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Brazilin Induces Apoptosis and G2/M Arrest via Inactivation of Histone Deacetylase in Multiple Myeloma U266 Cells

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ABSTRACT: Although brazilin [7,11b-dihydrobenz(*b*)indeno[1,2-*d*]pyran-3,6a,9,10(6*H*)-tetrol] isolated from *Caesalpinia sappan* was known to have various biological activities, including anti-inflammation, antibacteria, and antiplatelet aggregation, there is no report yet on its anticancer activity. In the present study, the anticancer mechanism of brazilin was elucidated in human multiple myeloma U266 cells. We found that brazilin significantly inhibited the activity of histone deacetylases (HDACs), transcription factors involved in the regulation of apoptosis and cell cycle arrest in U266 cells. Consistently, brazilin enhanced acetylation of histone H3 at Lys 23, indicating activation of histone acetyltransferase (HAT), and also suppressed the expressions of HDAC1 and HDAC2 at both protein and mRNA levels. Additionally, brazilin significantly increased the number of sub-G1 cell population and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells undergoing apoptosis and also activated caspase-3 and regulated the expression of Bcl-2 family proteins, including Bax, Bcl-x_L, and Bcl-2 in U266 cells, indicating that brazilin induces apoptosis through the mitochondria-dependent pathway. Interestingly, cell cycle analysis revealed that brazilin induced G2/M phase arrest along with apoptosis induction. Consistently, brazilin attenuated the expression of cyclin-dependent kinases (CDKs), such as cyclin D1, cyclin B1, and cyclin E, and also activated p21 and p27 in U266 cells. Furthermore, HAT inhibitor anacardic acid reversed activation of acetyl-histone H3 and cleavage of PARP induced by brazilin, while pan-caspase inhibitor Z-VAD-FMK001 did not affect the expression of HDAC induced by brazilin that brazilin mediates apoptosis via inactivation of HDAC in U266 cells. Notably, brazilin significantly potentiated the cytotoxic effect of standard chemotherapeutic agents, such as bortezomib or doxorubicin, in U266 cells. When our findings are taken together, they suggest that brazilin has potential as a chemotherapeutic agent alone or in combination with an anticancer agent for multiple myeloma treatment.

KEYWORDS: *Brazilin, HDAC, apoptosis, multiple myeloma, U266*

INTRODUCTION

Multiple myeloma (MM) is a clonal neoplasm of plasma cells derived from the B-lymphocyte lineage ranging from monoclonal gammopathy of undetermined significance (MGUS) to plasma cell leukemia.[1] It involves malignant plasma cells progressively infiltrating the bone marrow and producing a monoclonal immunoglobulin (Ig) (M protein).[2] Management of MM has been improved by introducing several new agents, such as bortezomib (Velcade, a proteasome inhibitor), thalidomide, and the thalidomide analogue lenalidomide (Revlimid, immune modulator) either by the agent monotherapies (bortezomib) or in combination (thalidomide or lenalidomide) with dexamethasone.[3]

Recent evidence suggests that the epigenome, which regulates gene expression, may be a promising therapeutic target in MM. Indeed, epigenomic dysregulation of DNA methylation and histone acetylation is a molecular target for cancers, including MM.[4] In this regard, histone deacetylase (HDAC) inhibitors have been suggested as a valuable targeted therapy to induce DNA hyperacetylation by inhibiting the removal of acetyl groups from amino tails on histone proteins, thereby derepressing silenced genes, including tumor suppressor genes.[5] For instance, Vorinostat (SAHA), the first HDAC inhibitor, has been approved by the U.S. Food and Drug Administration (FDA) for cutaneous T-cell lymphoma in 2006.[6] However, first, phase I and II studies demonstrate that pan-HDAC inhibitors may also cause numerous side effects, such as bone marrow depression, diarrhea, weight loss, taste disturbances, electrolyte changes, disordered clotting, fatigue, and cardiac arrhythmias.[7] Hence, it will be necessary to develop new HDAC inhibitors with less side effects and higher anticancer efficacy.

Brazilin [7,11b-dihydrobenz(*b*)indeno[1,2-*d*]pyran-3,6a,9,10(6*H*)-tetrol] (Figure 1A) is a chemical compound that is derived from *Caesalpinia sappan*.[8] *C. sappan* has been used as a beverage or food colorant in several Asian countries. Previously, several studies reported the biological activities of brazilin, such as hypoglycemic activity,[9] antihepatotoxicity,[10] inhibition of protein kinase C activity,[11] antiplatelet aggregation,[12] induction of immunological tolerance,[13] and anti-inflammatory activity.[14] However, anticancer activity of brazilin has not been reported until now. Thus, in the present study, we investigated the effect of brazilin on apoptosis and the HDAC-related signaling in MM cell line U266 using cytotoxicity assay, terminal deoxynucleotidyl transferase dUTP nick end labeling

