Natural Plant Extract Tubeimoside I Promotes Apoptosis-Mediated Cell Death in Cultured Human Hepatoma (HepG2) Cells

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Tubeimoside I (TBMS I), an extract from Chinese herbal medicine Bolbostemma paniculatum (MAXIM.) Franquèt (Cucurbitaceae) has been shown as a potent anti-tumor agent for a variety of human cancers, but yet to be evaluated for hepatoma that is highly prevalent in Eastern Asian countries including China. Here, we examined in vitro the cytotoxic effects of TBMS I on human hepatoma (HepG2) and normal liver (L-02) cell lines. We also investigated TBMS I-induced molecular events related to apoptosis in HepG2 cells. The results show that TBMS I inhibited the proliferation of both HepG2 and L-02 cells in a dose- and time-dependent manner, but HepG2 cells appeared more sensitive to the agent. When exposed to TBMS I for 24, 48 and 72 h, IC50 for HepG2 cells versus L-02 cells were 15.5 vs. 23.1, 11.7 vs. 16.2, 9.2 vs. 13.1 (μM, p<0.01), respectively. TBMS I induced cell shrinkage, nuclear condensation and fragmentation, cell cycle arrest at the G2/M phase, mitochondrial membrane disruption, release of cytochrome c from the mitochondria, activation of caspase 3 and 9, and shifting Bax/Bcl-2 ratio from being anti-apoptotic to pro-apoptotic, all indicative of initiation and progression of apoptosis involving mitochondrial dysfunction. Taken together, these results indicate for the first time that TBMS I potently inhibited growth in HepG2 cells by mediating a cascade of apoptosis signaling pathways. Considering its sensitivity of HepG2 cells, preferential distribution in the liver and natural product origin, TBMS I therefore may have a great potential as a chemotherapeutic drug candidate for hepatoma.

Key words: tubeimoside I; hepatoma HepG2 cell line; cytotoxicity; mitochondrial dysfunction; apoptosis

Hepatoma is one of the most prevalent malignant tumor types and the third leading cause of cancer-related death worldwide. Each year, approximately 550000 new cases of hepatoma are reported worldwide, representing more than 5% of all human cancers. The incidence is particularly high among Eastern Asian and African regions, but also rapidly rising in other parts of the world such as the United States. Despite the development and use of multimodality therapies including chemotherapy, the clinical outcome of hepatoma treatment remains unsatisfactory, with usually less than 7% of the 5-year overall survival rate. This may be in part due to ineffectiveness of the currently available chemotherapeutic drugs. Therefore, novel effective chemotherapeutic agents are desired, particularly those derived from natural products because of their intrinsic advantages.

One such agent is tubeimoside I (TBMS I), a constituent isolated from tuber of Bolbostemma paniculatum (MAXIM.) Franquèt (Cucurbitaceae) that has long been used as a traditional Chinese herbal medicine to treat a wide variety of illnesses including inflammation and snake venom. In the early 1980’s, TBMS I was isolated and identified as a triterpenoid saponin with a unique macrocyclic structure as shown in Fig. 1.

Subsequently, it has been shown that TBMS I could be a potent anti-tumor agent by inducing apoptosis in a variety of human cancer cell lines including promyelocytic leukemia (HL-60) cells, cervical cancer (HeLa) cells, and nasopharyngeal carcinoma (CNE-2Z) cells. However, TBMS I so far has not been well studied for its anti-tumor activity against hepatoma, even though that TBMS I is known to preferentially distribute in the liver during in vivo metabolism, and thus might better target liver cancer, or hepatoma.

Therefore, we evaluated TBMS I for its cytotoxicity to cultured human hepatoma cells or normal liver cells, from HepG2 or L-02 cell lines, respectively. We also investigated apoptosis-associated molecular events as potential mechanisms responsible for the cytotoxic effect of TBMS I on HepG2 cells. We found that compared to normal L-02 cells, TBMS I could inhibit proliferation of HepG2 cells more potently, which was paralleled by induction of several important signaling pathways associated with apoptosis, suggesting that TBMS I may be a potent promoter of apoptosis-mediated cell death in HepG2 cells and thus a particularly good candidate
for developing novel chemotherapeutic drugs for treatment of hepatoma.

