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ORIGINAL ARTICLE

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Role of mitochondria and mitochondrial cytochrome c in tubeimoside I-mediated apoptosis of human cervical carcinoma HeLa cell line

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Abstract *Background*: Tubeimoside I (TBMS1), a triterpenoid saponin, isolated from the tubers of *Bolbostemma paniculatum*, showed potent antitumor and antitumor-promoting effects. The objective of this study is to investigate the role of mitochondria and mitochondria cytochrome c in TBMS1-mediated apoptosis of human cervical carcinoma HeLa cell line. *Methods*: Viability of HeLa cells was measured by MTT assay. Apoptotic induction by TBMS1 was determined by fluorescence microscopy, flow cytometry and gel electrophoresis of fragmented DNA. Mitochondrial transmembrane potential ($\Delta\psi$m) was assayed by flow cytometry. Cytochrome c (Cyt c) was detected by Western blotting. *Results*: The results showed that Cyclosporin A (CsA) partly protected HeLa cells from growth inhibitory effect of TBMS1, and partly countered the ability of TBMS1 to rapidly induce apoptosis in HeLa cells, and that TBMS1 decreased $\Delta\psi$m and induced Cyt c release by a mechanism inhibited by CsA, and that TBMS1 induced apoptosis of HeLa cells dose-dependently in accordance with increase of cytosolic Cyt c. *Conclusions*: TBMS1 opens the permeability transition (PT) pore, thereby decreasing $\Delta\psi$m, releasing Cyt c from mitochondria, and further causing a series of events consistent with established mechanistic models of apoptosis.

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Introduction

"Tu-Bei-Mu", *Bolbostemma paniculatum* (Maxim.) Franquet (Cucurbitaceae) is a traditional Chinese medicinal plant, which was listed in the Supplement to the Compendium of Materia Medica, compiled in 1765, at the time of the Qing Dynasty. Its stem tuber was employed, for example, in the treatment of breast carcinoma, mammary abscess, cyclomastopathy and tuberculosis of lymph node, in ancient times, and was very effective in resolving masses and reducing swellings [1]. Some physicians of traditional Chinese medicine in Sichuan and Shaanxi provinces (China) used to apply it to treatment of esophagus carcinoma and stomach cancer with considerable efficiency during the 70s of last century, but the side-effects, such as nausea, vomiting, etc., were quite serious. TBMS1, a bisdesmosidic triterpenoid saponin whose sugar chains are connected by 3-hydroxy-3-methylglutaric acid to form a unique macrocyclic structure, was isolated from the tubers of *B. paniculatum* (Fig. 1) [2, 3]. Our previous studies demonstrated that TBMS1 had potent antitumor and antitumor-promoting effects [4, 5, 6], and induced cell cycle arrest and apoptosis in human promyelocytic leukemia (HL-60) cells [7], human low differentiated nasopharyngeal cacinoma (CNE-2Z) cells [8], and HeLa cells [9]. However, the cellular and molecular mechanisms by which TBMS1 leads to cell death remain to be elucidated.

Mitochondria play an important role in the initiation of apoptosis [10, 11]. A major player in this process is the mitochondrial permeability transition pore (mPTP), a non-specific pore that opens in the inner membrane. The early stage of apoptosis is characterized by a rupture in the $\Delta\psi$m preceding signs of DNA fragmentation [12]. This reduction in $\Delta\psi$m is

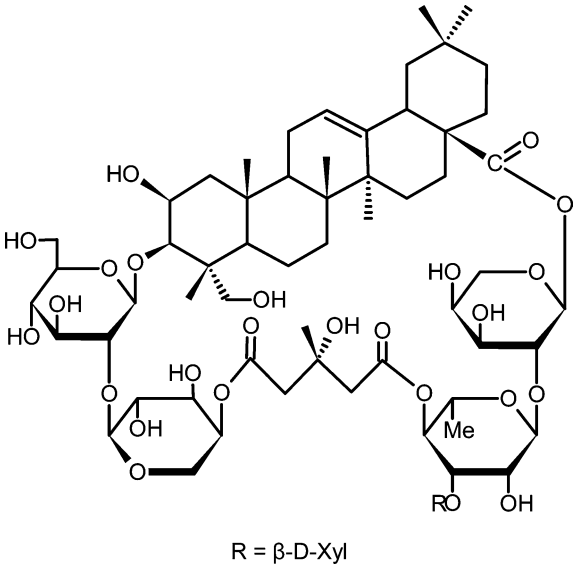

Fig. 1 Chemical structure of tubeimoside I (TBMS1)

believed to result from the opening of megachannels or pores, referred to as the mPTP. The mPTP is located at the contact sites between the inner and outer mitochondrial membranes and is a protein complex formed by the valtage-dependent anion channel (VDAC), members of the pro- and antiapoptotic BAX-BCL2 protein family, cyclophilin D (CyPD), and the adenine nucleotide (ADP/ATP) translocators (ANTs) [13, 14, 15]. In isolated mitochondria or in intact cells in vitro, mPTP can be blocked by CsA (a ligand of matrix CyPD). Specifically, the release of different proteins that are usually present in the intermembrane space of these organelles has been observed during the early stages of apoptotic cell death. Among these proteins are apoptosis-inducing factor, adenylate kinase-2 (AK-2), Smac/DIABLO, and Cyt c [16, 17, 18, 19]. Cyt c is localized in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus triggers the release of Cyt c from the mitochondria into the cytosol. Once in the cytosol, Cyt c interacts with its adaptor molecule, Apaf-1, resulting in the processing and activation of pro-caspase-9 in the presence of dATP [20]. The Cyt c/Apaf-1 complex activates caspase-9, which in turn then cleaves and activates pro-caspase-3 and -7; these effector caspases are responsible for the cleavage of various proteins leading to biochemical and morphological features characteristic of apoptosis [21].

The pathway of apoptosis mentioned above is referred to as the mitochondrial or Cyt c pathway of apoptosis. To determine whether TBMS1-induced apoptosis in HeLa cells is mediated through mitochondria or Cyt c pathway, it is of significance to investigate the effect of TBMS1 on the release of Cyt c from mitochondria into the cytosol in HeLa cells.

Materials and methods

Collection of " Tu-Bei-Mu" and preparation of TBMS1

"Tu-Bei-Mu" was collected at Shaanxi Province (China) and identified by Professor Quan Y (Department of Botany, Shaanxi Provincial Academy of Traditional Chinese Medicine and Pharmacy, China). A voucher specimen (No. 097) has been deposited in the Guangdong Provincial Key Laboratory of Marine Materia Medica, Zhanjiang Ocean University, 524025 Zhanjiang, China. TBMS1 was isolated from tubers of *B. paniculatum* (Maxim.) Franquet (Cucurbitaceae) by a modification of the methods reported previously [2, 3]. Its chemical structure was established by Kong et al [3]. The purified TBMS1 (purity >98.5%) was dissolved in distilled water and stored at −20°C.

