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# Induction of Apoptosis by 1-(2-Hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione through Reactive Oxygen Species Production, GADD153 Expression, and Caspases Activation in Human Epidermoid Carcinoma Cells

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This study examined the growth inhibitory effects of the structurally related $\beta$-diketones compounds in human cancer cells. Here, we report that 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB) induces growth inhibition of human cancer cells and induction of apoptosis in A431 cells through modulation of mitochondrial functions regulated by reactive oxygen species (ROS). ROS generation occurs in the early stages of HMDB-induced apoptosis, preceding cytochrome *c* release, caspase activation, and DNA fragmentation. The changes occurred after single breaks in DNA were detected, suggesting that HMDB induced irreparable DNA damage, which in turn triggered the process of apoptosis. Up-regulation of Bad and p21; down-regulation of Bcl-2, Bcl-$X_L$, Bid, p53, and fatty acid synthase; and cleavage of Bax were found in HMDB-treated A431 cells. Glutathione and *N*-acetylcysteine (NAC) suppress HMDB-induced apoptosis. HMDB markedly enhanced growth arrest DNA damage inducible gene 153 (GADD153) mRNA and protein in a time- and concentration-dependent manner. NAC prevented up-regulation of GADD153 mRNA expression caused by HMDB. These findings suggest that HMDB creates an oxidative cellular environment that induces DNA damage and GADD153 gene activation, which in turn helps trigger apoptosis in A431 cells.

**KEYWORDS: HMDB; apoptosis; cytochrome *c*; caspase-9; caspase-3; poly(ADP-ribose) polymerase; antioxidant; GADD153; reactive oxygen species**

## INTRODUCTION

Cancer chemoprevention is the use of pharmacological or natural agents to inhibit the development of invasive cancer or reverse the process of carcinogenesis. It could be the most direct process to reduce morbidity and mortality from cancerous disease (*1*−*3*). A large number of chemopreventive and chemotherapeutic agents, from natural products, have been used as a promising strategy to fight against cancer by inducing apoptosis in malignant cells (*3*, *4*).

Apoptosis is defined as a type of cell death, involving the concerted action of a number of intracellular signaling pathways, including members of the caspases family of cytsteine proteases, stored in most cells as zymogens or procaspases (*5*). Proteolytic cleavage of procaspases is an important step leading to caspase activation, which in turn is amplified by the cleavage and activation of other downstream caspases in the apoptosis cascade (*6*, *7*). The two main apoptotic pathways, the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, are activated by caspase-8 and caspase-9, respectively. Caspase-8 is recruited as a death-inducing signaling complex only when death receptors such as Fas or the tumor necrosis factor receptor bind to specific multimeric ligands. In contrast, caspase-9 is activated when cytochrome *c* is released into cytoplasm from the mitochondrial intermembranous space. Activated caspase-8 and caspase-9 activate executioner caspases, including caspase-3, which in turn cleaves a number of cellular proteins that include structural protein, nuclear proteins, cytoskeletal proteins, and signaling molecules (*6*). Moreover, the mitochondrial pathway is regulated by the Bcl-2 family of proteins, including antiapoptotic proteins such as Bcl-2 and Bcl-$X_L$ and proapoptotic proteins such as

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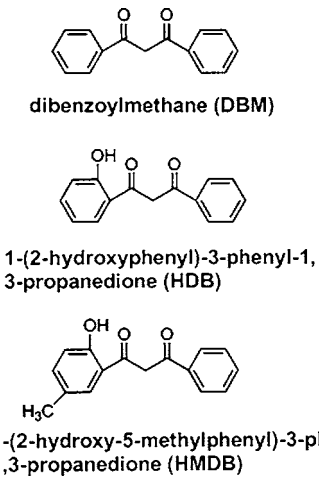


**Figure 1.** Chemical structures of DBM, HDB, and HMDB.

Bad, Bid, Bim, Bax, and Bak (*8*). Recent studies to the endoplasmic reticulum (ER) as a third subcellular compartment were implicated in apoptotic execution induced by ER stress (*9*−*11*). The ER stress-induced cell death modulator is a CCAAT/enhancer-binding protein (CEBP) homology protein (CHOP)/growth arrest, and DNA damage inducible gene 153 (GADD153), known as CHOP, is a member of the CEBP family of transcription factors (*12*). Expressed at low levels in proliferating cells, it is strongly induced in response to stresses that result in growth arrest or cell death, including oxidant injury (*13*), DNA damage agents such as peroxynitrite (*14*), UV radiation (*15*), anticancer drugs treatment (*16*), and ER stress (*12*, *17*). Recent studies suggest that GADD153 plays a central role in the apoptosis induction by overexpression of GADD153, of vector-transfected cells (*18*), including the dephosphorylation of the proapoptotic protein Bad (*19*) and down-regulation Bcl-2 expression (*17*). Therefore, the early events leading to initiation of apoptosis are triggered by gene expression of GADD153 and are not merely a consequence of apoptosis.

Our previous experiments showed that 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB) (**Figure 1**) inhibited proliferation and induced apoptosis in human colorectal carcinoma cells (*20*). In this study, we further selected dibenzoylmethane (DBM), 1-(2-hydroxyphenyl)-3-phenyl-1,3-propanedione (HDB), and HMDB for evaluation of anticancer activities in various human cancer cells. HDB and HMDB are identical in structure to DBM and similar to curcumin in that they possess a $\beta$-diketone (1,3-diketone), except that they possess a hydroxyl group and methyl group on the aromatic rings, respectively. It is interesting to note that, on the basis of our present studies, HMDB is far more potent than HDB and DBM of inhibiting the proliferation and inducing apoptosis in A431 cells. However, to date, no information is available regarding the mechanisms of the effects of HMDB on human epidermoid carcinoma A431 cells. Therefore, the mechanism of apoptosis was investigated. This included studies on the effects of HMDB on the activation of reactive oxygen species (ROS) production and caspase cascade, expression of Bax, Bad, Bcl-2, Bcl-$X_L$, and Bid, as well as changes in expression of p21, p53, fatty acid synthase (FAS), and GADD153 gene in A431 cells.

## MATERIALS AND METHODS

**Cell Culture and Chemicals.** The human epidermoid carcinoma cell line A431 (American Type Culture Collection [ATCC]) was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented wihth 10% fetal bovine serum. The human AGS gastric carcinoma cell lines (CCRC 60102) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Hep 3B and Hep G2 cell lines were derived from human HCC (ATCC HB-8064 and HB-8065). The HT 29 cell line was isolated from human colon adenocarcinoma (ATCC HTB-38). Cell lines were grown at 37 °C in 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium for A431, Hep 3B, Hep G2, MCF-7, and HeLa cells, DMEM/F12 for AGS cells, and RPMI for HT-29 cells, supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, 100 units/mL of penicillin and 100 $\mu$g/mL of streptomycin) and 2 mM L-glutamine (Gibco BRL). 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, and were dissolved in dimethyl sulfoxide (DMSO). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO). The inhibitors of caspase-3 (z-Val-Ala-Asp-fluoromethyl ketone, z-VAD-FMK) were purchased from Calbiochem (La Jolla, CA).