MATERIALS AND METHODS

Materials Tubeimoside I was purchased from the National Institute for Control of Pharmaceutical and Biological Products (purity >98%, HPLC, Beijing, China). One millimolar stock solution of TBMS I was prepared in phosphate buffered saline (PBS) and stored at -20°C. The stock solution was freshly diluted to the indicated concentrations with culture medium before use. Cell culture medium, RPMI-1640, and fetal bovine serum (FBS) were purchased from Biological Industries (Hyclone, U.S.A.), and Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China), respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, antibiotics, trypsin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Annexin V-FITC/propidium iodide (PI) double staining kit, rhodamine 123 fluorescent dye, and caspase-3, -8 and -9 activity assay kits were purchased from Key Gene (Nanjing, China), Hoechst 33258, Tubulin-Tracker Red, the pan caspase inhibitor Z-VAD-FMK, an enhanced chemiluminescence (ECL) kit and cell cytotoxicity to cultured liver cells (HepG2) and normal liver cell line (L-02) were obtained from the Institute of Cytology, Chinese Academy of Sciences (Shanghai, China). Bicinchoninic acid (BCA) protein assay kit was purchased from Bote corp. (Beijing, China). Rabbit anti-cytochrome c, Bcl-2, Bax and β-actin antibodies were purchased from Cell Signaling Technology (MA, U.S.A.). Horse radish protein (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) was obtained from Boster (Wuhan, China). All other chemicals were obtained from Huili Chemical Reagent Co., Ltd. (Chongqing, China).

Cell Culture Human hepatocellular carcinoma cell line (HepG2) and normal liver cell line (L-02) were obtained from American Type Culture Center (ATCC, Manassas, VA, U.S.A.). Additional human hepatocellular carcinoma cell line with high potential for metastasis (HCCLM3) and normal liver cell line derived from normal tissue aside tumor in a 35-year-old Chinese female liver cancer patient (QSG-7701) were obtained from the Institute of Cytology, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum (FBS). Cultured cells were maintained in a humidified incubator at 37°C in the presence of 5% CO2. The culture medium was changed every 2 d. Cells for assay were harvested by detachment with a solution of 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA).

TBMS I Cytotoxicity to Cultured Liver Cells TBMS I cytotoxicity to cultured HepG2 cells, L-02 cells, HCCLM3 cells, or QSG-7701 cells were evaluated in terms of proliferative inhibition determined by the relative absorbance of MTT dye by the cells. In brief, cells (1×10⁴ cells/well) were seeded in 96-well tissue culture plates and then cultured in RPMI-1640 growth medium for 24 h. Subsequently, the medium was replaced with RPMI-1640 growth medium containing designated concentrations of TBMS I (5, 10, 15, 20, 30, 40, 90 μM). Cells treated with sham containing equal volume of cell culture medium but no TBMS I (0 μM) were used as control in every experiment throughout this study. After exposure to TBMS I for 24, 48 and 72 h, MTT dye was added into each well at a final concentration of 0.5 mg/ml, and the plates were incubated for an additional 4 h at 37°C. The insoluble formazan produced by the living cells in response to MTT dye was collected and dissolved in dimethyl sulfoxide (DMSO) and measured with an ELISA reader (Bio-Rad, U.S.A.) at the wavelength of 492 nm. Each plate contained multiple wells for a given experimental condition and multiple wells of control. The procedure was replicated three times.

TBMS I-Induced Morphological Changes of the Cell, Nucleus and Microtubules TBMS I-induced morphological change of the HepG2 cell was evaluated by phase contrast optical microscopy. In brief, cells were prepared similarly as described above, but in 24-well cell culture plates at 4×10⁴ cells/well. Thereafter the cells were treated with either sham or TBMS I at 10, 20, 30 μM, respectively, for 24 h. Subsequently, cells were examined and photographed by a phase contrast microscope (Leica, Germany). The morphological change of the HepG2 cell’s nucleus or microtubules in response to TBMS I treatment was evaluated by fluorescent visualization with either Hoechst 33258, or Tubulin-Tracker Red (a red fluorescent probe for microtubules), respectively. Briefly, cells were prepared similarly as above but on 20 mm diameter coverslips, and then treated with either sham or 20 μM TBMS I for 24 h before staining with Hoechst 33258, or 3 h before staining with Tubulin-Tracker Red. Subsequent to the treatment of sham or TBMS I, cells were washed in PBS and fixed with 4% paraformaldehyde for 10 min. For nucleus staining, the fixed cells were incubated with Hoechst 33258 fluorescent dye (5 mg/ml) for 10 min, and then washed and dried before use. For microtubules staining, the fixed cells were first permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min and then rinsed three times in PBS, and subsequently incubated with Tubulin-Tracker Red for 1 h and then washed and dried before use. The fluorescently labeled cells were examined by fluorescence microscopy (Leica, Germany).

