

Natural plant extract tubeimoside I induces cytotoxicity via the mitochondrial pathway in human normal liver cells

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Abstract. Tubeimoside I (TBMS I) is a natural compound extracted from *Bolbostemma paniculatum* (Maxim.) Franquet (*Cucurbitaceae*), a traditional Chinese herbal medicine widely used for the treatment of inflammation. Recently, it has been suggested that TBMS I may be a potent anticancer agent for a variety of human cancers. However, TBMS I is known to distribute preferentially in the liver, and thus may harm normal liver cells if it is delivered systemically for cancer treatment. This safety concern warrants careful evaluation of the hepatotoxicity of TBMS I to normal liver cells, which to date has not been carried out. Here, we report the cytotoxic effects of TBMS I on one type of normal liver cells (L-02 cells), and the associated molecular events as underlying mechanisms. Cultured human normal liver L-02 cells were treated with TBMS I at concentrations of 0, 15 and 30 μ M for 24, 48 and 72 h, respectively. Subsequently, the cell survival rate was evaluated by the MTT dye method, and several key molecular events associated with apoptosis were assayed, including mitochondrial depolarization, release of cytochrome c (cyt-c), activation of caspases, and the balance between Bax and Bcl-2 protein expression. Our results indicate that TBMS I inhibited the proliferation of L-02 cells in a dose- and time-dependent manner. The TBMS I-induced growth inhibition of L-02 cells was accompanied by the collapse of mitochondrial membrane potential, release of cyt-c from the mitochondria to the cytosol, activation of caspase-9 and -3, decrease of anti-apoptotic protein Bcl-2 levels and increase of the pro-apoptotic protein Bax levels, all indicative of apoptosis through the mitochondrial pathway. Taken together, these results confirm that TBMS I has a significant apoptotic effect

on normal liver L-02 cells, which may be significant to its clinical applications.

Introduction

Tubeimoside (TBMS), or the tuber of *Bolbostemma paniculatum* (Maxim.) Franquet (*Cucurbitaceae*), is a herb that has long been used in traditional Chinese medicine, and was listed in the *Supplement to the Compendium of Materia Medica* published in early 1765 (1). TBMS is most widely used for the treatment of illnesses, such as inflammation and snake venoms, but it has also been reported to show potent anti-tumor activity (2). Such antitumor activity in part motivated the successful isolation of TBMS I, a triterpenoid saponin whose chemical structure is shown in Fig. 1 (3,4). Subsequent studies confirmed that TBMS I inhibits the growth of cultured cancer cells of several human cancer cell lines, including the human promyelocytic leukemia (HL-60), nasopharyngeal carcinoma CNE-2Z (CNE-2Z) and HeLa cell lines (5). These studies suggest that TBMS I is a candidate novel antitumor drug, despite its side effects on the digestive system causing nausea and vomiting (6). Furthermore, at the molecular level, the TBMS I-induced growth inhibition of cancer cells may well be mediated through apoptosis-associated processes, including microtubule depolymerization (7), prolonged endoplasmic reticulum stress (8) and mitochondrial dysfunction, which leads to decreased expression of anti-apoptotic proteins, increased expression of pro-apoptotic proteins and release of cytochrome c (8,9).

Notably, an *in vivo* pharmacokinetic study in an animal model indicates that TBMS I preferentially distributes in the liver as compared to other vital organs, such as the heart, brain and kidney. This suggests that the liver may be the primary target of TBMS I toxicity, and thus may determine both the therapeutic efficacy and side effects of this potentially important drug molecule. To date, however, TBMS I has not been evaluated in terms of its hepatotoxicity either *in vivo* or *in vitro*. Therefore, the present study was designed to examine the effects of TBMS I on the cell growth of L-02 cultured normal human liver cells. Additionally apoptosis-associated molecular events were investigated as the potential mechanisms responsible for the cytotoxic effect of TBMS I on L-02 cells. Together, this will provide useful new information