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co. Newborn calf serum (NCS) was purchased from Sijiqing Organism Engineering Materials Co. Ltd (Hangzhou, China). CsA, ethyleneglycol bis (2-aminoethylether) tetraacetic acid (EGTA) and rhodamine123 (R123) were obtained from Sigma Chemical Co. Stock solution of CsA was prepared in DMSO (the highest final DMSO concentration used was 0.1%.) and stored at −20°C. Mouse monoclonal anti-Cyt c antibody (clone 7H8.2C12) was from Pharmingen Corp. Rabbit monoclonal anti-β-actin antibody was purchased from Santa Cruz. Goat anti-mouse IgG-HRP antibody and goat anti-rabbit IgG-HRP antibody were purchased from Beijing Zhongshan Biotechnology Co. Ltd. Nitrocellulose membrane was the product of Pall Corp. Super West Pico kit was obtained from Pierce Co.

Cell line and cell culture

Human cervical carcinoma HeLa cell line (HeLa cells) was grown at 37°C in DMEM supplemented with 10% heat-inactivated NCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, and maintained under humidified 95% air/5% CO_2 at 37°C.

MTT assay

MTT assay was performed in HeLa cells to measure the cytotoxicity of TBMS1 to tumor cells and protective effect of CsA against the cytotoxicity of TBMS1 to tumor cells [22]. Single-cell suspensions were prepared and seeded into 96 well microculture plates with 1.0×10^4 cells/ml (90 μl/well). Cells were cultured for 12 h before addition of CsA and/or TBMS1. CsA and/or

TBMS1 was diluted into DMEM and added to each well in a volume of 10 μl. Cells were incubated at 37°C for the time indicated. MTT solution (5 mg/ml) was aliquoted to each well in a volume of 20 μl, and 5 h later 100 μl of the solubilization solution (10% SDS, 5% isobutyl alcohol, 0.012 M HCl (w/v/v)) was added into each well. The plates were allowed to stand overnight in the incubator in a humidified atmosphere. Absorbance at 570 nm (A_{570}) was determined for each well using an ELISA reader. Control wells contained all of the agents presented in the treated wells except TBMS1 and CsA. Each experimental point was performed in three replicates. The 50% inhibitory concentration (IC_{50}) was determined from dose-response data from at least three experiments.

Morphological analysis by fluorescence microscopy

TBMS1-induced apoptosis was analyzed by acridine orange/ethidium bromide (AO/EB, Sino-American Biotechnology Co.) double fluorescent staining. 0.5×10^5 cells/well were seeded in a 6-well plate and treated with 30 μM TMBS1 or 2 μM CsA + TBMS1 for 24 h at 37°C. In CsA + TBMS1 groups, CsA was added to culture medium at a final concentration of 2 μM, and 30 min later, TBMS1 added at a final concentration of 30 μM and then incubated for 24 h**.** For the AO/EB procedure, cells were washed with phosphate buffered saline (PBS), rinsed in 92 μl PBS, and then 8 μl of AO/EB solution (one part of 100 μg/ml AO in PBS, one part of 100 μg/ml EB in PBS) was added [23]. Cells were analyzed in a fluorescence microscope (DMIRB, Leica) using a fluorescein filter and a 40×objective.

Flow cytometric determinations of phosphatidylserine externalization (annexin V binding)

Cells were treated with test compounds and indicated as described for AO/EB assays, and then harvested cells. Harvested cells were washed twice with PBS, counted, and suspended in binding buffer at a density 1×10^6 cells/mL (Annexin V-FITC Staining Kit, BD Biosciences Pharmingen). Hundred microliters of this cell suspension was stained with 5 μl of annexin V conjugated to fluorescein isothiocyanate (FITC) and 10 μl of propidium iodide (PI, 20 μg/ml) for 15 min in the dark followed by the addition of 400 μl of PBS, Cells were immediately analyzed by flow cytometry (Epics XL, Coulter Co.). Typically, 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for annexin and PI, respectively.

Gel electrophoresis for isolation of DNA fragmentation

For DNA fragmentation analysis, HeLa cells were exposed to TBMS1 (25, 50 μM) or 2 μM CsA + TBMS1 (25, 50 μM) for 24 h. DNA was isolated from treated or untreated cells. Attached and floating cells were harvested, washed twice with ice-cold PBS, and lysed in 1 ml lysis buffer A (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, and 0.5% Triton X-100 (v/v)). The cell lysates were centrifuged at 12,000 rpm for 15 min in a microcentrifuge. The supernatant was incubated for 2 h at 56°C with 0.1 mg/ml proteinase K. The DNA was extracted with phenol/chloroform (1:1, v/v), and then precipitated with 2 volumes of absolute ethanol. After centrifugation for 5 min at 12,000 rpm and 4°C, the DNA pellet was washed with 500 μl of 70% ethanol and resuspended in 20 μl of TE-buffer (10 mM Tris/HCl, pH 8.0, 1 m*M* EDTA containing 20 μg/ml RNase), and incubated overnight at 4°C. Each DNA sample was then analyzed on a 1% agarose gel containing 0.1 mg/ml EB. The same amount of DNA, as assessed by spectro-photometric measurement, was loaded in each lane. DNA was visualized by transillumination in UV light and photographed [24].

Morphological analysis by electron microscopy

HeLa cells grown in 75-mm culture flask were treated with 20 μM TBMS1 or 2 μM CsA + 20 μM TBMS1 for 12, 24 h. Attached and floating cells were harvested, washed with ice-cold PBS. The samples were fixed overnight at 4°C in 2.5% glutaradehyde with 1% NCS in 0.2 M PBS, pH 7.2–7.4, and rinsed three times in the PBS and incubated in 1% osmium tetroxide for 2 h at room temperature. After fixation, infiltration, embedding, and hardening, ultrathin sections were cut with knife. The sections were picked up onto 300-mesh grids and stained with 2% uranyl acetate and Reynold′s lead citrate. Stained grids were viewed with a JEM 1200-EX electron microscope operating at 80 kV.

Flow cytometric analysis of mitochondrial transmembrane potential ($\Delta\psi$m)

Cells treated with or without test compounds were trypsinized and washed twice with PBS, and resuspended in 500 μl PBS with 10 mg/L R123. After incubation at 37°C for 30 min, cells were washed once with PBS and analyzed immediately by flow cytometry. At least 10,000 events were collected per sample.