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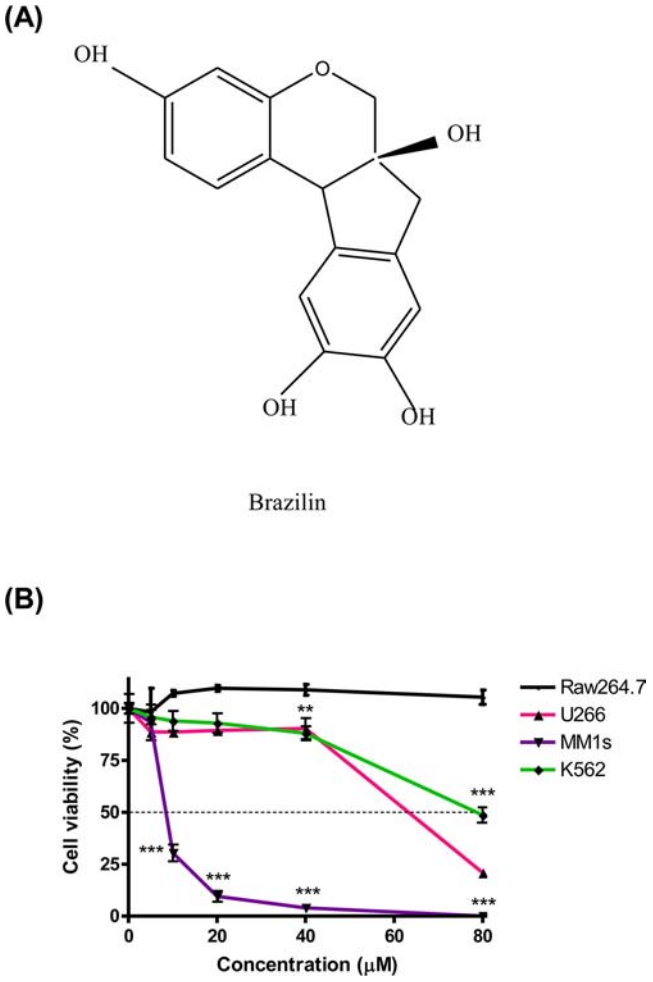

Figure 1. Effect of brazilin on the viability of MM cells. (A) Chemical structure of brazilin. Molecular weight = 286.28. (B) Cytotoxicity of brazilin was evaluated by the MTT assay on MM cells (U266 and MM1s), leukemia cell line (K562), and mouse macrophage cells (RAW264.7). Cells were treated with various concentrations of brazilin (0, 10, 20, 40, 60, or 80 μM) for 24 h. Data are presented as the mean ± SD.

(TUNEL) assay, cell cycle analysis, western blotting, reverse transcription-polymerase chain reaction (RT-PCR), and HDAC activity assay.

MATERIALS AND METHODS

Cell Culture. Human MM cell line (U266 and MM1s), human leukemia cell line (K562), and mouse macrophage RAW264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). U266, MM1s, and K562 cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 2 μM L-glutamine, and penicillin/streptomycin. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 μM L-glutamine, and penicillin/streptomycin (Gibco, Carlsbad, CA).

Cytotoxicity Assay. Cytotoxicity of brazilin (ICN Pharmaceuticals, Irvine, CA) was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO). Cells were seeded on 96-well microplates at a density of 2×10^4 cells per well and treated with various concentrations of brazilin (0, 10, 15, 20, 30, 40, 60, or 80 μM) for 24 h. The cells were incubated with MTT solution (1 mg/mL) for 2 h and MTT lysis buffer [20% sodium dodecyl sulfate (SDS) and 50% dimethylformamide] overnight. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA) at 570 nm. Cell viability was calculated as the percentage of viable cells in the brazilin-treated group versus the untreated control by the following equation: cell viability (%) = $(OD_{brazilin} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$.

Western Blot Analysis. Cells were lysed in RIPA buffer [50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholic acid−Na, 1 M ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail]. Protein samples were quantified using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA), separated by electrophoresis on 6−15% SDS−polyacrylamide gel electrophoresis (PAGE) gels, and electrotransferred onto Hybond ECL transfer membranes (Amersham Pharmacia, Piscataway, NJ). After blocking with 1−5% nonfat skim milk, the membranes were probed with antibodies for Mcl-1_L, cleaved caspase-3, caspase-9, PARP, acetyl-histone H3 (Lys 23) (Cell Signaling, Beverly, MA), HDAC1, HDAC2 (Millipore, Temecula, CA), Bcl-2, Bcl-x_L, Bax, cyclin D1, cyclin E, cyclin B1, p21, p27 (Santa Cruz Biotechnologies, Santa Cruz, CA), and β-actin (Sigma-Aldrich Co., St. Louis, MO), and then exposed to horseradish peroxidase (HRP)-conjugated secondary anti-mouse or anti-rabbit antibodies (AbD Serotec, Raleigh, NC). Protein expression was examined using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia, Piscataway, NJ).

HDAC Activity Assay. HDAC activity was analyzed using the HDAC assay kit (Millipore, Temecula, CA) following the instructions of the manufacturer. Cell lysates (20 μg of nuclear extract) from the cells treated with brazilin were incubated with HDAC assay substrate for 1 h at 37 °C. Activator solution was then added, and the mixture was incubated for another 15 min at room temperature. Fluorescence was measured using a fluorescence microplate reader (Molecular Devices Co., Sunnyvale, CA) at 405 nm.

