Tubeimoside-1 induces G2/M phase arrest and apoptosis in SKOV-3 cells through increase of intracellular Ca\textsuperscript{2+} and caspase-dependent signaling pathways

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Abstract. Tubeimoside-1 (TBMS1) extracted from Bolbostemma paniculatum (Maxim), is a traditional Chinese herb with anticancer potential. It induces apoptosis in a number of human carcinoma cell lines, but the mechanism has remained unclear. In the present study, we investigated the pro-apoptotic activity of TBMS1 against SKOV-3 cell lines and the underlying mechanisms. Treatment with TBMS1 resulted in dose- and time-dependent inhibition of proliferation, led to arrest in phase G\textsubscript{2}/M of the cell cycle and increased the levels of intracellular Ca\textsuperscript{2+}. Furthermore, TBMS1 up-regulated the levels of the glucose-regulated protein 78/immunoglobulin heavy chain binding protein (GRP78/Bip), C/EBP homologous protein (CHOP), Bax, and cleaved caspase-3 and down-regulated the levels of Bcl-2. It was shown to be linked to activation of the extracellular signal-regulated kinase (ERK) 1 and 2 signal transduction pathway. A decrease in Bcl-2/Bax ratio with increased expression of caspase-3, and intracellular Ca\textsuperscript{2+} provide compelling evidence that TBMS1-induced apoptosis is mediated by the mitochondrial pathway. The results of the present study suggest that TBMS1 has immense potential in cancer prevention and therapy based on its antiproliferative and apoptosis inducing effects.

Introduction

Ovarian cancer remains not only the commonest but also the most lethal gynaecological malignancy (1). Although most patients initially respond to surgery and adjuvant chemotherapy, the majority will experience disease recurrence. Therefore, research has to focus on the discovery of novel chemotherapeutics with increased activity and response rates to assist in the management of ovarian cancer. Tubeimoside-1 (TBMS1), a triterpenoid saponin extracted from Bolbostemma paniculatum (Maxim) Franquet, is the main component with anticancer potential (2). It induced arrest of the cell cycle and apoptosis in human low differentiated nasopharyngeal carcinoma (CNE-2Z) cells (3), human promyelocytic leukemia (HL-60) cells (4) and HeLa cells (5), respectively. However, there is no available information to address how TBMS1 affects human ovarian cancer cells in vitro and the mechanisms of TBMS1 induced cell death remain to be elucidated.

Apoptosis, a genetically controlled mode of cell death, is of critical importance for the removal of potentially dangerous cells, including precursor tumor cells (6). Ca\textsuperscript{2+} may act as a pro-apoptotic agent, and both intracellular Ca\textsuperscript{2+} depletion or overload may trigger apoptosis (7). It has been reported that elevation of the intracellular Ca\textsuperscript{2+} level is regarded as an important mediator of apoptosis (8). Staurosporine treatment of PC12 cells resulted in both a rapid (min) and prolonged (h) elevation of intracellular Ca\textsuperscript{2+} (9). The endoplasmic reticulum is a critical site responsible for the regulator of intracellular calcium homeostasis. Various stresses, including expression of mutant proteins, extreme environmental conditions and alterations in redox status cause calcium release from the lumen of the ER which is known as abnormal function of ER the so-called ER stress. When functions of the ER are severely impaired, transcriptional activation of the CHOP is involved in apoptotic events (10). GRP78/Bip is also upregulated under stress conditions (11). So, high level expression of CHOP and GRP78/Bip can be recognized as biomarks of ER stress. Because ER stress leads to increase in cytosolic calcium levels (12) and Ca\textsuperscript{2+} increase is associated with apoptosis signaling (9), it is an excellent strategy in chemotherapy that induce ER stress.

In the present study, we investigated the possible pathway that TBMS1 induced for apoptosis of SKOV-3 cells. We show in SKOV-3 cells that calcium release from the lumen of the ER was dependent on Bax/Bcl-2 activation. We further report that signaling from CHOP, GRP78/Bip or MAPK were activated by TBMS1.