Mitochondrial preparation and Cyt c release from isolated mitochondria

The rats (male, strain SD) were starved for 12 h before mitochondrial preparation. Approximately 0.6 g of rat liver pieces were flushed with PBS on ice and homogenized with a glass Dounce homogenizer in 3 ml of mitochondrial buffer (MB) (210 mM mannitol, 70 mM

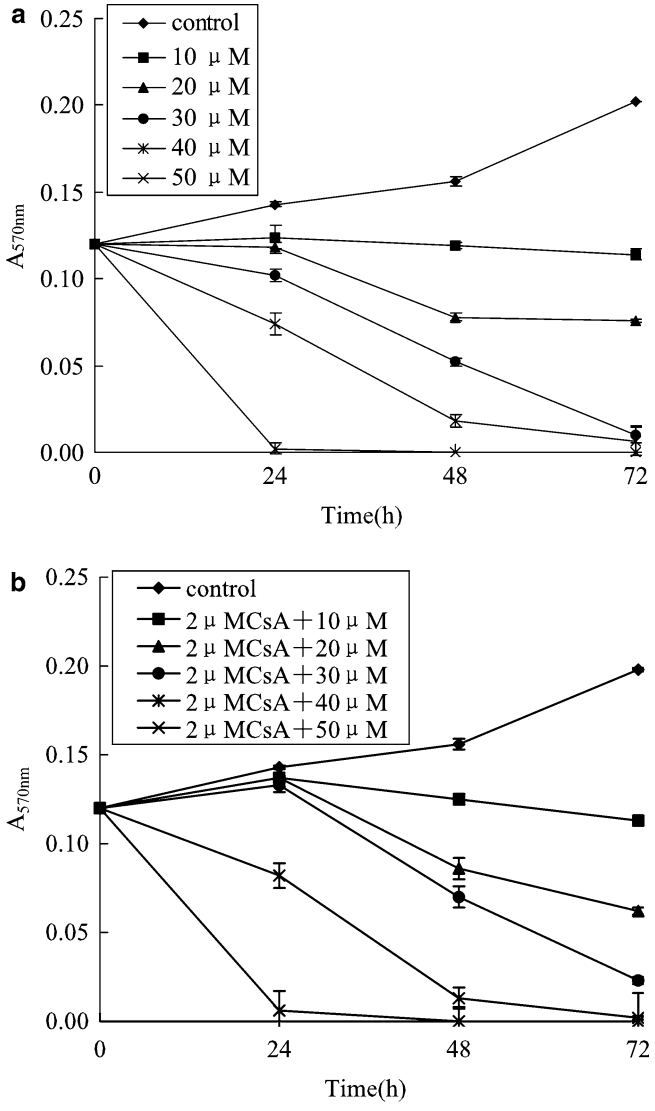

Fig. 2 Dose- and time-response of TBMS1 on growth of HeLa cells. HeLa cells were treated with various concentrations of TBMS1 (**a**) or 2 μM CsA + TBMS1 (**b**) for different time intervals. The cell proliferation was determined by MTT assay. The values are expressed as means ± SE of three independent experiments

sucrose, 20 mM Hepes-KOH, pH 7.5, and 1 mM EDTA containing 0.45% bovine serum albumin (BSA)). The homogenate was centrifuged at 1,300*g* for 10 min at 4°C. The pellet was dissolved in 1.5 ml MB containing 0.45% BSA and centrifuged for 10 min at 1,300*g*. All collected supernatants were recentrifuged at 17,000*g* for 15 min at 4°C for precipitation of mitochondria. Mitochondria pellets were resuspended in 3 ml of buffer B (210 mM mannitol, 70 mM sucrose, 10 mM succinate, 5 mM KCl, 0.1% BSA, 1 mM EGTA, 1 mM dATP, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride (PMSF) and 10 mM HEPES-KOH, pH 7.5). Mitochondria pellets were recentrifuged at 17,000*g* for 15 min at 4°C. Mitochondrial pellets were washed twice with buffer B, and resuspended in 300 μl of the same buffer. Protein concentration of mitochondrial fraction was determined by Lowry's method. Cyt c release was assayed as described previously with some modifications [25]. Briefly, mitochondria (100 μg protein) were incubated with various concentrations of TBMS1 with or without 2 μM CsA in a 100-μl assay buffer at 30°C for 1 h or incubated with 30 μM TBMS1 with or without 2 μM CsA at 30°C for the time indicated. At the end of incubation, mitochondria were pelleted by centrifugation at 20,000*g* for 15 min at 4°C. The resulting supernatant was analyzed with anti-Cyt c antibody by Western blotting.

Western blot analysis of Cyt c

Cells treated with or without test compounds were harvested by centrifugation at 1,000*g* for 5 min at 4°C. The cell pellets were washed twice with ice-cold PBS. For mitochondria enrichment, cells were suspended in 500 μl of ice-cold 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose [26]. Cells were homogenized with a 16-gauge needle and 1-ml syringe, and the homogenates were centrifuged at 750*g* for 5 min at 4°C. The supernatants were then centrifuged at 12,000*g* for 20 min at 4°C. The pellets were lysed with 100 μl of cell lysis buffer. The supernatant from this spin was taken as the cytosol fraction. Protein concentration was determined by Lowry's method. 50 μg protein/lane was loaded onto 12% SDS polyacrylamide gels, but 25 μg/lane for Western blot analysis of Cyt c of mitochondrial fraction. Separated protein was transferred to nitrocellulose membrane, and stained with Ponceau S to ensure equal protein loading. The membranes were blocked with 5% (w/v) fat-free milk in TTBS buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% (v/v) Tween-20) for 60 min and probed with the appropriate antibodies overnight at 4°C. Antibody against Cyt c was added in a final dilution of 1:1,000 in 5% fat-free milk in TTBS buffer. Antibody against *β*-actin was added in a final dilution of 1:500 in 5% fat-free milk in TTBS buffer. After being washed with TTBS, membranes were incubated with goat anti-mouse IgG-HRP antibody or goat anti-rabbit IgG-HRP antibody for 60 min. Proteins were detected using the Super West Pico kit.

Statistical analysis

Data were summarized as means ± SE. Student's *t* test was used for multiple comparisons between groups. *P* values less than 0.05 were considered to be statistically significant.

