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Tubeimoside V (1), a new cyclic bisdesmoside from tubers of *Bolbostemma paniculatum*, functions by inducing apoptosis in human glioblastoma U87MG cells

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Abstract—Malignant glioblastoma is one of the most common malignant tumors in the neurological system. Tubeimoside V (**1**), a new cyclic bisdesmoside from tubers of *Bolbostemma paniculatum*, appears to exhibit various biological activities, including antitumor effect, but the function and mechanism of this new agent on glioblastoma cells has not previously been determined. In the present study, we investigated the proliferation change of human glioblastoma U87MG cells exposured to different concentrations (0.9–14.8 μM) of Tubeimoside V (**1**) for a certain time. The results showed that Tubeimoside V (**1**) significantly suppressed U87MG cell proliferation in a time- and dose-dependent manner (IC_{50} = 3.6 μM). Flow cytometric analysis of DNA in U87MG cells showed that Tubeimoside V (**1**) induces the prominent appearance of a sub-G1 peak in the cell cycle suggestive of apoptosis. Furthermore, U87MG cells' treatment with Tubeimoside V (**1**) resulted in nuclear condensation with apoptotic bodies observed by both fluorescence and electron microscopy. The result of annexin V/PI assay showed that phosphatidylserine externalization began after treatment, and then increased in the following 24 h. Molecular changes explored through Western-blot staining showed Tubeimoside V (**1**) decreased the expression levels of Bcl-2 protein and increased the expression levels of Bax protein. The novel findings suggest that the cytotoxic actions of Tubeimoside V (**1**) toward U87MG cells result from the induction of cell apoptosis. Overall, our data demonstrate that Tubeimoside V (**1**) is an efficient apoptotic killing agent of glioblastoma cells and suggest that this mechanism may play a critical role in anti-tumor chemotherapy.

Malignant glioblastoma is one of the most common malignant tumors in the neurological system.[1] Glioblastoma is an incurable brain tumor that usually causes death within 2 years after conventional therapies consisting of surgery, radiation, and chemotherapy. With concurrent chemotherapy, median survival can be prolonged for only a few weeks; 2-year survival, however, is increased by 8.6%.[2,3] The success of chemotherapy of glioblastoma patients is hampered by the problem of drug-resistance, and a call for discovery of more effective agents to treat glioblastoma is becoming increasingly urgent.[4]

The tuber of *Bolbostemma paniculatum* (Maxim.) Franquet (*Cucurbitaceae*), a Chinese folk medicine named as 'Tu Bei Mu,' was listed in the Supplement to the Compendium of Materia Medica, compiled in the Qing Dynasty. Tubeimosides I, II, and III, isolated from the folk medicine, are the only three examples of saponins having novel cyclic structures with dicrotalic acid bridge and the name 'cyclic bisdesmoside' has been proposed for this type of saponin.[5] All the three compounds showed significant antitumor, anti-inflammatory, and antitumor-promoting effects. Tubeimoside V (**1**) is a new minor constituent from the ethanol extracts of tubers of *B. paniculatum*.[6–8] But the function of this new agent on human glioblastoma cells has not previously been determined.

Keywords: Tubeimoside V (**1**); Apoptosis; Human glioblastoma; U87MG cell; Bcl-2; Bax.
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In this study, we chose the human glioblastoma cell line U87MG as an in vitro model to explore the effects of Tubeimoside V (**1**) on glioblastoma cell growth and apoptosis to evaluate the value of the agent in glioblastoma therapy. These findings suggest that Tubeimoside V (**1**) may further develop as a novel anticancer drug.

Human glioblastoma (U87MG) cells used in this study were grown as a monolayer culture in DMEM (Dulbecco's modified Eagle's medium) (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL) and antibiotics in a humid atmosphere containing 5% CO_2.

Tubeimoside V (**1**), a new cyclic bisdesmoside, was isolated from tubers of *B. paniculatum* with molecular formula as $C_{64}H_{100}O_{30}$ (molecular weight: 1236).[9] Structure of this compound is shown in Figure 1. Stock solutions were prepared in DMSO and stored at −20 °C and were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 0.5%, so as not to affect cell growth. To evaluate the effects of Tubeimoside V (**1**), cells were incubated with either control medium or medium containing different concentrations of the sample. The controls were 0.1% DMSO.