Key words: tubeimoside-1, apoptosis, endoplasmic reticulum stress, mitochondrial dysfunction, SKOV-3 cells
Materials and methods

Reagents and antibodies. Tubeimoside 1 (TBMS1) (C_{43}H_{64}O_{29}) was extracted from *Bolbostemma paniculatum* (Maxim.) Franquet by our laboratory. The following were used: 3-(4,5-dimethylthiazol-2-thiazyl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), thapsigargin and tunicamycin (Sigma, St. Louis, MO, USA); ERK1/2 inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and fluo-3 AM (Invitrogen Corporation, Carlsbad, CA, USA); Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and Hoechst 33258 (Key-Gen Biotech Co., Ltd., Nanjing, China); 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma); TRIzol (Sangon Biological Engineering Technology & Services, Shanghai, China); Polymerase chain reaction (PCR) mixture (Takara Biotechnology, Dalian, China); anti-p38 MAPK, anti-phospho-p38 (p-p38) MAPK, anti-ERK1/2, and anti-phospho-ERK1/2 (p-ERK1/2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Bax, anti-Bcl-2, anti-GRP78/Bip, anti-CHOP and anti-caspase-3 (Cell Signaling Technology, Beverly, MA, USA); RPMI-1640 medium and fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA).

Cell lines and cell culture. Human ovarian carcinoma cell lines SKOV-3 were kindly donated by pathology institute of Chongqing Medical University. Cells were grown as a monolayer in 50-cm² tissue culture flasks and passed every 2-3 days. Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37°C in a humidified atmosphere 5% CO₂. For most experiments, when cells reached sub-confluence, they were pretreated with culture medium containing different concentrations of TBMS1 for 24 h, and then were used for further assays.

Cell viability assay. Cell viability was evaluated by the MTT reduction assay. In brief, SKOV-3 cells were seeded at a density of 7.5x10⁴ cells/well in 96-well microtiter plates containing 150 µl of 1640 medium with 10% FBS. After growing to sub-confluence, the cells were exposed to various doses of TBMS1 (2, 4, 8, 16 µM) and incubated for 24 h. Then incubated with MTT (5 mg/ml) in culture medium for 3 h at 37°C. After that, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 100 µl of DMSO. The absorbance was measured at 490 nm using a Sunrise Remote Microplate Reader (Grodlg, Austria), and then normalized the value to the control (untreated cells).

Apoptosis analysis with flow cytometry. SKOV-3 cells were grown in 25-cm² culture flasks in the presence of indicated concentrations of TBMS1 for 24 h. Cells were trypsinized, centrifuged, washed in PBS and then double-stained by using an Annexin V-FITC apoptosis detection kit according to the manufacturer’s specifications. Samples were incubated at room temperature for 15 min in the dark with Annexin V and PI. The fluorescence in cells was quantitatively analyzed at an emission wavelength of 530 nm and an excitation wavelength of 480 nm using a Vantage SE flow cytometer with fluorescence activated cell sorting (FACS) system (Becton-Dickinson, San Jose, CA, USA).

Flow cytometric evaluation of cell proliferation. The non-fluorescent dye CFSE can be cleaved by intracellular esterase to form fluorescent conjugates with amines and the dyeprotein adducts are equally inherited by daughter cells after cell division. Therefore, proliferation activity of cells can be accurately evaluated by the average fluorescent intensity, which is negatively correlated with the divided cell number. For flow cytometric evaluation of cell proliferation, SKOV-3 cells were grown in 25-cm² culture flasks in the presence of indicated concentrations of TBMS1 for 24 h. Then, the culture medium was washed away and cells were labeled with CFSE (40 µM) in RPMI-1640 medium (10% FBS) for 5 h at 37°C. After the treatment, cells were washed, harvested, resuspended with PBS and analyzed on a flow cytometer as before.

Flow cytometry for cell cycle analysis. SKOV-3 cells growing in 25-cm² culture flasks were harvested, washed and fixed with ice cold alcohol (75%) for >24 h. After further washing twice, cells were incubated with PBS (pH 7.4) containing RNase (5 U) and PI (50 µg/ml) for 15 min at 37°C. Flow cytometry was performed using a FACS vantage SE flow cytometer.