TBMS I-Induced Early and Late Apoptosis TBMS I-induced apoptosis in either the HepG2 or the L-02 cell was first investigated using Annexin V-FITC/PI double staining and flow cytometry. Annexin V-FITC is a green fluorescence-labeled protein that specifically binds to phosphatidylserine (PS), and PI is a red fluorescent molecule that non-specifically binds to DNA. In normal viable cells, neither Annexin V-FITC is accessible to PS, nor PI is accessible to DNA because PS is only located inside the cell whose membrane excludes entry of the staining agents. However, during apoptosis the normally intracellular PS is evaginated to the outer surface of the membrane, and thus can be specifically stained with Annexin V-FITC. On the other hand, at late stage of apoptosis the integrity of both cell and nuclear membranes is disrupted, and PI can thus enter the cell and bind to the exposed DNA. Accordingly, the early or late apoptotic cell could be detected by whether the cell expresses Annexin V-FITC alone (Annexin V-FITC+/PI-) or both Annexin V-FITC and PI (Annexin V-FITC+/PI+), respectively. Briefly, subsequent to either sham or TBMS I exposure (15 or 30 μM for 24 h), 1×10⁴ cells were harvested, washed twice with ice-cold PBS, and pelleted. The cells were then resuspended in 500 μl of the binding buffer followed by addition
of 5 μl Annexin V-FITC conjugate and 5 μl PI buffer, all from the Annexin V-FITC/PI double staining apoptosis kit (Beyotime, China), and further incubated at room temperature for 15 min in the dark. Then the cells were transferred to the FACScan flow cytometer with proprietary Cell Quest software (Becton Dickinson, San Jose, CA, U.S.A.), and the number of cells with either Annexin V-FITC+/PI− or Annexin V-FITC+/PI+ were then obtained automatically.

**TBMS I-Induced Cell Cycle Change** TBMS I-induced cell cycle change in HepG2 cells was analyzed by measuring the cellular DNA content using cytometry. Briefly, subsequent to exposure to either sham or TBMS I (15 or 30 μM for 24 h), 1×10⁶ cells were harvested, washed and fixed in 70% ice cold ethanol at 4°C for 2 h. Afterwards, the cells were washed twice and stained with PI solution (50 μg/ml of PI, 100 μg/ml RNase, and 0.1% Triton X-100 in PBS) at 37°C in the dark for 30 min. Then the cells were transferred to the FACScan flow cytometer with proprietary Cell Quest software (Becton Dickinson, San Jose, CA, U.S.A.), and the fluorescence intensity emitted from the PI–DNA complex was acquired automatically.

**TBMS I-Induced Mitochondrial Membrane Depolarization** We then investigated whether TBMS I would cause mitochondrial membrane depolarization, a known event associated with apoptosis. The depolarization was detected using a fluorescent probe of rhodamine 123. In normal viable cells, rhodamine 123 is quickly sequestered by the mitochondrial matrix due to the mitochondrial membrane polarization, and emits strong fluorescence. During apoptosis, however, the integrity of mitochondrial membrane is disrupted, which leads to the depolarization of the membrane and opening of mitochondrial permeability transition pores, and then release of the normally sequestered rhodamine 123. After washing off the released rhodamine 123, the depolarized (apoptotic) cells exhibit greatly decreased or even diminished emission, and thus can be detected using flow cytometry.

In brief, subsequent to either sham or TBMS I exposure (15 or 30 μM for 24 h), 1×10⁶ cells were harvested and washed twice with PBS and incubated with rhodamine 123 (1 μg/ml) at 37°C for 10 min. The cells were then transferred to a FACStar flow cytometer with proprietary ModFit software (Becton Dickinson, San Jose, U.S.A.), and the number of cells with or without mitochondrial membrane depolarization was determined automatically.

**TBMS I-Induced Release of Cytochrome c from Mitochondria** We then investigated whether TBMS I induced release of cytochrome c, a known intermediate of apoptosis, from mitochondria into the cytosol. The cytosolic, and mitochondrial fractions of cytochrome c were extracted from the cell with respective extraction buffers containing either dithiothreitol (DTT) or phenylmnesufonyl fluoride (PMSF) and protease inhibitors, both supplied in the cell mitochondrial isolation kit (Beyotime, China), and detected by Western blotting. In brief, subsequent to either sham or TBMS I exposure (10, 20, 30 μM for 24 h), 4×10⁶ cells were harvested, washed with ice-cold PBS, then centrifuged at 1000×g for 5 min at 4°C. The pelleted cells were resuspended in 1 ml of the cytosol extraction buffer and further incubated on ice for 10 min before being homogenized in an ice-cold tissue grinder. The homogenate was transferred to a 1.5 ml tube and centrifuged at 600 g for 10 min at 4°C. The supernatant was carefully collected and centrifuged again at 11000×g for 10 min at 4°C. This final supernatant was used as the cytosolic fraction, and the pellet was mixed with 100 μl of the mitochondrial extraction buffer and used as mitochondrial fraction, respectively. The cytochrome c expression in either the cytosolic or the mitochondrial fraction was detected by Western blotting.