In an attempt to identify the function of Tubeimoside V (**1**) on human glioblastoma cells, the proliferation of U87MG cells was evaluated by MTT assay after being treated with Tubeimoside V (**1**) at different concentrations (0.9–14.8 μM) for a certain time.[10] The results showed that Tubeimoside V (**1**), at doses ranging from 0.9 to 14.8 μM, significantly suppressed cell proliferation in a dose-dependent manner (Fig. 2). Furthermore, Tubeimoside V (**1**) reduced cell survival to 75% (IC_{25} = 2.6 μM), 50% (IC_{50} (inhibitory concentration 50%) = 3.6 μM), and 25% (IC_{75} = 7.0 μM). Whether the proliferation inhibition induced by Tubeimoside V (**1**) in U87MG cells was associated with apoptosis or cell lysis is still not clear.

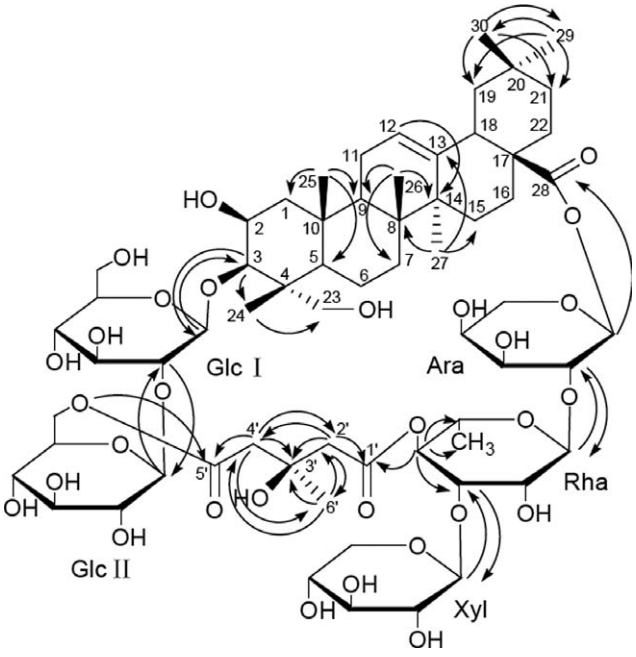

Figure 1. Structure of Tubeimoside V (**1**).

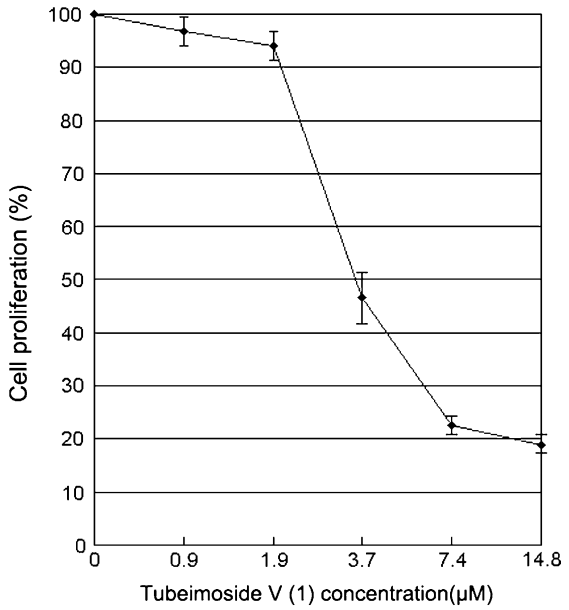

Figure 2. Inhibitory effects of Tubeimoside V (**1**) against human glioblastoma (U87MG) cell proliferation following 72 h. The growth-inhibitory activities were evaluated using MTT assays. Results are shown as mean values of three experiments (±standard deviation, n = 3). For further experiments four doses of Tubeimoside V (**1**) were selected that reduced cell survival to 75% (IC_{25} = 2.6 μM), 50% (IC_{50} = 3.6 μM), and 25% (IC_{75} = 7.0 μM).

