-3, or HMDB led to a significant ($p < 0.05$) increase, 4.2-, 4.7-, and 4.6-fold, respectively, in Bax protein levels. Additionally, a significant ($p < 0.05$) decrease in the protein level of Bcl-2, 0.5-, 0.3-, and 0.3-fold, respectively, was observed (**Figure 6A**). The Bax/Bcl-2 ratio increased in a time-dependent manner. A similar result was also obtained when using these three compounds to treat CH27 cells (**Figure 6B**).

Resveratrol, MR-3, and HMDB Stimulated Caspase-9 Activity in a Time-Dependent Manner. It is well-known that proteins of the Bcl-2 family play a pivotal role in apoptosis by affecting caspase activation (*21*). Caspase-9, an aspartic-acid-specific protease, is an initiator caspase linked to the mitochondrial death pathway, and it is activated during programmed cell death. To monitor the enzymatic activity of caspase-9 during apoptosis induced by resveratrol, MR-3 and HMDB, a specific fluorogenic substrate (Ac-LEHD-AFC) was used to detect caspase-9 activity. The results shown in **Figure 7** indicate that treatment with resveratrol, MR-3, and HMDB caused a significant ($p < 0.05$) time-dependent increase in caspase-9 activity for both the A549 (**Figure 7A**) and CH27 (**Figure 7B**) cell lines. Caspase-9 activity in the A549

Table 1. Effects of Resveratrol, MR-3, and HMDB on the Induction of Apoptosis and Necrosis in A549 and CH27 Cells

			resveratrol				MR-3				HMDB			
	concentration (μM)	C[a]	10	25	50	100	10	25	50	100	10	25	50	100
A549[b]	normal (%)	95.1	95.0	93.7	91.8	86.2	94.9	92.9	87.5	84.8	92.3	83.8	69.7	26.3
	early apoptosis (%)	1.7	1.6	3.0	3.9	8.5	1.4	1.7	7.7	6.7	4.4	5.7	23.9	5.0
	late apoptosis (%)	2.0	2.2	1.9	2.6	3.0	2.2	4.2	2.5	6.0	1.9	7.1	4.3	14.1
	necrosis (%)	1.2	1.2	1.4	1.8	2.4	1.6	1.2	2.3	2.5	1.5	3.4	2.2	54.7
CH27[b]	normal (%)	94.2	91.4	84.8	79.6	56.4	83.9	78.0	73.4	71.1	85.3	70.9	54.7	8.9
	early apoptosis (%)	1.0	2.4	3.0	4.8	14.3	4.9	5.4	7.5	6.1	2.5	4.3	3.8	22.4
	late apoptosis (%)	3.9	5.5	9.3	12.5	25.8	9.7	10.1	11.3	12.3	8.0	9.0	21.9	41.7
	necrosis (%)	0.7	0.5	2.8	3.0	3.5	1.5	6.3	7.5	10.4	4.0	15.6	19.6	26.8

[a] C = control group. [b] The cells were analyzed by flow cytometry after being stained with Annexin V-FITC and PI. The percentages of cells were calculated with the CELL Quest software.

