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Induction of Apoptosis by Hydroxydibenzoylmethane through Coordinative Modulation of Cyclin D3, Bcl-X_L, and Bax, Release of Cytochrome *c*, and Sequential Activation of Caspases in Human Colorectal Carcinoma Cells

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DBM (dibenzoylmethane) is a minor constituent of licorice that has antimutagenic activity. However, its other biological activities are not well-known. The structurally related β-diketones hydroxydibenzoylmethane (HDB) and hydroxymethyldibenzoylmethane (HMDB) were able to induce apoptosis in colorectal carcinoma COLO 205 cells. Thus, the effect of structurally related β-diketones on cell viability, DNA fragmentation, and caspase activity was assessed. The potency of these compounds on these features of apoptosis were in the order of HDB > HMDB > DBM in colorectal carcinoma COLO 205 cells. Here, we found that HDB-induced apoptotic cell death was accompanied by upregulation of cyclin D3, Bax, and p21 and down-regulation of Bcl-X_L, while HDB had no effect on the levels of Bcl-2 and Bad protein. These results indicate that HDB allows caspase-activated deoxyribonuclease to enter the nucleus and degrade chromosomal DNA and induces DFF-45 degradation. It is suggested that HDB-induced apoptosis is triggered by the release of cytochrome *c* into cytosol, procaspase-9 processing, activation of caspase-3 and caspase-2, degradation of PARP, and DNA fragmentation caused by the caspase-activated deoxyribonuclease through the digestion of DFF-45. The induction of apoptosis by HDB may provide a pivotal mechanism for its cancer chemopreventive action.

KEYWORDS: DBM; HDB; HMDB; apoptosis; cytochrome *c*; caspase-9; caspase-2; caspase-3; poly-(ADP-ribose) polymerase; DNA fragmentation factor; caspase-activated deoxyribonuclease

INTRODUCTION

Epidemiological studies have provided convincing evidence that dietary factors can modify the processes of carcinogenesis, including initiation, promotion, and progression of several types of human cancer (*1*). During the past decade, a large number of natural products and dietary components have been evaluated as potential chemopreventive agents (*2*). Dietary factors play an important role in human health and in the development of certain chronic diseases including cancer (*3*, *4*). Some foods contain antitumor compounds as well as mutagens and/or carcinogens (*5*). Recent research has also focused on the presence of minor constituents or nonnutrients, which possess antimutagenic and anticarcinogenic properties, in diets (*6*). Such compounds are candidates for chemopreventive agents against cancer development in humans.

Dibenzoylmethane (DBM) (**Figure 1**) is a minor constituent of licorice that has antimutagenic activity. DBM is similar to curcumin in that it possesses a β-diketone (1,3-diketone) moiety linking two phenyl groups. It has been demonstrated that DBM inhibits 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced breast tumorigenesis in mice (*7*). Singletary et al. (*8*) have shown that DBM has potent chemopreventive activity against DMBA-induced carcinogenesis in rat. The effect was attributed to, in part, the ability of DBM to inhibit DMBA−DNA adduct formation. Due to the antitumor effect of DBM, the structural analogue DBM and its derivative have received more attention, especially due to its potential use as a chemopreventive agent (*7*, *8*). In the present report, we selected HDB (hydroxydibenzoylmethane) and HMDB (hydroxymethyldibenzoylmethane)

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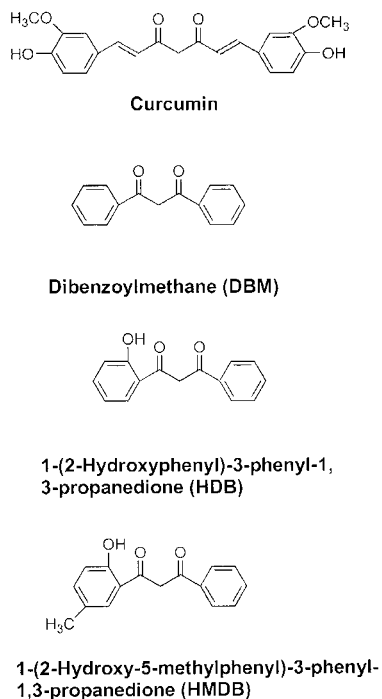

Figure 1. Structures of the DBM, HDB, HMDB, and curcumin.

for evaluation of anticancer activities. HDB and HMDB are identical in structure to DBM except that they possess a hydroxyl group and hydroxyl and methyl groups on one of the aromatic rings, respectively. It is interesting to note that, on the basis of our present studies, HDB and HMDB are far more potent than DBM in inhibiting tumor proliferation and in the induction caspase-3 and cell apoptosis.

