Apoptosis of Human Nasopharyngeal Cacinoma CNE-2Z Cell Line Induced by Tubeimoside I Isolated from Bolbostemma Paniculatum*

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Abstract **Aim** Tu-Bei-Mu″, Bolbostemma paniculatum (Maxim.) Franquet (Cucurbitaceae), a traditional Chinese medicinal plant, was listed in the Supplement to Compendium of Materia Medica, compiled in Qing Dynasty. Our prvious studies revealed that tubeimoside I(TBMS1), a triterpenoid saponin isolated from the tubers of Bolbostemma paniculatum, showed potent antitumor effect. This study was designed to investigate the effect of TBMS1 on the apoptosis of human NPC cell line CNE-2Z. **Methods** Cell growth inhibition mediated by TBMS1 was measured by MTT after treatment with TBMS1. The effect of TBMS1 on apoptotic induction in CNE-2Z cells was determined using flow cytometry, fluorescence microscopy, electron microscopy, and DNA agarose gel electrophoresis, respectively. Western blot analysis was performed to detect changes of apoptosis-related genes bcl-2 and bax protein expression. **Results** TBMS1 displayed growth inhibitory activity against CNE-2Z cells with IC_{50} values of 32.5, 20.7, 16.7 μmol/L for 24, 48, and 72 hours, and CNE-2Z cells showed typical apoptotic morphological features observed by fluorescent microscopy and electronic microscopy. In CNE-2Z cells occurred typical DNA ″Ladder″ bands after being exposed to TBMS1 (10μmol/L, 24, 48, 72 hours; 30, 40, 50, 60μmol/L, 12 hours; 50μmol/L, 1, 3,5, 12, 24 hours50, 60μmol/L). Sub-G1 peak was found using flow cytometry. When CNE-2Z cells were exposed to TBMS1(50 μmol/L, 12 hours), the apoptosis index was 72.8%. The down regulation and phosphorylation of bcl-2 (an inhibitor of apoptosis) were detected at 1 hour after the addition of TBMS1. In contrast, the levels of bax(death agonist) appeared to be significantly upregulated at 1 hour after the addition of TBMS1. **Conclusion** TBMS1 can induce the apoptosis of CNE-2Z cells, and the induction of apoptosis mediated by TBMS1 in CNE-2Z cells is closely associated with downregulation and phosphorylation of bcl-2, and bax activation.

Key Words: Bolbostemma paniculatum; Tubeimoside; Tubeimoside I; Apoptosis; Apoptosis-related gene; CNE-2Z cells

Nasopharyngea carcinoma (NPC) is a rare malignancy in most parts of the world, where the annual incidence of the disease is generally less than one per 100,000 population in either sex[1]. Among the handful of populations that are known to deviate from this low-risk pattern, the highest incidence is observed in southern Chinese who reside in central Guangdong Province and speak the Cantonese dialect[1,2] . Radiotherapy is the mainstay in the treatment of NPC. The overall 5-year survival rate is only 50-60%, and the main causes of therapeutic failure are loco-regional recurrence and distant metastasis. However, the combination of radio- and chemotherapy has improved the survival rate. Therefore, chemotherapy and discovery of new drugs for the treatment of NPC are essential and of special significance.

″Tu-Bei-Mu″, Bolbostemma paniculatum (Maxim.) Franquet (Cucurbitaceae), a traditional Chinese medicinal plant, was listed in the Supplement to Compendium of Materia Medica, compiled in Qing Dynasty[3]. Its stem tuber was applied for the treatment of the breast carcinoma in ancient times, and for the treatment of the esophageal cancer in Yanting county of Sichuan Province and in Shanxi province, during the seventies of last century, which has shown considerable efficiency, but the side-effects, such as nausea, vomiting, etc., were quite serious. TBMS1 was isolated from tubers of Bolbostemma paniculatum[4,5]. Our previous study demonstrated its antitumor and antitumor-promoting effects[6,8], and revealed that in TBMS1-treated human malignant tumor cell lines, the incorporation of

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^{3}H –thymidine into DNA fraction was suppressed, suggesting that normal synthesis and maturation of DNA were inhibited in those cells [8]. In general, when nuclear DNA is damaged, cells initiate a response that includes cell cycle arrest, apoptotic cell death, transcriptional induction of genes involved in DNA repair.