TUNEL Assay. DNA fragmentation was analyzed using the dead end fluorometric TUNEL assay kit (Promega, Madison, WI). U266 cells (3×10^5 cells/mL) were treated with or without 60 μM brazilin for 24 h and plated on poly-L-lysine-coated slides (Nalge Nunc International, Rochester, NY). The cells were fixed in 4% methanol-free formaldehyde solution [phosphate-buffered saline (PBS), pH 7.4] for 25 min at 4 °C and treated with terminal deoxyribonucleotidyl transferase (TdT) enzyme buffer containing fluorescein-12-dUTP for 1 h at 37 °C in the dark. The slides were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA) and visualized under an Olympus FluoView FV10i confocal microscope at 60× magnification.

RT-PCR. Total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer and reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). The cDNA was amplified by PCR using the synthesized specific primers (Cosmo, Seoul, Korea) with the following sequences: HDAC1, forward, 5′-CTC CTG TTT TTT TCA GGC TCC-3′; reverse, 5′-AGG AGA AGA CAG ACA GAG GGC-3′; HDAC2, forward, 5′-CCC TGA ATT TGA CAG TCT CAC C-3′; reverse, 5′-CAC AAT AAA ACT TGC CCA GAA AAA C-3′; and GAPDH, forward, 5′-TCA CCA TCT TCC AGG AGC GA-3′; reverse, 5′-CAC AAT GCC GAA GTG GTC GT-3′. The reaction was performed at 25 cycles of 92 °C for 2 min, 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, with extension at 72 °C for 5 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide (EtBr). The stained bands were visualized under ultraviolet (UV) light and photographed.

Cell Cycle Analysis. Cell cycle analysis was performed by propidium iodide staining (Sigma, St. Louis, MO). Cells were fixed in 70% ethanol, incubated with 0.1% RNase A in PBS at 37 °C for 30 min and resuspended in PBS containing 25 μg/mL propidium iodide (PI) for 30 min at room temperature. The stained cells were analyzed for DNA content by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) using the Cell Quest program (Becton Dickinson, Franklin Lakes, NJ).

Caspase-3 Activity Assay. Caspase-3 activity was measured using the caspase-3 colorimetric assay kit (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Cells were lysed

using lysis buffer [100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.4, 140 mM NaCl, and 1% protease inhibitor cocktail] and centrifuged at 13000*g* at 4 °C for 10 min. Supernatants were incubated with 200 μM DEVD-pNA or IETD-pNA and 5 mM dithiothreitol (DTT) at 37 °C for 2 h. The absorbance of enzymatically released pNA was measured by a microplate reader (Molecular Devices Co., Sunnyvale, CA) at 405 nm.

Combination Index (CI) Calculation. The CI was determined by the Chou−Talalay method and CalcuSyn software (Biosoft, Ferhuson, MO). A CI of less than 1 was considered synergistic.[15]

Statistical Analysis. Data were presented as the mean ± standard deviation (SD). The statistically significant differences between the control and brazilin-treated groups were calculated by Student's *t* test. All experiments were carried out at least 3 times.

RESULTS

Brazilin Exhibits the Cytotoxic Effect in Human MM Cell Lines. The cytotoxicity of brazilin (Figure 1A) against MM cells (U266 and MM1s), leukemia cells (K562), and RAW264.7 cells were evaluated by the MTT assay. Cells were treated with various concentrations of brazilin (0, 5, 10, 20, 40, or 80 μM) for 24 h. Brazilin significantly decreased the viability of U266, MM1s, and K562 cells in a dose-dependent manner but not RAW264.7 cells (Figure 1B).

Brazilin Inhibits the Activation and Expression of HDACs in U266 Cells. HDACs are enzymes that balance the activities of histone acetyltransferases (HATs) on chromatin remodeling and play essential roles in the regulation of gene transcription.[16] We examined whether or not brazilin can affect HDAC activation and expression in U266 cells. As shown in Figure 2A, brazilin significantly reduced the HDAC activity in a time-dependent manner. Consistently, brazilin enhanced the level of acetyl-histone H3 at Lys 23 in a time-dependent manner (Figure 2B). Furthermore, brazilin suppressed the expression of HDAC1 and HDAC2 at the levels of protein (Figure 2C) and mRNA (Figure 2D). However, the mRNA expressions of HDAC1 and HDAC2 were decreased from the early time, while their protein expressions were declined around 24 h post-incubation, which can be supported by the report from Hargrove and Schmidt[17] that individual mRNA and protein have different rates of degradation, because the average turnover for mRNA is about 10−20 h[18] but the half-life of protein is about 48−72 h. Next, we also comparatively evaluated the inhibition of HDAC in another MM cell (MM1s) and leukemia cell line (K562) by western blotting. In MM1s cells, HDAC1 was not detected but HDAC2 expression was decreased by brazilin. In contrast, brazilin did not affect the expression of HDAC1 and HDAC2 in K562 cells (Figure 2E), suggesting that inhibition of HDAC by brazilin may be more specific in MM cell lines, such as MM1s and U266 cells.