**Cell Survival Assay.** Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, human cancer cells were plated at a density of $1 \times 10^5$ cells/mL into 24 well plates. After overnight growth, cells were pretreated with a series of concentrations of DBM, HDB, and HMDB for 24 h. The final concentration of DMSO in the culture medium was <0.1%. At the end of treatment, 30 $\mu$L of MTT was added, and cells were incubated for a further 4 h. Cell viability was determined by scanning with an enzyme-linked immunosorbent assay reader with a 570 nm filter.

**DNA Extraction and Electrophoresis Analysis.** The A431 human cancer cells were harvested, washed with phosphate-buffered saline (PBS), and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM tris(hydroxymethyl)aminomethan (pH 8.0), and 10 mM ethylenediaminetetraacetic acied (EDTA) at 56 °C overnight and treated with RNase A (0.5 $\mu$g/mL) for 3 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl (25:24:1) before loading and was analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in Tris-borate/EDTA electrophoresis buffer. Approximately 20 $\mu$g of DNA was loaded into each well, visualized under UV light, and photographed (*21*).

**Acridine Orange Staining Assay.** Cells ($5 \times 10^5$) were seeded into 60 mm Petri dishes and incubated at 37 °C for 24 h. The cells were harvested after treatment for 24 h, and 5 $\mu$L of cell suspension was mixed on a slide with an equal volume of acridine orange solution (10 $\mu$g/mL in PBS). Green fluorescence was detected between 500 and 525 nm using an Olympus microscope (Olympus America, Inc., Lake Success, NY). Bright-staining condensed chromatin was detected in apoptotic cells.

**Flow Cytometry.** A431 cells ($2 \times 10^5$) were cultured in 60 mm Petri dishes and incubated for 24 h. The cells were then harvested, washed with PBS resuspended in 200 $\mu$L of PBS, and fixed in 800 $\mu$L of iced 100% ethanol at −20 °C. After they were left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 $\mu$g/mL RNase), and incubated at 37 °C for 30 min. Next, 1 mL of propidium iodide solution (50 $\mu$g/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

**ROS Production Determination.** Cells were treated with HMDB (100 $\mu$M) for different time periods, and dichlorodihydrofluorescein diacetate (DCFH-DA) (20 $\mu$M) or DHE (20 $\mu$M) was added into the medium for a further 30 min at 37 °C. ROS production was monitored by flow cytometry using DCFH-DA. This dye is a stable, nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells.

**Analysis of Mitochondrial Transmembrane Potential.** The change of the mitochondrial transmembrane potential was monitored by flow

cytometry. Briefly, A431 cells were exposed to HMDB (100 $\mu$M) for different time periods and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′- dihexyloxacarbocyanine [DiOC6(3)] (Molecular Probes, Eugene, OR). Fluorescence was measured after staining of the cells for 30 min at 37 °C. Histograms were analyzed using Cell Quest software and were compared with histograms of control untreated cells.

**Western Blotting.** The nuclear and cytosolic proteins were isolated from 431 cells after treatment with 60 $\mu$M for 0, 3, 6, 9, 12, and 24 h. The total proteins were extracted via the addition of 200 $\mu$L of gold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40, and 10 $\mu$g/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 $\mu$g of protein) were mixed with 5 × sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 12% SDS-polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was carried out on SDS-polyacrylamide gels. For electrophoresis, proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl and then immunoblotted with primary antibodies including anti-Bcl-2, anti-Bcl-$X_L$, anti-Bad, anti-Bax, anti-GADD153, anti-$\beta$-actin (Santa Cruz Biotechnology), anti-poly(ADP-ribose) polymerase (PARP) (UBI, Inc., Lake Placid, NY), anti-Bid, anti-p53, anti-FAS, anti-p21 (Transduction Laboratory, Lexington, KY), and anti-DNA fragmentation factor (DFF)45/inhibitor of caspase-activated DNase (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h, followed by secondary antibody conjugated with horseradish peroxidase. Detection was achieved by measuring the chemiluminescence of the blotting agent (ECL, Amersham Corp., Arlington Heights, IL), after exposure of the filters to Kodak X-Omat films. The mitochondria and cytosolic fractions isolated from cells were used for immunoblot analysis of cytochrome *c* as described (*22*). The cytochrome *c* protein was detected using anti-cytochrome *c* antibody (Research Diagnostic Inc., Flanders, NJ).

**Activity of Caspase.** Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 $\mu$g/mL pepstatin A, and 10 $\mu$g/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12000*g* for 20 min at 4 °C. The caspase activity in the supernatant was determined by a fluorogenic assay (Promega's CaspACE Assay System Corp., Madison, WI). Briefly, 50 $\mu$g of total protein, as determined by Bio-Rad protein assay (Bio-Rad Laboratories), was incubated with 50 $\mu$M substrate Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD) (caspase-1 specific substrate), Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (caspase-3 specific substrate), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) (caspase-8 specific substrate), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) (caspase-9 specific substrate) at 30 °C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 and emission at 460 nm using a fluorescence spectrophotometer (Hitachi, F2000).

**Assessment of DNA Damage.** The DNA damage level caused by HMDB was measured by Comet assay as previously described (*23*). After 2 h treatments of HMDB (100 $\mu$M) and $H_2O_2$ (200 $\mu$M), A431 cells were suspended in agarose gel, and the suspension was pipetted onto glass slides. Following treatment of the supported cells with alkaline detergent to remove cellular membranes, the resulting nucleoids were subjected to electrophoresis. Slides were then placed in a horizontal electrophoresis tank with freshly prepared cold electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH > 13) for 30 min at 4 °C and submitted to electrophoresis (20 V) for 15 min. The DNA of the nucleoids was stained with ethidium bromide to permit visualization using a fluorescence microscope at 100×, with an exciting filter of 515−560 nm and a barrier filter of 590 nm.

**Table 1.** Effect of Different Compounds on the Growth of Various Human Cancer Cells[a]

| | compounds $IC_{50}$ ($\mu$M) | | |
|---|---|---|---|
| cell line | DBM | HDB | HMDB |
| A431 | >100 | 89.18 ± 1.80 | 51.55 ± 1.66 |
| AGS | >100 | >100 | 54.53 ± 1.88 |
| HeLa | 68.04 ± 5.98 | 74.57 ± 0.15 | 53.54 ± 2.60 |
| Hep 3B | >100 | >100 | 78.50 ± 1.15 |
| Hep G2 | >100 | >100 | 63.26 ± 3.02 |
| HT-29 | >100 | >100 | 65.85 ± 9.56 |
| MCF-7 | >100 | >100 | 64.53 ± 1.73 |

[a] Cells were treated with various concentrations of selected compounds for 24 h. Cell viability then was determined by the MTT assay as described. Each experiment was independently performed three times and expressed as mean ± SE.