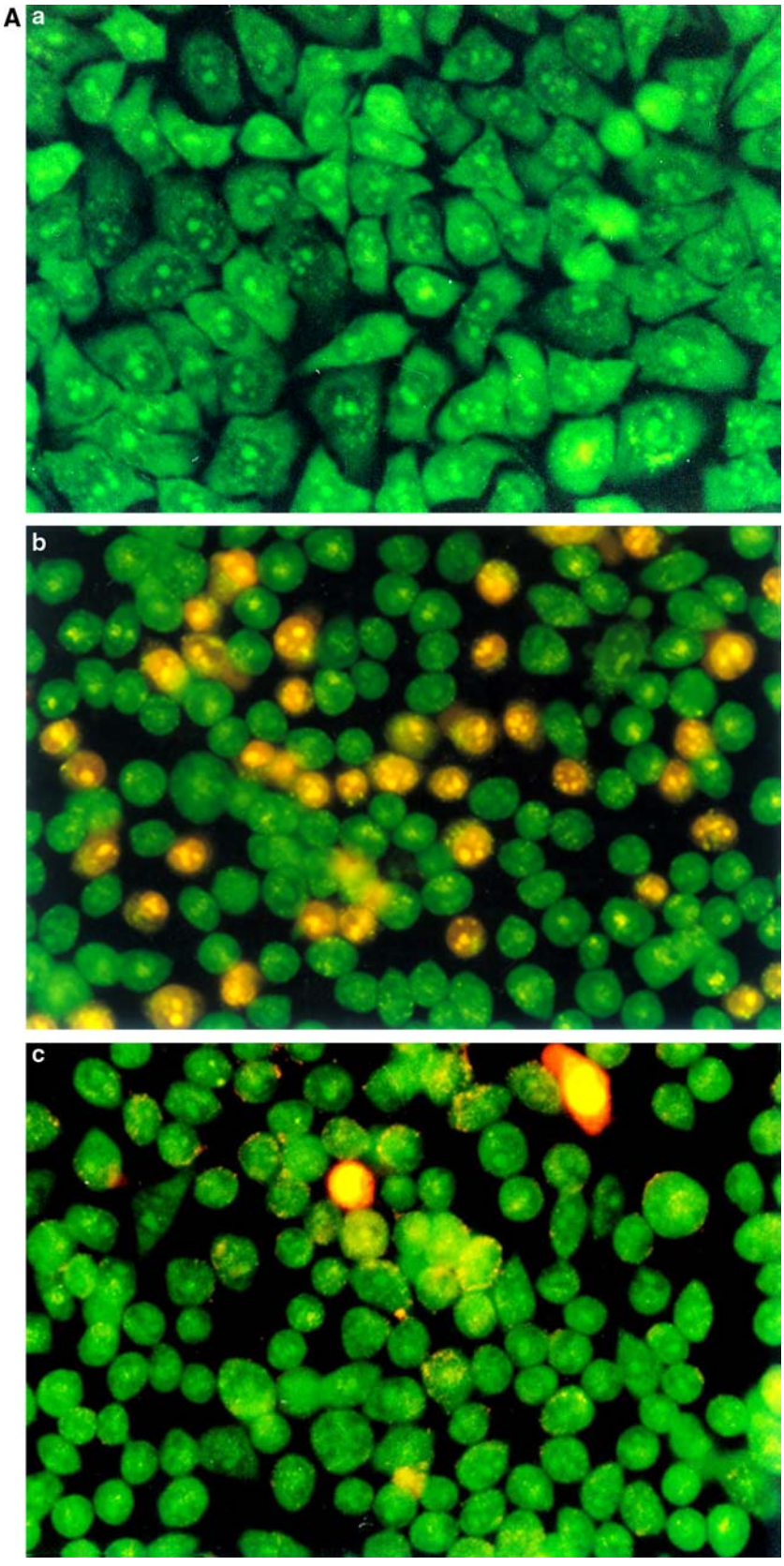

Fig. 3 Protective effect of CsA on TBMS1-induced apoptosis in HeLa cells by double staining with AO/EB and Annexin V/PI. (A) Cells were treated with 30 μM TBMS1 for 24 h and stained with AO/EB as described in ''Materials and methods''. Cells in which nuclei were *red* or *yellow–red* indicate apoptotic cells (original magnification×40). **a** Control (no TBMS1). **b** 30 μM TBMS1, 24 h. **c** 2 μM CsA + 30 μM TBMS1, 24 h. (B) Flow cytometric analysis of phosphatidylaerine externalization (annexin staining) with PI counterstaining. **a** Representative examples of flow cytometric histograms after 24 h of drug treatment. The appearance of cells with a high annexin signal and a low PI signal is characteristic of early apoptosis. Progression of apoptosis results in cells with a high annexin signal and a high PI signal (secondary necrosis). **b** Quantitation of early, late, and non-apoptotic cells after 24-h treatments with 30 μM TBMS1 or 2 μM CsA + 30 μM TBMS1. Means ± SE of three independent experiments