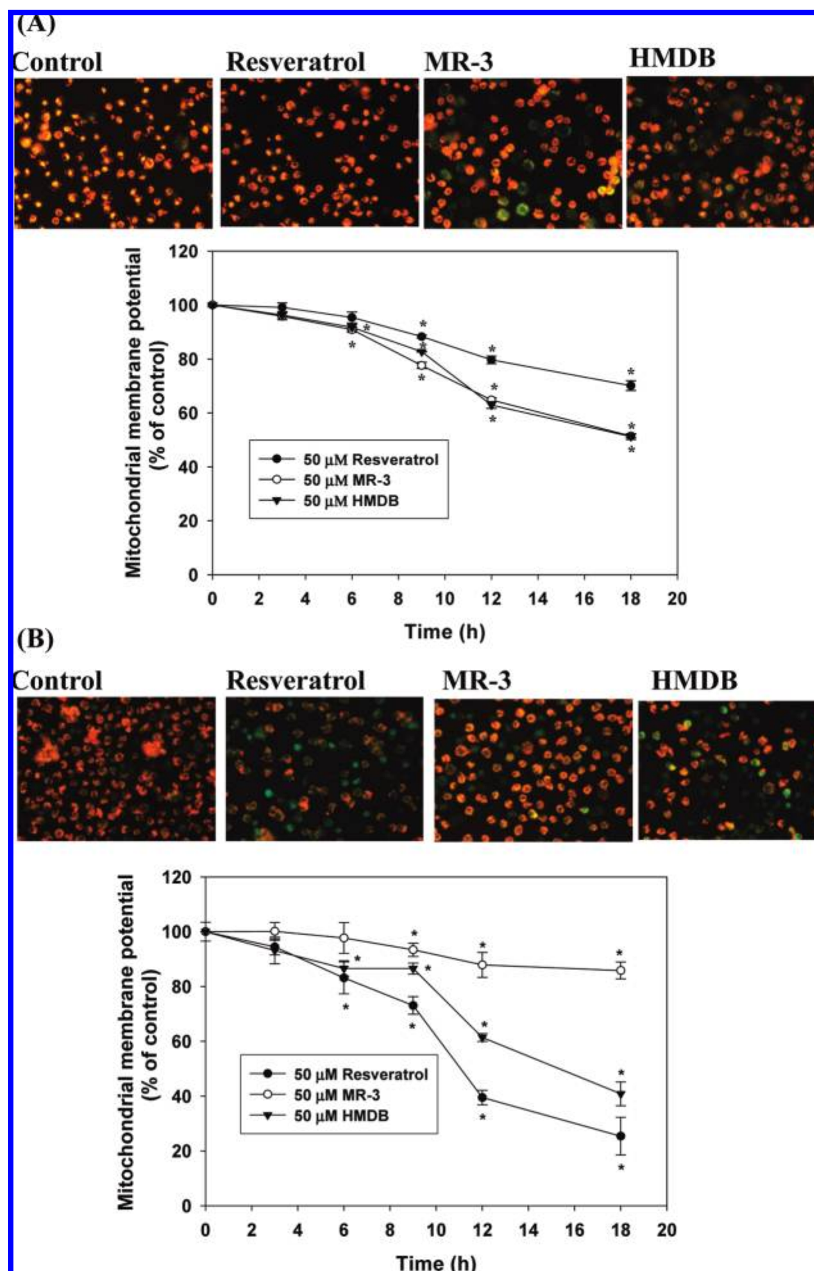

Figure 5. Effects of resveratrol, MR-3, and HMDB on mitochondrial membrane potential ($\Delta\psi_m$) in (A) A549 and (B) CH27 cells. Cells were treated with 50 μM resveratrol, MR-3, and HMDB for 0−18 h. (*) $p < 0.05$ compared to the control.

and CH27 cell lines was enhanced 2.4- and 3.4-fold, respectively, after treatment with HMDB for 18 h. In comparison to the resveratrol and MR-3, HMDB led to maximal caspase-9 activity in both cell lines.