**TBMS I-Induced Activation of Caspases** Since caspase-3, -8, -9 are known to mainly initiate and execute apoptotic process, we examined if they were activated in response to TBMS I exposure. The activity of caspase-3, -8, -9 was measured by a caspase activity assay kit (Key Gene, China). Briefly, subsequent to either sham, or 30 μM TBMS I exposure for 24 h, 3×10⁶ cells were harvested, washed twice with ice-cold PBS, then resuspended in the lysis buffer and left on ice for 60 min. The lysate was centrifuged at 6000×g at 4°C for 5 min. The supernatant was mixed with substrate peptides Ac-DEVD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA, respectively, which resulted in the release of pNA due to hydrolysis of respective peptide by caspase-3, -8, -9. The concentration of pNA which correlated with the activity of the caspase was determined, respectively, by the optical absorbance at 405 nm using an ELISA Reader (Bio-Rad, U.S.A.). We also tested if caspase inhibitor Z-VAD-FMK could inhibit the effect of TBMS I on the activity of caspases by exposing the cells to 30 μM TBMS I together with 50 μM Z-VAD-FMK, and then measuring caspase activity according to the above described method.

**TBMS I-Induced Change in Bax/B-Cell Lymphoma-2 (Bcl-2)** Apoptosis is also known to be critically determined by the balance between the Bcl-2 associated X protein (Bax) and the Bcl-2 proteins. We thus investigated the expression levels of Bcl-2 and Bax by Western blotting, and quantitated the ratio of these two proteins expressions as an apoptotic index. In brief, subsequent to either sham or TBMS I exposure (10, 20, 30 μM for 24 h), 4×10⁶ cells were harvested, washed in PBS, suspended in the lysis buffer containing 50 mM Tris (pH 7.4), 50 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5 mM DTT and 1% protease inhibitor cocktail and incubated on ice for 30 min. The lysate was centrifugation at 11000×g for 10 min at 4°C and the resultant supernatant was collected and then stored at −20°C until further use. The concentration of total proteins in the supernatant was determined by a bicinchoninic acid (BCA) protein assay kit (Biateke, China). Subsequently, equal volume aliquots of the supernatant containing same amount of total proteins were loaded into 12% SDS-polyacrylamide gel, the proteins were then separated electrophoretically and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, U.S.A.). After blocking with 5% non-fat milk and 1% tween-20 in TBS for 2 h at room temperature, the membranes were incubated with specific primary antibodies for Bax, Bcl-2 overnight at 4°C, followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Bax and Bcl-2 expressions were then detected using an enhanced chemiluminescence (ECL) kit (Beyotime, China).

**Statistical Analysis** Each experiment was repeated three times, and the result was presented as mean±standard deviation (S.D.). The results obtained under different experimental
conditions were evaluated using analysis of variance (ANOVA) followed by the Student's t-test, and differences were considered statistically significant when \( p < 0.05 \).

**RESULTS**

**TBMS I Differentially Inhibited Growth of Cells**

As shown in Fig. 2, TBMS I inhibited cell growth in a typical concentration- and time-dependent manner in HepG2, and L-02 cells. However, HepG2 cells appeared to be more sensitive to TBMS I as compared to their normal counterparts, particularly at the dose range of 10—30 \( \mu M \). When exposed to TBMS I for 24, 48, and 72 h, the IC\(_{50}\) for HepG2 cells was 15.5 \( \mu M \), 11.7 \( \mu M \), and 9.2 \( \mu M \), respectively, and significantly less than 23.1 \( \mu M \), 16.2 \( \mu M \), and 13.1 \( \mu M \) for L-02 cells \( (p < 0.01) \). To confirm this effect, we further tested two more less studied human liver cell lines, namely HCCLM3 and QSG-7701, for cytotoxicity of TBMS I. HCCLM3 is a hepatocellular carcinoma cell line with high potential of metastasis,\(^{17}\) and QSG-7701 is a hepatic cell line derived from cells isolated from normal tissue near tumor.\(^{18}\) After exposure to TBMS I for 24 h the survival rates of HCCLM3 cells, and QSG-7701 cells were similar to those of HepG2 cells, and L-02 cells, respectively \( (p > 0.05) \) in all cases except for 20, and 30 \( \mu M \) at which the survival rate in HCCLM3 cells was slightly different from that in HepG2 cells \( (41.2 \pm 1.7\% \text{ vs. } 35.3 \pm 1.5\% \text{ vs. } 22.1 \pm 0.3\%, \text{ respectively. } p < 0.05) \).