Flow cytometric evaluation of intracellular Ca²⁺ level. The non-fluorescent dye fluo-3 AM was used for intracellular Ca²⁺ detection. Upon diffusion into cells, fluo-3 AM can be cleaved into fluo-3 by endogenous esterases and trapped inside the cells. After binding to Ca²⁺, fluo-3 elicits fluorescent light, which is proportional to the intracellular Ca²⁺ level. In brief, after the drug treatment, cells were washed and incubated with RPMI-1640 medium (10% FBS) containing 0.5 µM of fluo-3 AM for 1 h at 37°C. Next, cells were washed, harvested, re-suspended with PBS and analyzed on a flow cytometer as before.

Hoechst staining. Cells were seeded into 96-well culture plates. After treating with various concentrations of TBMS1 for 24 h, cells were washed with PBS and then incubated in 100 µl medium containing 1 µg/ml Hoechst 33258 for 30 min at 37°C in darkness. Cells were then washed two times with PBS and immediately observed under a fluorescent microscope. The experiments were carried out three times.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from SKOV-3 cells using TRIzol (Takara). The quantity of RNA isolates was determined spectrophotometrically using a DNA/RNA Gene-Quant Calculator (Amersham Biosciences, USA). Reverse transcription was performed in 20 µl of reaction mixture containing 2 µg of total RNA, 5.0 units of AMV reverse transcriptase, 50 pmol of oligo-dT primer, 40 nmol of dNTP mixture, 40 units of RNase inhibitor, 4 µl of 5X RT buffer (Bioer, Hangzhou, China) at 42°C for 1 h and 95°C for 5 min. The following primers for RT-PCR were used: Bcl-2 (200 bp): sense 5'-GAA GGA ATG TTG CAT GAG TCG GAT C-3'; antisense: 5'-CCT AGA AAT GGA TCA TAT TGG AAC ATG T-3'. RT-PCR analysis was performed in 20 µl of reaction mixture containing 1 µl of cDNA reaction mixture, 10 nmol of dNTP mixture, 10 pmol of sense
and antisense primers, and two units of BioReady rTaq polymerase (Bioer). The thermal cycling program was as follows: 4 min at 94°C for initial denaturation; 30 cycles x30 sec at 94°C, 30 sec at 54°C, and 30 sec at 72°C for Bcl-2 and GAPDH; 30 cycles x30 sec at 94°C, 30 sec at 59°C, and 30 sec at 72°C for Bax. For PCR product analysis, 6 µl of each reaction mixture was electrophoresed on 1.5% agarose gel containing 1% Gold-View™. Band intensity was analyzed with Gelsdoc 2000 system (Bio-Red, USA) and presented as a percentage of GAPDH expression.

Quantitative real-time reverse transcription-PCR. Total RNA was extracted from SKOV-3 cells using TRIzol. Reverse transcription was performed in 10 µl of reaction mixture containing 0.5 µg of total RNA, 0.5 µl of primeScript RT mixture, 0.5 µl of oligo-dT primer, 0.5 µl random 6 mers, 2 µl of 5X RT buffer and the rest of volume was supplied by RNA free H₂O (Takara) at 37°C for 15 min and 85°C for 5 sec. Quantitative PCR was done in Bio-Rad CXF96 thermal cycler using SYBR-Green supermix (Bio-Rad Laboratories). The primers were used as follows: for Bip (70 bp): sense 5’-TCA GGG CAA CCG CAT CAC-3’; antisense 5’-CGC ATC GCC AAT CAG ACG-3’, CHOP (61 bp): sense 5’-GCT GGC TTG GCT G AC TGA-3’; antisense 5’-GCT CTG GGA GGT GCT TGT G-3’, β-actin (75 bp): sense 5’-AGC GCA TCG TCA CCA ACT G-3’, antisense 5’-GAG CCA CAC GCA GCT CTT T-3’. Concentration of mRNA was normalized to that of β-actin mRNA.