**Isolation of RNA and RT-PCR.** Total cellular RNA was isolated with an TRIzol Reagent kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. Changes in the steady state concentration of mRNA in GADD153 were assessed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA (2 $\mu$g) was converted to cDNA in a series of standard 10 $\mu$L reverse transcription reactions. DNA amplification was carried out in "Ready To Go" PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ). The amplification cycles were 95 °C for 30 s, 65 °C for 45 s, and 72 °C for 2 min. The PCR products were separated by electrophoresis on a 2% agarose after 30 cycles (422 bp GADD153 fragment; 294 bp $\beta$-actin fragment) and visualized by ethidium bromide staining. Amplification of $\beta$-actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primer, respectively; GADD153: 5′-GCACCTCCCA-GAGCCCTCACTCTCC-3′ and 5′-GTCTACTCCAAGCCTTCCCCCT-GCG-3′ and 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-TACATG-GCTGGGGTGTTGAA-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

## RESULTS

**Inhibition of Cell Proliferation in HMDB-Treated Human Cancer Cells.** A previous study showed that DBM and its analogues are potent reducers of COLO 205 cells survival (*20*). Here, we further examined the effects of DBM and its analogues (HDB and HMDB) on the growth of various human cancer cells. The structures of these compounds are illustrated in **Figure 1**. To assess the inhibitory effects of these compounds on the growth of human cancer cells, the cells were cultured for 24 h with or without compounds (5−100 $\mu$M), and the cell growth rates were determined by MTT assay. As shown in **Table 1**, HMDB decreased cell growth in cultured human cancer cells (A431, AGD, HeLa, Hep3B, HepG2, HT-29, and MCF-7) in a dose-dependent manner, assuming an $IC_{50}$ value of 51.55, 54.53, 53.54, 78.50, 63.26, 65.85, and 64.53 $\mu$M. As compared with DBM, HDB, and HMDB, HMDB more strongly inhibited A431 cell growth. Therefore, we further examined the cytotoxic effects of HMDB in A431 cells.

**HMDB Induces Apoptosis in Human Epidermoid Carcinoma A431 Cells.** Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosome degradation of DNA, and apoptotic body formation. In each case, nucleosomal DNA ladders (*24*, *25*), which are typical of apoptosis, are visible on agarose gels after staining with ethidium bromide. To investigate whether the cytotoxic effects of HMDB observed in A431 cells were due to the presence of apoptotic cell death, cells were treated with HMDB (20−100 $\mu$M) for 24 h and DNA fragmentation analyses were performed. As shown in **Figure**

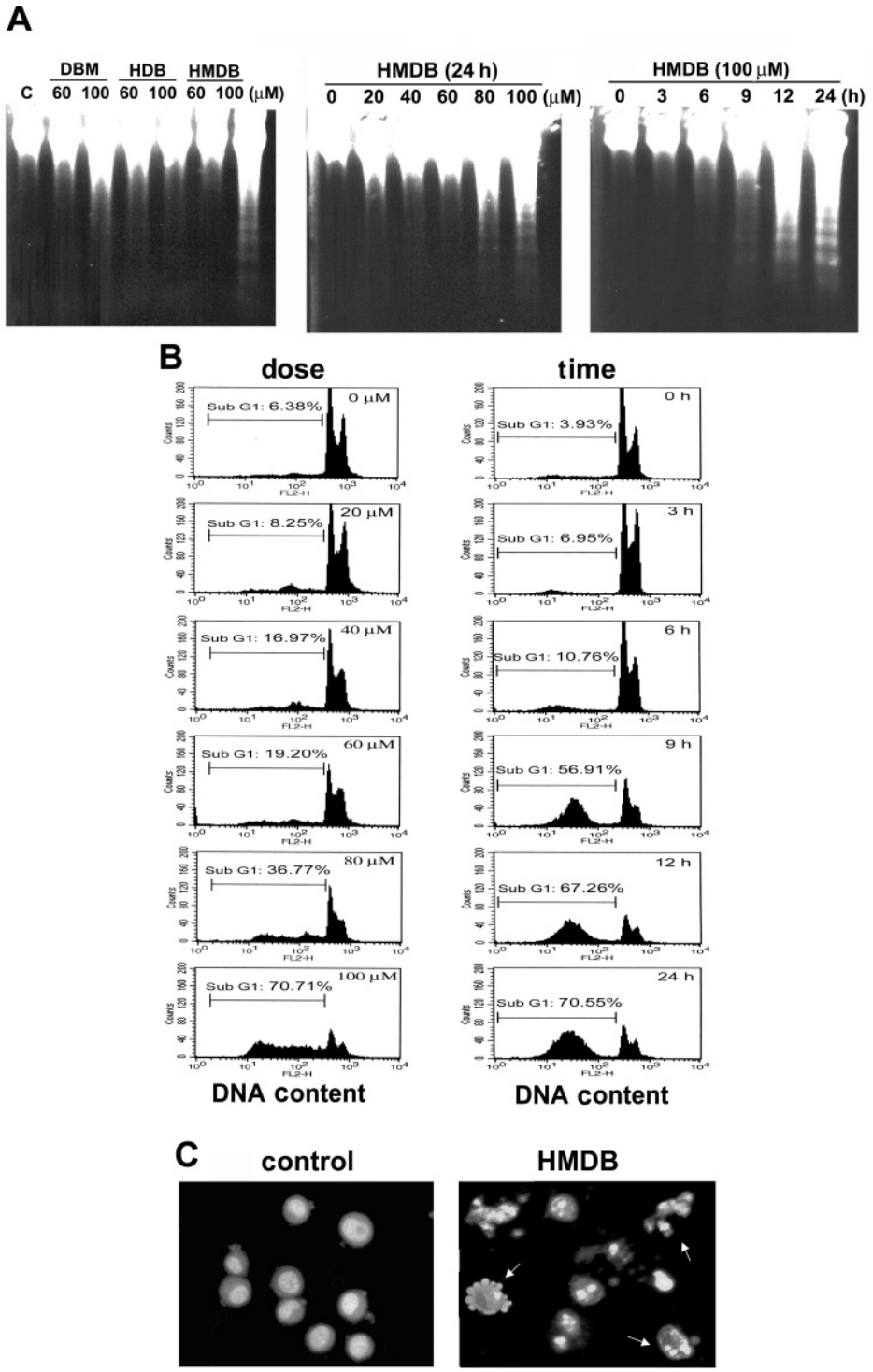


**Figure 2.** Induction of DNA fragmentation and chromatin condensation by HMDB in A431 cells. (**A**) A431 cells were treated with increasing doses of HMDB for 24 h or treated with 100 $\mu$M for the indicated time, and internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. Data shown are representative of three independent experiments. (**B**) Determination of sub-G1 cells in HMDB-treated A431 cells by flow cytometry. The method of flow cytometry used is described in the Materials and Methods. (**C**) A431 cells were treated with 0.05% DMSO as vehicle control or treated with 100 $\mu$M HMDB for 24 h, and cells were harvested and washed with PBS followed by staining with acridine orange. Nuclear staining was examined by fluorescence microscopy. The data presented are representative of three independent experiments.