Brazilin Induces G2/M Arrest and Regulated Cell-Cycle-Related Proteins in U266 Cells. Increasing evidence has shown that inhibition of HDACs reduces the growth of cancer cells by inducing cell cycle arrest and/or apoptosis.[19] Thus, the effect of brazilin on cell cycle progression was examined in U266 cells by flow cytometric analysis. Brazilin induced cell cycle arrest at the G2/M phase and apoptosis (peak time = 24 h) (Figure 3A). G2/M arrest by brazilin treatment was further confirmed by increased levels of cyclin-dependent kinase (CDK) inhibitors p21 and p27 in a time-dependent manner (Figure 3B). Especially, a dramatic increase of p27 expression was observed compared to p21 expression in brazilin-treated cells. Also, brazilin clearly suppressed cell cycle

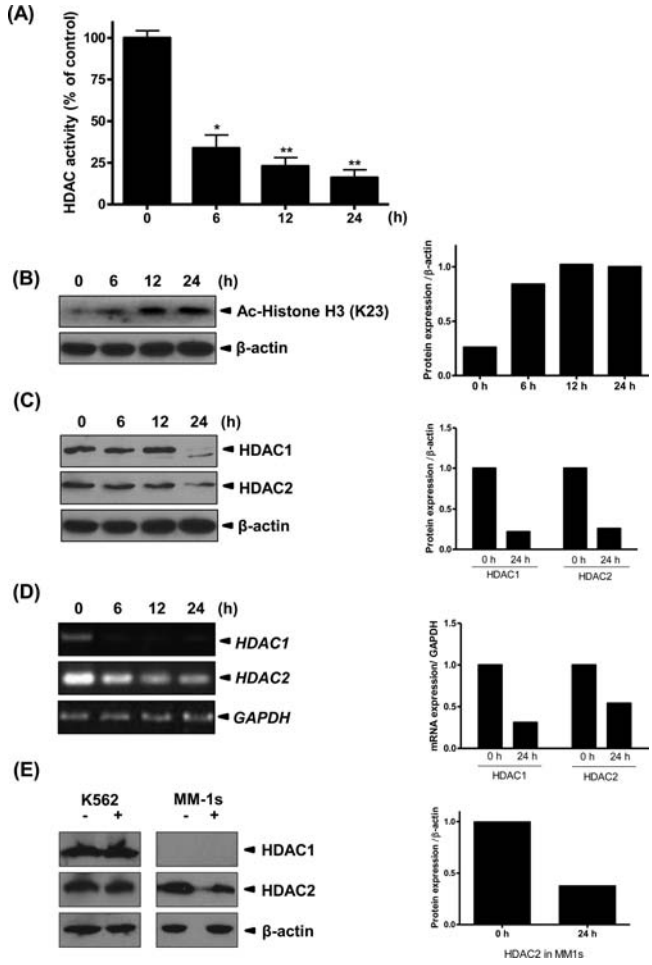

Figure 2. Effect of brazilin on activation and expression of HDACs in U266 cells. Cells were treated with brazilin (60 μM) for 0, 6, 12, or 24 h. (A) *In vitro* HDAC activity assay was performed using the colorimetric HDAC assay kit (Millipore). Data are presented as the mean ± SD. (*) $p < 0.05$ and (**) $p < 0.01$ versus the untreated control. (B) Cell lysates were prepared and subjected to western blotting with antiacetyl-H3 K23 antibody. (C) Western blotting was performed for the expression of HDAC1 and HDAC2. (D) mRNA expressions of HDAC1 and HDAC2 were analyzed by RT-PCR. (E) MM1s and K562 cells were treated with brazilin (0 and 80 μM, respectively) for 24 h.

regulatory protein cyclin D1 at 6 h post-incubation (Figure 3B). In contrast, levels of cyclin B1 and cyclin E were decreased after 24 or 48 h culture in U266 cells treated with brazilin (Figure 3C). Furthermore, we found that brazilin activated the G2 checkpoint-related proteins Chk1 and Chk2 (Figure 3D), indicating that brazilin induced G2/M arrest in U266 cells treated with brazilin.

Brazilin Induces Apoptotic Cell Death in U266 Cells. To explore whether the inhibitory effect of brazilin on HDACs is associated with apoptosis induction in U266 cells, we performed several *in vitro* apoptosis assays. As shown in Figure 4A, U266 cells treated with brazilin showed the increase in apoptotic morphological features, such as apoptotic bodies and cell shrinkage, under an inverted microscopy. A significant increase of TUNEL-positive U266 cells was observed in the treatment of brazilin (Figure 4B). Cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP) were gradually increased on a time-dependent manner in the presence of