Western blot analysis. For isolation of total protein extract, SKOV-3 cells were washed with ice-cold PBS and lysed in RIPA lysis buffer (50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 0.05 mM EDTA) for 15 min on ice, and cell lysate was centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was collected and protein content of extracted samples was measured using bicinchoninic acid protein assay kit (Bio-Med, Beijing, China). All samples were stocked at -80°C for further experiments. Levels of target proteins including caspase-3, Bcl-2, Bax, p-p38, p38, p-ERK1/2, ERK1/2, Bip, chop and β-actin were determined by Western blot analysis using the respective antibodies stated above. Briefly, total cell lysate was boiled in 5X loading buffer (125 mM Tris-HCl, pH 6.8, 10% SDS, 5% dithiothreitol, 50% glycerol and 0.5% bromochlorphenol blue) for 10 min. Equal amount of proteins (60 µg) was subjected to 8-15% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in PBS with 0.1% Tween-20 (PBST) for 1 h, and incubated with primary antibodies overnight at 4°C. Antibodies were detected by means of HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (ECL) and densitometric analysis was performed with the use of Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

Statistical analysis. All data are expressed as the means ± SD. Statistical analyses were performed by using the SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). Comparisons between groups were performed by Student’s t-test and one-way analysis of variance (ANOVA). Statistical significance was accepted at p<0.05.

Results

The effect of TBMS1 on SKOV-3 cell viability. To identify the proper concentration of TBMS1 to activate SKOV-3 cells, cells were treated with various concentrations of TBMS1 for 24 h, and cell viability was determined by MTT analysis (n=5) as described in Materials and methods. (B) Apoptosis is illustrated by 33258 staining for morphological proof. Cells were exposed to gradually increased concentration of TBMS1 (a-e: 0, 2-16 µM) and incubated for 24 h and then stained by Hoechst 33258 for 30 min at 37°C in the dark. The cells were observed under a fluorescent microscope (x10). The nuclei of apoptotic cells were condensed and fragmented. The bar graph shows the results of three independent experiments. *p<0.05, compared with vehicle-treated control group.

TBMS1 induces apoptosis in SKOV-3 cells. After treated with various concentrations of TBMS1 for 24 h, morphology of SKOV-3 cells was assayed. Fig. 1B shows that the condensed
chromatin of SKOV-3 cells induced by TBMS1 stained by Hoechst 33258 was brighter than that of vehicle-treated cells. It was shown that TBMS1 impaired DNA in cells.

Next, cells apoptosis, proliferation and cell cycle were detected by flow cytometry. As presented in Fig. 2, for the vehicle-treated control group 5.0% cells excluded PI and were positive for Annexin V-FITC binding, which represents apoptotic cells. The exposure to 16 µM of TBMS1 for 24 h gave rise to a 6.7-fold increase in apoptotic rate of SKOV-3 cells. (*p<0.05) (Fig. 2f). Effect of TBMS1 on cell proliferation was investigated using the intracellular dye CFSE. With successive cell division, the average fluorescence in daughter cells will be gradually lessened and indirectly reflect the proliferation activity. Results in Fig. 3A show that the fluorescent intensity in SKOV-3 cells was minimal in the vehicle-treated group and a noticeable increase was observed after TBMS1 exposure for 24 h (*p<0.05, vs. the vehicle-treated group). The blockade of cell cycle is often regarded as a directly triggering event for development of apoptosis (13). To test the role of TBMS1 in cycle progression of SKOV-3 cells, the cell cycle distribution was assessed by monitoring intensity of PI fluorescence. As indicated in Table I, in the vehicle-treated control group, 9.99±1.5% of the total cell number was displayed in the G2/M phase of the cell cycle. But, treatments with TBMS1 result in the cell number remaining in G2/M increased gradually. From these data, we concluded that TBMS1 blocked cell cleavage in phase G2/M of the cell cycle. It was demonstrated that TBMS1 induced apoptosis of SKOV-3 cells.

