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Mechanisms of Cancer Chemoprevention by Hop Bitter Acids (Beer Aroma) through Induction of Apoptosis Mediated by Fas and Caspase Cascades

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The bitter acids of hops (*Humulus lupulus* L.) mainly consist of α-acids, *β*-acids, and their oxidation products that contribute the unique aroma of the beer beverage. Hop bitter acids displayed a strong growth inhibitory effect against human leukemia HL-60 cells, with an estimated IC_{50} value of 8.67 *μ*g/mL, but were less effective against human histolytic lymphoma U937 cells. Induction of apoptosis was confirmed in HL-60 cells by DNA fragmentation and the appearance of a sub-G1 DNA peak, which were preceded by dissipation of mitochondrial membrane potential, cytochrome *c* release, and subsequent induction of pro-caspase-9 and -3 processing. Cleavages of PARP and DFF-45 were accompanied with activation of caspase-9 and -3 triggered by hop bitter acids in HL-60 cells. The change in the expression of Bcl-2, Bcl-X_L, and Bax in response to hop bitter acids was studied, and the Bcl-2 protein level slightly decreased; however, the Bcl-X_L protein level was obviously decreased, whereas the Bax protein level was dramatically increased, indicating that the control of Bcl-2 family proteins by hop bitter acids might participate in the disruption of mitochondrial integrity. In addition, the results showed that hop bitter acids promoted the up-regulation of Fas and FasL prior to the processing and activation of pro-caspase-8 and cleavage of Bid, suggesting the involvement of a Fas-mediated pathway in hop bitter acids-induced cells. Taken together, these findings suggest that a certain intimate link might exist between receptor- and mitochondria-mediated death signalings that committed to cell death induced by hop bitter acids. The induction of apoptosis by hop bitter acids may offer a pivotal mechanism for their chemopreventive action.

KEYWORDS: Hop bitter acids; α-acids; *β*-acids; apoptosis; caspase; mitochondria; Bcl-2; Bcl-X_L; Bax; Fas; FasL; Bid

INTRODUCTION

Beer is a popular beverage worldwide; it provides an appreciable fraction of calorie intake and is an indispensable daily food for many people. The characteristic bitter taste and aroma of pale beers is derived from hop bitter acids, which are extracted from female inflorescences (hop cones) of hops (*Humulus lupulus* L.) and added to sweet wort during brewing. The bitter acids of hops mainly consist of α-acids, *β*-acids, and their oxidation products. Both α-acids and *β*-acids are a mixture of homologues and analogues and introduced the names n-, co-, and ad-humulone and n-, co-, and ad-lupulone, respectively (**Figure 1**) (*1*). Hop bitter acids are shown to have various potent biological activities, including inhibition of angiogenesis (*2*), suppression of cyclooxygenase-2 gene transcription (*3*), induction of differentiation in myelogenous leukemia cells (*4*), inhibition of bone resorption (*5*), antioxidation (*6*), inhibition of tumor promotion by phorbol ester (*7*), antibacterial action (*8*), and induction of apoptosis (*9*). The antioxidative and antitumor properties provide potential mechanisms for hop bitter acids as a chemotherapeutic or chemopreventive agent.

Cancer chemoprevention is defined as the use of pharmacologic or natural agents to prevent the metabolic activation of procarcinogens and inhibit or delay the processes of tumor initiation, promotion, and progression (*10*). Long-term deficiency of chemopreventive agents leads to tumorigenesis (*11*−*13*). The mechanisms providing the basis for the use of chemopreventive agents include alteration of differentiated states of cells, which are characteristic of premalignant lesions before they become invasive and metastatic, blockade of preneoplastic cell expansion or induction of an apoptotic state in cells, intervention of metabolic activation of carcinogens by scavenging reactive oxygen species (ROS), which may activate procarcinogens or inhibit cytochrome p450 isoenzymes, which are responsible for the metabolism of procarcinogens.

Apoptosis is a major type of physiological or pathological cell death, which is important in the control of cell numbers

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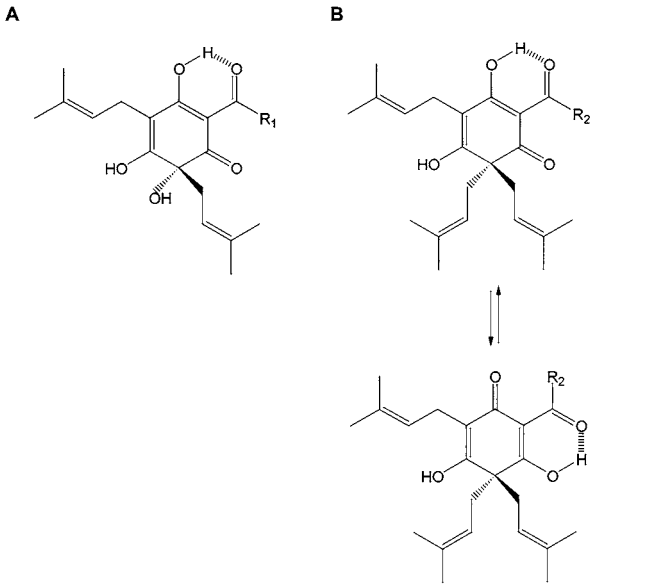

Figure 1. Chemical structures of hop bitter acids: (**A**) α-acids; (**B**) β-acids.

during development and proliferation and in the removal of damaged cells that threaten homeostasis. The mechanism of apoptosis is highly conserved from lower eukaryotes to mammals and exhibits a sophisticated network of tightly ordered molecular events that finally converge into the enzymatic fragmentation of chromosomal DNA, thereby driving a cell undergoing death (*14*). Apoptosis involves the activation of a family of cysteine proteases named caspases, which cleave a variety of cellular substrates that contribute to detrimental biochemical and morphological changes and eventual cell destruction (*15*, *16*). At least two pathways of caspase activation for apoptosis induction have been characterized. One is mediated by death receptors, including Fas, TNFR, DR3, DR4, and DR5 (*17*). These receptors are cell surface proteins and belong to the TNF receptor superfamily (*18*). Activation of death receptors (Fas) by cross-linking with their natural ligands (Fas ligand) induces apoptosis in sensitive cells (*19*). Death receptor ligands characteristically initiate signaling via receptor oligomerization and recruitment of specialized adaptor proteins followed by proteolysis and activation of procaspase-8, and activated caspase-8 directly cleaves and activates caspase-3, which in turn cleaves other caspases (e.g., caspase-6 and -7) for activation (*20*−*23*). The other pathway, driven by Bcl-2 family proteins, which may be anti-apoptotic (Bcl-2 and Bcl-X_L) or pro-apoptotic (Bax, Bak, and Bid), regulates cell death by controlling mitochondrial membrane permeability during apoptosis. Upon apoptosis, pro-apoptotic proteins, such as Bax and Bak, translocate to mitochondria and accelerate the opening of the mitochondria porin channel that leads to cytochrome *c* release, thereby triggering the cascade of caspase activation (*24*). In addition, activated caspase-8 can cleave Bid, a member of the Bcl-2 familiy, to generate 15 kDa truncated Bid (tBid), which translocates to the mitochondrial membrane and results in the release of cytochrome *c* and, thus, activation of the downstream caspases (*25*, *26*). Aberrant control of the balance of cell numbers may arise from malignant transformation, and induction of apoptosis in transformed cell population suppresses the development of cancer (*27*). The induction of apoptosis by the natural phytochemicals in malignant cells validates a promising strategy for human cancer chemoprevention (*28*, *29*).