In order to investigate the mechanism of Tubeimoside V (**1**) on U87MG cells, flow cytometry analysis of DNA content was performed to detect the changes in the cell cycle distribution.[11] Results showed that treatment for 12 h and 24 h with 2.6, 3.6, and 7.0 μM of Tubeimoside V (**1**) induced S phase arrest (Fig. 3). The percentages of cells in G2/M phase decreased. It is important to note that the highest concentration of Tubeimoside V (**1**) (7.0 μM) induced a significant increase in 'sub-G1 peak' cell-fraction (Fig. 3). The results showed that Tubeimoside V (**1**) significantly suppressed U87MG cell proliferation in a time- and dose-dependent manner.

To confirm Tubeimoside V (**1**) functioned by proliferation inhibition of U87MG cells through apoptosis, chromatin condensation and nuclear fragmentation typical for apoptosis induction were visualized by fluorescence microscopy of Hoechst 33342-stained cells 3, 6, 12, and 24 h after treatment with 7.0 μM Tubeimoside V (**1**) in U87MG cells (Fig. 4).[12] Morphological analysis of cell characteristics observed under fluorescence microscope showed that cells started to change their shape (they shrunk and started to round up) 3 h after the continuous treatment with Tubeimoside V (**1**). Three different types of alterations were detected: (a) weak and irregularly shaped marginal chromatin condensation; (b) highly condensed chromatin of nucleus that was