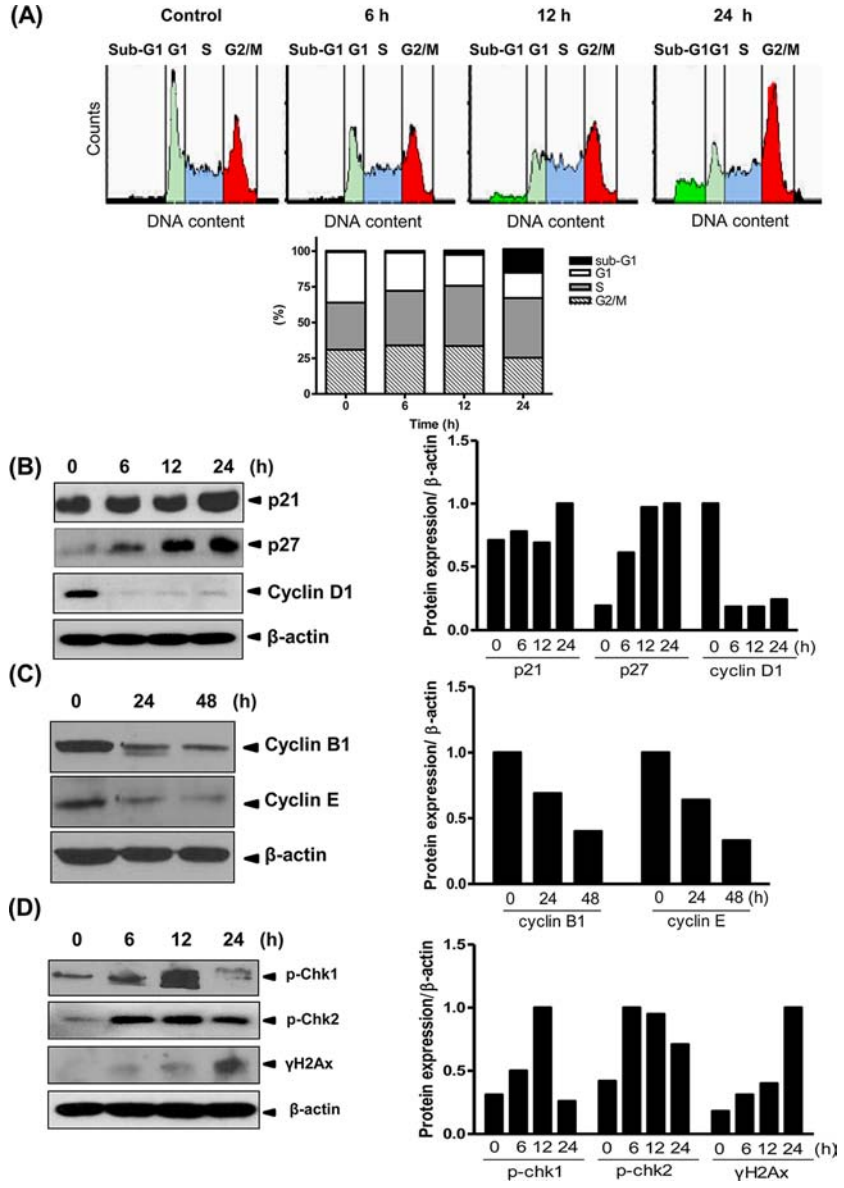

Figure 3. Effect of brazilin on cell cycle progression in U266 cells. (A and B) Cells were treated with brazilin (60 μM) for 0, 6, 12, or 24 h. (A) After fixing in 75% ethanol, the cells were stained with PI[34] and the cell cycle was analyzed by flow cytometry. (B) Cell lysates were prepared and subjected to western blotting for p21, p27, and cyclin D1. (C) Cells were treated with brazilin (60 μM) for 0, 6, 12, 24, or 48 h. Western blotting was performed with cyclin B1 and cyclin E. (D) Cells were treated with brazilin (60 μM) for 0, 6, 12, or 24 h. Western blotting was conducted to determine the phosphorylation of Chk1 and Chk2 and the expression of γH2Ax.

brazilin by western blotting (Figure 4C). The *in vitro* caspase-3 enzymatic activity assay further confirmed the involvement of caspase-3 in brazilin-induced apoptosis (Figure 4D). Brazilin also suppressed anti-apoptotic proteins, such as Bcl-x_L and Bcl-2 but not Mcl-1_L (Figure 4E). Furthermore, we used HAT inhibitor anacardic acid to explore that brazilin-induced apoptosis is associated with HDAC inhibition. As shown in Figure 5A, anacardic acid reversed acetylation of histone H3 as well as PARP cleavage induced by brazilin in U266 cells, indicating that HDAC inhibition is importantly involved in brazilin-induced apoptosis in U266 cells. To investigate whether or not inhibition of HDAC induced by brazilin can be affected by inhibitor of caspase, we used Z-VAD-FMK001, a pan-caspase inhibitor. As shown in Figure 5B, the pan-caspase inhibitor did not affect the inhibition of HDACs in brazilin-induced U266 cells, implying that brazilin mediates apoptosis via inactivation of HDAC and caspase does not mediates HDAC inhibition induced by brazilin in U266 cells (Figure 5B).

Brazilin Potentiates the Efficacy of Standard Chemotherapeutic Agents in U266 Cells. Bortezomib and doxorubicin are the FDA-approved chemotherapeutics for the treatment of MM patients.[20,21] We determined whether brazilin can potentiate the effect of standard chemotherapeutic agents, such as bortezomib and doxorubicin. As shown in panels A and B of Figure 6, the viability of U266 cells was significantly reduced in combinations of brazilin (40 μM) and bortezomib (Figure 6A) or doxorubicin (Figure 6B) compared to either drug alone. To evaluate the synergistic effect between brazilin and these agents, CI values were calculated using CalcuSyn software.[15] Here, we found that brazilin obviously showed the synergistic effect on cell death in combination of bortezomib or