Apoptosis, a morphologically distinct form of programmed cell death, is an evolutionary highly conserved phenomenon that plays an important role in the regulation of cellular activities in eukaryotes (*9*). Apoptosis is also the main response of cells to chemotherapeutic agents. A family of cytosolic proteases, the caspases, stored in most cells as zymogens, plays an essential role in the execution of apoptosis. Proteolytic cleavage activates the initiating zymogen, which in turn triggers sequential proteolytic activation of each successive procaspase in the apoptosis cascade (*10*−*12*). In recent years, it has become abundantly clear that caspase-9 activity is required for apoptosis induced by different stimuli (*13*). Specifically, damage to mitochondria results in the release of cytochrome *c*, which together with Apaf-1(apoptotic protease activating factor-1) and dATP lead to the recruitment and activation of pro-caspase-9 (*14*−*15*). Apaf-1 was the first identified mammalian homologue of Ced-4 that couples cytochrome *c* to activation of caspase-9 (*16*). Current study showed that directed expression of cyclin D3 and caspase-2 in human cells potentiated apoptosis compared with expression of caspase-2 alone. Cyclin D3 expression increased the amount of cleaved (active) caspase-2. The results are consistent with the idea that an interaction with cyclin D3 may stabilize caspase-2 and suggest that a physical interaction between cyclin D3 and caspase-2 connects the genetic networks that govern cell-cycle progression with those that govern cell death (*17*). In addition, apoptosis is regulated by Bcl-2 family proteins, including anti-apoptoic proteins such as Bcl-2 and Bcl-X_L and pro-apoptotic proteins such as Bax and Bak (*18*). Most evidence suggests that each cell type has a set point for the ratio of Bcl-2 family dimers, such as levels of Bcl-2 to Bax, which gauges the sensitivity of a cell toward survival or apoptosis. In many cells, survival or death depends on the altered expression level of death inhibitor to death promoter, respectively. Furthermore, it appears that a range of molecular affinities exist which control the interactions between family members, such as Bcl-2 (or Bcl-X_L) for Bax to promote cell survival or Bax homodimer formation to promote cell death (*19, 20*). Current evidence suggests that Bcl-2 acts upstream of caspse-3 activation, at the level of cytochrome *c* release, to prevent apoptosis (*21*−*23*).

In this study, we first examined the antiproliferative effects of dibenzoylmethane and its analogues on human colorectal carcinoma cells. Our results clearly demonstrate that these compounds can induce apoptosis in a dose-dependent manner in COLO 205 cells. The potency of these compounds on these features of apoptosis were in the order of HDB > HMDB > DBM in COLO 205 cells. We further evaluated the molecular mechanisms of apoptotic effects induced by HDB. To elucidate the anticancer mechanism of HDB, we investigated the change of the Bcl-2 protein family and caspase in HDB-induced apoptosis in human COLO 205 cancer cells.

MATERIALS AND METHODS

Cell Culture and Chemicals. The cell lines COLO 205 (CCL-222; American Type Culture Collection) was developed from a poorly differentiated human colon adenocarcinoma. Cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY), 1× penicillin/streptomycin (10000 units of penicillin/mL and 12 mg/mL streptomycin)−1 mM glutamine (GIBCO BRL, Grand Island, NY), and kept at 37 °C in a humidified 5% CO_2 incubator. Curcumin was purchased form E. Merck Co. (Darmstadt, Germany). Dibenzoylmethane, 1-(2-hydroxyphenyl)-3-phenyl-1,3-propanedione (HDB) and 1-(2-hydroxy-5-methylphenyl)-3-pheyl-1,3- propanedione (HMDB) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The purities of chemicals used in this study, curcumin, DBM, HDB, and HMDB, were 97%, 98%, 98%, and 97%, respectively, and were dissolved in dimethyl sulfoxide (DMSO). The inhibitors of caspase-3 (Z-Asp-Glu-Val-Asp-fluoromethyl ketone, Z-DEVD-FMK) and caspase-1 (acetyl-Tyr-Val-Ala-Asp-aldehyde, Ac-YVAD-CHO) were purchased from Calbiochem (La Jolla, CA).

DPPH free Radicals Scavenge Activity. α,α−Diphenyl-*β*-picryl-hydrazyl (DPPH) was purchased from Sigma (St. Louis, MO). DPPH (150 *µ*M in 60% of absolute alcohol) was mixed with different concentrations of tested compounds. Absorption at 517 nm was measured using an UV−visible spectrophotometer (Hitachi, U2000). The decrease in absorbance was shown when tested compounds possessed free radicals scavenge activity.

Cell Survival Assay. Cell viability was assayed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT). Briefly, COLO 205 cells were plated at a density of 1×10^5 cells/mL into 24-well plates. After overnight growth, cells were pretreated with a series of concentrations of DBM, HDB, and HMDB for 48 h. The final concentrations of dimethyl sulfoxide in the culture medium were <0.1%. At the end of treatment, 30 *µ*L of MTT was added, and cells were incubated for a further 4 h. Cell viability was obtained by scanning with an ELISA reader with a 490-nm filter.

DNA Extraction and Electrophoresis Analysis. COLO 205 human cancer cells were harvested, washed with PBS, and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM Tris (hydroxymethyl) aminomethane (pH 8.0), and 10 mM EDTA at 56 °C for overnight and treated with RNase A (0.5 *µ*g/mL) for 3 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25/24/1) before loading and analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in Tris-borate/EDTA electrophoresis buffer (TBE). Approximately 20 *µ*g of DNA was loaded in each well and visualized under UV light and photographed. (*24*).