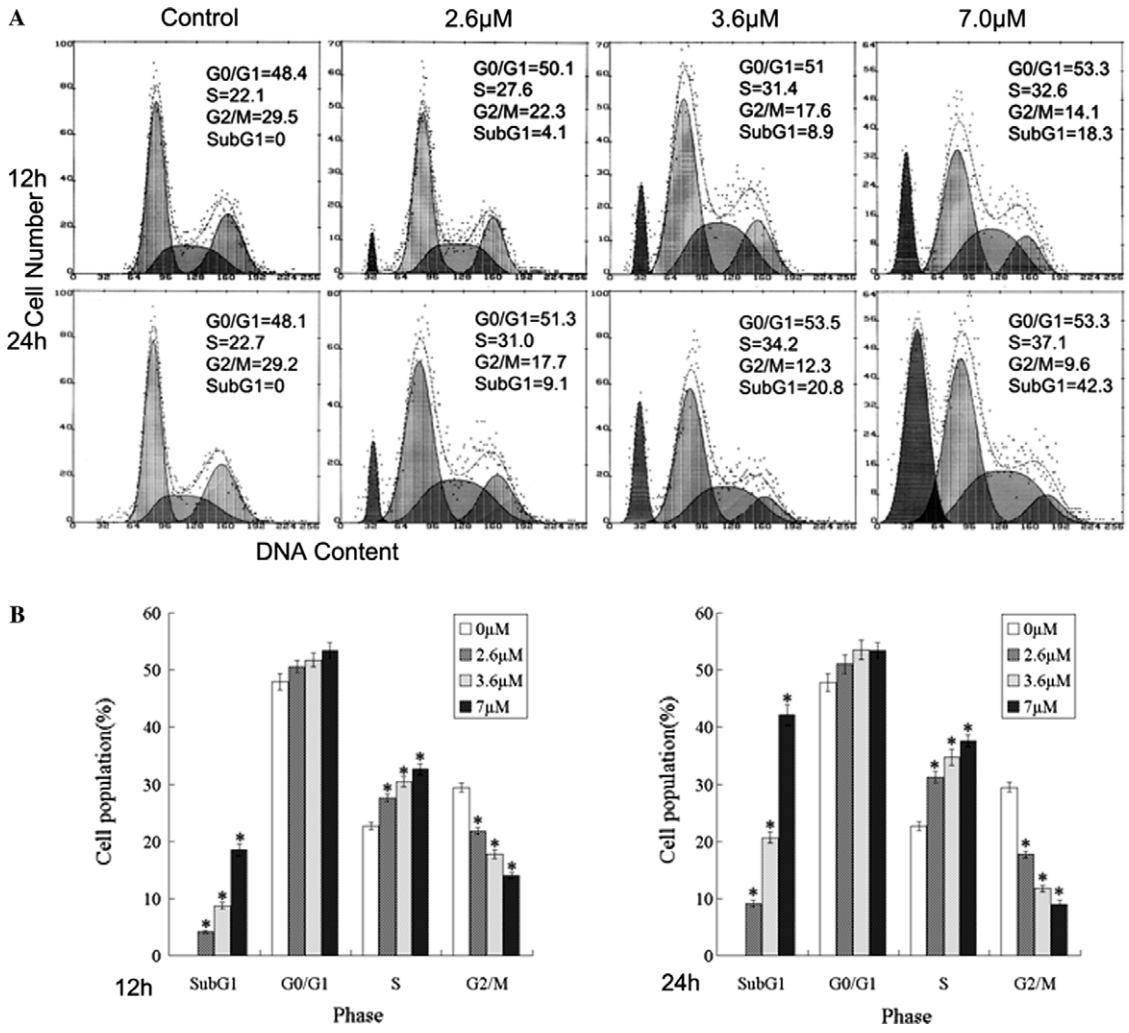

Figure 3. The distribution of cell cycle in Tubeimoside V (**1**)-treated U87MG cells. Cells were treated with control (0.1% DMSO), 2.6, 3.6, and 7 μM Tubeimoside V (**1**) for 12 h and 24 h. (A) The cell cycle distribution was analyzed by flow cytometry and the data are presented as histograms in which the cell number (*y*-axis) is plotted against DNA content (*x*-axis). (B) Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and Tubeimoside V (**1**)-treated cells as analyzed by Dunnett's test ($^{*}p < 0.05$).

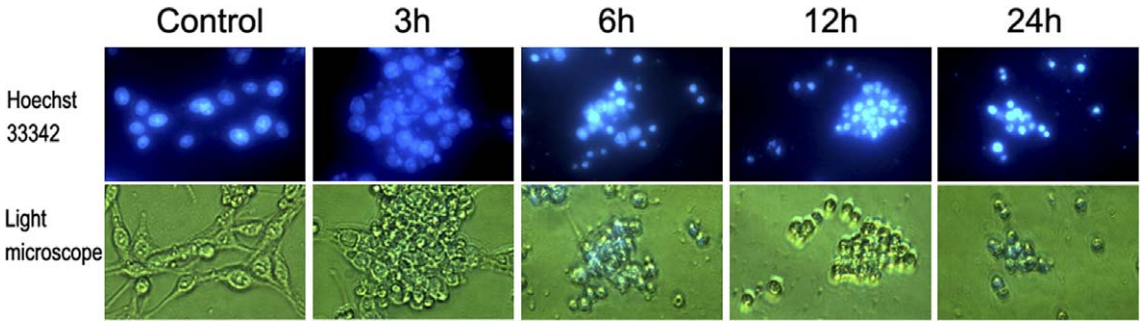

Figure 4. Morphological alterations of U87MG cells treated with Tubeimoside V (**1**). Chromatin condensation and nuclear fragmentation typical for apoptosis induction were visualized by fluorescence microscopy of Hoechst 33342-stained U87MG cells 3, 6, 12 and 24 h after treatment with 7.0 μM Tubeimoside V (**1**). The nuclei of U87 cells showed the characteristic morphological changes of apoptosis, including nuclear condensation, boundary aggregation and split, even nuclear fragmentation.

inverted in one side; (c) relatively compact and irregularly shaped marginal chromatin condensation.[13,14] These changes were typical for apoptosis that is characterized by the condensation of chromatin to compact and simple globular geometric figures.

As to morphology, intracellular damage caused by incubation with Tubeimoside V (**1**) (7.0 μM) for 24 h was investigated by electron micrographs.[15] After 24 h of incubation with 7.0 μM Tubeimoside V (**1**), some cells still appeared normal, whereas others exhibited

dramatic morphological alterations characteristic of apoptosis. The ultrastructure of Tubeimoside V (**1**)-treated U87MG cells showed Tubeimoside V (**1**) induced morphological changes characteristic of apoptosis. Disappearance of microvilli, cell shrinkage, and chromatin condensation without disruption of organelles (Fig. 5); and untreated U87MG cells exhibited fairly intact morphology. Numerous apoptotic bodies, which were membrane-enclosed vacuoles that had budded off the cytoplasmic extension, were also detected in Tubeimoside V (**1**)-treated U87MG cells by light microscopic observation.[16,17]

Externalization of phosphatidylserine (PS), the hallmarks of apoptosis, was also found in alternative types of programmed cell death.[18] To determine whether such processes were induced due to Tubeimoside V (**1**) treatment, U87MG cells were treated for 3, 6, 12, and 24 h with 7.0 μM of Tubeimoside V (**1**). The result of annexin V/PI assay showed that PS externalization began after treatment, and then increased in the following 24 h (Fig. 6).