The apoptotic effect of humulone is well-documented (*9*), yet little is known of the mechanism by which humulone induces apoptosis, and the apoptotic effect of the relative compounds of humulone involving α- and β-acids is uncharacterized. The present study used human leukemia cell line HL-60 and U937 to characterize hop bitter acids-induced apoptosis, and the cell death-inducing mechanisms by which hop bitter acids triggered apoptosis in HL-60 cells were explored. Understanding the effects of hop bitter acids on cell death may have implications for the treatment or prevention of some types of leukemia.

MATERIALS AND METHODS

Materials. Hop bitter acids containing 49.39% of α-acids and 24.94% of β-acids were obtained from Versuchsstation Schweizerischer Brauereien, Zürich, Switzerland, and dissolved in dimethyl sulfoxide (DMSO) prior to use. The antibodies to Bax and DFF-45 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-caspase-3, -8, and -9, anti-Bid, cytochrome *c*, and poly(ADP-ribose) polymerase (PARP) antibodies were from PharMingen (San Diego, CA); anti-Bcl-2, Bcl-X_L, Fas, and Fas ligand (FasL) antibodies were purchased from Transduction Laboratory (Lexington, KY). Secondary antibodies used were fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (Chemicon), horseradish peroxidase-conjugated anti-mouse, and rabbit IgG. Propidium iodide (PI) and rhodamine 123 were obtained from Sigma (St. Louis, MO). Fluorogenic peptide substrates Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) specific for caspase-3 acitvity and Ac-Ile-Glu-Thr-Asp-AMC (Ac−IETD-AMC) specific for caspase-8 activity were purchased from Bachem (King of Prussia, PA).

Cell Culture. Human leukemia cell lines HL-60 (promyelocytic cells) and U937 (histocytic cells) obtained from American Type Culture Collection were maintained in RPMI-1640, supplemented with 15% (HL-60) or 10% (U937) fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY) and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell Survival Assay. HL-60 or U937 cells (2×10^5 cells/mL) were plated in 12-well tissue culture plates. After overnight growth, cells were treated with a series of concentrations of hop bitter acids, and the final concentrations of DMSO in the culture medium were <0.1%. Following 24 h of incubation with various concentrations of hop bitter acids, the cell viability was assayed with a Luminescent ATP detection assay kit (Packard BioScience B.V.). Briefly, 50 μL of cell lysate was used to assay the production of luminescent light caused by the reaction of ATP with added luciferase and D-luciferin. Luminescence was measured on a Top Counter microplate scintillation and luminescence counter (Packard 9912V1, Meriden, CT) in single photon counting mode for 0.1 min/well, following a 2-min adaptation in the dark.

DNA Extraction and Electrophoresis Analysis. HL-60 or U937 cells (2×10^5 cells/mL) 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 3 h and treated with RNase A (0.5 μg/mL) for another 2 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 TBE buffer (Tris-borate/EDTA electrophoresis buffer). Approximately 20 μg of DNA was loaded in each well and visualized under UV light and photographed.

Flow Cytometric Cell Analysis. Cell cycle distribution was analyzed by flow cytometry as follows. At each time point, cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol for at least 2 h at −20 °C. Fixed cells were washed with PBS, incubated with 1 mL of PBS containing 0.5 μg/mL RNase A and 0.5% Triton X-100 for 30 min at 37 °C, and then stained with 50 μg/mL propidium iodide. The stained cells were analyzed using an FACScan

laser flow cytometer (Becton Dickinson, San Jose, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME).

Mitochondrial Membrane Potential. HL-60 cells were cultured and allowed to reach exponential growth for 24 h before treatment. The cells were harvested for 15 min, 30 min, and 17 h after treatment with 50 μg/mL of hop bitter acids. Changes in MMP were measured by uptake of the lipophilic cation rhodamine 123 into mitochondria (*30*). Untreated control cells were used to determine the normal uptake of this cation, and the percentage of treated cells with a low MMP was then calculated. Briefly, the cells were pelleted by centrifugation at 800*g* for 10 min at room temperature and washed once in PBS. The cells were resuspended in 1 mL of rhodamine 123 (10 μg/mL) for 30 min at room temperature, washed with PBS twice, and resuspended in PBS. The samples (10^4 events) were analyzed for fluorescence (FL1 detector, filter 430/30 nm band-pass) using a FACScan (Becton Dickinson, San Jose, CA). Histograms were analyzed using Cell Quest software and compared with histograms of control untreated cells.

Fractionation of Cell Extracts. Mitochondrial and cytosolic (S100) fractions were using differential centrifugation (*31*−*34*). Briefly, HL-60 cells were treated with hop bitter acids or vehicle (DMSO) control for indicated times. At the end of the treatment, cells were harvested, washed twice in ice-cold PBS, and resuspended in homogenizing buffer [250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiolthione, 17 μg/mL phenylmethanesulfonyl fluoride (PMSF), 8 μg/mL aprotinin, 2 μg/mL leupeptin (pH 7.4)] and incubated on ice for 30 min. Cells were passed through a needle 10 times. Unlysed cells, large plasma membrane pieces, and nuclei were pelleted by centrifugation at 750*g* for 10 min. The supernatant was spun at 10000*g* for 15 min. This pellet was resuspended in homogenizing buffer and represents the mitochondrial fraction. The remaining supernatant was spun at 100000*g* for 1 h. The supernatant from this final centrifugation represents the cytosolic (S100) fraction. The determination of cytochrome *c* release was performed by Western blot as described above.

Western Blot Analysis. HL-60 cells were treated with various concentrations of hop bitter acids for 24 h or indicated times. Cells were harvested and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% NP-40; 0.5 mM PMSF; and 0.5 mM dithiothreitol) for 30 min at 4 °C. Equal amounts of total cellular proteins (50 μg) were resolved by SDS−polyacrylamide gel electrophoresis (PAGE) (8% for PARP; 10% for caspases-8, Bid, and DFF-45; 12.5% for caspases-3 and caspase-9; 15% for Bcl-2, Bcl-X_L, Bax, and cytochrome *c*), transferred onto poly(vinylidene difluoride) (PVDF) membranes (Amersham, Arlington, IL), and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham).