Resveratrol, MR-3, and HMDB Activated Caspase-3 and Caused Cleavage of PARP. Caspase-9 is activated early in the apoptotic cascade by cytochrome *c*. Activated caspase-9 then stimulates the proteolytic activity of other downstream caspases, including caspase-3. Activation of caspase-3 leads to the cleavage of numerous proteins, one of which is poly(ADP-ribose) polymerase (PARP). To determine the effect of resveratrol, MR-3, and HMDB on the protein levels of caspase-3 and PARP in A549 and CH27 cells, cytosolic extracts from treated cells were evaluated by Western Blot analysis. As shown in **Figure 8**, treatment of the cells with 50 μM resveratrol, MR-3, and HMDB for 3−18 h significantly ($p < 0.05$) stimulates caspase-3 expression (panels **A-1** and **B-1** of **Figure 8**) and activity (panels **A-2** and **B-2** of **Figure 8**), in a time-dependent manner. Treating A549 cells with resveratrol, MR-3, and HMDB increased levels of activated caspase-3 by 4.9-, 9.5-, and 9.8-fold, respectively, compared to the control. Treatment of CH27 cells with resveratrol, MR-3, and HMDB also increased the levels of activated caspase-3 by 2.2-, 1.5-, and 3.5-fold, respectively, compared to the control. In addition, treatment with resveratrol, MR-3, and HMDB significantly ($p < 0.05$) induced PARP cleavage in A549 and CH27 cells, in a time-dependent manner.

DISCUSSION

The aims of this study were to evaluate the apoptotic effects and molecular mechanisms of resveratrol, DBM, and their analogues on human lung cancer cells. The anti-proliferative (IC_{50}) and the apoptotic induction (AC_{50}) activities of resveratrol were variable based on different modifications to its structure (*22*). The findings from Minutolo et al. (*23*) and Simoni et al. (*24*) demonstrated that treatment with MR-3, a methylated resveratrol derivative, yielded higher cytotoxicity than resveratrol in

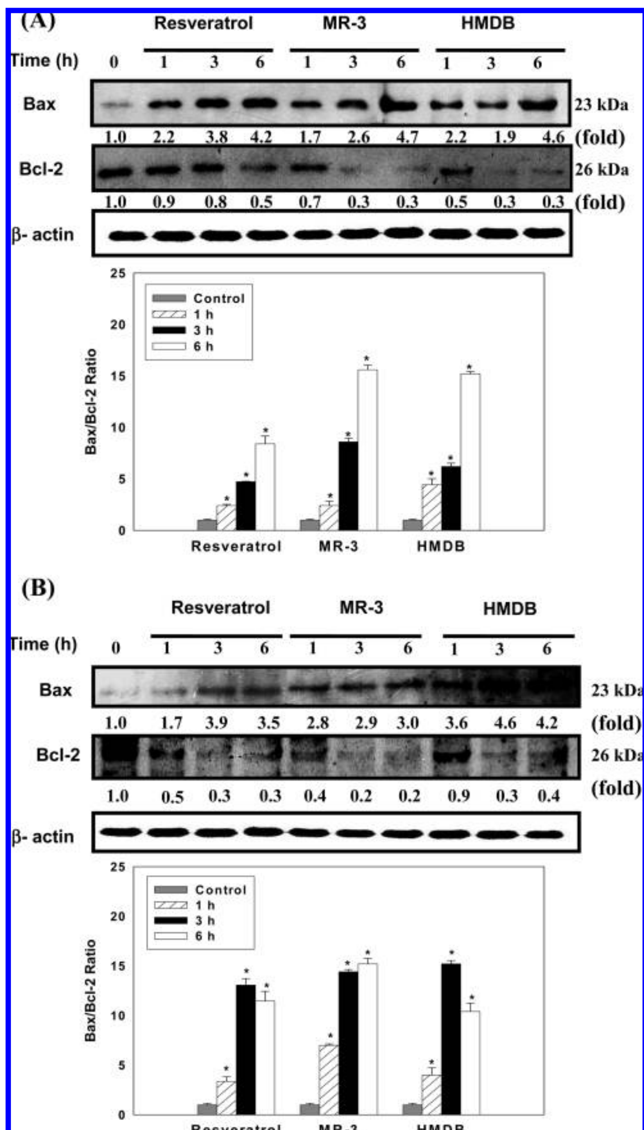

Figure 6. Effects of resveratrol, MR-3, and HMDB on the protein levels of Bcl-2 and Bax in (A) A549 and (B) CH27 cells. Cells were untreated or treated with 50 μM resveratrol, MR-3, or HMDB for 1, 3, and 6 h. (∗) $p < 0.05$ compared to the control. (#) $p < 0.05$ compared to each other compound at the same dose.