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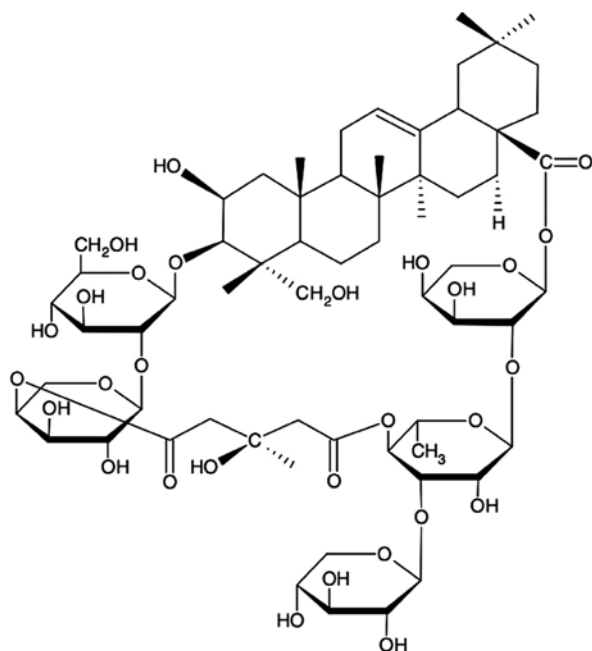


Figure 1. Chemical structure of TBMS I.

concerning not only the effects, but also the safety, of TBMS I as a cancer treatment agent for clinical use.

Materials and methods

Materials. TBMS I was purchased from the National Institute for Control of Pharmaceutical and Biological Products (purity >98%; HPLC, Beijing, China). A stock solution (1 mM) of TBMS I was prepared in 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, Logan, UT, USA) and Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China), respectively. 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) dye, antibiotics, trypsin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V-FITC/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, an enhanced chemiluminescence (ECL) kit and cell mitochondria isolation kit were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). The bicinchoninic acid (BCA) protein assay kit was purchased from Bioteke Corporation (Beijing, China). Rabbit anti-cytochrome c, Bcl-2, Bax and β -actin antibodies were purchased from Cell Signaling Technology (MA, USA). Horseradish protein (HRP)-conjugated goat anti-rabbit IgG was obtained from Boster (Wuhan, China). All other chemicals were obtained from Huili Chemical Reagent Co., Ltd. (Chongqing, China).

Cell culture. The normal liver L-02 cell line was obtained from the American Type Culture Center (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS. The cultures were maintained in a humidified incubator at 37°C in the presence of 5% CO_2 . The culture medium was changed every 2 days. Cells for assay were detached by a solution of 0.25% trypsin and 0.02% EDTA.

MTT assay. TBMS I cytotoxicity to cultured L-02 cells was evaluated by the MTT assay. Briefly, cells (1×10^4 cells/well) were seeded in 96-well cell 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 and $90 \mu\text{M}$). Cells treated with sham containing equal volumes of cell culture medium but no TBMS I ($0 \mu\text{M}$), were used as a control in each experiment throughout the study. After exposure to TBMS I for 24, 48 and 72 h, MTT dye was added to each well at a final concentration of 0.5 mg/ml, and the insoluble formazan produced by the living cells in response to the MTT dye was collected and dissolved in DMSO and measured with an ELISA reader (Bio-Rad, USA) at a wavelength of 492 nm.

TBMS I-induced morphological changes in the cell and nucleus. TBMS I-induced morphological changes in the L-02 cells was evaluated by phase contrast optical microscopy. In brief, cells were prepared similarly to as described above, but in 24-well cell culture plates at 4×10^4 cells/well. Thereafter, the cells were treated with either sham or TBMS I at 15 and $30 \mu\text{M}$, respectively, for 24 h. Subsequently, the cells were examined and photographed under a phase contrast microscope (Leica, Germany). The morphological change of the L-02 cell nucleus in response to TBMS I treatment was evaluated by fluorescent visualization with Hoechst 33258 staining. Briefly, cells were prepared similarly to as above, but on 20-mm diameter coverslips, and then treated with either sham or $20 \mu\text{M}$ TBMS I for 24 h. After exposure to TBMS I, the cells were washed in PBS, fixed with 4% paraformaldehyde for 10 min and then incubated for 10 min with Hoechst 33258 fluorescent dye (5 mg/ml). The cells were then washed, dried and examined by fluorescence microscopy (Leica).