Assay of Caspase Activity. HL-60 cells were treated with 50 μg/mL hop bitter acids at indicated times and then 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 PMSF, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12000*g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promega's CaspACE Assay System Corp., Madison, WI). Briefly, 100 μg of total protein, as determined by bicinchoninic acid assay (Promega Corp.), was incubated with 50 μM substrate Ac-DEVD-AMC (caspase-3 specific substrate) or Ac-IETD-AMC (caspase-8 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 F-4500).

Flow Cytometric Analysis of Fas and Fas Ligand Expression. In brief, a total of 5×10^6 cells were treated with 50 μg/mL hop bitter acids for the indicated time periods. At the end of incubation, cells were washed twice with PBS and resuspended in1% bovine serum albumin in PBS. The primary antibody (Fas or FasL) was added and incubated for 1 h at 4 °C. Then, cells were washed twice with PBS and incubated with a fluorescein isothiocyanate-labeled rabbit anti-mouse IgG for 1 h at 4 °C followed by washing with PBS. Finally, cells were fixed in 1% paraformaldehyde in PBS for 30 min at 4 °C and analyzed on a flow cytometer (Becton Dickinson, San Jose, CA).

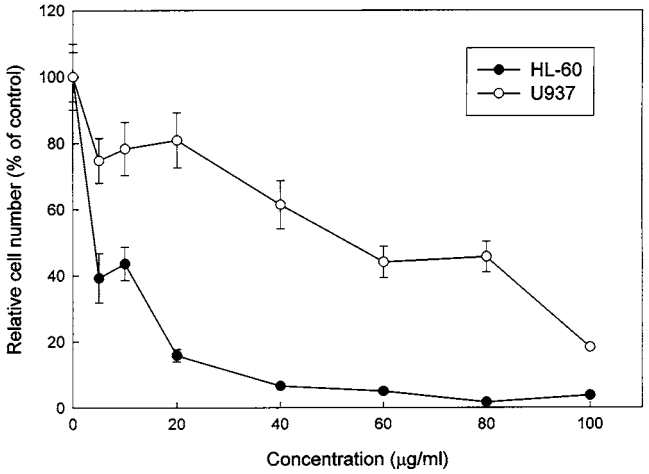

Figure 2. Effects of hop bitter acids on cell viability. HL-60 and U937 cells were treated with either 5 μL/mL of DMSO as vehicle control or various concentrations of hop bitter acids for 24 h. Cell viability then was determined by Luminescent ATP detection assay kit, as described under Materials and Methods. Data are represented as means ± SE for three determinations.

RESULTS

Hop Bitter Acids Caused Dose-Dependent Reduction in Leukemic Cell Viability. We first investigated the effect of hop bitter acids on cell viability; human leukemia cell lines HL-60 and U937 were treated with different concentrations of hop bitter acids. After 24 h of treatment, the live cells were determined by means of an ATP content test as described under Materials and Methods. As shown in **Figure 2**, hop bitter acids appeared to be a potent inhibitor of HL-60 cell viability with an IC_{50} of 8.67 μg/mL, and inhibition of the cell viability was found to be dose-dependent. However, U937 cells were more resistant to hop bitter acids, with an IC_{50} value of ~58.87 μg/mL.

Hop Bitter Acids Induced DNA Fragmentation of Leukemic Cell Lines. To determine whether the inhibition of cell growth by hop bitter acids resulted from the induction of apoptosis, DNA fragmentation, a hallmark of apoptosis, was demonstrated by incubating HL-60 or U937 with different concentrations of hop bitter acids for 24 h. DNA fragmentation became apparent at 20 μg/mL (in HL-60, **Figure 3A**, left panel) or 60 μg/mL (in U937, **Figure 3B**, left panel) of hop bitter acids treatment, and these DNA fragmentation responses were dose-dependent. When cells were treated with 50 μg/mL of hop bitter acids, DNA ladders were just visible as early as 6 h in HL-60 cells (**Figure 3A**, right panel) or 12 h in U937 cells (**Figure 3B**, right panel) after treatment, and gradually increasing DNA fragmentation was observed from 6 to 24 h. Because HL-60 cells were more sensitive to hop bitter acids than U937 cells, we further used HL-60 cells as a model to study the mechanism by which hop bitter acids triggered apoptotic cell death.

A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA (*35*), was detected in cells that were treated with hop bitter acids (50 μg/mL), washed, and stained with propidium iodide. As shown in **Figure 4**, the percentages of apoptotic HL-60 cells (right column) were gradually increased after 0, 6, 12, 18, and 24 h of incubation with hop bitter acids compared to untreated cells (5 μg/mL of DMSO, left column). Unlike the timing of the appearance of the DNA ladder, the peak of apoptosis did not appear until 12 h after incubation with hop bitter acids, and the relative levels of apoptotic cells after 12, 18, and 24 h of hop bitter acids treatment were 18.76,

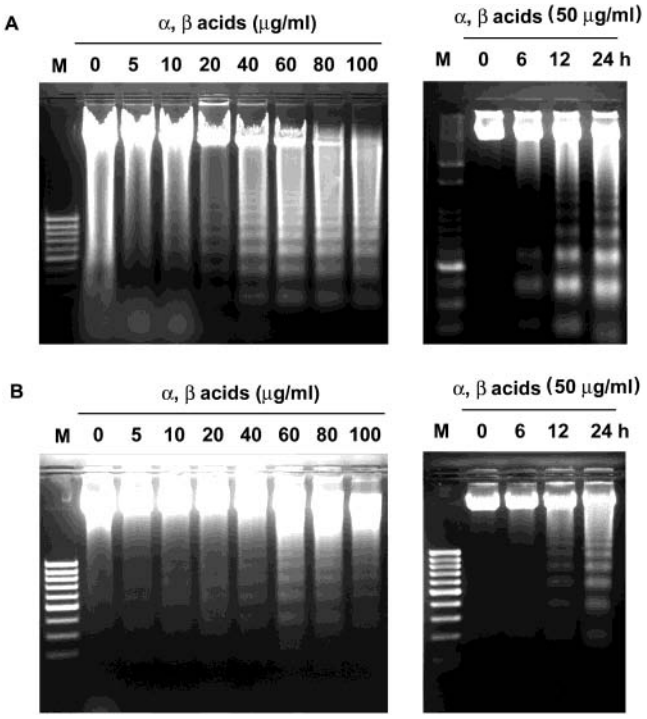

Figure 3. Induction of DNA fragmentation by hop bitter acids: (**A**) HL-60 or (**B**) U937 cells were treated with various hop bitter acids as indicated for 24 h or treated with 50 μg/mL hop bitter acids for indicated times, and internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. M, 100 base pair DNA ladder size marker.