In particular, the involvement of the Bcl-2 gene in apoptosis was reported in several studies of anticancer drugs.[19,20] The role of Bcl-2 in Tubeimoside V (**1**)-induced apoptosis of human glioblastoma U87MG cells was evaluated by Western-blot analysis.[21] The results showed Bcl-2 expression was down-regulated after treatment with 2.6, 3.6, and 7.0 μM Tubeimoside V (**1**) for 6 and 24 h, while Bax expression was up-regulated (Fig. 7).

Little improvement in prognosis for patients diagnosed with glioblastoma has been realized over the past half-century. Present therapeutic options include steroids, resective surgery, radiation therapy, and chemotherapy. Tubeimoside V (**1**), a new cyclic bisdesmoside from tubers of *B. paniculatum*, appears to inhibit the proliferation of some kind of cancer cells, but its mechanism and function on human glioblastoma cells has not previously been determined. Our present findings provide the identifications of proliferation inhibition by Tubeimoside V (**1**) on human glioblastoma cell line. The antitumor effects of Tubeimoside V (**1**) may result from multiple mechanisms of action, such as interfering with cell cycle progression and inducing apoptosis.

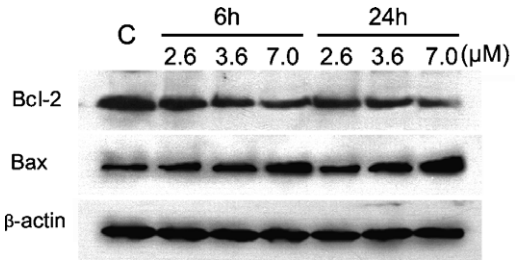

Figure 7. Protein analysis by Western blot: Human glioblastoma U87MG cells were treated for 6 and 24 h with control (C), 2.6, 3.6, and 7.0 μM Tubeimoside V (**1**). Proteins were extracted, then Bcl-2 and Bax expressions were analyzed by Western blot.

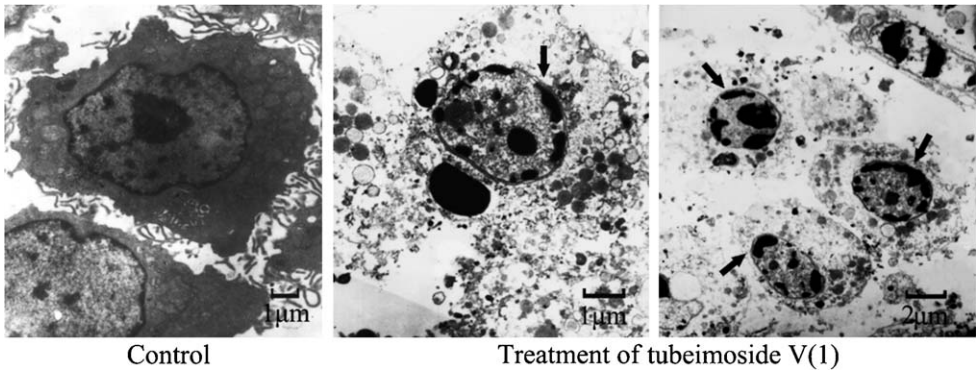

Figure 5. Ultrastructure changes of U87MG cells treated with Tubeimoside V (**1**) by electron micrographs. Control cells (5000×); 7.0 μM Tubeimoside V (**1**) for 24 h (7500× and 4000×). Black arrows indicate chromatin condensation.