TBMS I-induced early and late apoptosis. TBMS I-induced apoptosis was first investigated using Annexin V-FITC/PI double staining and flow cytometry. Briefly, subsequent to either sham or TBMS I exposure (15 or $30 \mu\text{M}$ for 24 h), 1×10^6 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 the addition of 5 μl Annexin V-FITC conjugate and 5 μl PI buffer, all from the Annexin V-FITC/PI double staining apoptosis kit, 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, USA), and the number of cells with either Annexin V-FITC⁺/PI⁻ or Annexin V-FITC⁺/PI⁺ were obtained automatically.

TBMS I-induced mitochondrial membrane depolarization. Next, we 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 and flow cytometry. In brief, subsequent to either sham or TBMS I exposure (15 or $30 \mu\text{M}$ for 24 h), 1×10^6 cells were harvested and washed twice with PBS, then incubated with rhodamine 123 (1 $\mu\text{g/ml}$) at 37°C for 10 min. The cells were then transferred to a FACStar flow cytometer with proprietary ModFit software (Becton

Dickinson, San Jose, CA, USA), and the number of cells with or without mitochondrial membrane depolarization was determined automatically.

TBMS I-induced release of cytochrome c from mitochondria. The cytosolic and mitochondrial fractions of cytochrome c were extracted from the cell with respective extraction buffers containing either dithiothreitol (DTT) or phenylmethanesulfonyl fluoride (PMSF) and protease inhibitors, both supplied in the cell mitochondrial isolation kit, and detected by Western blotting. In brief, subsequent to either sham or TBMS I exposure (15 and 30 μ M for 24 h), 4×10^6 cells were harvested, washed with ice-cold PBS, then centrifuged at $1,000 \times g$ 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 $600 \times g$ for 10 min at 4°C . The supernatant was carefully collected and centrifuged again at $11,000 \times g$ for 10 min at 4°C . This final supernatant was used as the cytosolic fraction, and the pellet was mixed with 100 μ l of the mitochondrial extraction buffer and used as the mitochondrial fraction. Cytochrome c expression in the cytosolic or mitochondrial fraction was detected by Western blotting.

TBMS I-induced activation of caspases. The activity of caspase-3, -8 and -9 was measured by a caspase activity assay kit. Briefly, subsequent to either sham or TBMS I exposure (30 μ M for 24 h), 3×10^6 cells were harvested, washed twice with ice-cold PBS, then resuspended in lysis buffer and left on ice for 60 min. The lysate was centrifuged at $6,000 \times g$ at 4°C for 5 min. The supernatant was mixed with the substrate peptides Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, respectively, which resulted in the release of pNA due to the hydrolysis of each respective peptide by caspase-3, -8 and -9. The concentration of pNA correlating with the activity of the caspase was determined by the optical absorbance at 405 nm using an ELISA Reader (Bio-Rad).

TBMS I-induced change in Bax/Bcl-2. The expression levels of Bcl-2 and Bax were investigated by Western blotting. In brief, subsequent to either sham or TBMS I exposure (15 and 30 μ M for 24 h), 4×10^6 cells were harvested, suspended in 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% SDS, 0.5 mM DTT and 1% protease inhibitor cocktail, and incubated on ice for 30 min. The lysate was centrifuged at $11,000 \times g$ 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 the bicinchoninic acid (BCA; Bioteck, China) method. Subsequently, equal-volume aliquots of the supernatant containing equal amounts of total proteins were separated by SDS-PAGE gel, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) and probed with specific primary antibodies for Bax and Bcl-2, followed by incubation with corresponding HRP-conjugated secondary antibodies. Bax and Bcl-2 expression was then detected using an enhanced chemiluminescence (ECL) kit (Beyotime, China).