Western Blotting. The nuclear and cytosolic proteins were isolated form COLO 205 cells after treatment with 100 *µ*M HDB for 0, 4, 8,

12, 24, 36, and 48 h. The total proteins were extracted by 200 μL of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10 000*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 μg 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% bromphenol blue. The mixtures were boiled at 100 °C for 5 min and subjected 12% SDS-polyacrylamide minigels at a constant current of 20 mA. Subsequent electrophoresis was ordinarily carried out on SDS-polyacrylamide gels. Following 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-cyclin D3, anti-α-tubulin (Santa Cruz Biotech.), anti-PARP (UBI, Inc., Lake Placid, NY), anti-p21 (Transduction Lab. Lexington, KY), and anti-DFF45/ inhibotor of caspase-activated DNAse (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h. Detection was achieved by measuring the chemiluminescence of 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. The cytochrome *c* protein was detected by 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 (pH7.5), 5 mM MgCl, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12 000*g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promeaga's CaspACE Assay System Corp., Madison, WI). Briefly, 50 μg of total protein, as determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany), was incubated with 50 μM substrate Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD-AMC), Ac-Val-Asp-Val-Ala-Asp-AMC (Ac-VDVAD-AMC), Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), Ac-Val-Glu-Ile-Asp-AMC (Ac-VEID-AMC), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) at 30 °C for 1 h. The release of methyl-coumaryl-7-amine (AMC) was measured by excitation at 360 and emission at 460 nm using a fluorescence spectrophotometer (HITACHI, F2000).

RESULTS

Antioxidative Properties of DBM and Its Analogues. The antioxidant activity of the four compounds (shown in **Figure 1**) was assayed with DPPH free radical. Of these compounds tested, curcumin was the most potent antioxidant over the same concentration range (**Figure 2A**), whereas DBM, HDB, and HMDB were shown to be less active DPPH scavengers (**Figure 2B**).

Treatment with DBM, HDB, and HMDB Cause Dose-Dependent Reduction in Cell Survival. We first tested the effect of DBM, HDB, and HMDB on cell viability. Human colorectal carcinoma cells were treated with different concentrations of DBM, HDB, and HMDB. After 48 h of treatment, the number of live cells was determined by means of MTT test. As shown in **Figure 3**, HDB appeared to be more potent inhibitor of cell viability, with an IC_{50} of 15 μM, than HMDB (IC_{50} of 19 μM) and DBM (IC_{50} of 31 μM).

Effect of DBM, HDB, HMDB, and Curcumin on DNA Fragmentation of COLO 205 Cells. Physiological cell death is characterized by apoptotic morphology, including chromatin

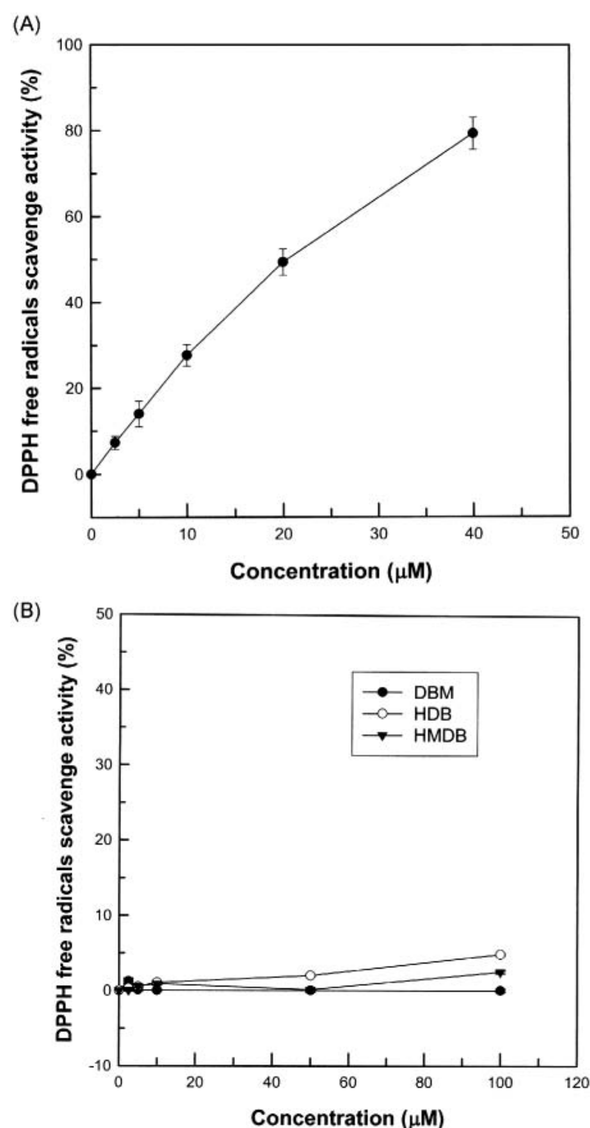

Figure 2. DPPH scavenging activity of DBM, HDB, HMDB, and curcumin. Antioxidatvie properties of tested compounds. DPPH free radical scavenge activity of (A) curumin, (B) DBM, HDB, and HMDB. Various concentrations of these compounds were mixed with 150 μM (final) DPPH dissolved in 60% alcohol. Absorption at 517 nm was measured immediately; free radical scavenge activity was expressed, correlating to the decrease in OD 517 nm value. Data were represented as means ± SE for three determinations.