The induction of apoptosis is a major action mechanism of many common cytotoxic agents, and the dysregulation of apoptotic pathways can play a significant role in the growth and therapeutic responsiveness of cancer cells[9,10] . The regulation of cell growth is a homeostatic balance between stimulatory and inhibitory signals. Negative growth control by tumor suppressor genes, differentiation factors, and programmed cell death(apoptosis) factors, provides alternative strategies for treatment of malignancies and other diseases. Among them, apoptosis factors are a highly attractive and widely studied area to search for more effective agents for treatment of human cancers. Variety of in vivo and in vitro studies published in recent years suggested that many chemotherapeutic agents could induce apoptotic cell death in different cancer cells. Furthermore the sensitivity of a cell to cancer chemotherapy is determined in part by how readily the tumor cells undergo apoptosis.

In the present study, we confirmed that CNE–2Z cells did indeed undergo TBMS1–induced apoptosis, as determined by flow cytometry, fluorescence and electron microscopy, and DNA fragmentation assay, and revealed that the induction of apoptosis mediated by TBMS1 in CNE –2Z cells is closely associated with low bcl–2 expressing level and bcl–2 phosphorylation, and overexpression of bax.

MATERIALS AND METHODS

Collection of "Tu–Bei–Mu" and Preparation of TBMS1 "Tu –Bei –Mu" was collected at Shanxi Province (China) and identified by Professor Quan Y (Department of Botany, Shanxi 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. TBMS1 was isolated from tubers of Bolbostemma paniculatum (Maxim.) Franquet (Cucurbitaceae) by a modification of the methods reported previously[4,5] . Its chemical structure was established by Kong et al.[5] (Fig.1).

Chemicals 3 –(4,5 –dimethylthiazol –z –yl) –2,5 –diphenyl–tetrazolium bromide (MTT), RNase, propidium iodide (PI), Hoechst 33342 and Triton X–100 were purchased from Sigma, and newborn calf serum (NCS) was obtained from Sijiqing Biological Material Co.(Hangzhou, China). All other chemicals used were of reagent grade.

Cell Line and Cell Culture The poorly differentiated NPC CNE–2Z cell line was derived from a Cantonese patient and established by Gu et al. [11]. CNE–2Z cells were grown in RPMI 1640 medium supplemented with 10% heat–inactivated NCS, 100 u/ml penicillin and 100 μg/ml streptomycin, and maintained under humidified 95%air/5%CO_2 at 37℃ in monolayer.

MTT Assay MTT assay was performed in CNE –2Z cells to measure the cytotoxicity of TBMS1 to tumor cells[12] . Single–cell suspensions were prepared and seeded into 96 well microculture plates with 2.0×10^8 cells/L (90μl/well). Cells were cultured for 12 hours before addition of TBMS1. TBMS1 was diluted with RPMI 1640 medium and added to each well in a volume of 10μl. Cells were incubated at 37℃ for the time indicated. MTT solution (5mg/ml) was aliquoted to each well in a volume of 20μl, and 5 hours later 100μl of the solubilization solution (10%SDS–5% isobutyl alcohol–0.012mol/L HCl (w/v/v)) was added into each well. The plates were allowed to stand overnight in

each well using an ELISA reader. Control wells contained all of the agents presented in the treated wells except TBMS1. Each experimental point was performed in three replicates. The 50% inhibitory concentration (IC50) was determined from dose- and time-response data from at least three experiments.

Fluorescence Microscopy The occurrence of apoptosis or necrosis was visualized using a modified PI-Hoechst 33342 double staining assay. Cells were plated at a density of 1×10^6/ml in six well plates and allowed to incubated overnight. Cells were cultured for 12 hours before addition of TBMS1. TBMS1 was diluted with RPMI 1640 medium and added to each well in a volume of 10 μl. Cells were incubated at 37℃ for the time indicated. Then the Cells were collected by centrifugation at 1300r/min. 0.5ml of fresh growth medium was added to each tube and mixed. At the same time, Hoechst 33342 dye was added to the solution to a final concentration of 10g/ml. Cells were water-bathed at 37℃ for 15 min. Medium was aspirated, followed by resuspension in growth medium containing 50 μg/ml PI dye and icebath for 15 min. The cells were then spun and resuspended in 100 μl of PBS. Apoptotic cells were scored as those that exhibited the DNA condensation characteristic of apoptotic cells, visible upon Hoechst 33342 staining, but did not stain intensively with PI. Necrotic cells were scored as those staining substantially above background levels with PI. The cells remaining on cover-slips were photographed on a fluorescence microscope sequentially using filters for both Hoechst 33342 dye and PI, at a magnification of ×200.