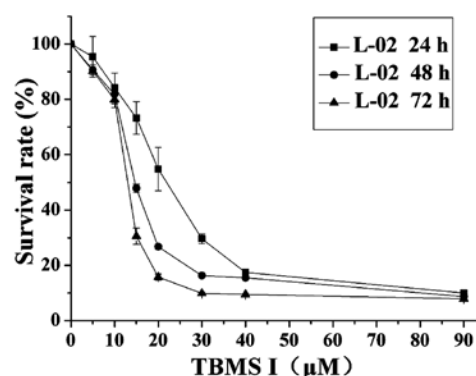


Figure 2. Viability of cultured L-02 cells in response to TBMS I treatment. L-02 cells were treated with either sham (0 μ M) or TBMS I at concentrations of 5, 10, 15, 20, 30, 40 and 90 μ M for 24, 48 and 72 h (** $p < 0.01$, $n = 18$).

Statistical analysis. Each experiment was repeated three times, and the results are presented as the means \pm standard deviation (SD). 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 inhibited the growth of L-02 cells. As shown in Fig. 2, TBMS I inhibited the growth of L-02 cells in a typical concentration- and time-dependent manner. When exposed to TBMS I for 24, 48 and 72 h, the IC_{50} , the dose at which 50% of the L-02 cells did not survive was 23.06, 16.20 and 13.10 μ M, respectively.

TBMS I induced morphological changes in the cell and nucleus. In the absence of TBMS I, L-02 cells had a common spindle shape with intact nuclei and almost uniform Hoechst 33258 (blue) staining. In the presence of TBMS I (15 and 30 μ M), the cells were observed to exhibit increasing shrinkage and detachment from the substrate as the exposure dose and duration increased (Fig. 3A from left to right), indicating an increasing extent of apoptosis in these cells. The change of cell shape in response to TBMS I exposure was accompanied by morphological changes in the nuclei. After exposure to TBMS I (20 μ M, 24 h), the nuclei appeared to have brighter Hoechst 33258 staining, but more significantly, condensed and fragmented chromatin, indicating apoptosis (Fig. 3B from left to right).

TBMS I induced early and late apoptosis. As shown in Fig. 4A, when L-02 cells were treated with TBMS I at 0, 15 and 30 μ M for 24 h, there were increases in the number of both early (Annexin V-FITC⁺/PI⁻) and late (Annexin V-FITC⁺/PI⁺) apoptotic cells. The quantitative percentage of early apoptotic cells increased from 1.29% at 0 μ M to 2.10% at 15 μ M, and 30.36% at 30 μ M, while late apoptotic cells increased from 1.37% at 0 μ M to 3.43% at 15 μ M, and 15.50% at 30 μ M (Fig. 4B).

TBMS I induced breakdown of the mitochondrial membrane and release of cytochrome c. When treated with TBMS I for 24 h

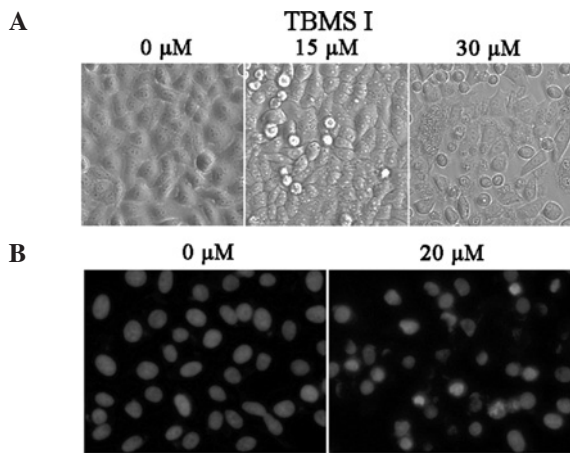


Figure 3. Morphological changes in L-02 cells and nuclei in response to TBMS I treatment. (A) Phase contrast images of cells after being treated for 24 h with either sham (0 μ M), 15 or 30 μ M of TBMS I. (B) Fluorescence photomicrograph of the L-02 cell nuclei stained with Hoechst 33258 after treatment of the cells for 24 h with either sham (0 μ M) or 20 μ M of TBMS I (magnification, x200).