<table>
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<tr>
<th>Groups (mM)</th>
<th>$G_0/G_1$ (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
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<td>Control</td>
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<td>20.40±3.06</td>
<td>9.99±1.50</td>
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<td>22.69±3.40</td>
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<td>20.15±3.02</td>
<td>17.76±2.66</td>
</tr>
<tr>
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<td>55.378±3.31</td>
<td>24.32±3.65</td>
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Cells were treated with different concentrations of TBMS1 (2, 4, 8, 16 µM) for 24 h, harvested and labeled with PI, and then analyzed by measuring the fluorescence intensity of PI. Values are the means ± SD. (n=3). *p<0.05, **p<0.01 compared to the vehicle-treated control group.

Table I. Flow cytometry for cell cycle analysis of SKOV-3 cells treated with various concentrations of TBMS1.

Cell apoptosis induced by TBMS1 is relevant to ER stress. It was reported that an increase of cytosolic Ca$^{2+}$ concentration subsequently initiated the occurrence of cell apoptosis. Ca$^{2+}$ plays an important role in regulation of cell growth and death. We determined the effect of TBMS1-induced Ca$^{2+}$ influx in SKOV-3 cells by flow cytometry. Cells were treated with various concentrations of TBMS1 (2, 4, 8, 16 µM) for 24 h. As shown in Fig. 3B, TBMS1 exposure significantly increased

Figure 2. Effect of TBMS1-induced apoptosis in SKOV-3 cells. SKOV-3 cells were harvested and labeled with a combination of Annexin V-FITC and PI, and then analyzed by flow cytometry. The figures show representative flow cytometric histograms of vehicle-treated cells (a), cells exposed to various concentrations of TBMS1 (b-c: 2-16 µM) for 24 h. Results are expressed as the means ± SD (n=3). *p<0.05 compared to vehicle-treated control group.
the Ca\(^{2+}\) influx in SKOV-3 cells, compared to the vehicle-treated group (p<0.05). This indicated that TBMS1 induced the increase of intracellular Ca\(^{2+}\) concentration.

To evaluate whether the change of Ca\(^{2+}\) concentration was relevant to endoplasmic reticulum (ER) stress we determined ER stress. Glucose-regulated protein 78/Bip (GRP78/Bip) is an ER-resident protein that plays a central role in the ER stress response (14). C/EBP homologous protein (CHOP) is one of the components of the ER stress-mediated apoptosis pathway. Thus, we evaluated first the expression of Bip and CHOP mRNA by real-time PCR and protein levels by Western blot analysis. We used thapsigargin (TG) and tunica-myacin (TM) as the positive controls, which are both triggers of ER stress. The mRNA (Fig. 4A) and protein levels (Fig. 4B) of Bip and CHOP were increased after treatment by TBMS1 (16 µM) (p<0.05). This demonstrated that TBMS1 induced ER stress which was relevant to increase of cytosolic Ca\(^{2+}\) concentration.

TBMS1 up-regulates Bax and down-regulates Bcl-2 expression at transcription and translation levels in SKOV-3 cells. The transcription factor, CHOP, which is induced by ER stress at the transcript level, was reported to induce apoptosis mediated by down-regulation of Bcl-2 (15) and by up-regulation of Bax (16). In the previous experiment, we demonstrated CHOP was induced after treated with TBMS1. Therefore, we examined mRNA expression of Bcl-2 by RT-PCR analysis in SKOV-3 cells. Cells were treated with various doses of TBMS1 (2, 4, 8, 16 µM) for 24 h. As expected, Bcl-2 mRNA level was significantly decreased, compared to vehicle-treated control group (p<0.05) (Fig. 5A).

We also measured the mRNA expression of Bax, a pro-apoptotic protein in Bcl-2 family. As indicated in Fig. 5A, the exposure to different concentrations of TBMS1 for 24 h gave rise to ~1.5-2.5-fold increase in expression of Bax mRNA in cells (p<0.05).

The effect of TBMS1 at protein levels of Bax and Bcl-2 was also tested by Western blot analysis. As indicated in Fig. 5B, cells in the vehicle-treated group showed low level of expression of Bax, which was remarkably induced by TBMS1 treatment for 24 h (p<0.05). Contrary to Bax, the expression of Bcl-2 was significantly decreased when cells were treated by TBMS1 (Fig. 5B). The result of protein expression was similar with the change of mRNA levels of Bax and Bcl-2.