**2A**, significant DNA ladders were observed in A431 cells after 80 $\mu$M HMDB treatment for 24 h. After treatment with 100 $\mu$M HMDB, digested genomic DNA was evident at 12 h (**Figure 2A**). To investigate the induction of a sub-G1 cell population, a hallmark of apoptosis, the DNA content of A431 cells treated with HMDB for various periods and concentrations was analyzed by flow cytometry (**Figure 2B**). Cells were treated with HMDB (0−100 $\mu$M) and stained with propidum iodide. As seen in **Figure 2B**, the percentages of apoptotic A431 cells were 6.38, 8.25, 16.97, 19.20, 36.77, and 70.71% after incubation with 0, 20, 40, 60, 80, and 100 $\mu$M for 24 h and 3.93, 6.95, 10.76, 56.91, 67.26, and 70.55% after 0, 3, 6, 9, 12, and 24 h of incubation, respectively, with HMDB (100 $\mu$M), respectively. To characterize the cell death induced by HMDB, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding agent, acridine orange. Within 24 h

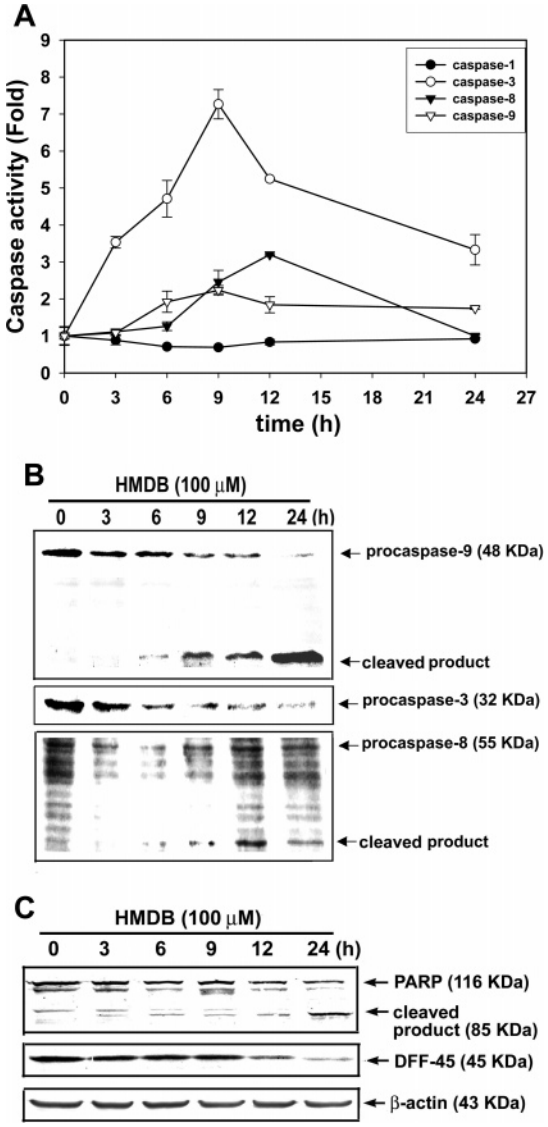


**Figure 3.** Induction of caspase activities, PARP cleavage, and DFF-45 degradation in A431 cells. (**A**) A kinetics of caspase activation in A431 cells. Cells were treated with 100 $\mu$M for different times. Caspase activities were analyzed as described in the Materials and Methods. Data represent means ± SD for three determinations. (**B**) Western blot analyses of procaspase-9, -3, and -8 in A431 cells treated with 100 $\mu$M HMDB for different times. Degradation of procaspase protein represents its activation. (**C**) Cleavage of PARP and DFF-45 induced by HMDB was time-dependent. A431 cells were treated as indicated and analyzed by Western blotting as described in the Materials and Methods.

of treatment with 100 $\mu$M HMDB, cells clearly exhibited significant morphological changes and chromosomal condensation, which is indicative of apoptotic cell death (**Figure 2C**). Such results imply that the cytotoxic action of HMDB was due to its ability to induce apoptosis.

**HMDB Induced the Cleavage and Activation of Caspase Activity.** The caspases are believed to play a main role in causing apoptotic response by cleaving or degrading several cellular substrates (*6*, *26*). To monitor the enzymatic activity of caspase-1, -3, -8, and -9, the caspase activity was measured following treatment of A431 cells with 100 $\mu$M HMDB for various times. As shown in **Figure 3A**, HMDB induced a dramatic increase in caspase-3 activity to approximately 7.2-fold after 9 h of treatment. Furthermore, both caspase-8 and -9 were time dependently activated by HMDB, but the data showed a very low level of caspase-1 activity after HMDB treatment. **Figure 3B** shows a gradual decrease in the level of pro-caspase-9, -3, and -8, indicating that caspases were activated after 6 h of HMDB treatment. The cleavage of procaspase-9 and procaspase-8 was detected at 6 h in HMDB-treated A431 cells. A time-dependent proteolytic cleavage of caspases with an increase of the cleavage fragment was associated with the activity of caspase.

Activation of caspase-3 causes the cleavage of PARP, a hallmark of apoptosis, to produce an 85 kDa fragment during apoptosis (*27*). As was already described, ICAD is a mouse homologue of human DFF-45. Caspase-3 cleaves DFF-45, and once caspase-activated deoxyribonuclease (CAD) is released, it can enter the nucleus, where it degrades chromosomal DNA to produce interchromosomal DNA fragmentation (*28*, *29*). **Figure 3C** shows that exposure of A431 cells to HMDB causes the degradation of 116 kDa PARP to 85 kDa fragments and degradation of DFF-45 protein. The protein cleavages were associated with activation of caspase-3.

**Involvement of Mitochondrial Dysfunction, ROS Production, and Release of Cytochrome *c* from Mitochondria to Cytosol in HMDB-Induced Apoptosis.** It recently became clear that apoptosis involves a disruption of mitochondrial membrane integrity that is decisive for the cell death process. We next evaluated the effects of HMDB on the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the release of mitochondrial cytochrome *c* into cytosol. We measured $\Delta\Psi_m$ fluorescence using a DiOC6(3) probe monitored via flow cytometry. As shown in **Figure 4A**, which compares A431 cells exposed to HMDB and control cells, the DiOC6(3) fluorescence intensity shifted to the left from 123.45 to 82.94 and 59.31 in HMDB-induced apoptotic A431 cells at 1 and 3 h, respectively. The loss of $\Delta\Psi_m$ did not change until exposure of HMDB for 1 h was markedly decreased. These results demonstrate that HMDB causes a decrease in mitochondrial transmembrane potential in A431 cells. ROS have been shown to play an important role in the induction of apoptosis. Results of flow cytometry analysis using DCFH-DA and DHE as fluorescent ROS, $H_2O_2$, and $O_2$ indicators show an increase in intracellular peroxide levels in HMDB-treated A431 cells. Increases of intracellular peroxide levels by HMDB were detected at 0.5 h and clearly increase the mean of DCFH-DA fluorescence intensity from 312.36 to 566.21 and DHE fluorescence intensity from 84.96 to 121.88. These data indicate that the increment of ROS might play a role as an early mediator in HMDB-induced apoptosis. These findings show that HMDB has an effect on mitochondrial function and accumulation of ROS. The features are cues for the induction of apoptosis. Caspase-9 binds to Apaf-1 in a cytochrome *c* and dATP-dependent fashion to become active and, in turn, cleaves and activates caspase-3 (*30*). As shown in **Figure 4B**, the release of mitochondrial cytochrome *c* into the cytosol was detected at 6 h in HMDB-treated A431 cells. Further treatment of A431 cells with HMDB caused a highly significant and dose-dependent increase of cytochrome *c* into cytosol. Therefore, these results suggest that mitochondrial dysfunction caused cytochrome *c* to be the cascade between caspase-9 and caspase-3.