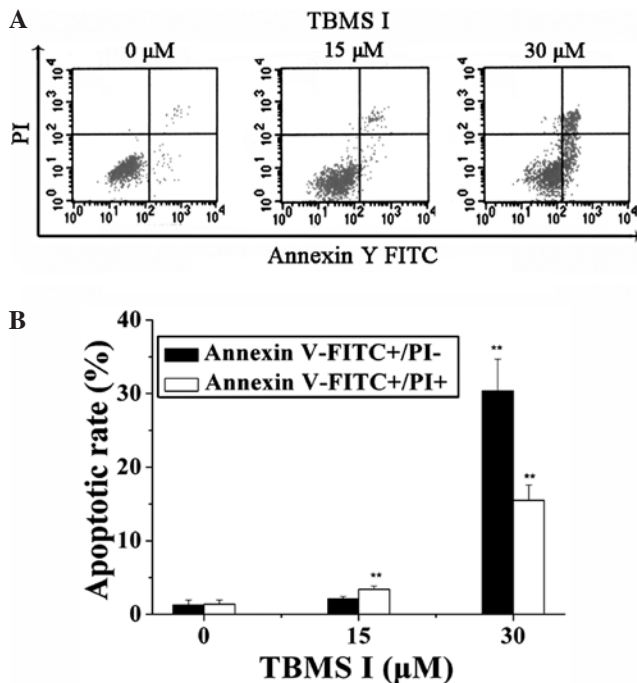


Figure 4. Flow cytometric results and quantitative percentage of the number of cells expressing either Annexin V-FITC⁺/PI⁻ (early apoptosis) or Annexin V-FITC⁺/PI⁺ (late apoptosis). (A) Flow cytometric results showing that there were increasing numbers of cells expressing either Annexin V-FITC⁺/PI⁻ (early apoptosis) or Annexin V-FITC⁺/PI⁺ (late apoptosis) when the cells were treated with increasing concentrations of TBMS I for 24 h. (B) Quantified percentage or apoptotic rate of Annexin V-FITC⁺/PI⁻ cells (solid bar) and Annexin V-FITC⁺/PI⁺ cells (open bar), indicating that the early apoptotic cells dominated the response of the L-02 cells to TBMS I at a high concentration (** $p < 0.01$, $n = 3$).

at increasing concentrations (0, 15 and 30 μ M), the number of depolarized cells increased, as shown in Fig. 5A. The quantitative percentage of depolarized cells increased from 2.30% at 0 μ M to 5.21% at 15 μ M and 33.28% at 30 μ M, respectively (Fig. 5B), which indicated an increasing number of cells with