MDA-MB-231 (a human breast cancer cell line) (IC_{50} = 1.2 μM) and HL-60 (a human promyelocytic leukemia cell line) cells (IC_{50} = 2.5 μM). Piceatannol (3,5,3′,4′-tetrahydroxy-*trans*-stilbene), a hydroxylated resveratrol derivative, induced apoptosis in the human melanoma cell line, SK-Mel-28, cells at a low concentration of 1 μM and arrested the cell cycle in the G_2 phase (*25*). It was reported that DBM can inhibit cell proliferation of HeLa (a human cervical cancer cell line) (IC_{50} = 68 μM) and COLO205 (a human colonic carcinoma cell line) cells (IC_{50} = 31 μM). HDB and HMDB, analogues of DBM, also have an inhibitory effect on cell proliferation of A431 (a human epithelial carcinoma cell line) (IC_{50} = 89 and 52 μM, respectively) and COLO205 cells (IC_{50} = 15 and 19 μM, respectively) (*26*, *27*). As mentioned above, it was interesting to note that the methylated or hydroxylated polyphenols might have a higher pharmaceutical activity and bioavailability than the nonmethylated or nonhydroxylated counterparts. In the current study, results from the MTT and LDH leakage assays clearly indicated that MR-3 and HMDB at a range of concentrations from 5 to 100 μM could effectively inhibit cell population growth and induce cell injury in the A549 and CH27 cell lines. Our findings further indicate that the trends obtained using LDH leakage to assess cytotoxicity (**Figure 3**) were consistent with the data obtained using flow cytometry (**Figure 4**) to examine cellular apoptosis (PI staining method) induced by resveratrol, DBM, and their analogues.

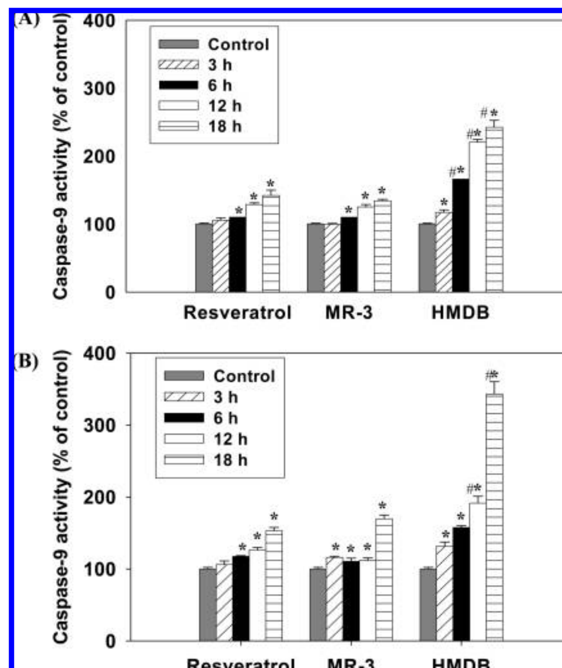

Figure 7. Effects of resveratrol, MR-3, and HMDB on caspase-9 activity in (A) A549 and (B) CH27 cells. Cells were untreated or treated with 50 μM resveratrol, MR-3, or HMDB for 3, 6, 12, and 18 h. (∗) $p < 0.05$ compared to the control.