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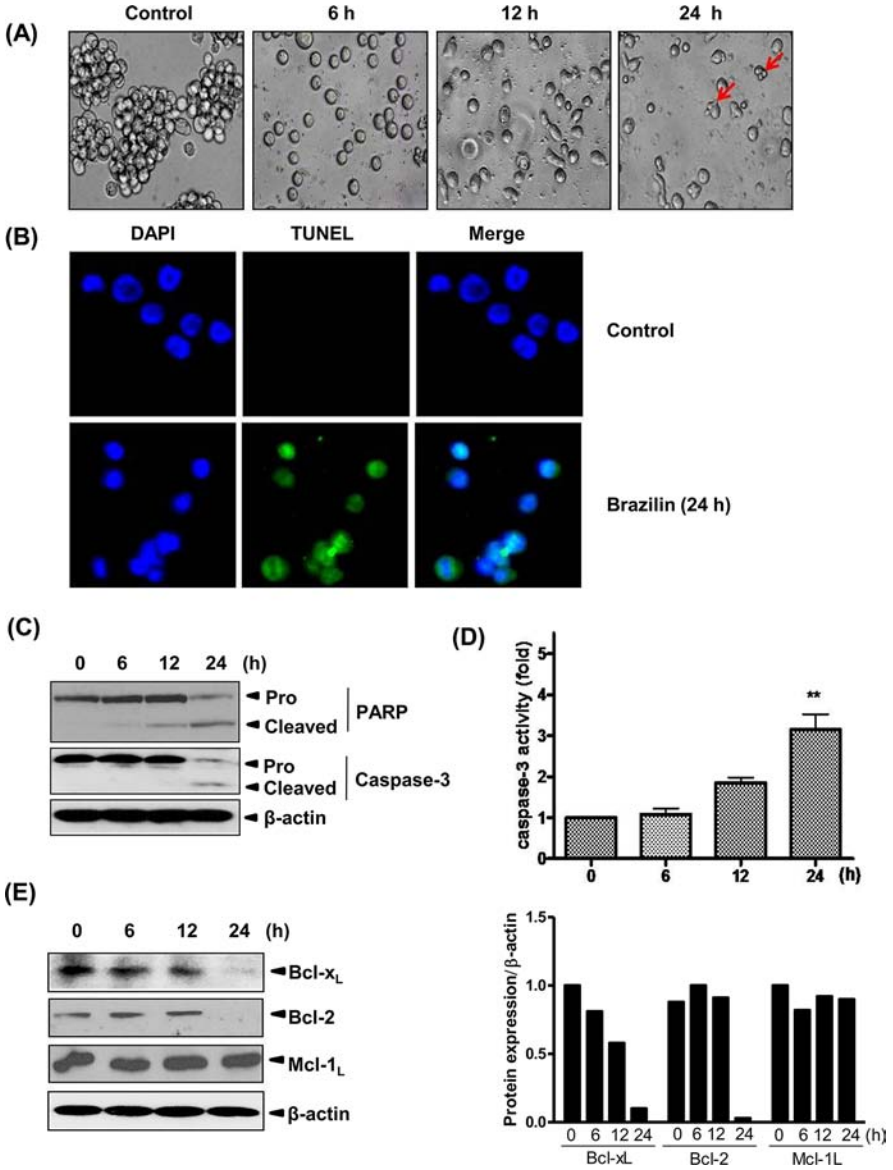

Figure 4. Effect of brazilin on apoptosis induction in U266 cells. (A) Cells were treated with brazilin (60 μM) for 0, 6, 12, or 24 h. Cell morphological changes were observed under inverted microscopy. (B) TUNEL staining was performed in the cells treated with or without brazilin (60 μM) for 24 h and visualized by an Olympus FluoView FV10i confocal microscope. Arrows indicate TUNEL (FITC)-stained cells. (C–E) Cells were treated with brazilin (60 μM) for 0, 6, 12, or 24 h. (C) Cell lysates were prepared and subjected to western blotting for PARP and caspase-3. (D) Fluorigenic caspase-3 activity assay was performed using luminometric assays. All data are presented as the mean ± SD of a minimum of 3 or more replicates. (**) $p < 0.01$ versus the untreated control. (E) Western blotting was carried out to determine the expression of Bcl-x_L, Bcl-2, and Mcl-1.

doxorubicin, with CI > 1 (0.754 and 0.314 in co-treatment with brazilin + bortezomib and brazilin + doxorubicin, respectively) (Figure 6C).

■ DISCUSSION

Alterations in tumor suppressor genes or oncogenes are not always due to mutations. They may also be due to transcriptional regulation by epigenetic mechanisms, including DNA methylation or demethylation and/or histone acetylation or deacetylation. The balance between histone acetylation and deacetylation, mediated by HATs and HDACs, respectively, is usually well-regulated, but the balance is often upset in diseases, such as cancer.[22] A total of 18 human HDACs have been identified and divided into 4 subfamilies according to sequence homologies. Of the subfamilies, HDAC1, HDAC2, HDAC3, and HDAC8 (class I) and HDAC4 (class II) are highly expressed in various cancer cells, such as prostate and gastric.[23,24] Therefore, targeting HDACs using HDAC inhibitors has been considered as an attractive approach for cancer therapy.