condensation, membrance blebbing, internucleosome degradation of DNA, and apoptotic body formation. In each case, nucleosomal DNA ladders (*24*), which are typical of apoptosis, were visible on agarose gel after staining with ethidium bromide. The induction of DNA fragmentation was demonstrated by incubating COLO 205 cells with different concentrations of DMB analogues for 48 h (**Figure 4**), and then the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation ladder was found in ethidium-stained gels, at 25 μM HDB treatment, and this DNA fragmentation response was dose-dependent. When cells were treated with 50 μM DMB and HMDB, DNA ladders were just visible 48 h after treatment. In contrast, COLO 205 cells showed only slightly induced DNA fragmentation by curcumin treatment. When human colorectal carcinoma COLO 205 cells were treated with 100 μM HDB, DNA ladders were just visible 24 h after treatment, and increasing DNA fragmentation was observed from 24 to 48 h (**Figure 4B**).

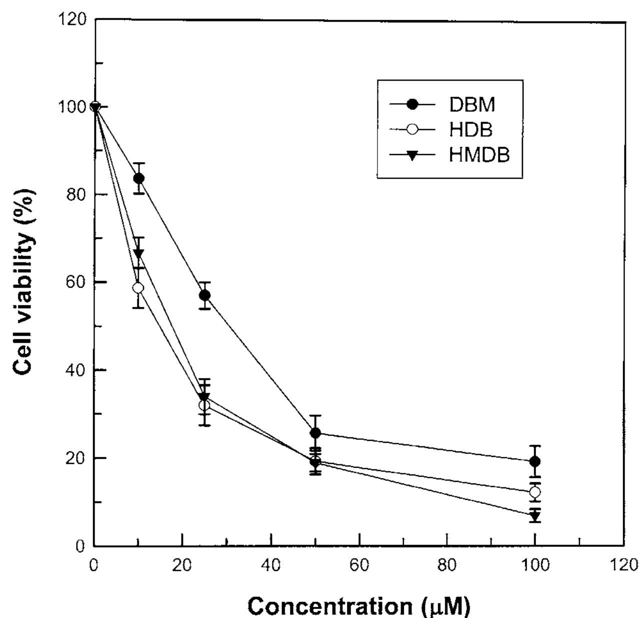

Figure 3. Effect of DBM, HDB, and HMDB on the cell survival of COLO 205 cells. COLO 205 cells were treated with these compounds at different concentrations for 48 h. COLO 205 cells were treated with 0.1% DMSO as vehicle control. Cells viability then was determined by MTT assay, as described under Materials and Methods. Data were represented as means ± SE for three determinations.

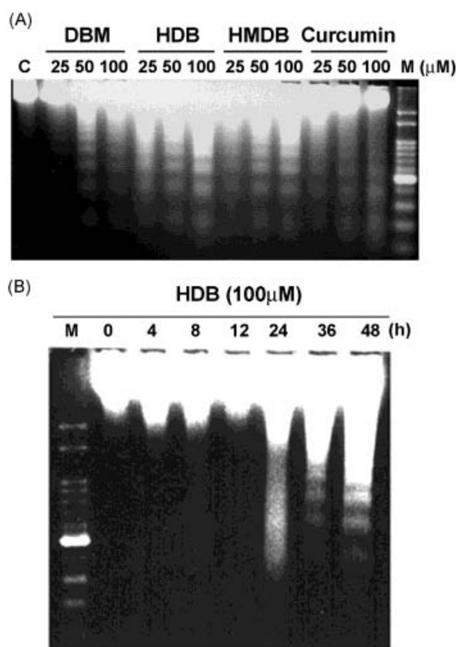

Figure 4. Effect of DBM, HDB, HMDB on DNA fragmentation of COLO 205 cells. Induction of DNA fragmentation in COLO 205 cells by DBM, HDB, and HMDB. (A) Treated with these compounds of different concentration for 48 h. (B) Time-dependent increasing doses of fragmentation ladders induced by 100 μM HDB. Agarose gel analysis of DNA fragmentation was performed. C, control; M, DNA ladder maker.