37.85, and 46.44%, respectively. Taken together, these results indicated that hop bitter acids induced typical apoptosis in HL-60 cells.

Disruption of Mitochondrial Membrane Potential Accompanied Cytochrome *c* Release and Cleavage of Caspase-9 in Hop Bitter Acids-Induced Apoptosis. The alteration of mitochondrial function is linked to apoptosis, and a decreasing mitochondrial membrane potential ($\Delta\Psi$m) is associated with mitochondrial dysfunction (*36*). Therefore, we investigated the change of $\Delta\Psi$m in hop bitter acids-induced apoptosis. HL-60 cells were treated with 50 μg/mL of hop bitter acids for 30 min and 17 h and then exposed to the lipophilic rhodamine 123 that is taken up by mitochondria and undergoes a red shift in emission spectrum during changes in $\Delta\Psi$m. **Figure 5A** displays that a reduction in $\Delta\Psi$m was detected at 30 min and clearly changed at 17 h after exposure to hop bitter acids (the means of fluorescent intensity were shifted from 87 and 70 to 105% compared to control cells, respectively).

Disruption of mitochondrial membrane potential ($\Delta\Psi$m) results from the opening of permeability transition pores, causing a local disruption of the outer mitochondrial membrane, and leads to the release of soluble intermembrane proteins, including cytochrome *c*, and the cytochrome *c* release contributes to the activation of caspase-9 and subsequently causes apoptosis. To determine whether the reduction of $\Delta\Psi$m induced by hop biter acids could cause the release of cytochrome *c*, we next evaluated the effect of hop bitter acids on the mitochondrial cytochrome *c* release into the cytosol. Subcellular fractions of HL-60 cell lysates harvested for indicated times with 50 μg/mL of hop bitter acids treatment were separated as described under Materials and Methods and then the release of cytochrome *c* was determined by Western blotting. **Figure 5B** shows that cytochrome *c* release into cytosol was detected at 12 h after hop bitter acids treatment relative to the gradual decrease of mitochondrial cytochrome

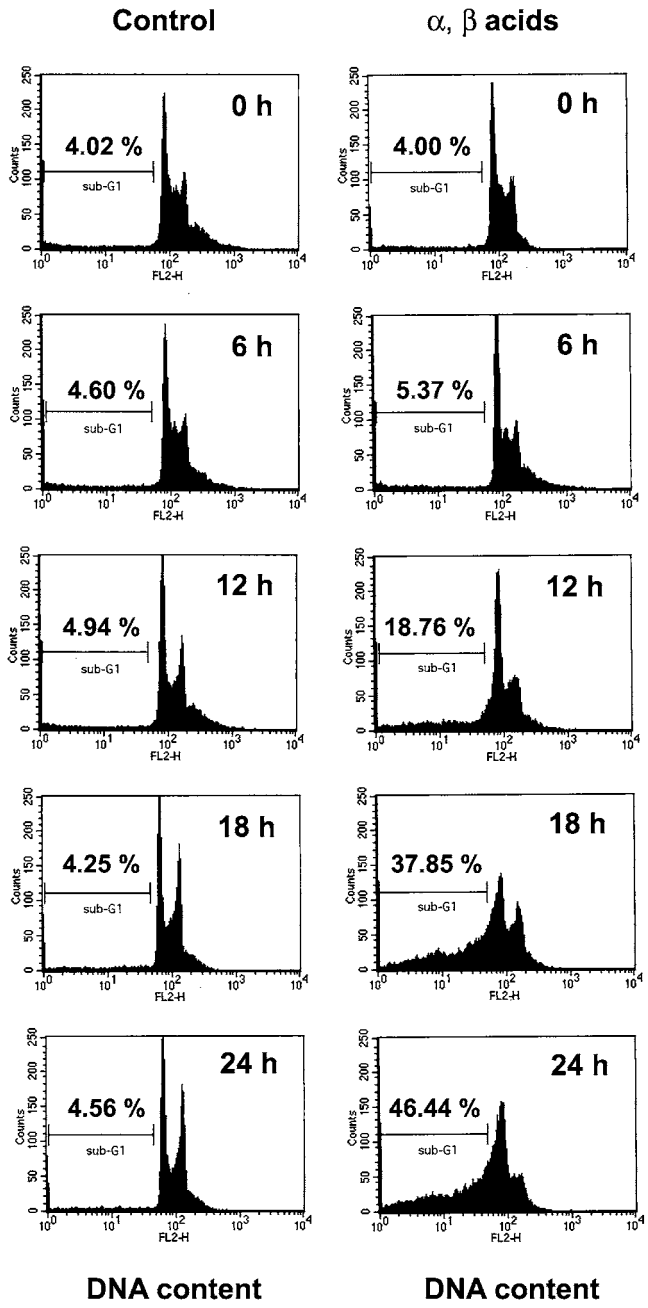

Figure 4. Determination of sub-G1 cells in control and hop bitter acids-treated HL-60 cells by flow cytometry. HL-60 cells were treated with 50 μg/mL hop bitter acids and 5 μg/mL DMSO as control at different time periods, and the ratio of sub-G1 cells in hop bitter acids-treated HL-60 cells was analyzed by flow cytometry. Sub-G1 represents apoptotic cells with a lower DNA content.

c. To further demonstrate whether the cytochrome *c* release resulting from hop bitter acids treatment would subsequently result in the cleavage and activation of caspase-9, the cleavage of pro-caspase-9 was detected at various time points after 50 μg of hop biter acids treatment. Consistent with the timing of the cytochrome *c* release, the cleavage of pro-caspase-9 sequentially occurred in HL-60 cells exposed in a time-course-dependent manner (**Figure 5C**). These observations suggested that an apoptosis-inducing mechanism via mitochondria triggered by hop bitter acids operates in HL-60 cell lines.