Because sodium butyrate (NaBu) was the first HDAC inhibitor to be discovered in the late 1970s, being initially found to have antitumor activity by inducing cell differentiation, various HDAC inhibitors with different structures and potencies have been synthesized or purified from natural sources, and their effects as anticancer drugs are now widely documented.[25] In 2006, suberoylanilide hydroxamic acid (SAHA), also called Vorinostat, was the first HDAC inhibitor approved by the FDA for the treatment of cutaneous T-cell lymphoma.[6] Development of HDAC inhibitors for the

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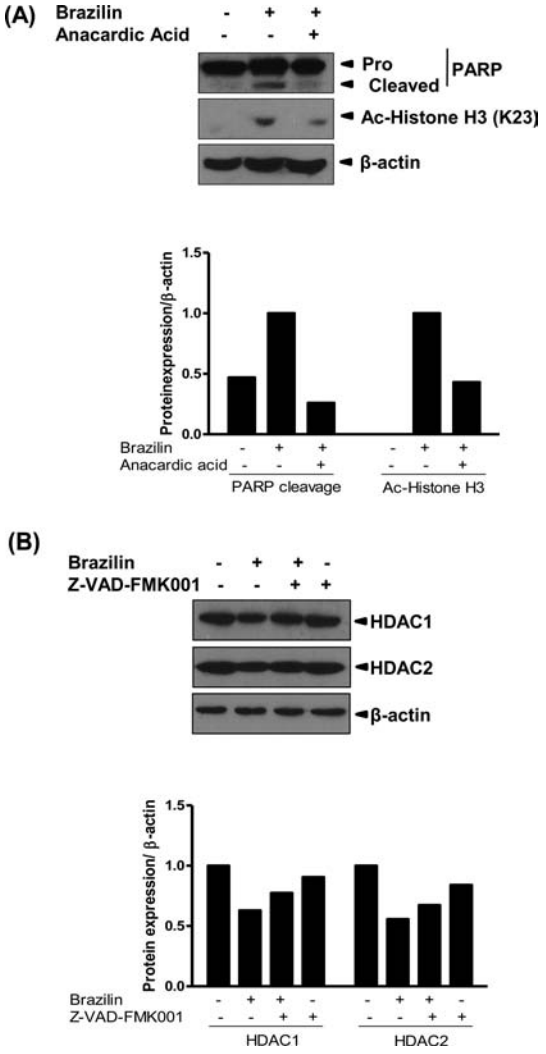

Figure 5. Effect of anacardic acid on brazilin-induced apoptosis in U266 cells. (A) Cells were exposed to brazilin (60 μM) and/or anacardic acid (125 nM) for 24 h. Cell lysates were prepared and subjected to western blotting for acetyl-histone H3 (K23) and cleaved PARP. (B) Cells were treated with brazilin (60 μM) and/or Z-VAD-FMK001 (20 μM) for 24 h. Cell lysates were prepared and subjected to western blotting for HDAC1, HDAC2, and β-actin.

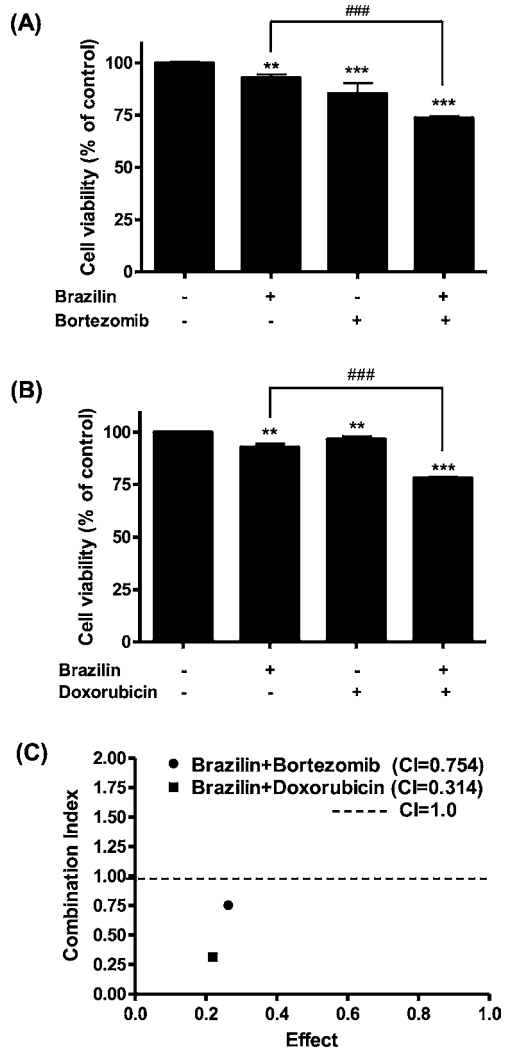

Figure 6. Synergistic effect of brazilin and anticancer drugs in U266 cells. (A and B) Cells were treated with brazilin (40 μM) and/or (A) bortezomib (20 nM) or (B) doxorubicin (1 μM) for 24 h. The cytotoxicity was determined by the MTT assay. All data are presented as the mean ± SD of a minimum of 3 or more replicates. (**) $p < 0.01$ and (***) $p < 0.001$ versus the untreated control. (###) $p < 0.001$ versus the brazilin-treated group. (C) CI between brazilin and doxorubicin or bortezomib was determined by the Chou−Talay method and CalcuSyn software (Biosoft, Ferhuson, MO).