Electron Microscopy Cells were treated with 50 μmol/L of TBMS1 for 24, 48 hours. For transmission electron microscopy, after 2 rinses in 0.1 mol/L PBS, the cells were first fixed in o.1 mol/L PBS containing 2.5% glutaraldehyde at a pH of 7.5. They were then fixed in 0.1 mol/L PBS containing 1% osmium tetroxide(OsO_4) at a pH of 7.2. Samples were rinsed two times in distilled water and dehydrated in an ethanolic series culminating in two changes of 100% acetone. Samples were then placed in a mixture of Spur resin and acetone (1:1) for 30 min, followed by 2 hours in 100% resin with 2 changes. Finally, samples were placed in fresh 100% resin in molds and polymerized at 70℃ for 8 hours to 3 days. The cells were embedded into Epon 812(TAAB, Berkshire, United Kingdom) and sliced into 60 nm sections. The ultrathin sections were contrasted with uranyl acetate and lead citrate and then examined with a transmission electron microscope[13].

Analysis of Cell Cycle Progression. CNE-2Z cells were seeded at a density of 5×10^8 cells/ml of medium in 6-well culture plates. TBMS1-treated and control cells were collected after the indicated time by centrifugation and fixed with 75% ethanol. After washing with ice-cold PBS, cells were suspended in about 0.3ml of PBS, 0.5ml of PI solution containing 0.1% Triton X-100, Rnase 20 mg/L, and then PI 50 mg/L were added. The cells were kept at 4℃ for 30 min. The suspension was filtered through 50μm nylon mesh, and the DNA content of stained nuclei was analyzed by flow cytometry [14]. Cell cycle was analyzed by using Multicycle-DNA Cell Cycle Analyzed Software.

DNA Isolation and Gel Electrophoresis. After treatment, cell cultures were incubated at 37℃ for the time indicated. DNA was isolated from TBMS1-treated and control cells. Cells were washed twice with PBS and centrifuged for 5min at 800×g. Cell pellets were re-suspended at 1×10^7 cells/ml and subjected to lysis and proteolytic digestion in a buffered solution containing 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8.0), 0.5%(v/v) Triton X-100 and 100μg/ml proteinase K, and were incubated for 2-3 hours at 50℃. Crude DNA was extracted with phenol-chloroform (1:1,v/v) for 10min, followed by centrifugation at 12,000×g for 5min. The supernatants containing fragmented DNA were precipitated overnight with 2 volumes of absolute ethanol and 1/10 volume of 3 mol/L NaAc (pH 5.2) at -20℃. DNA precipitates were recovered by centrifugation at 12,000×g for 5 min, dried at room temperature for 15min, re-suspended in 50 μl TE buffer containing 10mmol/L Tris-HCl (pH8.0), 1 mmol/L EDTA (pH 8.0) and 20μg/ml Rnase and water-bathed at 37℃ for 30 min. DNA concentration was calculated by determining absorption at 260nm. DNA electrophoresis was carried out in 1.5% agarose gel. Before electrophoresis, loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40%(w/v) saccharose) was added to each sample. Approximately 10μg DNA was loaded into each well and electrophoresis was carried out in 0.5×TBE buffer at pH 8.0 (45 mmol/L Tris boric acid, 1 mmol/L EDTA). DNA ladder marker was applied to provide size markers. The migration time was 4 hours at 45 v and 15 mA, After the gel

was soaked in TBE containing 0.5μg/ml EB(ethidium bromide), DNA was visualized by transillumination in UV light and photographed[15].

Western Blot Analysis. For Western blotting, CNE-2Z cells cultured in culture bottles were harvested after the indicated time. After being washed with ice-cold PBS twice, cells were lysed for 30 min on ice in appropriate volume ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl at pH 8.0, 0.02% NaN_3, 0.1% SDS, 1% NP-40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 100 μg/ml PMSF(phenylmethylsulfonyl-fluoride). Debris was removed by centrifugation for 15 min at 12, 000×g, 4℃. Protein concentrations were determined using Bradford´s method. Soluble protein was separated by electrophoresis on 10% SDS polyacrylamide gels and blotted onto nitrocellulose filter. The filters were first stained to confirm uniform transfer of all samples and then incubated in blocking solution (5% nonfat dry milk in PBS) for 2 hours at room temperature. After being blocked, the filters were reacted with monoclonal antibody anti-bcl-2 or anti-bax at a dilution of 1:1000 for 2 hours at room temperature, followed by extensive washes with PBS twice and TBST twice. Filters were then incubated with horseradish peroxidase-conjugated secondary antibody (1:1000, Amersham) for 1 hour, washed with TBST, and developed using the Super Signal West Pico kit.