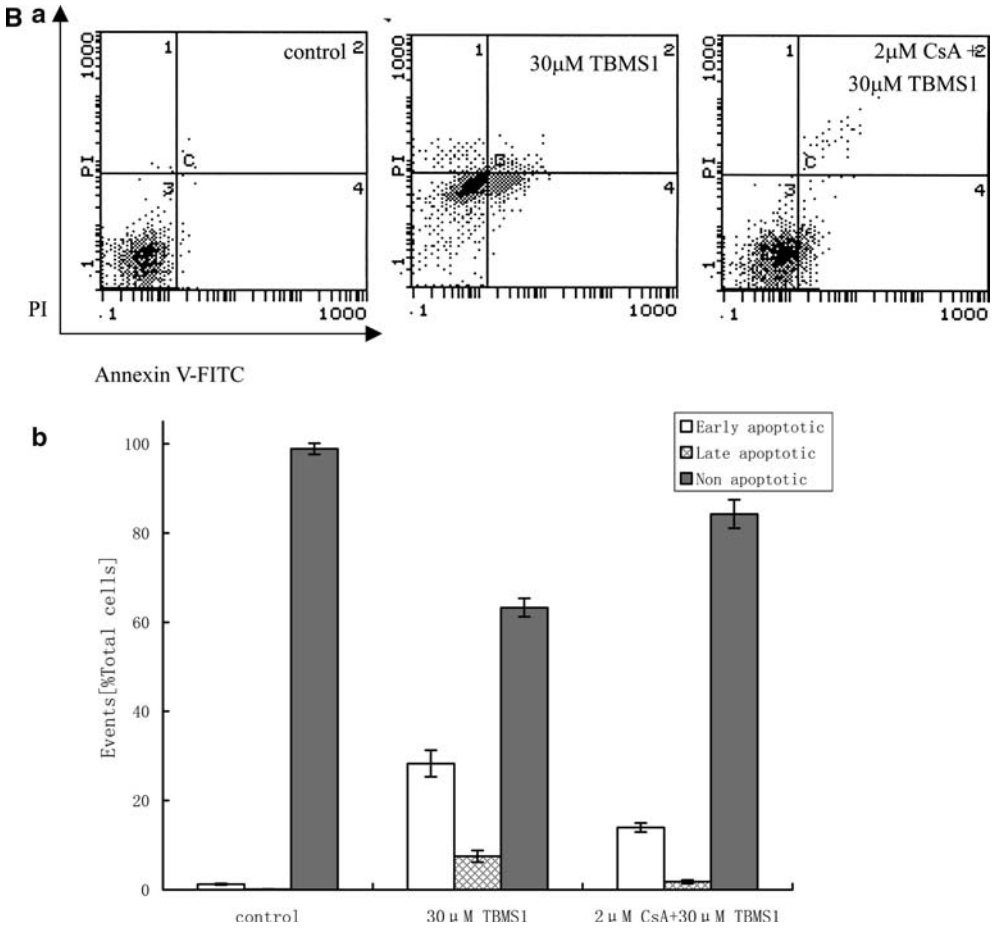

Fig. 3 (Contd.)

Results

Protective effect of CsA against TBMS1-induced growth inhibition of HeLa cells

Inhibition of cell growth by TBMS1 or CsA (2 μM) + TBMS1 was observed following treatment of different doses of TBMS1 for the time indicated. As shown in Fig. 2, the inhibitory activity of TBMS1 was dose- and time-dependent, and the estimated IC_{50} values after 24, 48, 72 h of treatment were 34.8, 27.1, and 17.1 μM. However, the estimated IC_{50} value after 24 h of CsA (2 μM) + TBMS1 was 41.7 μM. The results indicated that CsA partly protected HeLa cells from growth inhibitory effect of TBMS1.

Morphological analysis by fluorescence microscopy

Morphological analysis of cell death was investigated using AO/EB staining for fluorescence microscopy. AO is a vital dye that stains both live and dead cells, and EB stains only cells that have lost membrane integrity. Live cells appear uniformly green, whereas

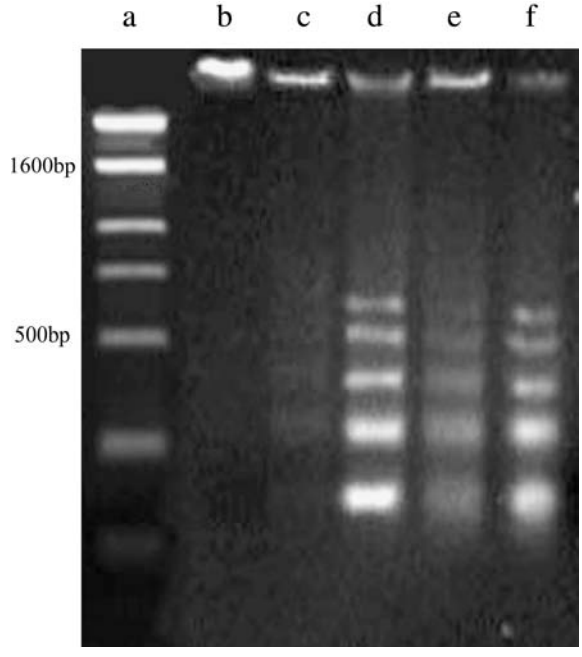

Fig. 4 Induction of DNA fragmentation in response to TBMS1 or CsA + TBMS1 exposure. **a** Marker. **b** Control (no TBMS1). **c** 2 μM CsA + 25 μM TBMS1, 24 h. **d** 25 μM TBMS1, 24 h. **e** 2 μM CsA + 50 μM TBMS1, 24 h. **f** 50 μM TBMS1, 24h