treatment of cancer is still ongoing, and 80 phase I and II clinical trials are currently underway to validate these drugs alone or in association with other therapies in patients with hematological or solid tumors.[26,27]

Tumor cells are more sensitive and undergo growth arrest by inhibition of differentiation and induction of apoptosis, whereas normal cells are relatively resistant to the treatment with HDAC inhibitors.[28] The molecular regulation of HDAC inhibitors has been thought to be related to altered gene expression and to changes in non-histone proteins via regulation at the epigenetic and post-translational modification levels, respectively. In the present study, we found the inhibitory effect of brazilin from *C. sappan* on HDACs in MM cell line U266. Brazilin significantly inhibited the transcriptional activity of HDAC as well as the expression of HDAC1 and HDAC2 but not other HDAC subfamilies (data not shown) at protein and mRNA levels. Consistently, brazilin significantly increased the level of acetyl-histone H3 at K23, implying that brazilin has potential as a HDAC inhibitor.

In many tumor cell lines, HDAC inhibitors cause upregulation of the cell cycle gene p21, blocking the cyclin/CDK complexes, leading to cell cycle arrest and inhibiting differentiation.[29] HDAC inhibition modulates the balance between pro- and anti-apoptotic proteins, causing tumor cell death.[30] HDAC inhibition upregulates the intrinsic and/or extrinsic apoptosis pathways through the induction of the pro-apoptotic genes, such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and death receptor (DR) 5,[31] respectively. Also, hyperacetylation stabilizes the p53 protein, promoting both cell cycle arrest and the expression of pro-apoptotic genes.[32] Similar to the post-translational modification of p53, HDAC inhibition by HDAC inhibitors increases the stability and transcriptional activity of RUNX3, which induces p21 and Bim, leading to cell cycle arrest and apoptosis of tumor cells.[29] Likewise, our data showed that brazilin induced apoptosis by increasing the sub-G1 portions and TUNEL-

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positive cells, activating caspase-3 and downregulating the Bcl-2 anti-apoptotic family proteins, such as Bcl-2 and Bcl-x_L, in U266 cells. Also, brazilin mediated cell cycle arrest at the G2/M phase and significantly elevated the levels of CDK inhibitors, such as p21 and p27, in U266 cells. Consistently, brazilin suppressed the cell-cycle-related proteins, such as cyclin D1, cyclin B1, and cyclin E. Importantly, anacardic acid (HAT inhibitor) treatment disturbed PARP cleavage and acetyl-histone H3 induced by brazilin, implying that brazilin induces apoptosis by inhibiting HDAC activation in U266 cells.

Drug combination is most widely used in treating the most dreadful diseases, such as cancer and acquired immune deficiency syndrome (AIDS). The main aims are to achieve a synergistic therapeutic effect and dose and toxicity reduction and to minimize or delay drug resistance.[15] HDAC inhibitor therapy for hematologic and solid tumors usually adopt combined therapy with other conventional agents that can produce synergistic or additive effects. In one phase I trial, Vorinostat in combination with paclitaxel or carboplatin was used to treat 25 patients with advanced solid tumors. A total of 11 patients showed partial responses, and a total of 7 patients showed a stable disease,[33] demonstrating that HDAC inhibitors have promising antitumor activity when they used in combination with other chemotherapeutics. Several groups reported that HDAC inhibitors have been used in advanced solid tumors or hematologic cancer patients in combination with the DNA methylation inhibitor azacitidine, the differentiating agent all-*trans*-retinoic acid, or proteosome inhibitor bortezomib.[34] In addition, Whitesell et al. reported that HDAC inhibition leads to the loss of HSP90 chaperone function and enhanced degradation of client proteins, such as Bcr-Abl, ErbB2/neu, and Fms-like tyrosine kinase (FLT3), suggesting the possibility of potential synergistic effects between HDAC inhibitors and imatinib, traztuzumab, or FLT3 inhibitors.[35] In light of these events, we investigated whether brazilin has potential as a combinational chemotherapeutic agent with other conventional drugs. Co-treatment of brazilin and bortezomib or doxorubicin significantly reduced the viability of U266 cells with a synergistic effect (<1) by CI between brazilin and bortezomib or doxorubicin.

In conclusion, our results suggest that brazilin could be a potential therapeutic intervention agent alone or in combination with current drugs for MM as a novel inhibitor of HDAC, regulating apoptosis and cell cycle arrest. Further studies will be required to confirm the antitumor activity of brazilin by *in vivo* experiments in the near future. We also have to elucidate the role of brazilin in metabolites and pharmarcokinetic and toxicological studies. Because brazilin isolated from *C. sappan* has been traditionally used as a food and beverage colorant in Indonesia, brazilin-containing plants can be usefully applied as anticancer functional food in the future.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

brazilin, [7,11b-dihydrobenz(*b*)indeno[1,2-*d*]pyran-3,6a,9,10(6*H*)-tetrol]; CDK, cyclin-dependent kinase; HDAC, histone deacetylase; HAT, histone acetyltransferase; MM, multiple myeloma; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; RT-PCR, reverse transcription-polymerase chain reaction

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