Statistics. Values were expressed as $\bar{x}\pm s$, and statistical significance was assessed using t-test. P values less than 0.05 were considered to be significant.

RESULTS

Inhibition of HeLa cell growth by TBMS1 All the experiments were repeated at least three times. Inhibition of cell growth was observed following treatment of different doses of TBMS1 for 24, 48, 72 hours. The inhibitory activity was dose- and time-dependent (Fig.2), and the estimated IC_{50} values after 24, 48, 72 hours of TBMS1 treatment were 32.5, 20.7, 16.7 μmol/L.

Morphological analysis by fluorescence microscopy. The occurrence of apoptosis was verified by Hoechst- and PI-staining, which detects chromatin condensation, one of the hallmarks of apoptotic cell death [16]. Obvious differences were observed in the nuclei of TBMS1-treated and -untreated CNE-2Z cells after staining with Hoechst and PI. The Hoechst and PI dyes stained morphologically normal nuclei bluish, whereas TBMS1-treated cells demonstrated reddish and smaller nuclei. These changes in nuclear morphology, which are observed after 5 hours of 50μmol/L TBMS1 treatment, reflected chromatin condensation and nuclear shrinkage (Fig. 3).

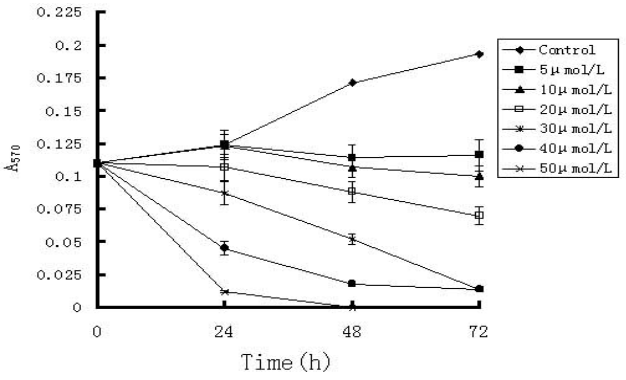

Fig. 2 Dose and time-response of tubeimoside I on growth of CNE-2Z cells. CNE-2Z cells were treated with various concentration of tubeimoside for different time intervals. The cell proliferation was determined by MTT assay. The values are expressed as means± S of 3 independent experiments.
-◆-, Control; —■—, 10μM, —▲—, 15μM; —□— 20μM ; -□-25μM; —●—, 30μM; --×-- 35μM.

Morphological analysis by electron microscopy. The occurrence of apoptosis was also verified by higher magnification of an individual CNE-2Z cell. CNE-2Z cells were treated with 50 μmol/L TBMS1 for 5 hours, and then CNE-2Z cell in the middle stage of TBMS1-induced apoptosis displayed vacuolization of cytoplasm components, condensation and margination of nuclear chromatin against nucleus envelope, clear outline of nucleus membrane and intact cell membrane (Fig. 4B). CNE-2Z cell in the late stage of TBMS1-induced apoptosis exhibited vacuolization of cytoplasm components, obvious condensation and spot- and lump-shaped margination of nuclear chromatin against nucleus envelope, disruption of cell membrane, and leakage of cytoplasm components (Fig. 4C). These changes in cell morphology reflected the features observed in most tumor cells undergoing apoptosis induced by anti-tumor agents.

Effect of TBMS1 on cell cycle. To assess the effect of TBMS1 on the cell cycle, CNE-2Z cells were treated with 10μmol/L TBMS1 for 24, 48, 72 hours or with 50μmol/L TBMS1 for 1-24 hours, respectively, and then hypotonically lysed in the presence of propidium iodide to stain the nuclear