In the analysis of cell apoptosis using PI staining, treatment of A549 and CH27 cells with resveratrol, DBM, and their analogues induced cellular apoptosis in a dose-dependent manner (**Figure 4**). Resveratrol, MR-3, and HMDB, the three characteristic polyphenols in PI staining, were used in the subsequent experiments. Annexin V-FITC binds to phosphatidylserine on the cell surface and can be used to detect the early stages of apoptosis. After the treated cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry, a dose-dependent increase in the early apoptotic cell population was observed (**Table 1**). The results revealed that resveratrol, MR-3, and HMDB could induce apoptotic cell death in the A549 and CH27 cell lines. However, treatment with MR-3 did not significantly increase the percentages of A549 and CH27 cells undergoing apoptosis compared to those treated with resveratrol. From a cell cycle analysis with PI staining, we found that treatment with MR-3 could increase the fraction of cells in the sub-G_1 phase (an apoptotic phenomenon) and cause an accumulation of cells in the G_2/M phase (data not shown). It was thus hypothesized that MR-3 might inhibit cell growth of the A549 and CH27 cell lines through induction of apoptosis and regulation of the cell cycle.

Mitochondria play an essential role in cell death signal transduction because the opening of the permeability transition pore and the collapse of the mitochondrial membrane potential result in the rapid release of caspase activators, such as cytochrome *c*, into the cytoplasm. In general, a change in the externalization of membrane phosphatidylserine follows the reduction of mitochondrial membrane potential. Detection of the mitochondrial membrane potential provided an early indication of the initiation of cellular apoptosis. In the current study, the A549 and CH27 cell lines treated with resveratrol, MR-3, and HMDB showed a reduced mitochondrial membrane potential, in a time-dependent

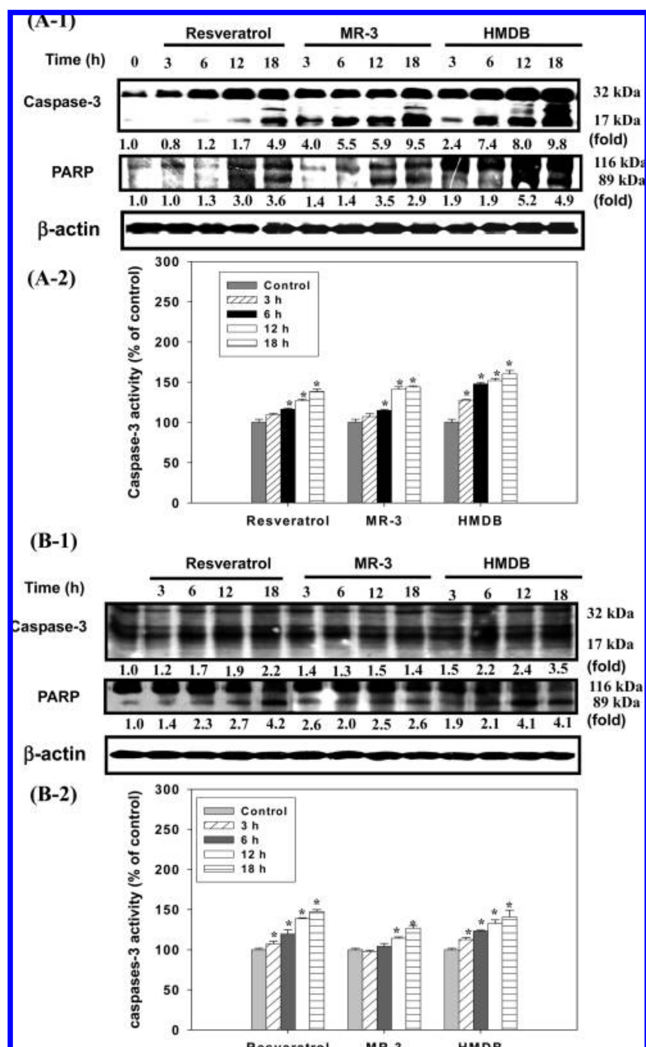

Figure 8. Effects of resveratrol, MR-3, and HMDB on the protein levels of caspase-3 and cleaved PARP (A-1 and B-1) and on the activity of caspase-3 (A-2 and B-2) in (A) A549 and (B) CH27 cells. Cells were untreated or treated with 50 μM resveratrol, MR-3, or HMDB for 3, 6, 12, and 18 h. (∗) $p < 0.05$ compared to the control.

manner (**Figure 5**). This result might suggest that apoptosis was initiated by treatment with these compounds.