**TBMS I-Induced Morphological Changes of the Cell, Nucleus and Microtubules**

In the absence of TBMS I, HepG2 cells appeared in common spindle shape (Fig. 3A) with intact nuclei appearing in almost uniform staining of Hoechst 33258 (blue, Fig. 3E) and bright cytoplasmic microtubules radiating from center to periphery of the cell (Fig. 3G). In the presence of TBMS I, the cells were observed to exhibit increasing shrinkage and detachment from the substrate as the exposure dose and duration increased. For example, after exposed to TBMS I for 24 h, the cells appeared to be somewhat shrank and retracted from their neighbors at 10 \( \mu M \) (Fig. 3B), significantly shrank at 20 \( \mu M \) (Fig. 3C) and some of the cells were even observed to be floating in the culture medium (Fig. 3D), indicating increasing occurrence of apoptosis in these cells. The change of cell shape in response to TBMS I exposure was accompanied by morphological changes in nuclei and microtubules. After exposure to TBMS I \( (20 \mu M, 24 \text{ h}) \), the nuclei appeared with brighter staining of Hoechst 33258, but more importantly, condensed and fragmented chromatin, indicating apoptosis (Fig. 3F). And exposure to TBMS I \( (20 \mu M, 3 \text{ h}) \) resulted in apparent decrease in the fluorescent intensity, membrane translocation, and amorphous appearance in the cytoplasm of the microtubules labeled by Tubulin-Tracker Red, as well as cell round-up, all indicating tubulin depolymerization (Fig. 3H).

**TBMS I Induced Apoptosis and Cell Cycle Arrest**

When HepG2 cells were treated with TBMS I for 24 h at increasing concentrations \( (0, 15, 30 \mu M) \), there were increases of both early (Annexin V-FITC+/PI-) and late (Annexin V-FITC+/PI+) apoptotic cells as shown in Fig. 4A. The percentage of early apoptotic cells seemed indifferent from that of some of the cells were even observed to be floating in the culture medium (Fig. 3D), indicating increasing occurrence of apoptosis in these cells. The change of cell shape in response to TBMS I exposure was accompanied by morphological changes in nuclei and microtubules. After exposure to TBMS I \( (20 \mu M, 24 \text{ h}) \), the nuclei appeared with brighter staining of Hoechst 33258, but more importantly, condensed and fragmented chromatin, indicating apoptosis (Fig. 3F). And exposure to TBMS I \( (20 \mu M, 3 \text{ h}) \) resulted in apparent decrease in the fluorescent intensity, membrane translocation, and amorphous appearance in the cytoplasm of the microtubules labeled by Tubulin-Tracker Red, as well as cell round-up, all indicating tubulin depolymerization (Fig. 3H).

![Fig. 2. Cytotoxicity of TBMS I on Cultured HepG2 Cells and L-02 Cells](image)

HepG2 cells were treated with either sham \( (0 \mu M) \) or TBMS I at concentration of 5, 10, 15, 20, 30, 40, 90 \( (\mu M) \) for 24, 48, and 72 h, while L-02 cells were treated with TBMS I at the same concentrations but only for 24 h. The cytotoxicity was assessed by MTT method and quantified as the percentage of viable cells, or survival rate, after TBMS I treatment \( (**p < 0.01, n=18) \).

![Fig. 3. TBMS I-Induced Morphological Changes in the HepG2 Cell, Nucleus and Microtubules](image)

(A—D) Phase contrast images of cells after being treated for 24 h with either sham \( (0 \mu M) \), or TBMS I of increasing concentration at 10, 20, 30, and 50 \( \mu M \). (E, F) Fluorescence photomicrographic images of HepG2 cell nuclei stained with Hoechst 33258 after the cells being treated for 24 h with either sham \( (0 \mu M) \), or 20 \( \mu M \) TBMS I \( (100\times \text{ mag.}) \). (G, H) Fluorescence photomicrographic images of HepG2 cell microtubules stained with Tubulin-Tracker Red fluorescent probe after cells being treated for 3 h with either sham \( (0 \mu M) \), or 20 \( \mu M \) of TBMS I \( (400\times \text{ mag.}) \).
late apoptotic cells at low concentrations of TBMS I (e.g. ≤15 μM), but at high concentrations (e.g. ≥30 μM), early apoptotic cells were in much greater percentage as compared to late apoptotic cells (Fig. 4B), suggesting that TBMS I at high concentrations may predominately promote early apoptosis. TBMS I induced similar trend of apoptotic process in L-02 cells, but with less total apoptotic cells (Fig. 4B), which was consistent with results of proliferation inhibition.