**Effect of HMDB on the Expression of Bcl-2 Family, $p21^{Cip1/WAF1}$, p53, and FAS Protein in A431 Cells.** Several gene products are known to be important in controlling the apoptotic process. The imbalance of expression of anti- and proapoptotic proteins after stimulation is one of the major mechanisms underlying the ultimate fate of cells in the apoptotic process. We examined the expression of proapoptosis proteins,

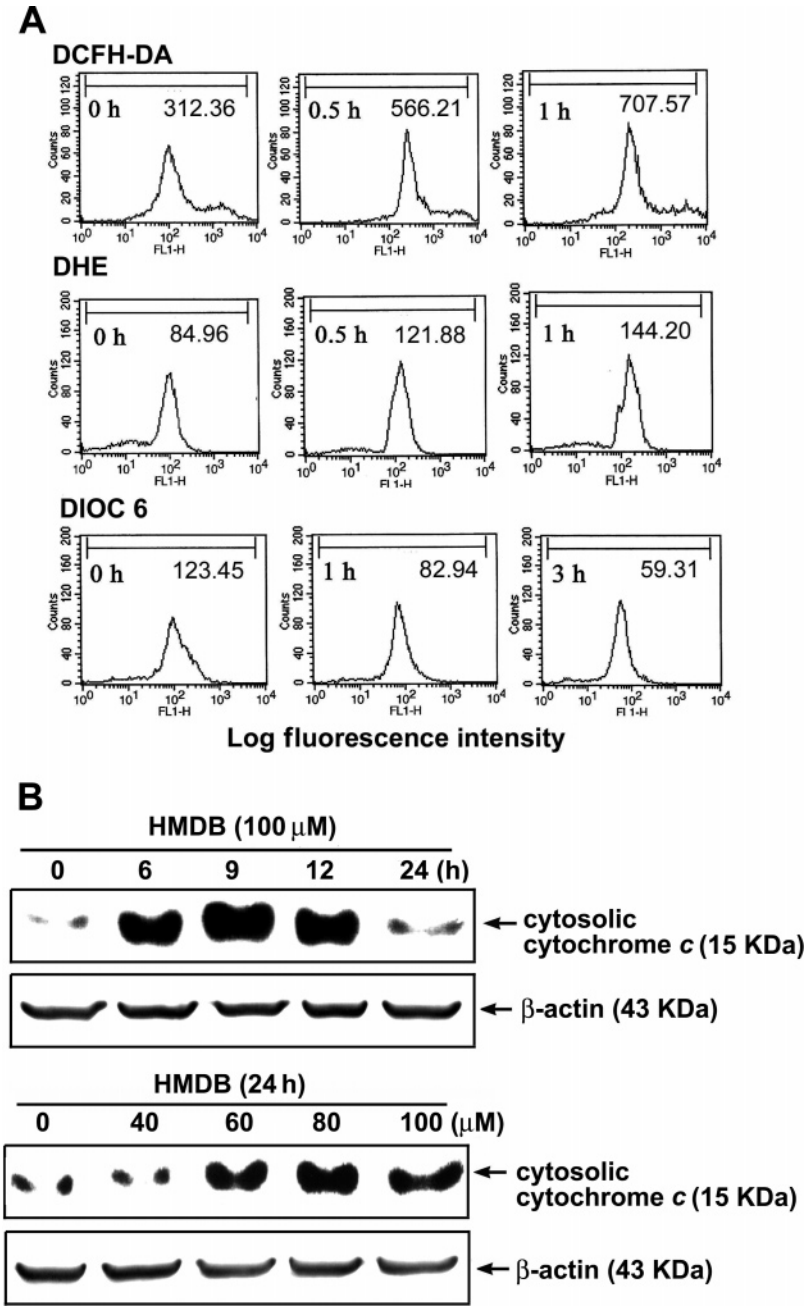


**Figure 4.** Induction of ROS generation, mitochondrial dysfunction, and cytochrome *c* release in HMDB-induced apoptosis. (**A**) A431 cells were treated with 100 $\mu$M HMDB for indicated times and were then incubated with DCFH-DA (20 $\mu$M), DHE (20 $\mu$M), and 3,3′-dihexyloxacarbocyanine (40 nM), respectively, and analyzed by flow cytometry. Data are presented as log fluorescence intensity. (**B**) A431 cells were treated with 100 $\mu$M HMDB for different times (0, 6, 9, 12, and 24 h) or different doses of HMDB (0, 40, 60, 80, and 100 $\mu$M) for 24 h. Subcellular fractions were prepared as described in the Materials and Methods, and cytosolic cytochrome *c* was detected by cytochrome *c* antibody. This experiment was repeated three times with similar results.

Bax, Bad, and Bid, at different time points in HMDB-treated cells. It has been reported that during drug-induced apoptotic cell death, Bax can be cleaved by caspases and calpains and then translocated to mitochondria upon introduction of apoptosis (*31*). As shown in **Figure 5A** (upper panel), Bax protein was cleaved to produce a small fragment in HMDB-treated A431 cells. There was a marked increase of Bad expression and a slight decrease of Bid levels after HMDB treatment. We next examined the expression of antiapoptotic proteins, Bcl-2 and Bcl-$X_L$, of various times after HMDB treatment. The exposure of A431 cells to 100 $\mu$M HMDB resulted in down-regulation of Bcl-2 and Bcl-$X_L$ (**Figure 5A**). In response to HMDB, p21 expression in A431 cells increased in a time-dependent manner. In contrast, there was a decreased in p53 expression. A431 cells expressed mutant p53 in HMDB-treated cells (**Figure 5B**). Recent studies demonstrated that chemical inhibitors of FAS inhibit growth and induce apoptosis in several cancer cells (*25*, *26*). As shown in **Figure 5C**, exposure of A431 cells to 100 $\mu$M HMDB resulted in down-regulation of FAS. Such results imply that FAS might play an important role in the HMDB-induced apoptosis observed in the A431 cells.