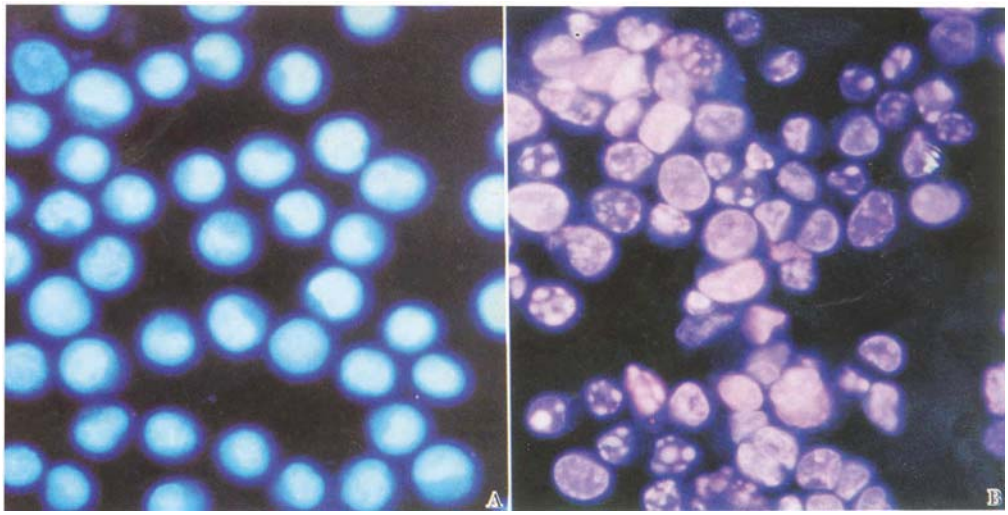

Fig. 3 Morphological analysis by fluorescence microscopy
A: Control, ×200; B: CNE-2Z cells treated with 50μmol/L tubeimoside 1 for 5h, ×200.

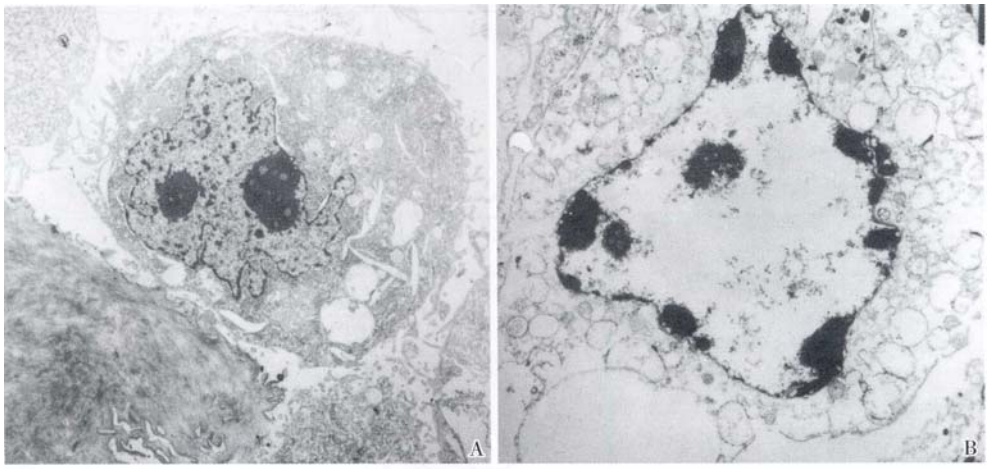

Fig. 4 Morphological analysis by electron microscopy
CNE-2Z cells were treated with 50 μmol/L tubeimoside 1 for 5h.
A: Control, ×5000; B: CNE-2Z cell in the middle stage of tubeimoside 1-induced apoptosis, ×5000;
C: CNE-2Z cell in the late stage of tubeimoside 1-induced apoptosis, ×5000.

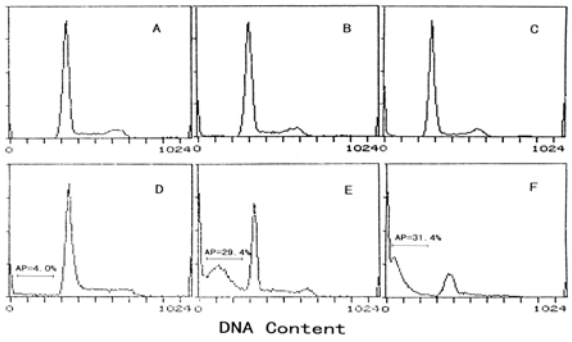

Fig.5 Flow cytometric analysis of cell cycle distribution in CNE-2Z cells treated with 10μmol/L tubeimoside for 24, 48, 72h. A, B, C: CNE-2Z cells untreated for 24, 48, 72h; D, E, F: CNE-2Z cells treated with 10μmol/L tubeimoside 1 for 24, 48, 72 h.