Cell cycle analysis by flow cytometry demonstrated that exposure of HepG2 cells to increasing concentration of TBMS I (0, 15, 30 μM, 24 h) led to a decreasing percentage of cells in G1 phase, but an increasing percentage of cells in G2 phase (9.8, 30.9, 33.8%, respectively) and cells in apoptosis (Fig. 5). These results suggest that TBMS I inhibited HepG2 cell proliferation via G2/M phase arrest in a dose-dependent manner.

TBMS I Induced Breakdown of Mitochondrial Membrane, and Release of Cytochrome c  As shown in Fig. 6A, when HepG2 cells were treated with TBMS I for 24 h at increasing concentrations (0, 15, 30 μM), there were increasingly more cells expressing rhodamine 123 emission below the threshold (M1), indicating increasing number of cells with mitochondrial membrane depolarization, or breakdown. The quantitative percentage of depolarized cells increased from 7.3% at 0 μM to 14.2% at 15 μM, and 65.9% at 30 μM, respectively (Fig. 6B). In parallel to mitochondrial membrane breakdown, the protein expression of cytochrome c decreased in the mitochondrial fraction, while increased in the cytosolic fraction (Fig. 6C), indicating release of the protein from mitochondria into the cytosol.

TBMS I Activated Caspase-3 and -9  As shown in Fig. 7A, when HepG2 cells were treated with TBMS I at 30 μM for 24 h the activities of caspase-3 and -9 were significantly increased while little change was observed in the activity of caspase-8. As compared to cells treated with sham, the activity of caspase-3, and -9 in the cells treated with TBMS I increased by 3.0±0.2 fold, and 3.1±0.1 fold (p<0.01). However, the activation of caspase-3 and -9 induced by TBMS I was largely inhibited when HepG2 2 cells were treated with 30 μM TBMS I together with 50 μM Z-V AD-FMK. In the presence of the pan caspase inhibitor (Z-VDAD-FMK), the TBMS I-induced activation of caspase-3, and -9 was reduced from 3.0±0.2 fold to 1.6±0.1 fold (p<0.01), and 3.1±0.1 fold to 1.5±0.1 fold (p<0.01), respectively. In addition, the presence of Z-VDAD-FMK also dramatically prevented the TBMS I-induced cell death as shown in Fig. 7B. As compared to cells treated with 30 μM TBMS I for 24 h, cells treated with 30 μM TBMS I with 50 μM Z-VDAD-FMK for 24 h exhibited a more than 2 fold increase of cell survival rate (42.9±2.6% vs. 20.5±0.3%, p<0.01).

TBMS I Changed Balance between Bax and Bcl-2  When exposed to TBMS I at increasing concentrations, the...
Bcl-2 protein expression increased, but the Bax protein expression decreased as compared to the control. More importantly, the ratio of Bax/Bcl-2 increased with increasing TBMS I concentration, indicating that TBMS I up-regulated Bax expression and down-regulates Bcl-2 expression (Fig. 8).

Fig. 6. TBMS I-Induced Depolarization of Mitochondrial Membrane

(A) The upper panel from left to right shows flow cytometric results of HepG2 cells after being treated for 24 h with either sham (0 μM), 15 μM TBMS I, or 30 μM TBMS I. There were an increasing number of cells expressing rhodamine 123 staining below the threshold (M1), or depolarized cells, as the concentration of TBMS I increased. (B) The lower left panel shows the quantified percentage of depolarized cells versus concentration of TBMS I. (+p < 0.05, and **p < 0.01, n=3). (C) The lower right panel shows the Western blotting results of cytochrome (cyt) c protein expressions in either cytosol or mitochondria versus the concentration of TBMS I. After TBMS I treatment for 24 h, cyt c increased in cytosol, but decreased in mitochondria as the concentration of TBMS I increased.