HDB Stimulated Caspase Activity in a Dose- and Time-Dependent Manner. Caspases are believed to play a central role in mediating various apoptotic responses. To monitor the enzymatic activity of caspase during DBM analogue-induced apoptosis, we used six fluorogenic peptide substrates: Ac-YVAD-AMC is a specific substrate for caspase-1, Ac-VDVAD-AMC specific for caspase-2, Ac-DEVE-AMC, specific for caspase-3, Ac-VEID-AMC specific for caspase-6, Ac-IEAD-AMC specific for caspase-8, and Ac-LEHD-AMC specific for caspase-9. Caspase activities were measured following treatment of COLO 205 cells with different concentrations of HDB, DBM, and HMDB for 48 h. As shown in **Figure 5A**, the induction of caspase-3 activity by HDB paralleled the dose-dependent pattern of apoptosis. Activities of HMDB also followed a dose-dependent pattern. In contrast, DBM only slightly induced caspase-3 activity. We also monitored the activation of caspase-2, caspase-6, caspase-8, and caspase-9, respectively, during compound-induced apoptosis. As illustrated **Figure 5B** and **5D**, HDB induced a dramatic increase in caspase-9 and caspase-2 activities in treated COLO 205 cells. In contrast to the increase in DEVD-specific activity, negligible caspase-8 (**Figure 5C**) and caspase-6 (**Figure 5E**) activities were observed. Caspase-3 is time-dependently activated by HDB (**Figure 5F**). HDB induced a rapid rise in caspase-3 activity to approximately a 9-fold increase after the addition of HDB for 48 h. As showed in **Figure 5F**, HDB (100 μM) induced a dramatic increase in caspase-2 and caspase-9 activities in a time-dependent manner.

Treatment with DBM, HDB, and HMDB Caused Degradation of PARP, an Endogenous Substrate of Caspase-3, and Cleavage of DFF-45, an Inhibitor of Endonuclease. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is poly-(ADP-ribose) polymerase (PARP). The cleavage of PARP is the hallmark of apoptosis. PARP (116 kDa) is cleaved to product an 85-KDa fragmentation during apoptosis (*25*). We examined the cleavage of PARP in DBM, HDB, and HMDB-induced apoptosis. We determined the cleavage of PARP with DMB, HDB, and HMDB (100 μM) for 24 and 48 h. As shown in **Figure 6A**, the amounts of cleaved product were higher in HDB than in DBM and HMDB treated cells. Treatment of COLO 205 cells with 100 μM HDB for more than 24 h caused a time-dependent proteolytic cleavage of PARP, with accumulation of the 85-KDa fragments and concomitant disappearance of the full-size 116-KDa protein (**Figure 6B**). These data are consistent with DNA fragmentation and caspase-3 activity. We further explored the possibility that activation of caspase-3 may also induce DFF-45 protein degradation. Treatment of COLO 205 cells with 100 μM HDB caused proteolytic cleavage DFF-45 at 24 h (**Figure 6C**). The activation of caspase-3 was also observed 24 h later (**Figure 5F**) with a time course that paralleled with the cleavage of DFF-45. With apoptosis, nearly all of the DFF-45 was degraded at 36 h. As already described, ICAD is a mouse homologue of human DFF-45, caspase-3 cleaves DFF-45, and, once caspase-activated deoxyribonuclease is released, it can enter the nucleus, where it degrades chromosomal DNA (*26*, *27*).

Time-Dependent Effect of HDB on Expression of Bcl-X_L, Bax, p21, and cyclin D3. Several gene products are known to be important in controlling the apoptotic process. To determine if HDB has an effect on the expression levels of these gene products, a similar time course experiment was performed, and cellular lysates were analyzed by western blotting. As shown in **Figure 7A**, exposure of COLO 205 cells to 100 μm HDB resulted in down-regulation of Bcl-X_L. In contrast, the Bax protein began to increase after 4 h of incubation. The expression levels of p21 protein began to increase after 8 h of incubation. A previous report (*28*) indicated that caspase-2 acts upstream of mitochondria to promote cytochrome *c* release during etoposide-induced apoptosis. Cyclin D3 expression increased the amount of cleaved (active) caspase-2 (*17*). As shown in **Figure 7B**, HDB markedly up-regulated the level of cyclin D3, which are associated with caspase-2 activation.