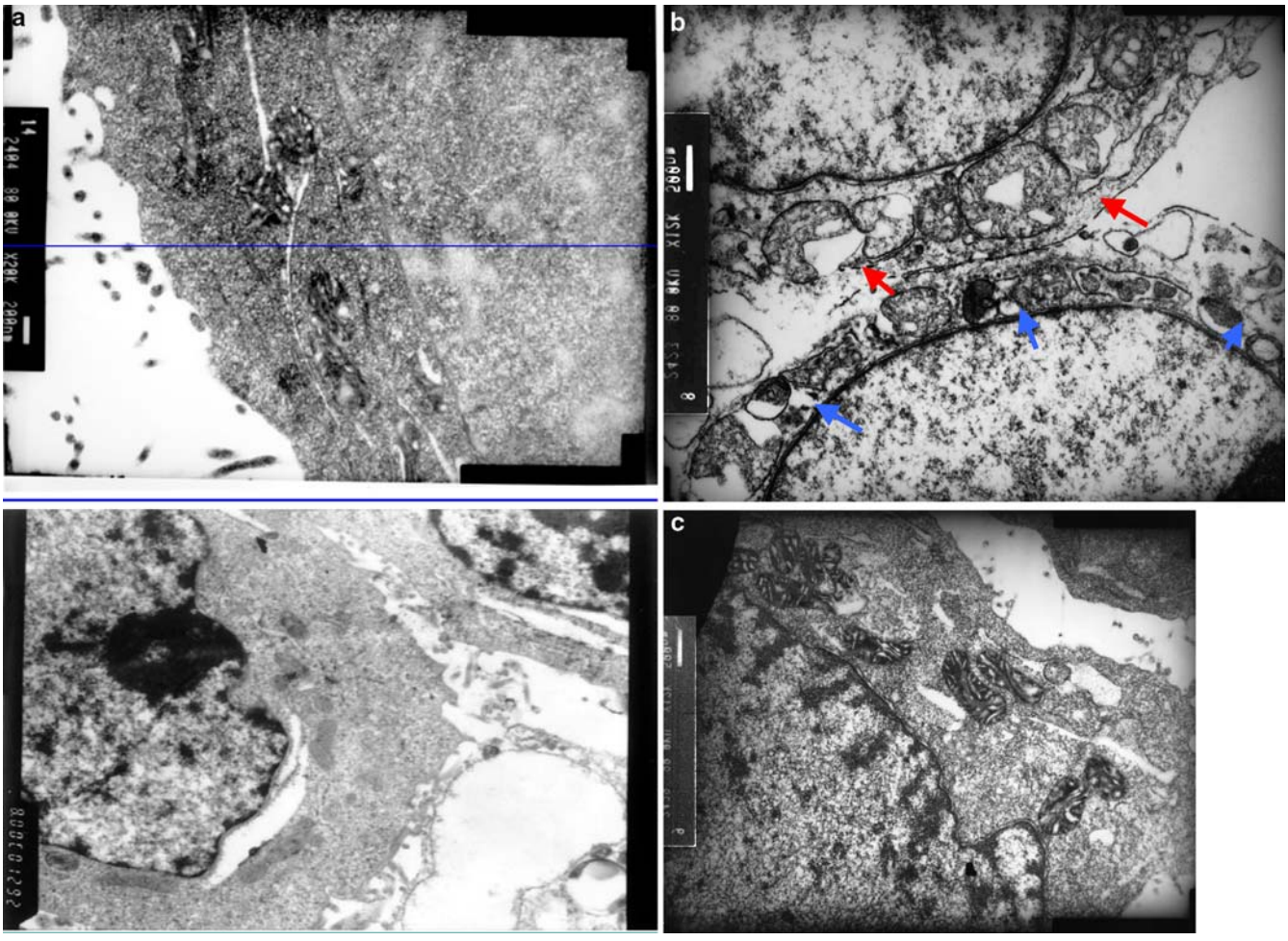

Fig. 5 Protective effect of CsA on TBMS1-induced swelling of mitochondria in HeLa cells by electron microscopy. Cells were treated with TBMS1 or CsA + TBMS1 and then processed for electron microscopy as described in "Materials and methods". **a** Control (no TBMS1). Mitochondrial membrane was intact and cristae were arranged in the form of concentrical ring or vertical line, congested and clear. **b** Cells treated with TBMS1 (20 μM, 12 h), mitochondria were swollen obviously. Mitochondrial membrane was vague or partly ruptured and cristae were obviously loose and dissolved, and vacuolization in mitochondria appeared (*red arrow*), and some mitochondria has high electrically dense and the cristae disappeared (*blue arrow*). **c** Cells treated with CsA (2 μM, 30 min) and then with TBMS1 (20 μM, 12 h). Previous treatment of CsA diminished damage at mitochondrial cristae and matrix induced by TBMS1, and the mitochondrial structure was basically normal. Mitochondria were orderly arranged, mitochondrial membrane was basically intact, cristae were congested and there was no vacuolization

late apoptotic cells stain orange with dots in the nuclei as a consequence of chromatin condensation. Necrotic cells were also stained orange, but these have a nuclear morphology resembling that of viable cells with no condensed chromatin. Obvious differences were observed in the nuclei of TBMS1-treated, CsA + TBMS1-treated and untreated HeLa cells after staining with AO/EB. AO/EB dyes stained morphologically normal nuclei green (Fig. 3A-a), whereas 30 μM TBMS1-treated cells demonstrated yellow, smaller and shrunken nuclei. These changes in nuclear morphology, which were observed after 24 h of 30 μM TBMS1 treatment, reflected chromatin condensation and nuclear shrinkage (Fig. 3A-b). However, in comparison with HeLa cells treated with TBMS1, HeLa cells treated with CsA (2 μM, 30 min) and then with TBMS1 (30 μM, 24 h) demonstrated lighter yellow, and slight nuclear shrinkage (Fig. 3A-c). The results indicated that CsA partly protected HeLa cells from apoptotic induction by TBMS1.

Loss of plasma membrane asymmetry during apoptosis

Changes in plasma membrane phospholipids, such as externalization of phosphatidylserine residues in the outer plasma membrane, are a characteristic marker of early apoptotic events [27, 28]. Phosphatidylserine externalization can be conveniently detected by fluoresceinated annexin V binding. Counterstaining with PI, which detects cells with compromised cell membrane integrity, allows one to distinguish among necrotic, early-apoptotic, and late-apoptotic cells [29, 30] (Fig. 3b).

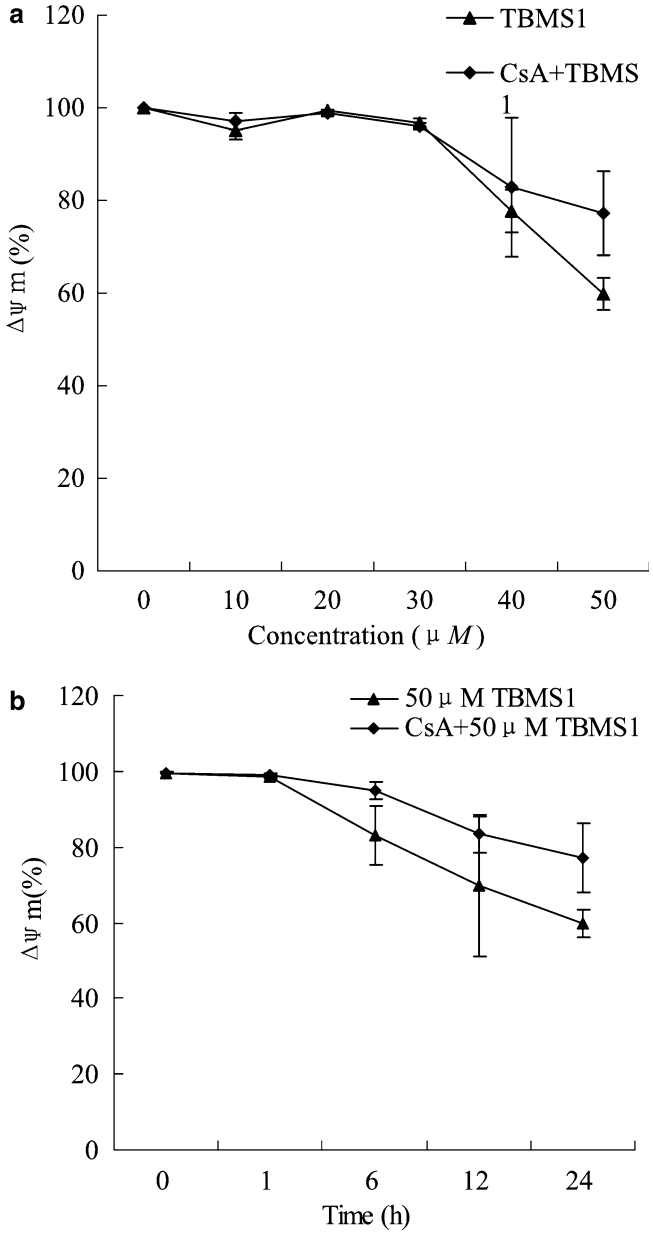

Fig. 6 Reversal of TBMS1-induced decline of Δψm by CsA in HeLa cells. **a** Cells treated with various concentrations of TBMS1 and with or without CsA. With the increase of concentrations of TBMS1, Δψm accordingly decreased, but previous addition of CsA partly countered decline of Δψm induced by TBMS1. **b** Cells treated with 50 μM TBMS1 with or without CsA. A time-dependent Δψm decline can be observed following 50 μM TBMS1 treatment for 1, 6, 12, 24 h, but previous addition of CsA partly countered decline of Δψm induced by TBMS1

Representative flow cytometric histograms(Fig. 3B-a) illustrated profound shifts in annexin V and PI signals in HeLa cells following 30 μM TBMS1 treatment for 24 h. The appearance of cells with a high annexin signal and a low PI signal is characteristic of early apoptosis. The progression of apoptosis results in cells with a hight annexin signal and a high PI signal characteristic of late apoptosis (secondary necrosis).