Fig. 7. TBMS I-Induced Activation of Caspase-3, -8, -9 in HepG2 Cells, and Inhibition of Caspase Activation and Cell Death

(A) After treatment with either sham (0 μM), 30 μM TBMS I, or 30 μM TBMS I + 50 μM Z-VAD-FMK, the activity of caspase-3, and -9 was greatly elevated in the cells treated with 30 μM TBMS I (solid bar) as compared to those treated with sham (open bar) (**p < 0.01, TBMS I-treated cells vs. sham-treated cells, n=18), which was inhibited by about half the amount in the cells treated with 30 μM TBMS I + 50 μM Z-VAD-FMK (gray bar) (##p < 0.01, TBMS I-treated cells vs. TBMS I + Z-VAD-FMK-treated cells, n=18). (B) After treatment with 30 μM TBMS I + 50 μM Z-VAD-FMK for 24 h, the cell viability assessed by MTT assay was 42.9 ± 2.6%, a 2 fold increase from that of the cells treated with 30 μM TBMS I (solid bar, ##p < 0.01, TBMS I-treated cells vs. TBMS I + Z-VAD-FMK-treated cells, n=18).

Fig. 8. TBMS I-Induced Regulation of Bax and Bcl-2 in HepG2 Cells

The lower panel shows the Western blotting results of protein expressions of Bax, and Bcl-2 versus the concentration of TBMS I. After TBMS I treatment for 24 h, the protein expression of Bax increased, but that of Bcl-2 decreased with increasing TBMS I concentration. The upper panel shows the ratio of quantified protein expression of Bax to that of Bcl-2 versus the concentration of TBMS I. (**p < 0.01, n=3).

Bcl-2 protein expression increased, but the Bax protein expression decreased as compared to the control. More importantly, the ratio of Bax/Bcl-2 increased with increasing TBMS I concentration, indicating that TBMS I up-regulated Bax expression and down-regulates Bcl-2 expression (Fig. 8).
DISCUSSION

The primary finding of this study is that TBMS I inhibited proliferation of both hepatocellular carcinoma cells (HepG2 cells, HCCLM3 cells) and normal hepatic cells (L-02 cells, QSG-7701 cells) in a concentration- and time-dependent manner, and TBMS I was much more potent to suppress growth of HepG2 cells and HCCLM3 cells as compared to L-02 cells and QSG-7701 cells. We also observed that TBMS I caused various changes in HepG2 cells including abnormal cell shape, nuclear condensation and fragmentation, tubulin depolymerization, cell cycle arrest at the G2/M phase, loss of mitochondrial membrane potential, cytochrome c release, activity increase of both caspasases 9 and 3, decreased expression of Bcl-2 protein and increased expression of Bax protein, all indicative of apoptosis.

Under the experimental conditions, we found that the IC50 in all cases was greater than 10 µg/ml. This is another indication that the cytotoxic effect of TBMS I was due to inhibition of cell proliferation, rather than direct cell killing according to the standard for drug evaluation. Importantly, the dose-response curves demonstrate that TBMS I preferentially inhibited proliferation of the HepG2 cell as compared to normal L-02 cells. When exposed to a low dose TBMS I (15 µM) for 24 h, TBMS I caused nearly 2 fold more cell death in HepG2 cells than in normal liver cells, suggesting that cancer cells may be more sensitive to the cytotoxicity of this chemical agent. We also found that the dose response curve of HCCLM3 cells, and QSG-7701 cells to TBMS I treatment was not significant different from that of HepG2 cells, and L-02 cells, respectively. Thus, it is reasonable to assume that study of TBMS I effects on HepG2 cells may be to some extent sufficient for in vitro evaluation of this agent as liver cancer drug.

Although TBMS I has been shown to inhibit proliferation of several types of tumor cell in vitro by promoting apoptotic processes and endocytopenic reticulum stress in the cell, it has not been evaluated, to our best knowledge, for its anti-tumor effect on hepatoma cells. Here, we instead studied the in vitro cytotoxic effects of TBMS I on either cancerous or normal cells of the same type, and demonstrated that at least to TBMS I, the cancerous cells were more sensitive as compared to their normal counterparts. While the underlying mechanisms are not fully understood, this sensitivity difference between hepatoma and normal liver cells to TBMS I together with the preferential distribution of TBMS I in the liver provide encouraging evidence that TBMS I may be especially useful as a potential chemotherapeutic agent for human hepatoma.

As regards the molecular mechanisms of TBMS I-induced growth inhibition of cancer cells, several pathways have been proposed and investigated previously in various cancer types including anti-microtubule polymerization, apoptosis induced by prolonged endoplasmic reticulum stress, and apoptosis involving mitochondrial dysfunction. So far, apoptosis with mitochondrial involvement attracted the most attention, and our study follows this line of research and the findings are largely consistent with previous reports of other cancer types. In hepatoma cell line, TBMS I induced similar processes of apoptosis, confirming that TBMS I inhibited hepatoma cell proliferation by mediating apoptosis instead of direct cell killing. In principle, tumor growth is determined by imbalance of cell proliferation and apoptosis. Although high-dose chemotherapy and radiation could cause cell necrosis and thus eradicate tumor cell, they may also cause irreversible damage to normal cells. Therefore, anti-cancer agents that generally achieve therapeutic purposes by inducing apoptosis in cells sensitive to them are highly desirable.