Cleavage and Activation of Pro-caspase-3 Associated with the Degradation of PARP and DFF-45 in Hop Bitter Acids-Induced Apoptosis. Because activation of caspase-9 is neces-

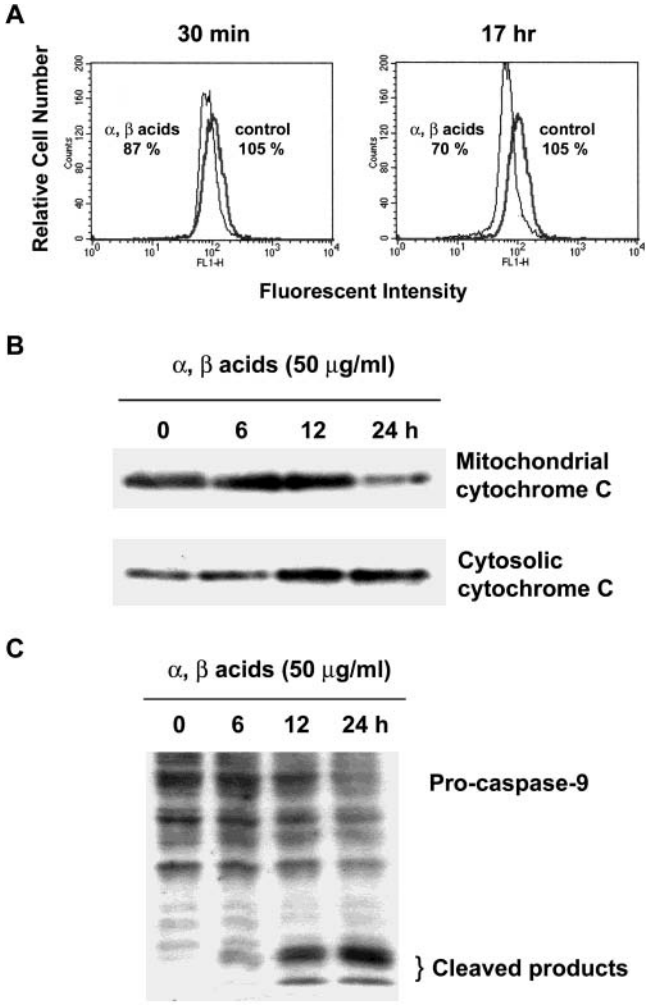

Figure 5. Induction of mitochondria dysfunction and cytochrome *c* release followed by the cleavage of caspase-9 in hop bitter acids-induced apoptosis. (**A**) HL-60 cells were treated with 50 μg/mL hop bitter acids at 30 min and 17 h, and relative $\Delta\Psi$m measured by fluorescent emission from rhodamine 123 taken up by mitochondria. The heavy line shows the profile of control cells, and the light line represents the treated cells. The numbers represent the mean of the relative fluorescent intensity. (**B**) HL-60 cells were treated with 50 μg/mL hop bitter acids at indicated time periods. Subcellular fractions were prepared as described under Materials and Methods, and cytochrome *c* was detected by cytochrome *c* antibody. (**C**) Cell lysates of HL-60 after 50 μg/mL hop bitter acids treatment were harvested at the indicated times, and equal amounts of protein (50 μg) from each sample were subject to Western blotting analysis and probed for caspases-9.

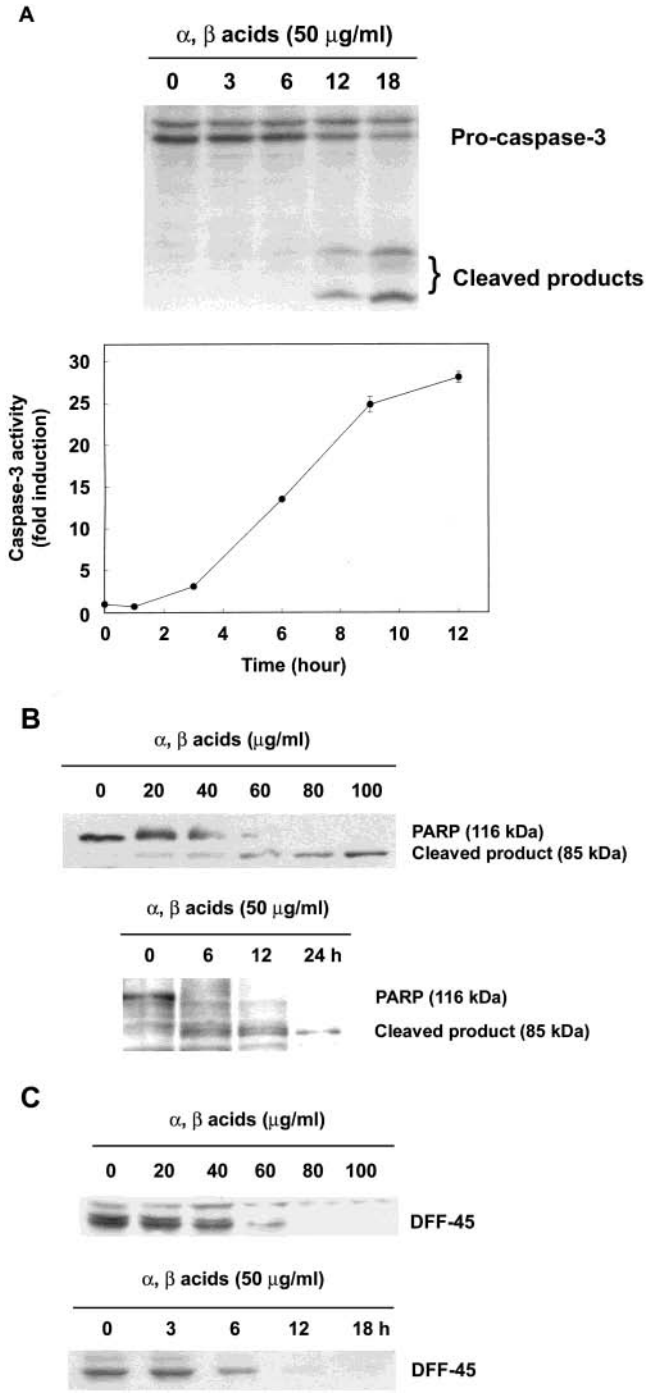

Figure 6. Cleavage and activation of caspases-3 resulted in the processing of caspase-3 substrates during hop bitter acids-induced apoptosis: (**A**) cleavage of caspases-3 (upper panel) and kinetics of caspase-3 activation (lower panel) in hop bitter acids-treated HL-60 cells; (**B**) dose-dependent (upper panel) and time-dependent (lower panel) cleavage of poly(ADP-ribose) polymerase induced by hop bitter acids; (**C**) dose-dependent (upper panel) and time-dependent (lower panel) cleavage of DFF-45 induced by hop bitter acids. HL-60 cells were treated with various concentrations of hop bitter acids for 24 h or with 50 μg/mL hop bitter acids for the indicated times, and processing of procaspase-3, DFF-45, and PARP was detected by Western blotting analysis using specific antibodies against caspases-3, DFF-45, and PARP. Caspase-3 activity assay was performed as described under Materials and Methods.

sary for the processing and activation of caspase cascades, we next evaluated whether the processing and activation of caspase-3, which was considered to play a central role in many types of stimuli-induced apoptosis (*23*, *37*), were associated with the activation of caspase-9 in hop bitter acids-induced apoptosis. As shown in **Figure 6A**, caspase-3 was time-dependently activated by hop bitter acids and the cleavage form of caspase-3 could be seen at 12 h correlated with the gradual induction of caspase-3 activity after the addition of hop bitter acids.