**ROS Production Involved in HMDB-Induced Apoptosis.** Recently growing evidence indicates that ROS plays an important role in the induction of apoptosis. As shown in **Figure 6A**, pretreatment with catalase (CAT, C), superoxide dismutase (SOD, S), and CAT combined with SOD did not suppress

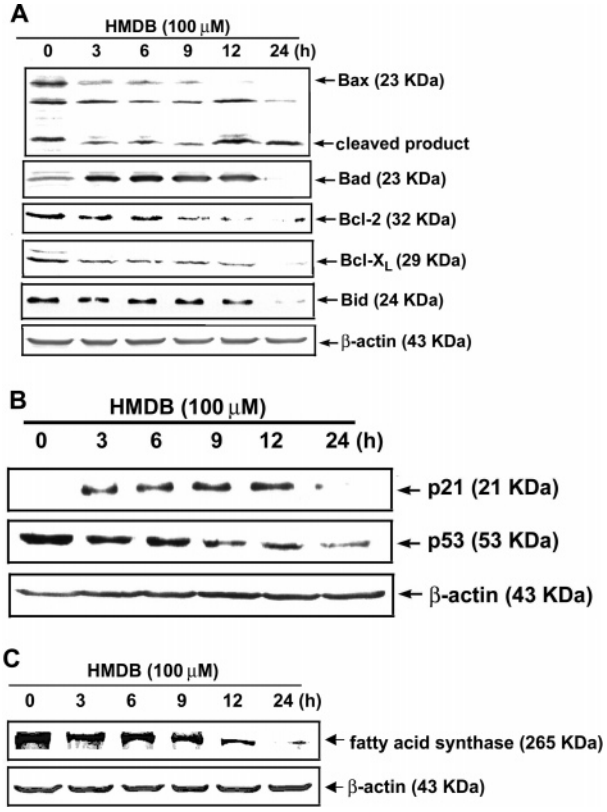


**Figure 5.** Effect of HMDB on Bcl-2 protein family, p21, p53, and FAS expression in HMDB-treated A431 cells. A431 cells were treated with (**A**) or (**B**, **C**) 100 $\mu$M HMDB for indicated amounts of time. The expression of Bax, Bad, Bcl-2, Bcl-$X_L$, and Bid (**A**), p21 and p53 (**B**), and FAS (**C**) was analyzed by Western blotting as described in the Material and Methods. This experiment was repeated three times with similar results.

HMDB-induced DNA fragmentation in A431 cells, whereas z-VAD-FMK (Z), a universal caspase inhibitor, suppressed DNA fragmentation. However, as a result, in **Figure 4A**, an excessive accumulation of ROS was induced in HMDB-treated cells. Therefore, antioxidants such as allopurinol (ALL), CAT, DMSO, diphenylene iodonium (DPI), glutathione (GSH), *N*-acetylcysteine (NAC), SOD, vitamin C (Vit C), and vitamin E (Vit E) were used in the present study to investigate if ROS production is an essential event for HMDB-induced apoptosis of A431 cells. As shown in **Figure 6B**, pretreatment with GSH and NAC, but not ALL (an xanthine oxidase inhibitor), CAT, DMSO, DPI (an NADPH oxidase inhibitor), pyrrolidine dithiocarbamate (PDTC) (an NF$\kappa$B inhibitor), SOD, Vit C, and Vit E, protects A431 cells from HMDB-induced apoptosis. To determine if exposing A43l cells to HMDB induces DNA damage as an indicator of oxidative stress, cell were exposed to 100 $\mu$M HMDB for 2 h. The Comet assay was then performed to detect any single strand breaks in DNA (**Figure 6C**). As can be seen in the representative photos from fluorescence microscopy, the nucleoids of control cells (0 $\mu$M HMDB) were uniformly spherical in shape, reflecting the absence of DNA damage. In contrast, the nucleoids of HMDB-treated cells appeared as "comets", reflecting the presence of significant DNA damage as evidence by the typical "comet tail" that shows up when DNA single strand breaks exist with $H_2O_2$ (200 $\mu$M) as the positive control.

**Induction of GADD153 mRNA and Protein Expression by HMDB in A431 Cells.** Recently, several studies suggested that GADD153 triggers the critical early events leading to the initiation of apoptosis (*13*, *15*−*17*). Thus, the effect of HMDB on the GADD153 mRNA level in A431 cells was measured by RT-PCR analysis using gene specific primers (**Figure 7A**). After cells were exposed to HMDB (100 $\mu$M), GADD153 mRNA increased noticeably for 0.5 h (**Figure 7A**, upper panels) and up-regulated in a concentration-dependent manner (**Figure 7A**, lower panels). Next, we determined whether GADD153 protein expression correlates with GADD153 mRNA induction during HMDB-induced apoptosis. After being treated with HMDB, GADD153 protein expression became apparent after only 0.5 h and increased considerably with the time of incubation, suggesting that newly synthesized GADD153 mRNA was rapidly translated into GADD153 protein, which relates to GADD153 mRNA expression (**Figure 7B**). NAC, which is known to increase intracellular concentrations of the antioxidant GSH (*32*), was able to prevent the increased GADD153 mRNA expression caused by HMDB in A431 cells (**Figure 7C**).

## DISCUSSION

Previously, we observed that HMDB effectively inhibited cell growth and induced apoptosis in human colorectal carcinoma COLO 205 cells (*20*). Therefore, we further examined the effects of DBM and its analogues (HDB and HMDB) on the growth of various human cancer cells. The results show that HMDB more strongly inhibited A431 cell growth. To our knowledge, this is the first demonstration that HMDB inhibits growth and induces apoptosis in human epidermoid carcinoma A431 cells. The inhibitory effect of HMDB on cell growth dose not appear to be limited to A431 cells, as a similar inhibition was also observed in other cultured human cancer cells, such as AGS, Hep 3B, Hep G2, HT-29, and MCF-7 cells (**Table 1**). In this study, we clarified the molecular mechanism by which HMDB triggered A431 cells undergoing apoptosis. As shown in **Figure 2**, HMDB was the potent and rapid inducer of apoptosis, concurrent with DNA ladder, sub-G1 peak appearance, and chromatin condensation. Indeed, treatment with HMDB caused the activation of caspase-9, caspase-8, and caspase-3, but not caspase-1, associated with the degradation of PARP and DFF-45/ICAD, which preceded the onset of apoptosis. ROS are a family of active molecules including superoxide ($O_2^-$), peroxyl ($ROO^\bullet$), hydroxyl ($OH^\bullet$), and nitric oxide ($NO^\bullet$) that are involved in the modulation of biological cell functions. However, a large amount or sustained levels of ROS can result in the oxidation of biomolecules including lipid, protein, and DNA, resulting in cell damage leading to growth arrest, senescence, or death (*33*, *34*). Herein, A431 cells showed increasing ROS production within 30 min, reducing mitochondria membrane potential within 1 h, both caused by HMDB. The chemical structure of HMDB is similar to curcumin in that it possesses a $\beta$-diketone moiety, linking a phenyl and phenolic group, including a methyl group. Curcumin directly generated superoxide anion and $H_2O_2$ and, when copper was present, a hydroxyl radical as well (*35*, *36*). A possible fundamental explanation for why HMDA causes apoptosis could be that they induce oxidative DNA damage, as suggested by a previous study. Thus, the results suggest that HMDB induced apoptosis in A431 cells directly though ROS production. However, mitochondria have also been implicated as a source of ROS during apoptosis. Therefore, the increase in ROS in HMDB-treated cells was probably due to the effect of mitochondria cycling dioxygen through the electron transport assembly and generating ROS by one-electron-transfer. Mitochondria could be a main target of nonspecific damage through oxidative stress at the level of outer and inner membranes (*37*). Oxidative damage to the mitochondria membrane due to increased generation of ROS has been shown to play an