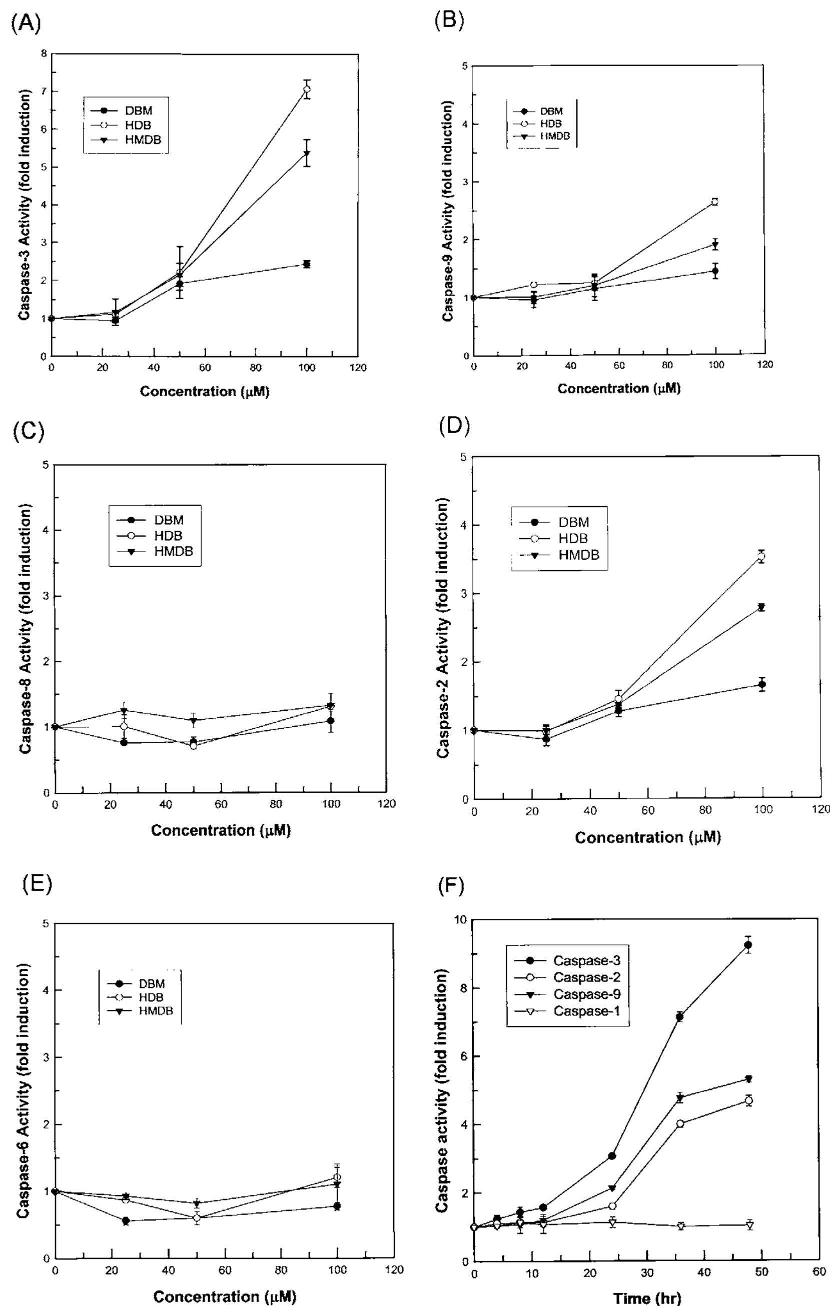

Figure 5. Effect of DBM, HDB, and HMDB on caspase activities. Cells were treated with these compounds of different concentration for 48 h. These compounds induced caspase-2, caspase-3, and caspase-9 activities by dose-dependent. Caspase activities were analyzed as described under Materials and Methods. Data represent means ± SE for three determinations. (A) capsase-3; (B) capsase-9; (C) caspase-8; (D) capsase-2; (E) caspase-6. (F) Kinetics of caspase activation. Cells were treated with 100 μM HDB for different time periods.

HDB Induction of Cytochrome *c* Release. The process of cell death may involve the release of cytochrome *c* from the mitochondria, which subsequently causes apoptosis by activation of caspases. Together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* released from mitochondria (*18*). Treatment of COLO 205 cells with 100 μM HDB caused a time-dependent release of cytochrome *c* into cytosol (**Figure 8A**). COLO 205 cells were exposed to different concentrations of HDB for 48 h. As shown in **Figure 8B**, the release of cytochrome *c* into cytosol was highly significant and dose-dependent. Therefore, these results suggest that mitochondrial dysfunction caused cytochrome *c* to be the cascade between caspase-9 and caspase-3.

A Caspase-3 Inhibitor, Z-DEVD-FMK, Abolishes Apoptosis Induced by HDB. The above result clearly indicates that caspase-3 protease is activated in response to the apoptosis induced by HDB. To determine whether the activation of caspase-3 is required for induction of cell death by HDB, we pretreated COLO 205 cells with caspase inhibitor. As shown in **Figure 9**, an inhibitor of caspase protease, Z-DEVD-FMK, was able to inhibit HDB-stimulated DEVD-specific activity and cell death in a dose-dependent manner. In contrast, Ac-YVAD-CHO, an inhibitor of caspase-1 activity, had little effect at similar concentrations. Thus, these data suggest that the induction of caspase-3 activity is a specific biochemical event brought about by apoptosis-inducing HDB.