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 produce an 85-kDa fragment during apoptosis (*38*). Caspase-activated DNase (CAD), also a substrate of caspase-3, pre-exists in living cells as an inactive complex with an inhibitory subunit, DNA fragmentation factor-45, DFF-45 (*39*). In apoptotic cells, activation of CAD occurs by means of caspase-3-mediated cleavage of DFF-45, leading to the release

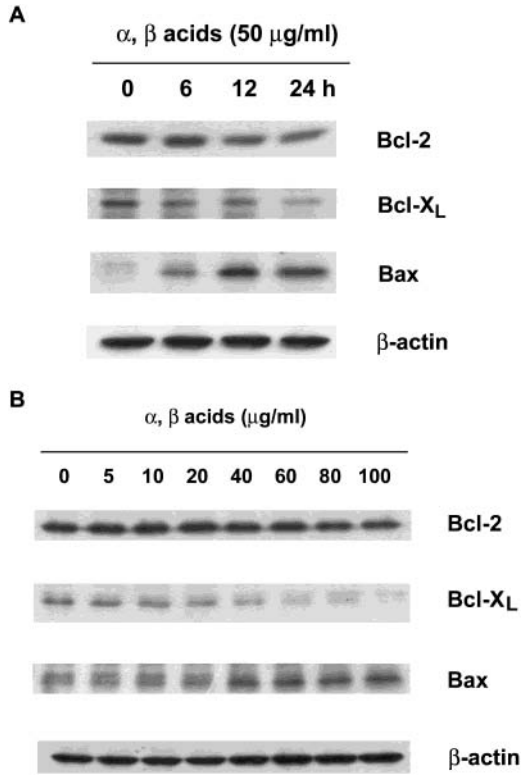

Figure 7. Effect of hop bitter acids on Bcl-2, Bcl-X_L, and Bax expression in HL-60 cells. HL-60 cells were treated with (**A**) 50 μg/mL hop bitter acids for indicated time points or (**B**) various concentrations of hop bitter acids for 24 h. Expression of Bcl-2, Bcl-X_L, and Bax was detected by Western blotting analysis using specific antibodies against Bcl-2, Bcl-X_L, and Bax, respectively.

and activation of CAD. Once CAD is released, it can translocate to the nucleus, where it degrades chromosomal DNA to produce interchromosomal DNA fragmentation (*40*−*42*). To examine the cleavage of PARP and DFF-45 following the activation of caspase-3, HL-60 cells were treated with various concentrations of hop bitter acids for 24 h or with 50 μg/mL of hop bitter acids for 3−24 h. Western blotting analysis showed that hop bitter acids caused a dose- and time-dependent proteolytic cleavage of PARP, with accumulation of the 85-kDa species and concomitant disappearance of the full-size 116-kDa protein (**Figure 6B**), and the cleavage of DFF-45 triggered by hop bitter acids also progressively increased in a dose- and time-dependent manner (**Figure 6C**), which paralleled with the activation of caspase cascades (**Figures 5C** and **6A**). Taken together, our results suggest that the mechanism by which hop bitter acids induced apoptosis involved the dissipation of $\Delta\Psi$m, resulting in cytochrome *c* release from mitochondria and subsequently activating the caspase cascades.

Effect of Hop Bitter Acids on the Expression of Bcl-2 Family Proteins in HL-60 Cells. The imbalance of expression of anti- and pro-apoptotic proteins after the stimulus is one of the major mechanisms underlying the ultimate fate of cells in the apoptotic process. We examined the expression of anti-apoptotic proteins, Bcl-2 and Bcl-X_L, for indicated times after 50 μg of hop bitter acids treatment. There was a slight decrease of Bcl-2 expression (**Figure 7A**, upper panel), but a significant change in the expression of Bcl-X_L was observed at 6 h after hop bitter acids treatment in HL-60 cells (**Figure 7A**, middle panel). We next examined the expression of a pro-apoptotic protein, Bax, which inserts into the outer membrane of mitochondria and forms a large channel, allowing the release of