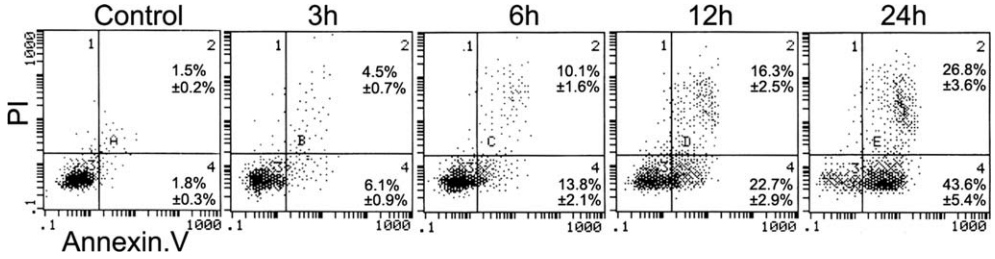

Figure 6. Apoptosis induced by Tubeimoside V (**1**) on U87MG cells. U87MG cells were treated for 3, 6, 12, and 24 h with 7.0 μM Tubeimoside V (**1**). PS externalization was determined by combined annexin V/PI assay. Cells stained positive for annexin and negative for PI represent those with intact membrane and externalized PS (percentages are indicated in the lower right panel). Cells stained positive with annexin and PI are those that lost membrane integrity (percentages are indicated in the upper right panel). Each value is the means ± SD of three determinations.

First, we detected the effect of Tubeimoside V (**1**) on U87MG cells through MTT assays. The results showed that Tubeimoside V (**1**) significantly suppresses the proliferation of U87MG cells. Therefore, MTT test is indicative for the number of viable cells, and it represents the sum of cytostatic and cytotoxic drug effects. To investigate the mechanism of this proliferation suppress, we performed the following experiments. Mammalian cells have evolved a complex defense network to maintain genomic integrity by inhibiting the fixation of permanent damage.[22] Cell-cycle checkpoints prevent cells with damaged genomes from undergoing DNA replication or mitosis. Therefore, we analyzed the influence of Tubeimoside V (**1**) on the distribution of the cell cycle. The results showed that Tubeimoside V (**1**) could induce accumulation of cells arrested at S phases of the cell cycle. Moreover, we have observed that Tubeimoside V (**1**) exhibits more potent effects on SubG1 peak as described in the results. SubG1 tests can indirectly reflect the partial condition of apoptosis.

When exposed to 3.6 and 7.0 μM Tubeimoside V (**1**) for 12 h and 24 h, the SubG1 ratio of U87MG cells was 8.7% ± 0.6%, 18.5% ± 1.1%, 20.6% ± 1.0%, and 42.1% ± 1.8%, respectively. These distinct actions on cell cycle progression and apoptosis suggest that Tubeimoside V (**1**) is able to trigger different signal transduction pathways, and to exert effects on two key processes during cancer treatment. Taken together, our data demonstrate Tubeimoside V (**1**) has a high capacity of suppressing cell proliferation and triggering apoptosis of human glioblastoma cells.

The results of fluorescence microscopy showed that these classical alterations of apoptosis were more obvious in 24 h treatment group than in 3, 6, and 12 h treatment group. Moreover, U87MG cells showed higher ratio of apoptosis cells contained typical characteristics at high concentrations of Tubeimoside V (**1**). These results were identical with the changes in the cell cycle analysis. Additionally, the ultrastructure of Tubeimoside V (**1**)-treated U87MG cells showed Tubeimoside V (**1**)-induced morphological changes characteristic of apoptosis under electron micrographs.

Analysis of annexin V/PI assay revealed that some cells lost their membrane integrity at the time when PS externalization occurred.[23] The results were consistent with the biochemical characteristics of apoptosis that PS externalization is an early and sensitive event during cascade of apoptosis. This observation confirmed the data obtained by morphological analysis.

Furthermore, we detected the expression of Bcl-2 and Bax in U87MG cells treated by Tubeimoside V (**1**). Oncogenes such as Bcl-2 are often involved in the development of tumors, and may be related to tumor cell drug resistance.[24] Our results showed that Tubeimoside V (**1**) not only induced U87MG cell apoptosis, but also drastically decreased Bcl-2 protein expression concurrently. The identification of the genes involved and their related signal transduction pathways critical to tumorigenesis awaits further study. Tubeimoside V (**1**) appears to induce apoptosis via down-regulation of the Bcl-2 protein. Bcl-2 family members are characterized by containing at least one of four Bcl-2 homology domains (BH1–BH4), and play important roles in regulating apoptosis.[25] Bcl-2 seems to influence the response to chemotherapy by inhibiting apoptosis induced by many cytostatic drugs including alkylating agents, topoisomerase inhibitors, and antimetabolites.[26] Bcl-2 family members directly control mitochondrial membrane permeability. Some of the members increase permeability (such as Bax), while others prevent it (such as Bcl-2).[27–29] Concerning mechanisms of apoptosis, several results revealed that the kind of apoptosis showed by Tubeimoside V (**1**) is commonly modulated, which is corroborated by up-regulation of Bax protein and down-regulation of Bcl-2 protein. Bcl-2 protein expression decreased with Tubeimoside V (**1**) treatment time, suggesting that Tubeimoside V (**1**) might play a role in inducing apoptosis of human glioblastoma U87MG cells by possibly down-regulating the expression of the Bcl-2 gene.