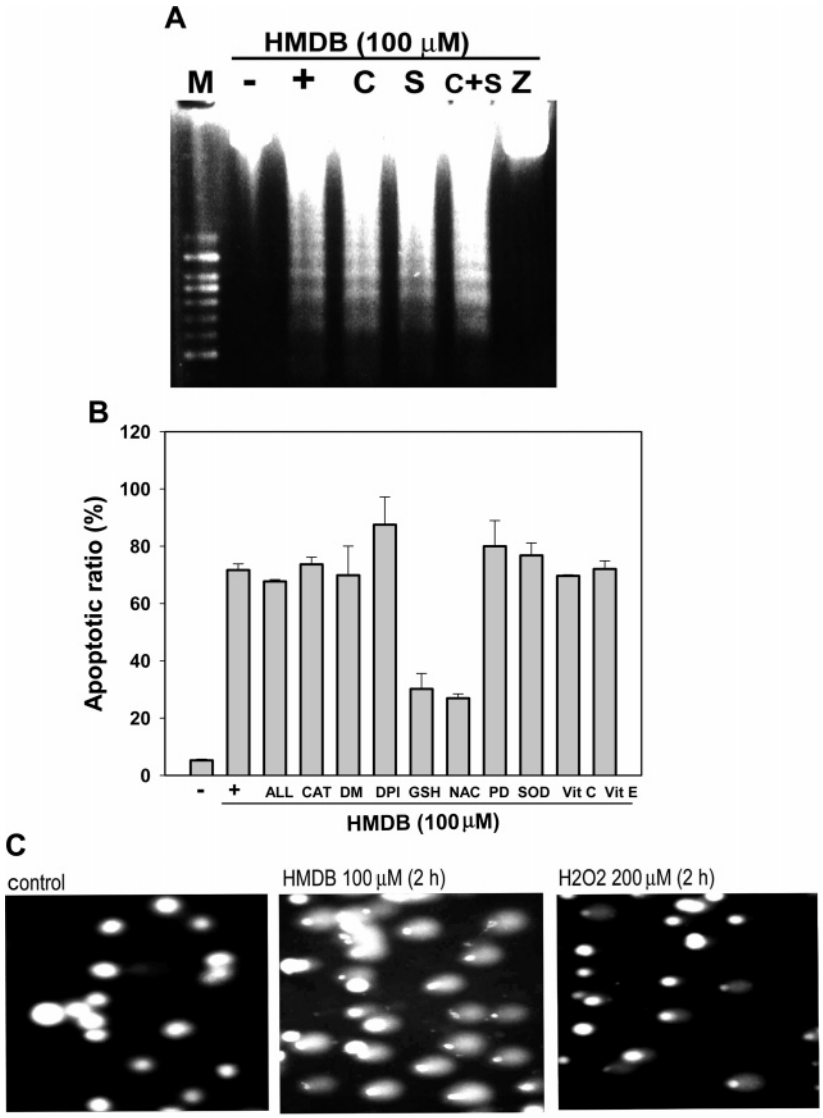


**Figure 6.** Effect of antioxidant on HMDB-treated A431 cells. (**A**) A431 cells were pretreated with 400 U/mL CAT (C), 200 $\mu$g/mL SOD (S), or C and S and 50 $\mu$M Z-VAD-FMK (Z) for 1 h followed by 100 $\mu$M HMDB for a another 24 h. DNA fragmentation was analyzed by 1.8% agarose electrophoresis. M, DNA marker. (**B**) The antioxidants GSH and NAC but not ALL, CAT, DMSO, DPI, PDTC, SOD, Vit C, and Vit E, attenuated HMDB-induced apoptosis according to flow cytometry. Cells were treated with ALL, CAT, DMSO, DPI, GSH, NAC, PDTC, SOD, Vit C, or Vit E for 1 h followed by HMDB (100 $\mu$M) treatment for 24 h. Sub-G1 cells in HMDB-treated A431 cells were determined by flow cytometry. The method of flow cytometry used is described in the Materials and Methods. Each value is presented as the mean ± SD of three independent experiments. ALL (100 $\mu$M), CAT (400 U/mL), DMSO (0.5%), DPI (40 $\mu$M), GSH (200 $\mu$M), NAC (20 mM), PDTC (40 $\mu$M), SOD (200 $\mu$g/mL), Vit C (2 mM), and Vit E (2 mM). (**C**) Assay of DNA damage with comet assay. Greater single strand breaks in DNA of A-431 cells exposed to 100 $\mu$M HMDB for 2 h. The Comet assay was then performed as described under the Materials and Methods to assess to extent of DNA damage. Similar results were produced by two additional different experiments.

important role in apoptosis. Herein, we demonstrated that HMDB could disrupt the functions of mitochondria at the early stages of apoptosis and subsequently coordinate caspase-9 activation, but not caspase-1, through the release of cytochrome *c*. The Bcl-2 family protein, whose membranes may be antiapoptosis or proapoptotic, regulate cell death by controlling the mitochondria membrane permeability during apoptosis (*38*, *39*). We, therefore, inferred that Bcl-2 family protein might participate in the event that controlled the change in mitochondria membrane potential and triggered cytochrome *c* release during apoptosis induced by HMDB. In our study, we found that HMDB down-regulated Bcl-2, Bcl-$X_L$, and Bid expression, up-regulated Bad protein, and cleaved Bax protein in HMDB-treated cells (**Figure 5**). It has been reported that p21 can mediate the response to DNA damage (*40*). In this study, HMDB induced an increase of p21 in a time-dependent manner, while the expression of p53, mutated in A431 cells, was decreased. These results suggest that the p21 protein may play a key role in p53-independent cell cycle arrest leading to apoptosis of A431 cells. Apparently, the p53 protein is not required for mediating apoptosis caused by HMDB in A431 cells. FAS is a key enzyme responsible for the de novo synthesis of long chain fatty acids for two-carbon precursors (*41*). Recent studies demonstrated that inhibitors of FAS, lower expression in human tissues but higher in a variety of human cancers, inhibit growth and induce apoptosis in several cancer cell lines in vitro and tumor xenografts in vivo (*42*, *43*). In the present study, we find down-regulation of FAS expression in HMDB-treated cells; this result would suggest that inhibition of FAS expression could be involved in HMDB-induced apoptosis.