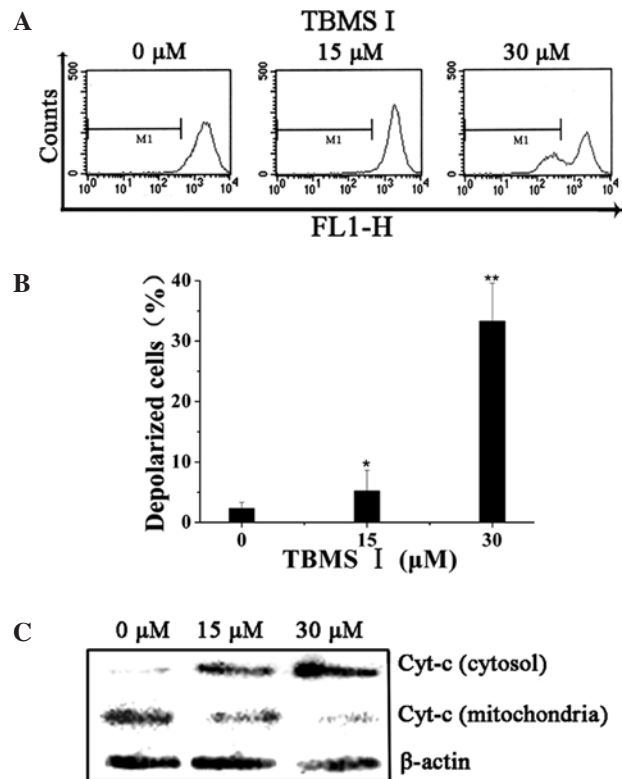


Figure 5. Mitochondrial membrane depolarization and release of mitochondrial cytochrome (cyt) c in response to TBMS I treatment. (A) Cells were treated with either sham (0 μ M) or TBST I at the concentrations indicated. Change in mitochondrial membrane potential was determined by flow cytometric analysis with rhodamine 123 staining. (B) Quantified percentage of depolarized cells vs. concentration of TBMS I (* $p < 0.05$ and ** $p < 0.01$, $n = 3$). (C) Western blotting results of cyt c protein expression in either the cytosol or mitochondria vs. the concentration of TBMS I. After TBMS I treatment for 24 h, cyt c increased in the cytosol, but decreased in the mitochondria as the concentration of TBMS I increased.

mitochondrial membrane depolarization or breakdown. In parallel to mitochondrial membrane breakdown, the protein expression of cytochrome c decreased in the mitochondrial fraction, but increased in the cytosolic fraction (Fig. 5C), indicating release of the protein from the mitochondria into the cytosol.

TBMS I activated caspase-3 and -9, and changed the balance between Bax and Bcl-2. When cells were treated with TBMS I at 30 μ M for 24 h, the activities of caspase-3 and -9 were significantly increased, but that of caspase-8 underwent little changed, as compared to cells treated with sham (0 μ M, 24 h) (Fig. 6). In addition, the protein expression of Bcl-2 increased with exposure to TBMS I at increasing concentrations (0, 15 and 30 μ M), while that of Bax decreased in the TBMS I-treated cells as compared to the control cells treated with sham. Of even greater note, the ratio of Bax/Bcl-2 protein expression increased from 0.36 to 1.39 when TBMS I concentration increased from 0 to 30 μ M, indicating that TBMS I up-regulates Bax expression and down-regulates Bcl-2 expression (Fig. 7).

Discussion

The primary finding of this study is that TBMS I inhibited L-02 proliferation in a concentration- and time-dependent manner,

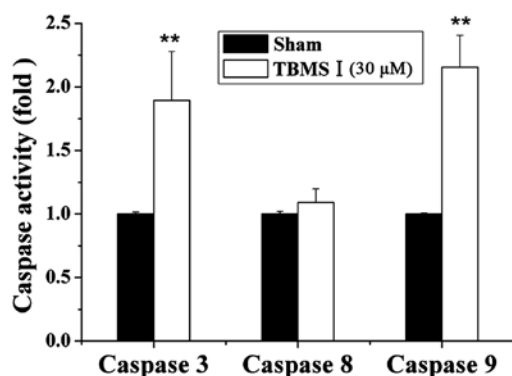


Figure 6. Activity of caspase-3, -8 and -9 in L-02 cells after treatment with either sham (0 μ M) (solid bar) or 30 μ M TBMS I (open bar) for 24 h (** p <0.01, n=6).

which is likely to be mediated through apoptosis-associated processes. The evidence of apoptosis induction by TBMS I in L-02 cells includes characteristic morphological changes in the cell and nucleus, such as cell shrinkage and nuclear condensation and/or fragmentation; the loss of mitochondrial membrane potential followed by the release of cytochrome c from the mitochondrial intermembrane space to the cytosol; activation of caspase-3 and -9; and the up-regulation of Bax protein and down-regulation of Bcl-2 protein.