The Bcl-2 family of proteins forms ion channels in biological membranes, and this ion channel regulates apoptosis by influencing the permeability of the intracellular membrane of mitochondria (*28*). It was proposed that the ratio between Bcl-2 and Bax is more important in the regulation of apoptosis than the level of each Bcl-2 family protein separately (*29*). Bax overexpression alone has been demonstrated to accelerate apoptotic cell death (*30*). Our data indicated that treatment with resveratrol, MR-3, and HMDB markedly decreased the protein levels of Bcl-2 and increased Bax protein expression. The Bax/Bcl-2 ratio also gradually increased with the treatment of higher concentrations of the compound (**Figure 6**). It was suggested that the protein levels of anti-apoptotic Bcl-2 decreased and pro-apoptotic Bax increased, which might play a key role in how these three compounds induce apoptosis in the A549 and CH27 cell lines.

It is well-known that proteins of the Bcl-2 family play a pivotal role in cells undergoing apoptosis by interfering with caspases and caspases are the key effectors of programmed cell death (*21*). The caspase cascade is initiated by the proteolysis of inactive procaspases, and it is propagated by the cleavage of downstream caspases and substrates, such as PARP (*28*). Caspase-9 is a member of the CED-3 family and bears high similarity to caspase-3. Caspase-3 is one of the key executioners of apoptosis. Cleavage of procaspase-3 by caspase-9 produces an active enzyme that is capable of cleaving PARP. PARP is a chromatin-bound enzyme, which, upon activation by DNA-strand breaks, catalyzes the successive transfer of ADP-ribose units from NAD to nuclear proteins. Cleavage of PARP results in its inactivation, slowing the DNA repair process and enhancing the apoptotic process. In the current study, the results showed that resveratrol-, MR-3-, and HMDB-induced apoptosis were controlled through activation of caspases. The compounds first enhanced caspase-9 activity in a time-dependent manner (**Figure 7**). Subsequent Western Blot analysis indicated progressive proteolytic cleavage of caspase-3 and PARP in A549 and CH27 cell lines (**Figure 8**).

In conclusion, this study indicates that the apoptotic effects of these compounds in A549 and CH27 cells were initiated by raising the Bax/Bcl-2 ratio, collapsing the mitochondrial membrane potential, and activating caspase-9 and caspase-3 to further cleave substrates, such as PARP. Resveratrol and DBM are two natural dietary compounds in food. The contents of resveratrol in grapes, peanuts, and red wine are 50−100 mg/g, 0.02−1.79 mg/g, and 13.4 mg/L, respectively. DBM is also a constitute in licorice, which is a common material for food seasoning. MR-3 and HMDB, methylated or hydroxylated polyphenols, were demonstrated to possess a higher pharmaceutical activity and bioavailability than resveratrol and DBM. MR-3 might arrest the cell cycle of CH27 cells in the G_2/M phase, which would inhibit cell growth. Among the compounds tested, HMDB might have the most potent ability to induce apoptosis. Inhibition of proliferation and induction of apoptosis are regulated by a large network of signaling pathways, which also involve a number of related apoptotic proteins, leading to possible targets for rational tumor therapy. Hence, resveratrol, DBM, and their analogues could be effective candidates for chemoprevention of lung cancer. However, further *in vivo* verification is necessary.

ABBREVIATIONS USED

DBM, dibenzoylmethane; HDB, 1-(2-hydroxyphenyl)-3-phenyl-1,3-propanedione; HMDB, 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione; LDH, lactate dehydrogenase; MR-3, 3,5,4′-trimethoxy-*trans*-stilbene; MOMP, mitochondrial outer membrane permeabilization; MR-5, 3,4,3′,4′,5′-pentamethoxystilbene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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