The quantitation of these effects (Fig. 3B-b) shows that a 24-h incubation with 30 μM TBMS1-induced profound apoptosis. Treatment with 2 μM CsA + 30 μM TBMS1 reduced the proportion of cells in the combined early and late apoptotic compartments, compared to treatment with 30 μM TBMS1. In particular, in the 2 μM CsA + 30 μM TBMS1-treated cultures, the early apoptosis compartment was diminished markedly. The differences between the early apoptosis compartments were significant ($P < 0.05$)

DNA fragmentation

Previous studies have shown that DNA fragmentation appeared following TBMS1 treatment in a dose- and time-dependent manner [9]. In this study, the same results were obtained on the induction of a ladder pattern typical of internucleosomal fragmentation by TBMS1, but CsA, a ligand of matrix cyPD, inhibited the activation of DNA fragmentation by TBMS1 (Fig. 4).

Ultrastructure of mitochondria

As shown in Fig. 5, CsA protected mitochondria against TBMS1-induced damage. In control group mitochondrial membrane was intact and cristae were arranged in the form of concentrical ring or vertical line, congested and clear (Fig. 5a). After exposure to 20 μM TBMS1 for 12 h, mitochondria were obviously swollen. Mitochondrial membrane was vague or partly ruptured and cristae were obviously loose and dissolved, and vacuolization in mitochondria appeared (red arrow), and some mitochondria has high electrically dense and the cristae disappeared (blue arrow) (Fig. 5b). 2 μM CsA diminished damage at mitochondrial crista and matrix induced by 20 μM TBMS1, and the mitochondrial structure was basically normal in CsA + TBMS1 treated group. Mitochondria were orderly arranged, mitochondrial membrane was basically intact, cristae were congested and there was no vacuolization (Fig. 5c).

Mitochondria involvement in TBMS1-induced apoptosis of HeLa cells

To examine the role of mitochondria in TBMS-induced apoptosis of HeLa cells, we analyzed the changes in MPT pore using the mitochondria dye R123 [31], a cationic fluorophore taken up by mitochondria as a result of their membrane potential. As shown in Fig. 6 a, b, TBMS1 decreased Δψm in a dose- and time-dependent manner, and CsA partly countered decline of Δψm induced by TBMS1.

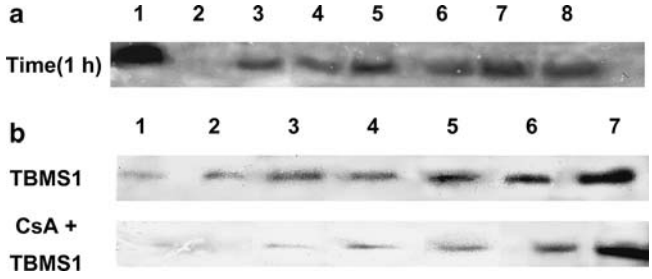

Fig. 7 Induction of Cyt c release from isolated rat mitochondria by TBMS1. **a** Dose-dependent course of Cyt c release. Lane 1: 2% Triton X-100; Lane 2: Control (no TBMS1); Lane 3, 5, 7: TBMS1 (10, 30, 50 μM), 1 h; Lane 4, 6, 8: 2 μM CsA + TBMS1 (10, 30, 50 μM), 1 h. **b** Time-dependent course of Cyt c release. Lane 1: Control (no TBMS1); Lane 2, 3, 4, 5, 6: 30 μM TBMS1 or 2 μM CsA + 30 μM TBMS1 (5, 10, 20, 40 min, 1 h); Lane 7: 2% Triton X-100

TBMS1 induces Cyt c release from isolated mitochondria

Considering the findings that TBMS1 induces $\Delta\psi$m decrease and Cyt c release in HeLa cells, we examined whether TBMS1 has direct effect on mitochondria. To this end, the isolated rat mitochondria were incubated with TBMS1. Release of Cyt c was analyzed by Western blotting. As shown in Fig. 7, TBMS1 induced a time- and dose-dependent release of Cyt c from mitochondria, which became evident within 1 h of incubation. However, TBMS1-induced Cyt c release was inhibited by previous addition of CsA, that is consistent with the results obtained in HeLa cells.

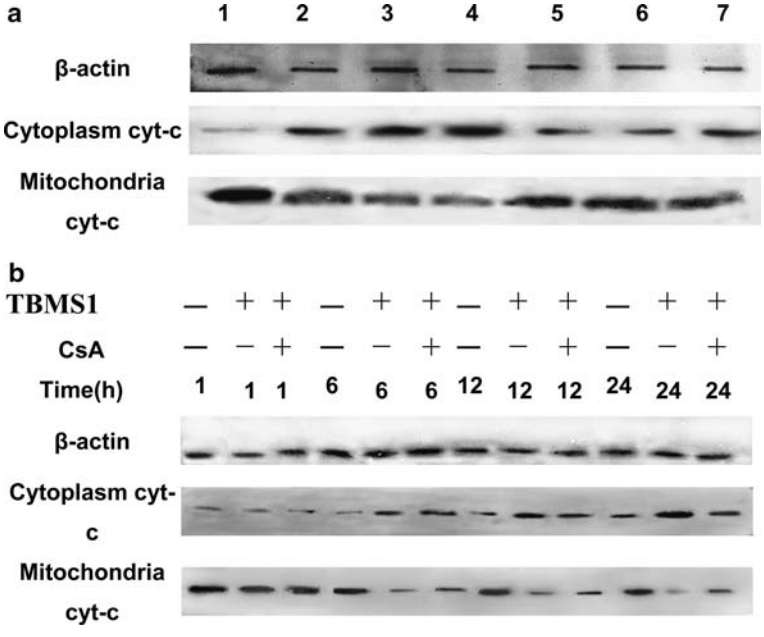

Fig. 8 Effect of treatment of TBMS1 on Cyt c level in HeLa cells by Western blotting. **a** Cells exposed to various concentrations of TBMS1 or 2 μM CsA + TBMS1 for 24 h. Lane 1: Control (no TBMS1); Lane 2, 3, 4: TBMS1 (10, 30, 50 μM), 24 h; Lane 5, 6, 7: 2 μM CsA + TBMS 1 (10, 30, 50 μM), 24 h. **b** Cells exposed to 30 μM TBMS1 or 2 μM CsA + 30 μM TBMS1 for 1, 6, 12, 24 h