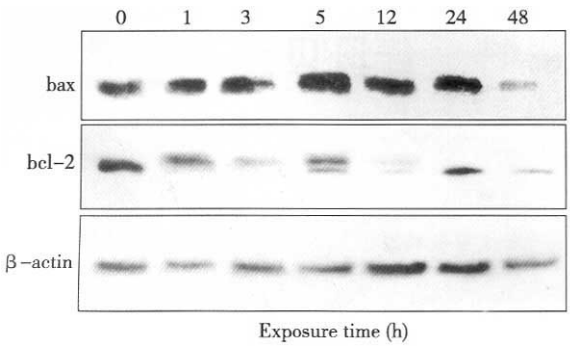

Fig.8 Western blot analysis of bcl-2 and bax in tubeimoside Ⅰ-treated CNE-2Z cells.
CNE-2Z cells treated with 50 μmol/L tubeimoside Ⅰ for 1, 3, 5, 12,24,48 h.
lane 1: control; lane 2: 1 h; lane 3: 3 h; lane 4: 5 h; lane 5: 12 h;lane 6: 24 h; lane 7: 48 h.

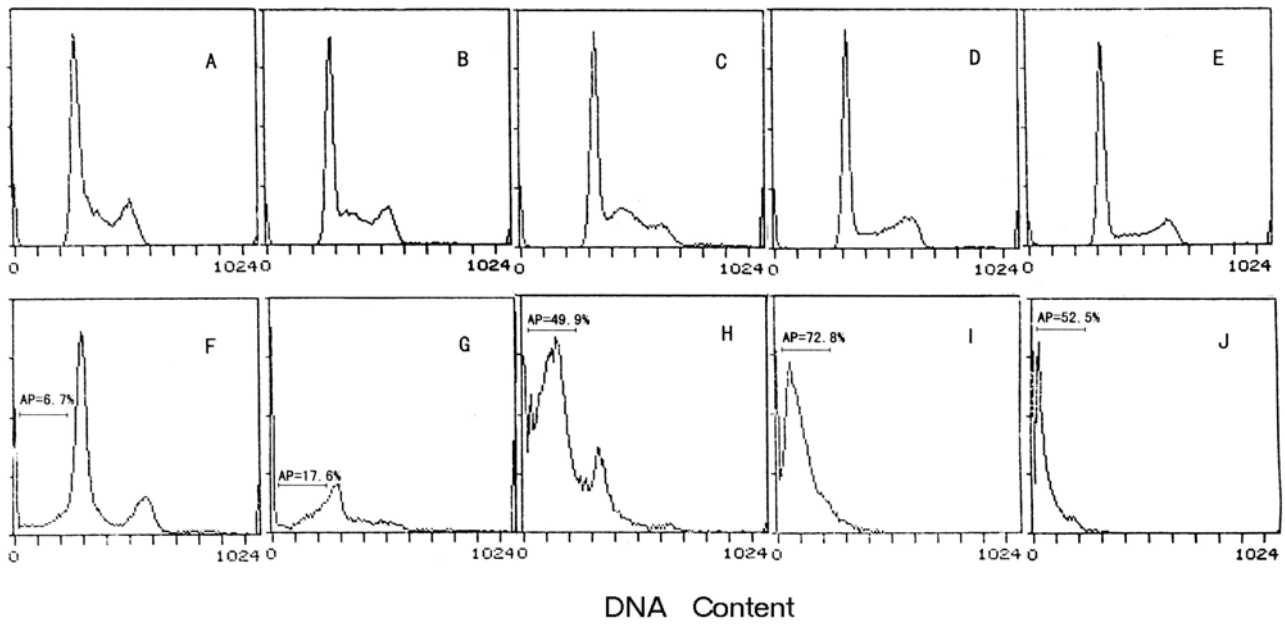

Fig. 6 Flow cytometric analysis of cell cycle distribution in CNE-2Z cells treated with 50 μmol/L tubeimoside Ⅰ for 1, 3, 5, 12, 24h.
A, B, C, D, E: CNE-2Z cells untreated for 1, 3, 5, 12, 24 h; F, G, H, I, J: CNE-2Z cells treated with 50μmol/L tubeimoside Ⅰ for 1, 3, 5, 12, 24 h.