TBMS1-induced apoptosis and activation of Bcl-2/Bax are relevant to the up-regulation of p-p38 MAPK level and the down-regulation of p-ERK1/2 level. Mitogen-activated protein kinase (MAPK) pathways have been implicated in the apoptosis induced by chemotherapeutic drugs (17). We assessed the effect of TBMS1 on phosphorylation of p38/MAPK and ERK1/2. SKOV-3 cells were treated with high (16 µM) or low (2 µM) concentration of TBMS1 for 24 h. As shown in Fig. 5D, TBMS1 exposure led to an increase in the level of p-p38. On the contrary, the level of p-ERK1/2 in SKOV-3 cells was decreased.

Figure 3. Flow cytometry for cell proliferation and Ca\(^{2+}\) influx analysis of SKOV-3 cells treated with TBMS1. (A) Cells were treated with various concentrations of TBMS1 (2, 4, 8, 16 µM) for 24 h, and then cell proliferation was performed by flow cytometry as described in Materials and methods. (B) After the treatment, intracellular Ca\(^{2+}\) levels in SKOV-3 cells was determined by flow cytometry as described in Materials and methods. The histograms presented the fluorescent intensities of SKOV-3 cells treated with vehicle, TBMS1. Data are expressed as means ± SD (n=3). *p<0.05 compared to the vehicle-treated group.
Figure 4. Effect of TBMS1 on mRNA and protein levels of endoplasmic reticulum-related signaling pathway. (A) Cells were treated with thapsigargin (TG) (100 nM), tunicamycin (TM) (3 µM), low and high concentrations of TBMS1 (2, 16 µM) respectively for 2 h. TG and TM were both endoplasmic reticulum stressors. GRP78/Bip and CHOP mRNA was determined by real-time RT-PCR as described. The histograms gave expression of the mRNA levels of GRP78/Bip and CHOP. (B) Cells were treated with thapsigargin (TG) (100 nM), tunicamycin (TM) (3 µM), low and high concentrations of TBMS1 (2, 16 µM), respectively, for 24 h. After the treatment, protein level of GRP78/Bip and CHOP was measured by Western blot analysis as described in Materials and methods. The amount of mRNA and proteins was normalized to that of β-actin. Data are expressed as means ± SD (n=3). *p<0.05 compared to the vehicle-treated group.

Figure 5. Effect of TBMS1 on mRNA and protein levels of mitochondria-related signaling pathway. Cells were treated with various concentrations of TBMS1 (2, 4, 8, 16 µM) for 24 h. After the treatment, (A) Bax and Bcl-2 mRNA levels were determined by RT-PCR analysis as described in Materials and methods. Protein levels of (B) Bax, Bcl-2 and (C) cleaved caspase-3 were measured by Western blot analysis as described. (D) p-p38: phosphorylated p38 MAPK; p-ERK: phosphorylated ERK. Cells were treated with high (16 µM) or low (2 µM) concentration of TBMS1 for 24 h. After the treatment, protein levels of p-p38 and p-ERK were determined by Western blot analysis. The amount of mRNA was normalized to that of GAPDH. The amount of all proteins was normalized to that of β-actin. Data are expressed as means ± SD (n=3). *p<0.05, **p<0.01 compared to the vehicle-treated group.
Because we had demonstrated that TBMS1 induced up-regulation of Bax and down-regulation of Bcl-2 in both transcription and translation levels, we presumed the activation of Bax was concerned with phosphorylation of p38, and the activation of Bcl-2 with phosphorylation of ERK1/2. SKOV-3 cells were pretreated with p38/MAPK inhibitor, SB203580, ERK1/2 inhibitor, PD98059, for 30 min and then exposed to TBMS1 (8 µM) for 24 h. As shown in Fig. 6B, the inhibitor of p38/MAPK, SB203580 suppressed the up-regulation of Bax and the inhibitor of ERK1/2, PD98059 suppressed the down-regulation of Bcl-2 induced by TBMS1. No difference was detected in expression of Bax and Bcl-2 between cells treated with the inhibitor alone and the vehicle group. From this experiment, we concluded the inhibitor of p38/MAPK suppressed the pro-apoptosis action of Bax and the inhibitor of ERK1/2 suppressed the anti-apoptosis action of Bcl-2.