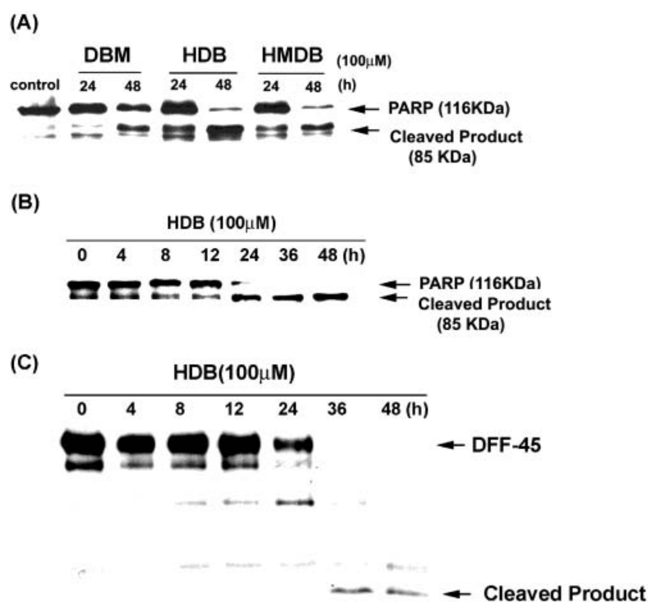

Figure 6. Cleavage of PARP and DFF-45 during DBM-, HDB-, and HMDB-induced apoptosis, respectively. (A) Time-dependent increases in the cleavage of PARP by these compounds. (B) Time course of PARP cleavage by 100 μM HDB. (C) Kinetics of DFF-45 cleavage by HDB. COLO 205 cells were treated as indicated and analysis by western blotting as described in Materials and Methods. This experiment was repeated three times with similar results.

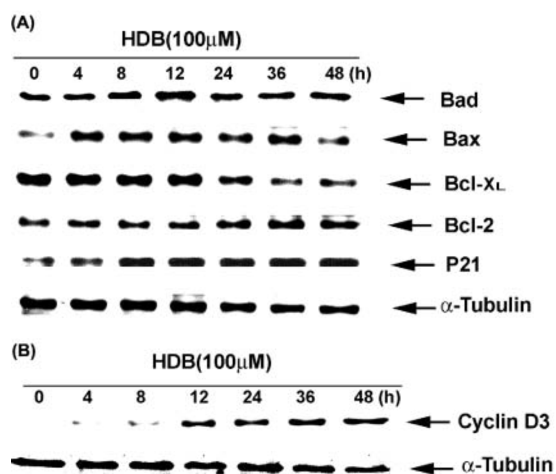

Figure 7. Western blot analysis for expression of Bcl-2 protein family, p21 ,and cyclin D3 in HDB-treated COLO 205 cells. Cells from each time point following 100 μM HDB treatment were analyzed. (A) Expression of Bad, Bax, Bcl-X_L, Bcl-2, and p21, respectively. (B) Cyclin D3. This experiment was repeated three times with similar results.

DISCUSSION

HDB (2-hydroxyldibenzoylmethane) is structurally related dibenzoylmethane. Both have a β-diketone moiety, have conjugated double bonds, and are metal-chelating agents. DBM was found to be critical for the ability to induce Phase 2 detoxification enzymes in murine hepatoma cells (*29*). DBM is a natural compound that has recently emerged as a potential chemopreventive agent. It has reported that dietary DBM is a potent inhibitor of chemically induced mammary carcinogenesis and PAH–DNA adduct formation in rodents (*7*, *30*). Christopher et al. (*31*) have shown that DBM has chemopreventive activity through phase I enzyme expression by modulation of AhR

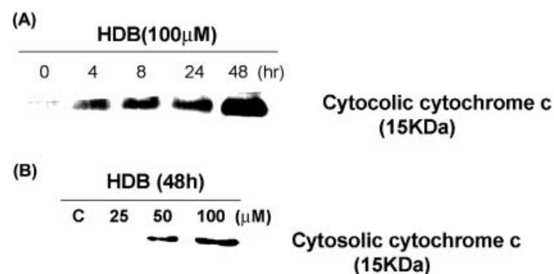

Figure 8. HDB-induced cytosolic cytochrome *c* release. (A) Time-dependent and (B) dose-dependent increases in the cytochrome *c* release by HDB. Cytochrome *c* was detected by cytochrome *c* antibody and detected as described under Materials and Methods. This experiment was repeated three times with similar results.

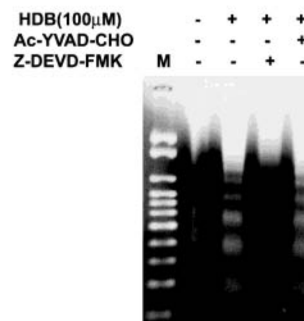

Figure 9. Suppression of HDB-induced DNA fragmentation by caspase-3 protease inhibitor. COLO 205 cells were pretreated for 2 h with caspase-3 inhibitors (Ac-DEVD-FMK) or with caspase-1 protease inhibitor (Ac-YVAD-CHO) and then treated with 100 μM HDB for 48 h, and agarose gel analysis of DNA fragmentation was performed. This experiment was repeated tree times with similar results. M, DNA ladder marker.

function. The present results demonstrate for the first time that HDB can induce apoptosis in human colorectal carcinoma cells. The induction of apoptosis occurred within several hours, consistent with the view that HDB induces apoptosis by activating the preexisting apoptosis machinery. Indeed, treatment with HDB caused an induction of caspase-3 activity and degradation of PARP and DFF-45 which precedes onset of apoptosis. Many lines of evidence demonstrated that activation of caspase is a central mechanism of apoptosis, and caspases are considered to be the "executioners" in the process of cell death (*32*). Through these domains the caspases interact with adaptor proteins that recruit them to specific "death complexes". In mammals, the death complexes include the Apaf-1/caspase-9 apoptosome and the FADD/caspase-8 death-inducing signaling complex (DISC). The apoptosome and DISC are thought to account for most caspase-dependent apoptosis. The upstream signaling pathways leading to assembly of the death complex are often called the mitochondrial (intrinsic) and receptor (extrinsic) pathways of apoptosis.