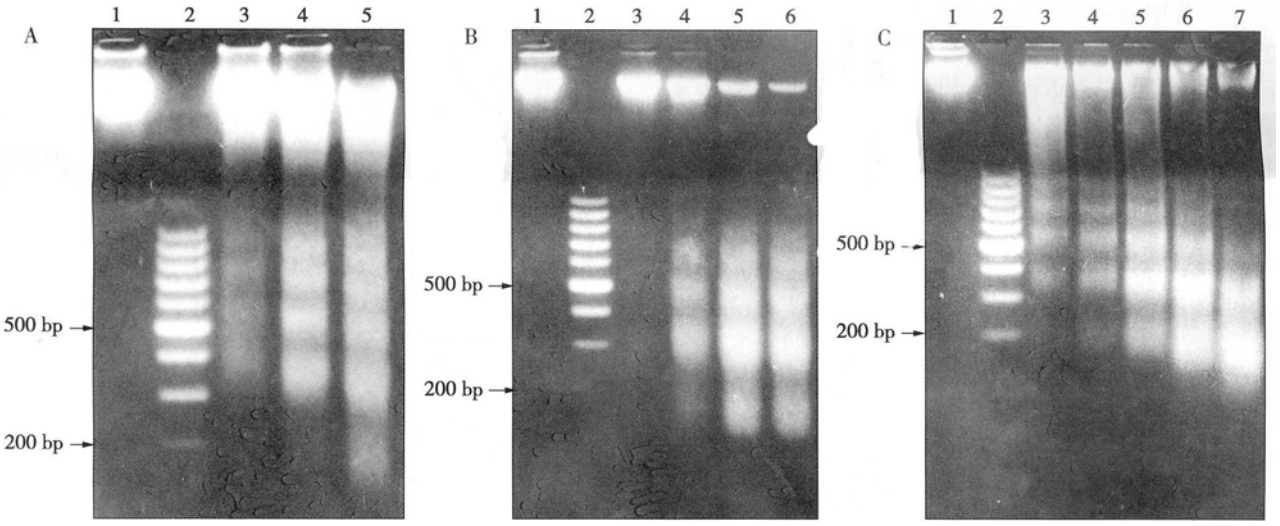

Fig. 7 DNA fragmentation in tubeimoside Ⅰ-treated CNE-2Z cells on agarose electrophoresis
A: CNE-2Z cells treated with 10 μmol/L tubeimoside Ⅰ for 24, 48, 72 h.
lane 1: control; lane 2: marker; lane 3: 24 h; lane 4: 48 h; lane 5, 72 h.
B: CNE-2Z cells treated with 30, 40, 50, 60 μmol/L tubeimoside Ⅰ for 12 h. lane 1: control; lane 2: marker; lane 3: 30 μmol/L; lane 4: 40 μmol/L; lane 5: 50 μmol/L; lane 6: 60 μmol/L.
C: CNE-2Z cells treated with 50 μmol/L tubeimoside Ⅰ for 1, 3, 5, 12, 24 h. lane 1: control; lane 2: marker; lane 3: 1 h; lane 4: 3 h; lane 5: 5 h; lane 6: 12 h; lane 7: 24 h

DNA. A fraction of TBMS1-treated cells appeared as a distinct peak below the G1 peak (sub-G1 population) compared with control untreated cells, and increased in dose- and time-dependent manner (Fig.5,6), and the apoptosis indices are 4.0%, 29.4% and 31.4% (10μmol/L, for 24, 48, 72 hours), and 6.7%, 17.6%, 49.9%, 72.8%, and 52.5% (50μmol/L, for 1, 3, 5, 12, 24 hours), respectively. The appearance of a sub-G1 population of cells is a potential indicator of apoptosis, thus showing that TBMS1 may inhibit the growth of CNE-2Z cells by inducing cell death. It should be noted that the sub-G1 population of CNE-2Z cells treated with 50μM TBMS2 for 24 hours was far less than that

Tab.1 Cell Cycle Distribution of CNE-2Z Cells Treated with 10μmol/L Tubeimoside (%, $\bar{x}\pm s$, n=3)

Cell cycle	24 hours		48 hours		72 hours	
	Control	Treatment	Control	Treatment	Control	Treatment
Sub-G_1	0.4±0.2	4.0±0.4[c]	2.0±0.1	29.4±0.3[c]	1.5±0.1	31.4±3.5[c]
G_0/G_1	70.3±2.8	64.3±0.1[a]	76.6±0.4	67.6±0.7[c]	77.5±2.7	67.7±2.2[b]
S	22.1±1.1	33.0±0.1[c]	16.6±0.2	27.0±0.7[c]	15.6±0.4	30.1±1.4[c]
G_2/M	7.7±0.4	2.6±0.1[c]	6.8±0.3	5.5±1.5	6.9±2.3	2.2±0.9[a]

Compared with control, [a]$P<0.05$, [b]$P<0.005$, [c]$P<0.001$

of CNE-2Z cells treated with 50μM TBMS2 for 12 hours, suggesting that a part of CNE-2Z cells could collapse from the treatment of TBMS2 (50μM, 24 hours).