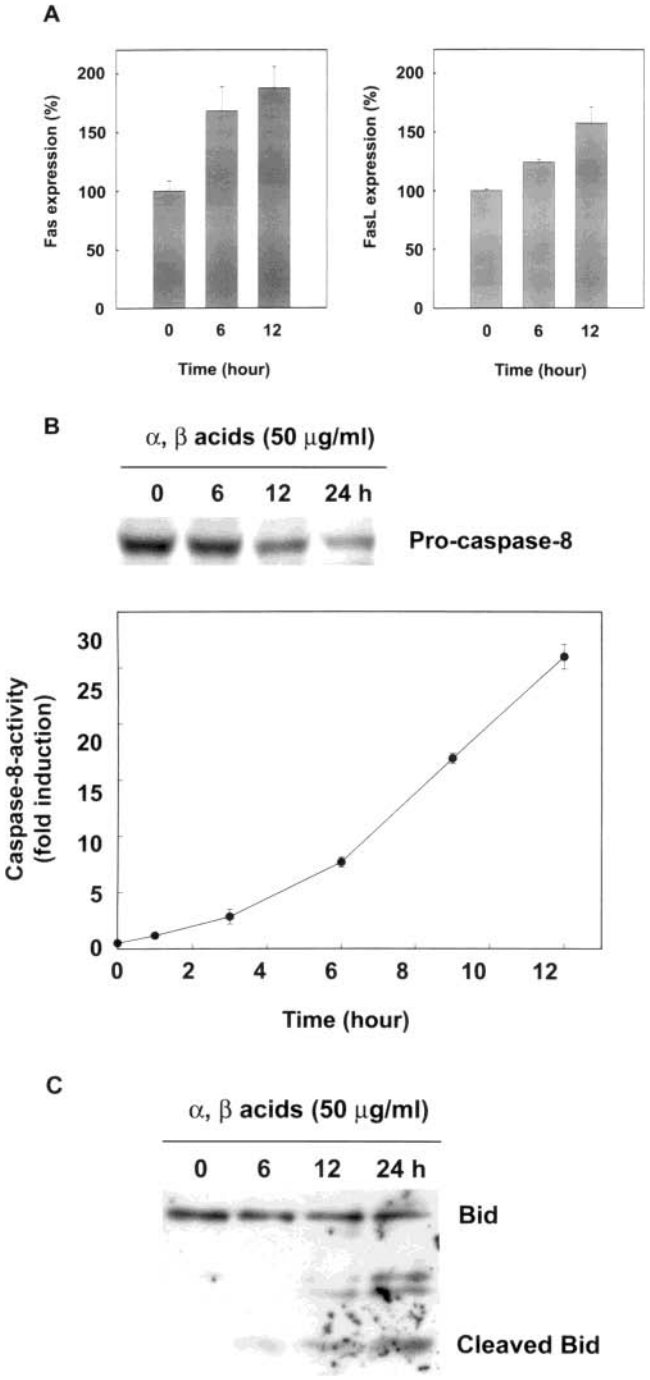

Figure 8. Increase in the expression of Fas and FasL leading to cleavage and activation of caspases-8 and Bid during hop bitter acids-induced apoptosis. (**A**) The cells were treated with 50 μg/mL hop bitter acids for the indicated periods. The expression of Fas and FasL was determined by flow cytometry as described under Materials and Methods. Data are presented as means ± SE for three determinations. (**B**, **C**) HL-60 cells were treated with 50 μg/mL hop bitter acids. Cell lysates were collected at indicated times, and equal amounts of protein (50 μg) from each sample were subjected to Western blotting analysis and probed for caspases-8 (**B**, upper panel) or Bid (**C**). Caspase-8 activity was assayed as described under Materials and Methods (**B**, lower panel).

cytochrome *c*; this process can be prevented by Bcl-2 or Bcl-X_L (*43*). The result shown in **Figure 7A** (lower panel) displays the time-dependent increase of Bax protein induced by hop bitter acids. The dose decrease of Bcl-X_L and dose increase of Bax were also observed in hop bitter acids-treated HL-60 cells,

although there was no obvious change in Bcl-2 protein levels in **Figure 7B**. These results indicated that there was no common mechanism regulating the Bcl-2 family proteins to respond with apoptotic cell death under hop bitter acids-stress in HL-60 cells.

Activation of Fas-Mediated Apoptotic Pathway by Hop Bitter Acids, Resulting in Activation of Caspase-8 and Cleavage of Bid. To assess whether hop bitter acids promoted apoptosis via receptor-mediated pathway, the Fas and Fas ligand (FasL) protein levels were determined on the surface of HL-60 cells exposed to hop bitter acids (50 μg/mL). HL-60 cells were immunofluorescence labeled with mAbs to the Fas and FasL and analyzed by flow cytometry. The result showed that hop bitter acids could stimulate the expression of Fas and FasL on the surface of HL-60 cells after treatment with hop bitter acids for up to 12 h (**Figure 8A**).

Engagement of Fas and FasL results in the clustering of intracellular death domains of Fas and recruits Fas-associated death domain (FADD) and pro-caspase-8 into the death-inducing signal complex (DISC), where caspase-8 is cleaved and activated (*20, 44*). To verify whether the activation of caspase-8 was associated with Fas and FasL production in response to hop bitter acids treatment, the cleavage and activation of caspase-8 were detected after treatment of HL-60 cells with 50 μg/mL hop bitter acids at the indicated time points. As shown in **Figure 8B**, the cleavage of pro-caspase-8 was observed after 6 h of incubation accompanied with the gradual increase of caspase-8 activity in a time-dependent manner, indicating that hop bitter acids could activate the Fas-mediated pathway to lead to the apoptosis in HL-60 cells. The change in $\Delta\Psi$m triggered by Fas and FasL is due to the activation of caspase-8, which then cleaves the pro-apoptotic protein, Bid, to produce the truncated Bid fragment (tBid), and tBid targets mitochondria, causing mitochondrial damage and amplifying apoptotic signals by activating the mitochondria pathway (*45*−*47*). To investigate whether the disruption of mitochondria was partially due to the tBid generated by active caspase-8, the presence of Bid cleavage in HL-60 cells during hop bitter acids-induced apoptosis was monitored by Western blotting. Consistent with the timing of pro-caspase-8 cleavage, Bid cleavage occurred at 6 h and the amount of cleavage fragment increased later (**Figure 8C**). These data suggested that the cleavage of Bid degraded by active caspase-8 may be one of the mechanisms that contributed to the activation of the mitochondrial pathway during hop bitter acids-induced apoptosis.

DISCUSSION

In this study, we clarified the molecular mechanism by which hop bitter acids triggered human leukemia HL-60 cells undergoing apoptosis. We found that hop bitter acids induced time- and dose-dependent apoptosis in HL-60 cells. DNA fragmentation appeared in HL-60 cells by low concentration of hop bitter acids (20 μg/mL). In addition, we found that the percentage of cells with sub-diploid DNA (indicative of apoptotic fraction) significantly increased after hop bitter acids treatment compared with DMSO vehicle control. In comparison with HL-60 cells, U937 cells were relatively less susceptible to the inhibitory effect of hop bitter acids. We suggest that this difference came from the varieties in their general ability to undergo apoptosis, but this issue must be elucidated in further studies.

Recent studies have suggested that mitochondria play a pivotal role in apoptosis triggered by many stimuli (*48*, *49*). Herein, we demonstrated that hop bitter acids could disrupt the function of mitochondria at the early stage of apoptosis and subsequently coordinate caspase activation through the release of cytochrome *c*. The Bcl-2 family proteins, whose members may be anti-apoptotic or pro-apoptotic, regulate cell death by controlling the mitochondria membrane permeability during apoptosis (*50*−*52*). We, therefore, inferred that the Bcl-2 family proteins might participate in the event that controlled the change in mitochondrial membrane potential and trigger cytochrome *c* release during apoptosis induced by hop bitter acids. In our study, we did not observe a clear difference of Bcl-2 expression after time-course or dose-dependent incubation of hop bitter acids in HL-60 cells (**Figure 7A**,**B**, upper panels). This suggested that Bcl-2 may not be a determinant factor in regulating hop bitter acids-induced apoptosis. However, we found down-regulation of Bcl-X_L expression and concomitant up-regulation of Bax expression under the same conditions (**Figure 7A**,**7B**, middle and lower panels). Consistent with a model in which the ratio of anti-apoptotic to pro-apoptotic proteins determines cellular susceptibility to apoptosis (*53*), the lower ratio of Bcl-X_L to Bax correlated inversely with the increase of incubation times after hop bitter acids treatment. These results suggested that the transcriptional regulation of Bcl-2 family proteins might be involved in the mechanism for the induction of apoptosis by hop bitter acids. It was reported that humulone could suppress TNFα-induced cyclooxygenase-2 expression by mediating NF-κB and NF-IL6 activity (*3*), and p53 can directly transactivate human Bax gene (*54*) that affects mitochondria function (*55*), raising the possibility that the expression of Bcl-X_L or Bax could be transcriptionally regulated in response to hop bitter treatment, but this issue should be elucidated. Although hop bitter acids could change the integrity of mitochondrial membranes by controlling the expression of Bcl-2 family proteins, we did not rule out the possibility that hop bitter acids could permeate into cells and directly target mitochondria to increase membrane permeability and decrease mitochondrial membrane potentials regardless of the change in Bcl-2 family protein levels.