On the other hand, our data showed that exposure of HepG2 cells to TBMS I for 24 h caused a shift of cell numbers in different phases of cell cycle. As TBMS I concentration increased from 0 to 30 µM, there were increasingly more cells in G2 phase and accompanied by a decreasing percentage of cells in G1 phase. These results indicate that TBMS I induced a G2/M phase cell cycle arrest in HepG2 cells, which was correlated with the progression of apoptosis in the HepG2 cells in response to TBMS I treatment. Furthermore, the fact that inhibition of caspasases with Z-VAD-FMK greatly reduced the efficacy of TBMS I to activate relevant caspasases strongly support that the effects of TBMS I on HepG2 cell proliferation was mediated via apoptosis signaling mechanisms.

Mitochondria are thought to play a pivotal role in apoptosis. The mitochondrial apoptotic pathway has been described as an important signaling pathway of apoptosis. In our present study, we observed that TBMS I treatment resulted in the loss of mitochondrial membrane integrity and thus release of cytochrome c from mitochondria in HepG2 cells. And the release of cytochrome c was accompanied by marked activation of caspase 9, and 3, which is consistent with the generally recognized apoptotic cascade that release of cytochrome c triggers formation of the apoptosome complex and activation of the initiator caspase-9, and leads to the proteolytic activation of caspase-3, the primary effector caspase of the cell. In the present study, caspase 8 was not activated by TBMS I. Although it remains unclear why caspase-8 may not be relevant to TBMS I-induced apoptosis cascade, our results were consistent with findings reported by Hsu et al. and Yao et al. Further study is required to fully elucidate the details of the mechanisms underlying TBMS I effect on hepatoma cells.

It is known that the apoptosis of mitochondrial apoptotic pathway is regulated by members of the Bcl-2 family proteins, and the Bcl-2 family can be divided into either anti-apoptotic (e.g., Bcl-2, Bcl-XL and Mcl-1) or pro-apoptotic (e.g., Bax, Bad, Bak, Bcl-Xs and NOXA) members. It is the balance between the pro-apoptotic and anti-apoptotic Bcl-2 family members that determines whether the cell survives or undergoes apoptosis. In our present study, TBMS I treatment induced in HepG2 cells a significant increase in Bax expression and a decrease of Bcl-2, and more importantly, the ratio of Bax expression to Bcl-2 expression gradually increased from 0.2 to 4.1 as TBMS I concentration increased from 0 to 30 µM, suggesting that TBMS I induced a changing balance between anti-apoptotic and pro-apoptotic protein members of the Bcl-2 family and eventually leads to promotion of apoptotic activities.

It should be noted that additional mechanisms may as well be involved in TBMS I-induced growth inhibition of hepatoma cells, particularly at low concentrations as pointed out by Wang and his colleagues that the effects of TBMS I
on mitochondria and mitochondrial cytochrome c have been observed mostly at rather high concentrations of TBMS I (>20 μM). Our results show that most events of the mitochondrial dysfunction induced by TBMS I in HepG2 cells appeared most markedly at concentration ≥15 μM, but relatively low at concentration <15 μM. Considering the discussion above, it strongly suggests that when exposed to low dose of TBMS I, other molecular mechanisms such as alteration of microtubule dynamics can not be ruled out to contribute to the inhibition of hepatoma cells. Since the core structure of TBMS I is reminiscent of a steroid molecule, it is reasonable to assume that TBMS I would behave similarly as its structural analogues to inhibit assembly of tubulin and thus cause microtubule depolymerization. Indeed, Ma et al. have demonstrated that TBMS I was able to inhibit binding of known tubulin ligands through competitively binding at colchicine binding site of tubulin. Here, we also demonstrated that TBMS I caused tubulin depolymerization and translocation to cell peripheral in the HepG2 cells, even for a short 3 h exposure time.

Taken together, we conclude that TBMS I was a potent agent to suppress growth of hepatoma cells in vitro. The growth inhibition was in large part mediated via apoptosis-associated mitochondrial dysfunction and regulation of Bel-2 signaling pathways as well as tubulin polymerization dynamics. Furthermore, TBMS I was more toxic to hepatoma cells than to normal liver cells. This difference in its cytotoxicity sensitivity, together with its preferential distribution in the liver and its origin of natural medicinal plant, suggest that TBMS I may be a preferred drug candidate for treating liver cancer.

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REFERENCE