DNA Fragmentation Previous studies have shown that DNA fragmentation is not an artifact of the process of lysis and is characteristic of endonuclease cleavage of DNA[17,18], and it is widely recognized that DNA fragmentation assay is one of standard endpoint techniques for measuring apoptosis[19]. In the present study, this technique was applied to CNE-2Z cells at multiple time points after their exposure to 50μmol/L TBMS1. Results of electrophoretic separation of DNA extracted from the cells are shown in Fig. 7. which illustrates our observation of dose- and time-dependent DNA fragmentation. In CNE-2Z cells occurred typical DNA "Ladder" bands after being exposed to TBMS1 (10 μmol/L, 24, 48, 72 hours; 30, 40, 50, 60μmol/L, 12 hours; 50 μmol/L, 1, 3, 5, 12, 24 hours).

Apoptosis-related genes After treatment with TBMS1, the protein levels of bcl-2 and bax were examined by Western blotting. Western blot analysis revealed that downregulation and phosphorylation of bcl-2 protein and upregulation of bax protein were induced by TBMS1 in CNE-Z cells, and that the phenomena were detected 1 hour after treatment of 50 μmol/L TBMS1(Fig. 8).

DISCUSSION

Our previous study revealed that in TBMS1-treated human malignant tumor cell lines, the incorporation of ^{3}H-thymidine into DNA fraction was suppressed, suggesting that normal synthesis and maturation of DNA were inhibited in those cells[8]. If DNA is irrevocably damaged by chemical insults, the cell undergoes a process called programmed cell death or apoptosis. From the TBMS1-induced morphological and biochemical changes, such as condensation and spot- and lump-shaped margination of nuclear chromatin, formation of vacuoles inside cytoplasm, disruption of cell membrane, the presence of sub-G1 population, and occurrence of typical DNA "Ladder" DNA bands, characterized by apoptosis, we came to the conclusion that apoptosis play important role in antitumor effect of TBMS1.

Programmed cell death play an indispensable role in the development and maintenance of homeostasis within all multicellular organisms. Genetic and molecular analysis from nematodes to humans has indicated that the pathway of cellular suicide is highly conserved. Although the capacity to carry out apoptosis appears to be inherent in all cells, the susceptibility to apoptosis varies markedly and is influenced by external and cell-autonomous events. Considerable progress has been made in identifying the molecules that regulate the apoptotic pathway at each level. Since its original isolation, bcl-2 expression has been documented in a variety of normal and transformed cell types[20, 21]. In some models, bcl-2 can promote cell survival by inhibiting the process of apoptosis[22, 23]. Bcl-2 family proteins are thought to play a major role in neoplastic development. Many tumor cells express anti-apoptotic proteins which allow these cells to persist. In addition, these proteins are overexpressed in some cancers which can result in resistance to chemotherapy. Thus anti-apoptotic bcl-2 family proteins have been targets in numerous studies. The first pro-apoptotic homolog of bcl-2 family, bax, was identified by coimmunoprecipitation with bcl-2 protein. Bax is a 21-kDa protein that shares homolog with bcl-2 clustered conserved regions including BH1 and BH2. When bax was overexpressed in cells, apoptotic death in response to a death signal was accelerated, earning its designation as a death agonist. When bcl-2 was overexpressed,

it heterodimerized with bax and death was repressed[24] Thus the ratio of bcl-2 to bax is important in determining susceptibility to apoptosis.

In sum, apoptosis is a very complex mechanism of cell elimination, and a variety of different mechanisms have been described to induce this phenomenon. To delineate the mechanism underlying the induction of apoptosis by TBMS1, it is clearly not sufficient to pick two proteins that are somehow involved in apoptosis and show changes in expression by Western blotting. Therefore, further analysis of upstream causes and downstream pathways and a study on the effect of TBMS1 on tubulin are in progress in our laboratory.

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