Bid, a BH3-domain-only member of the Bcl-2 family, is cleaved by caspase-8 following TNFα or FasL treatment (*25*, *26*), and the 15-kDa truncated Bid fragment (tBid) translocates to mitochondrial membranes, where it triggers the homooligomerization of Bax and Bak, resulting in the release of cytochrome *c* (*45*−*47*). Our results exhibited that Bid was cleaved (**Figure 8C**) following the Fas and FasL expression and cleavage of caspase-8 (**Figure 8A**,**B**) during hop bitter acids-induced apoptosis in HL-60 cells. They also indicated that hop bitter acids might dissipate mitochondrial membrane potential via a receptor-mediated pathway. However, recent findings suggested that caspase-8 activation and Bid cleavage may be executed in a receptor-independent and a caspase-3-dependent manner (*56*, *57*). In this model, a hierarchy of caspase activation has been established after the entry of cytochrome *c* to the cytosol. Caspase-9 appears to be the first activation in this context and next initiates processing of caspase-3. Upon activation by caspase-3, caspase-6 in turn promotes the activation of caspase-8. Thus, we speculated that such an apoptotic self-amplifying feedback loop might operate in hop bitter acids-treated HL-60 cells. In our study, caspase-9 and -3 cleavage fragments were detected (**Figures 5C** and **6A**), and a number of caspase-3 substrates such as PARP and DFF-45 were cleaved (**Figure 5B**,**C**), indicating the presence of mitochondria-mediated caspase-3 activity. Caspase-3 might result in caspase-8 activation and tBid generation (**Figure 8B**,**C**), which then relocates to the mitochondria to further disturb mitochondrial membranes and amplify the mitochondria-mediated pathway.

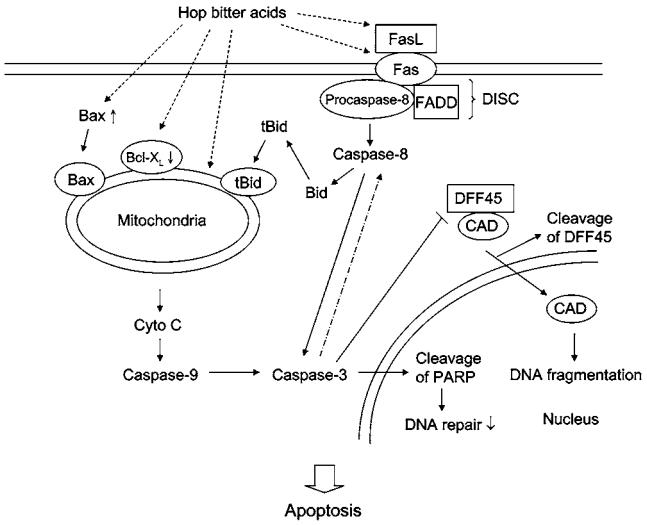

Figure 9. Schematic representation of action mechanism by which hop bitter acids induced apoptosis in HL-60 cells. Hop bitter acids might dissipate $\Delta\Psi$m, result in cytochrome *c* (Cyto C) release, and lead to the consequent activation of caspase-9 and -3 by triggering the translocation of pro-apoptotic Bcl-2 family members, Bax, to mitochondria or directly perturbing mitochondrial membrane. Hop bitter acids also down-regulated anti-apoptotic Bcl-2 family, Bcl-X_L, which blocks cytochrome *c* release from mitochondria. Alternatively, hop bitter acids affected death receptor (Fas and FasL) signaling that drove activation of caspase-8. Caspase-8 might directly activate the effector caspase-3 or indirectly disturb $\Delta\Psi$m by cleaving Bid, the truncated fragment (tBid) of which translocated to mitochondria, where it activated Bax and caused the release of cytochrome *c*. Active caspase-3 further cleaved DNA fragmentation factor 45 (DFF45), leading to the release of caspase-activated DNase (CAD) and internucleosomal cleavage of DNA and cleavage of PARP, which is responsible for DNA repair. A self-amplification loop might be set up between caspase-3 and caspase-8 (– • –). FADD, Fas-associating protein with death domain.

The receptor-mediated apoptotic pathway is another major pathway in activating caspase cascades. In our study, we observed the enhancement expression of Fas and FasL (**Figure 8A**) induced by hop bitter acids. It was likely that the cleavage of caspase-8 and Bid (**Figure 8B**,**C**) was efficiently activated by the direct death receptor activation. It is not known how to increase the expression of Fas and FasL by hop bitter acids. However, it was reported that mitochondrial dysfunction is directly associated with the promotion of Fas gene expression (*58*), suggesting that hop bitter acids might cause the damage of mitochondria via an unknown pathway and that this defect in mitochondria might directly or indirectly lead to the overexpression of Fas and FasL proteins.

In summary, our observations suggested that at least two possible mechanisms acted on the induction of apoptosis by hop bitter acids (**Figure 9**): In the mitochondria-mediated pathway, activation of caspase cascades responsible for apoptosis occurred following the loss of $\Delta\Psi$m and further cytochrome *c* release, which were partially initiated by the imbalance of Bcl-2 family proteins. Alternatively, in the receptor-mediated pathway, hop bitter acids might induce the engagement of Fas and FasL expression, forming the death-inducing signaling complex (DISC) and resulting in activation of caspase-8, which subsequently promoted proteolytic processing of caspase-3 and Bid. In addition, significant cross-linking between the mitochondria and death receptor signaling pathways might be achieved via a feedback loop involving caspase-3 and -8, as discussed above, or via an event in which tBid targeted the mitochondrial membrane.

In conclusion, our results clearly demonstrate that hop bitter acids triggered apoptosis in a dose- and time-dependent manner in HL-60 and U937 cells. Analyses of the expression of the Fas, FasL, and Bcl-2 family proteins, subcellular location of cytochrome *c*, and the status of various caspases suggested that apoptosis induced by hop bitter acids in HL-60 cells was mainly associated with Fas activation and mitochondrial dysfunctions. These results may provide a potential mechanism for hop bitter acids in cancer therapeutic and chemopreventive functions.

ABBREVIATIONS USED

PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; TNFR, tumor necrosis factor receptor; DR, death receptor; MMP; mitochondrial membrane potential; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; CAD, caspase-activated DNase.

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