Recently, Scott et al. reported that curcumin-induced apoptosis through the production of ROS, DNA damage, up-regulation

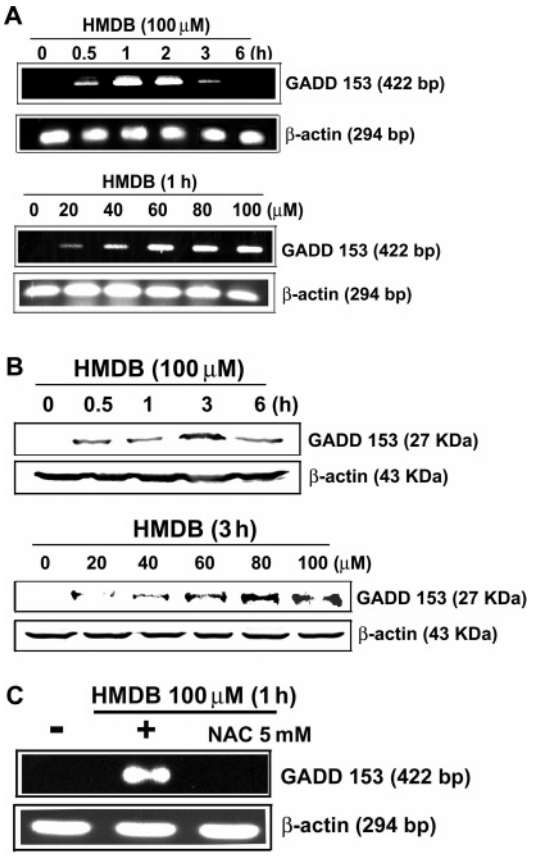


**Figure 7.** Up-regulation of GADD153 mRNA and protein expression by HMDB in A431 cells. (**A**) A431 cells were incubated with 100 $\mu$M HMDB for 0–6 h or 0–100 $\mu$M HMDB for 1 h. After incubation, total RNA was isolated for subsequent multiplex relative RT-RCR analysis using gene specific primers for the target gene, GADD153, and also the internal control gene, $\beta$-actin. (**B**) Cells were incubated with 0–100 $\mu$M HMDB for 3 h or 100 $\mu$M for 0–6 h. Whole cell lysates were prepared for subsequence Western blotting analysis of GADD153 protein and $\beta$-actin. (**C**) In testing free radical scavengers, cells were pretreated with 5 mM NAC for 1 h, prior to exposure to HMDB for 30 min. This experiment was repeated three times with similar results.

of the GADD153 gene, and the antioxidant PDTC and NAC, but neither α-tocopherol nor CAT, prevented GADD153 mRNA expression caused by curcumin (*44*). Therefore, we speculated that intracellular generation of ROS could be an important factor in HMDB-induced apoptosis. To verify this, we performed a test to determine antioxidants NAC and GSH but not other selected antioxidants that significantly inhibited HMDB-induced A431 apoptosis (**Figure 6**). The result is in agreement with reports that NAC inhibited curcumin-induced DNA damage and apoptosis. As the central novel finding in the present study, HMDB increased expression of the GADD153 gene, which has been acknowledged as a proapoptosis gene (*16*, *18*, *45*). Because the GADD153 gene is typically induced in response to cellular DNA damage and ER stress, it is suggested that HMDB-induced up-regulation of GADD153 mRNA could be an early transcriptional response to HMDB (**Figure 7**). Furthermore, increased GADD153 expression was essentially prevented by NAC, which is known to markedly increase intracellular concentration of reduced GSH (*32*), suggesting that a redox mechanism was involved in increasing GADD153 mRNA expression. The intracellular GSH:GSSG ratio is known to influence the expression of redox sensitive gene (*46*). Although the full significance of up-regulation of GADD153 gene expression in

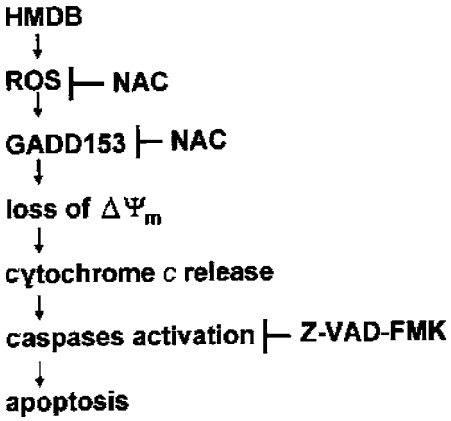


**Figure 8.** Tentative model for HMDB-induced apoptosis in A431 cells proposed in the present study. See the text for details.

HMDB-treated A431 cells is not known, the effect of HMDB on GADD153, in particular, might contribute to the capacity of HMDB-induced apoptosis. Thus, the sum of these results suggests that the GADD153 gene may be an important target gene for apoptotic cell death induced by HMDB. These findings suggest that GADD153 expression may modulate the sensitivity of A431 cells to apoptosis triggered by HMDB-induced DNA damage. Further studies will be required to elucidate how HMDB modulates expression of the GADD153 protein. Noteworthy, we did not rule out the possibility that HMDB could penetrate into cells, directly target ER, increase ER stress and mitochondria, and increase membrane permeability and decrease $\Delta\Psi_m$ accompanied by ROS production.

Taken collectively, the current findings can be interpreted to propose a temporal sequence of events regarding the effects of HMDB on A431 cells (**Figure 8**). The initial event induced by HMDB may induce ROS production and coordinative modulation of GADD153 gene expression, and the GADD153 protein would promote mitochondrial dysfunction (loss of mitochondrial membrane potential), resulting in cytochrome *c* release, caspases activation, and apoptotic death. However, other genes may also be involved in the cellular responses to HMDB exposure, an involvement that may eventually lead to the cells undergoing apoptosis. Further studies are needed to determine whether DNA damage and the GADD153 protein directly initiate apoptosis in A431 cells exposed to HMDB. Because induction of apoptosis is considered an important mechanism of prevention/treatment of cancer by chemopreventive agent(s), further elucidation of the mechanism during HMDB-mediated apoptosis would provide useful information concerning the basis for its use as a potential therapeutic and chemopreventic agent.

## ABBREVIATIONS USED

DBM, dibenzoylmethane; HDB, hydroxydibenzoylmethane; HMDB, 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; GADD153, growth arrest and DNA damage inducible gene 153; NAC, *N*-acetylcysteine; GSH, glutathione; FAS, fatty acid synthase; ALL, allopurinol; CAT, catalase; DCFH-DA, dichlorodihydrofluorescein diacetate; PDTC, pyrrolidine dithiocarbamate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DPI, diphenylene iodonium; ROS, reactive oxygen species; SOD, superoxide dismutase; Z-VAD-FMK, z-Val-Ala-Asp-fluoromethyl ketone.

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