However, we were unable to detect significant changes in the activities of caspase-1, caspase-6, and caspase-8 during HDB treatment. This raises the possibility that factors or proteases other than caspase-1, caspase-6, and caspase-8 are involved in the activation of caspase-3. Caspase-3 is activated by two sequential proteolytic events that cleaved a 32-kDa precursor at aspartic acid residues to generate an active heterodimer of 20- and 12-kDa subunits (*33*). In fact, in vitro studies have previously identified Apaf1, cytochrome *c*, and caspase-9 as participants in a complex important for caspase-3 activation. In vitro depletion of caspase-9 from cytosolic fractions resulted in the failure of caspase-3 activation (*26*). Taken together, these

data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* release from mitochondria. Release of cytochrome *c* from the mitochondria has been shown to be an almost universal phenomenon during apoptosis, although it is unclear whether the cytochrome *c*-mediated caspase cascade is triggered only by a few apoptotic stimuli or serves as a general amplification mechanism to accelerate cell death (*34*). In this study, we found that HDB induced the release of cytochrome *c* and the activation of caspase-9. However, the induction abilities of HMDB and DBM were lower.

In the present study, we also show the structurally related DBM can induce apoptosis in COLO 205 cells. However, to date, the mechanism of apoptosis is unclear. Thus we investigated the mechanism of apoptosis, the roles of the Bcl2 family members, and the caspase cascade in compound-induced COLO 205 cell apoptosis. After or at the time of the mitochondrial cytochrome *c* efflux, the caspases were activated and PARP was cleaved in HDB-treated cells. Upon treatment with HDB, the activation of caspases occurred after the release of mitochondrial cytochrome *c*. We first observed this 24 h after HDB treatment, and it appeared to precede the activation of caspase-2, −3, and −9. In contrast to the increase in caspase-3 activity, negligible caspase-1 was observed. Recent reports indicated that caspase-2 acts upstream of mitochondria to promote cytochrome *c* release during etoposide-induced apoptosis (*28*). Mendelson et al. indicated that cyclin D3 could help activate caspase-2. (*17*). During HDB treatment, we found the expression levels of cyclin D3 began to increase after 4 h of incubation and reached a maximum level between 8 and 12 h (**Figure 7**). The role of cyclin D3 protein in HDB-induced apoptosis is uncertain because we only could detect cyclin D3 accumulation in the COLO 205 cells. An important area of future research is identification of the genes that are involved in the apoptotic program of cell death. In fact, the findings that cell death occurs at a certain time and at certain locations during precise stages of normal development or metamorphosis implies that there are genes responsible for the occurrence of cell death. The best example of a cell death-associated gene is probably the bcl-2 gene (*35*). In our study, we found that the release of cytochrome *c* from mitochondria to cytosol as well as down-regulation of Bcl-X_L and an increase of Bax expression are early events during HDB-induced apoptosis.

In the present study, induced-apoptosis activity was examined. HDB was found to be more potent than DBM and HMDB. These results suggest that the *o*-hydroxyl group on the phenyl ring is essential for the apoptosis induction effect. In conclusion, the schematic representation of HDB-induced apoptosis cascade is described as the following. Treatment of human colorectal carcinoma cells with HDB induces cell death-transducing signals that activate caspase-2 by upregulated cyclin D3 and regulate the mitochondrial membrane permeability by down-regulation of Bcl-XL and upregulation of Bax and trigger cytochrome *c* release to cytosol. Upon entering the cytosol, cytochrome *c* triggers the caspase-9 pathway, then activates downstream executioner caspase-3, and consequently cleaves specific substrates (PARP and DFF-45), leading to apoptotic changes, nuclear condensation and DNA fragmentation. Taken together, our observations provide new information for the design of cancer chemopreventive agents and the study of these functional groups in the future. Furthermore, other genes may also be involved in the cellular responses to HDB exposure, an involvement that may eventually lead to the cells undergoing apoptosis.

ABBREVIATIONS USED

DBM, dibenzoylmethane; HDB, hydroxydibenzoylmethane; HMDB, hydroxymethyldibenzoylmethane; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; DMBA, 7, 12-dimethylbenz[*a*]anthracene; Apaf-1, apoptotic protease activating factor-1; ICAD, inhibitor of caspase-3-activated DNase.

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