Although TBMS I has been evaluated for its cytotoxicity to cancer cells using several human cancer cell lines (6,7,10), its cytotoxicity to normal cells has not been studied. Toxicity to normal cells or tissue is the major limitation to the use of any compound, including TBMS I, in clinical treatment. Therefore, we demonstrated for the first time the hepatocytotoxic effects of TBMS I on normal liver (L-02) cells. We also demonstrated that the TBMS I-induced hepatocytotoxic effects were associated with several important apoptotic processes at the cellular and molecular levels. At the molecular level in particular, the loss of mitochondrial membrane integrity was observed in the TBMS I-treated L02 cells first, followed by the release of cytochrome c from the mitochondria into the cytosol and the marked activation of caspase-3 and -9, all of which were consistent with what had been observed in TBMS I-treated cancer cells (9). The generally recognized apoptotic cascade involving mitochondrial signaling, that is, the release of cytochrome c, triggers the formation of the apoptosome complex and the activation of the initiator caspase-9, and leads to the proteolytic activation of caspase-3, the primary effector caspase of the cell (11,12).

Furthermore, our results revealed that TBMS I treatment induced a significant increase in the protein expression of Bax and a decrease in Bcl-2 in L-02 cells. It is known that the Bcl-2 protein family plays key roles in regulating apoptosis involving the mitochondrial signaling pathway, and the 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 (13,14). The pro-apoptotic proteins translocate from mitochondria into the cytoplasm and thus promote the release of pro-apoptotic factors and induce apoptosis, while the anti-apoptotic proteins, sequestered in the mitochondria, inhibit the release of pro-apoptotic proteins and factors from liposomes, and prevent apoptosis (15). It is the balance between these two groups of Bcl-2 family members that determines whether the

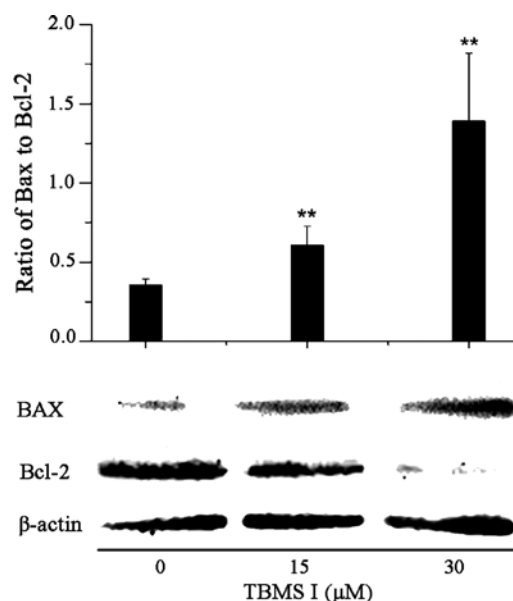


Figure 7. Regulation of Bax and Bcl-2 in L-02 cells by TBMS I. The lower panel shows the results of Western blotting showing the protein expression of Bax and Bcl-2 vs. the concentration of TBMS I. After TBMS I treatment for 24 h, the protein expression of Bax increased, while that of Bcl-2 decreased with increasing TBMS I concentration. The upper panel shows the ratio of quantified protein expression of Bax to that of Bcl-2 vs. the concentration of TBMS I (** p <0.01, n=3).

cell survives or undergoes apoptosis (16). The observation that TBMS I treatment resulted in a marked increase in the ratio of protein expression between the pro-apoptotic Bax and the anti-apoptotic Bcl-2 suggests that TBMS I may alter the balance between anti- and pro-apoptotic protein members in the cell, and eventually lead to the promotion of apoptotic activities.

Taken together, our data indicate that TBMS I induced apoptosis in L-02 cells via the mitochondrial pathway. As the first of its kind, this *in vitro* study not only addressed the issue of the hepatotoxicity of TBMS I to cultured normal liver cells, but the findings obtained may also provide important clues regarding the potential damage of TBMS I to the liver *in vivo*, or protection of the liver from such damage by selective reduction or blocking of the mitochondrial apoptotic signaling pathways.

Acknowledgements

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