Effects of TBMS1 on Cyt c level

The representative Western immunoblots showed that the monoclonal antibody for Cyt c detected a single band at an expected size of 15 kDa. Without TBMS1 treatment, most of the detectable Cyt c was in the mitochondria. After TBMS1 treatment, there was an increase in Cyt c level in the cytosolic fraction and an accordant decrease in Cyt c level in the mitochondrial fraction (Fig. 8 a, b). The increase of Cyt c in the cytosolic fraction correlated with the TBMS1 concentration. Importantly, as shown in Fig. 8, coincubation of CsA with TBMS1 inhibited the TBMS1-induced translocation of Cyt c in HeLa cells. Equal loading was confirmed by reprobing the blots with antibody against β-actin (Fig. 8 a, b).

Discussion

The mitochondrion has long been known both as a chemical powerplant and as a cellular compartment housing various biosynthetic pathways. However, studies on the function of mitochondrion in apoptotic cell death have revealed a versatility and complexity of these organelles previously unsuspected. The mechanisms proposed for mitochondrial involvement in cell death are diverse and highly controversial. In one model, mitochondria are seen as passive containers that can be made to leak out cytotoxic proteins. In other scenarios, however, certain more or less familiar aspects of mitochondrial physiology, such as oxidative phosporylation, generation of oxygen radicals, dynamic morphological

rearrangements, calcium overload, and permeability transition, are proposed to play crucial roles. Indeed, in recent years, multiple mechanisms have been proposed to explain mitochondrial function in cell death [32, 33, 34, 35]. Some of these involved aspects of mitochondrial physiology, such as the production of reactive oxygen species (ROS), opening of the PT pore, respiration [36, 37] or ATP synthase [38], to give just a few examples. On the other hand, growing evidence also supports the simple view of mitochondria as passive structures, acted upon by proteins that permeabilize the outer membrane to release apoptogenic proteins.

Another mechanism through which mitochondrial outer membranes can become permeabilized is through the mPTP [39]. PT involves the opening of a proteinaceous channel, known as the PTP, in the inner membrane which is believed to be comprised of complexes between CyPD and ANT protein in the inner membrane, associated with VDAC and the peripheral benzodiazepine receptor in the outer membrane. Sustained opening of the PTP allows the equilibration of ions between the matrix and cytoplasm, which implies a dissipation of the inner membrane potential, $\Delta\psi m$. This ionic redistribution leads to an osmotic swelling of the matrix and a consequent rupture of the outer membrane, because it cannot expand as much as the highly invaginated inner membrane.

CyclophilinsD has been proposed to sensitize the opening of the mPTP mainly based on the findings that CsA, a pseudo-CyPD substrate, hyperpolarizes $\Delta\psi m$ and inhibits apoptosis. However, overexpression of CyPD desensitizes HEK293 and rat glioma C6 cells to apoptotic stimuli [40]. Furthermore, CyPD can specifically repress ANT-1-induced apoptosis [41]. Importantly, CyPD is upregulated in human tumors of the breast, ovary, and uterus, suggesting that inhibition of PTP via upregulation of CyPD may play a role in tumorigenesis [41]. These observations strongly suggest that the inhibitory effect of CsA on Cyt c release and apoptosis is likely mediated through PTP-independent functions. Like CyPD, ANT has also been proposed to be a critical component of the PTP and apoptosis. Nevertheless, knocking out both ANT-1 and ANT-2 only marginally affects the mPTP as well as cell death induced by a variety of apoptotic stimuli [42], suggesting that ANTs are non-essential components of the mPTP and that they are dispensable for at least some forms of mPTP-associated cell death.

In apoptosis, coincident with the permeabilization of the outer mitochondrial membrane, there is typically a rapid reduction in the mitochondrial membrane potential [43]. Recent studies have shown that this loss of $\Delta\psi m$ reflects a block of respiratory function which is due to the cleavage and inactivation of electron transport chain constituents by activated caspases [44, 45, 46].

It is evident that the loss of Cyt c, an essential player in the respiratory chain, can inhibit the electron transfer process and thus lower ATP levels. Since Cyt c delivers electrons from Cyt bc_1 to Cyt c oxydase, the complete loss of Cyt c could similarly lead to accumulation of electrons in the chain and oxygen radical formation.

In the present study, the facts obtained from the experiments substantiate a critical role of mitochondria in TBMS1-induced apoptosis. We first confirmed that CsA partly protected HeLa cells from growth inhibitory effect of TBMS1, and the ability of TBMS1 to rapidly induce apoptosis in HeLa cells could be partly countered by CsA, and subsequently demonstrated that TBMS1 decreased mitochondrial membrane potential and induced Cyt c release by a mechanism inhibited by CsA, and furthermore proved that TBMS1 directly acted on mitochondria and induced apoptosis of HeLa cells dose-dependently in accordance with increases in cytosolic Cyt c.

One important point to be stressed is that the effects of TBMS1 on mitochondria in this study were observed at rather high concentrations of TBMS1, (>20 μM). This is in accordance with most of the data reported to date about the proapoptotic activity of TBMS1 in cancer cells [8, 9], with the exception of HL-60 cells [7], which are sensitive to low TBMS1 concentrations.

The other important point to be stressed is that the effects of TBMS1 on mitochondria in this study were observed at rather various concentrations of TBMS1, i.e., in 20–50 μM range, in the different experiments. For instance, effect of TBMS1 on ultrastructure of mitochondria was detected at 20 μM of TBMS1, but time-dependent $\Delta\psi m$ decline in HeLa cells induced by TBMS1 was not detected until at 30 μM of TBMS1. Therefore, this phenomenon may be based on different methodological approaches. Nevertheless, it is generally indispensable to select one main concentration to draw an interconnected conclusion.

Taken together, the findings mentioned above suggest that TBMS1 opens the PT pore, thereby decreasing membrane potential, releasing Cyt c from mitochondria, and further causing a series of events consistent with the established mechanistic models of apoptosis. The results suggest that a significant part of the proapoptotic effects of TBMS1 reported in the literatures could be partly explained by a direct action of TBMS1 on the mitochondria of target cells. Therefore, it is reasonable to conclude that the mitochondria are involved in TBMS1-induced apoptosis of HeLa cells, and the release of Cyt c to the cytosol may initiate a mitochondrial pathway of apoptosis.

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