To clarify the relationships between the phosphorylation of p38MAPK and ERK1/2 and cell apoptosis induced by TBMS1, we also pretreated SKOV-3 cells with p38/MAPK inhibitor SB203580, and ERK1/2 inhibitor PD98059, for 30 min and then exposed to TBMS1 (8 µM) for 24 h. Flow cytometric evaluation of apoptosis and cell proliferation. Results in Fig. 6A showed that the inhibitor of p38/MAPK decreased the apoptosis rate, compared to the TBMS1-treated group. The inhibitor of ERK1/2 decreased the fluorescence intensity, reflecting the state of cell proliferation, compared to the TBMS1-treated group.

Discussion

Compared to another gynecologic malignancy, cervical carcinoma, ovarian cancer diagnosed at early stage is more difficult, which is due to the deep location. So, the adjuvant therapy after surgery has notable importance. Chemical drug therapy, especially traditional Chinese herbal medicines is the focus at present. TBMS1 was employed, for example, in the treatment of breast carcinoma, mammary abscess, cyclomastopathy and tuberculosis of lymph node (18). In ancient times, and was very effective in applying it to treatment of esophagus carcinoma and stomach cancer. However, the anti-tumor effects of TBMS1 in ovarian cancer are mostly unknown. In this study, we identified effects of TBMS1 on ovarian cancer and the possible mechanism of apoptosis of SKOV-3 cells treated with TBMS1.

ER is the primary site for protein synthesis, folding and trafficking. Under a variety of stressful conditions, the accumulation of unfolded or misfolded proteins in the ER results in the onset of ER stress (19). Regulation of intracellular calcium (Ca^{2+}) levels is one of main functions of ER. Elevation of cytosolic-calcium levels or depletion of ER calcium stores represents typical responses of cells to various stimuli. Our study found that TBMS1 induces cytosolic calcium level elevation.
Bcl-2, both in mRNA and protein levels. Changes in the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins might activate downstream signals, for example, caspase-3, which would finally result in apoptosis. These results imply that mitochondrial dysfunction, at least in part, contributed to the cytotoxicity of TBMS1.

Mitogen-activated protein kinase (MAPK) signaling pathways regulate a range of cellular activities and have been implicated in the pathogenesis of several diseases. p38/MAPK is involved in regulating cellular responses to stress and cytokines (29). It has been reported that cell survival and apoptosis are regulated through the ERK1/2 and p38/MAPK pathways in various cancer cells (30). However, there have been no studies on the mechanism of the TBMS1 via ERK1/2 and p38/MAPK in SKOV-3 cells. To assess the role of ERK1/2 and p38/MAPK in the TBMS1-induced apoptosis, first, we detected phosphorylation of p38/MAPK and ERK1/2 by Western blot analysis, then cells were stimulated with the ERK1/2 specific inhibitor PD98059, and the p38/MAPK-specific inhibitor SB203580, we detected apoptosis and proliferation by flow cytometry. Data suggested that p38/MAPK promoted cell apoptosis, while ERK1/2 showed cell proliferation. Furthermore, after treated with specific inhibitor of p38/MAPK and ERK1/2, we found that p38/MAPK regulated Bax expression and ERK1/2 affected the role of Bcl-2. From this result, we presumed p38/MAPK was the upstream regulation factor of Bax and ERK1/2 located in the upstream of Bcl-2.

In summary, the modulation of ovarian cancer cell apoptosis is highly complex and involves multiple signal transduction, ER stress and mitochondrial dysfunction pathways. Based on these results, it can be concluded that TBMS1-induced SKOV-3 cell apoptosis correlated with ER stress, increased Bax expression via phosphorylation of p38/MAPK elevation and decreased Bcl-2 levels via phosphorylation of ERK1/2 reduction. Finally, the ratio of Bax and Bcl-2 proteins changed, which might activate the downstream signal caspase-3, which would finally result in the SKOV-3 cell apoptosis.

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References