In conclusion, Tubeimoside V (**1**) is capable of inhibiting growth and inducing apoptosis of human glioblastoma U87MG cells in a dose- and time-dependent manner. The antitumor effects of Tubeimoside V (**1**) may result from multiple mechanisms of action, such as interfering with cell cycle progression and inducing apoptosis, possibly by decreasing Bcl-2 protein expression. Though no evidence showed that Tubeimoside V (**1**) could directly cross the blood–brain barrier, there were many ways of chemotherapy (e.g., intracerebral microinfusion, indwelling balloon applicator, and locoregional chemotherapy) to treat glioblastoma. These indicated that the agent might hold promise as an anticancer agent. Based on the observation that Tubeimoside V (**1**) has significant anti-glioblastoma activity in cell culture, it deserves further evaluation of its potential preventative or therapeutic utility in vivo.

Acknowledgments

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10. MTT assay. 5×10^3 cells per well were seeded in 96-well plates. Different concentrations of this were then added to the cells 24 h after seeding. The cells were continuously treated for 72 h and then the MTT assay was performed as described.
11. Cell cycle analysis. U87MG cells were trypsinized, counted, centrifuged, and fixed in ethanol at certain time points after the treatment. Then cells were washed twice in PBS and centrifuged. Pellet was resuspended in a solution of RNase (0.02 mg/ml, Sigma, USA) and propidium iodide (PI, 0.02 mg/ml, Sigma, USA), and incubated at 4 °C for 30 min. Fluorescence of stained cells was measured for approximately 10,000–20,000 cells. Data were collected and analyzed. Results were expressed as a plot of fluorescence intensity vs cell number.
12. Hoechst staining. For apoptosis assays, apoptosis was analyzed after staining of the cells with Hoechst 33342 (Sigma–Aldrich) and subsequent fluorescence microscopy. In brief, cells were incubated with Hoechst 33342 at a final concentration of 1.5 μM for 10 min. Cell morphology was then determined by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss, Germany). Cells were analysed with 40× magnification and documented using a CCD camera device (Zeiss Axiocam MR).
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15. Electron microscope. The U87MG cell line was plated in 35 mm dishes and allowed to incubate overnight. Aliquots of Tubeimoside V (**1**) (10.0 μg/ml) were added into the culture dish for 24 h. At the end of incubation, cell samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 2% buffered osmium tetroxide for 2 h and dehydrated in ethanol. Specimens for transmission electron microscopy were embedded in epon. Thin sections were cut on an ultramicrotome and double stained with uranyl acetate and lead citrate. Electron micrographs were taken on an electron microscope (JEM-2000EX, JEOL, Japen) operating at 80 kV.
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18. AnnexinV/PI method. The appearance of phosphatidylserine (PS) on the extracellular side of membrane was evaluated with annexinV/PI method. At certain time points after the treatment, cells were trypsinized, counted, washed twice in ice-cold PBS, and resuspended in 1× binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Thereafter, 5 μl of annexin V-FITC (BD Pharmingen) and 10 μl of propidium iodide (50 μg/ml) were added to 100 μl of cell suspension and incubated for 15 min at room temperature protected from light. Finally 400 μl of binding buffer was added to the samples and handled ice-cold until they were analyzed on FACSCalibur (Becton–Dickinson) flow cytometer. Ten thousand cells were analysed per sample.
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21. Western blot analysis. Protein extracts of U87MG cells were prepared by lysing cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris HCl, pH 8), 10 mM EDTA, and 1 mM PMSF (Sigma, USA) for 30 min at 4 °C. Samples were then centrifuged for 15 min at 15,000*g*. Protein concentration in supernatant was determined. For each sample, 60 μg of protein was loaded on 12.5% SDS–polyacrylamide gel, electrophoresed, and transferred to nitrocellulose membrane (0.22 μm, Protran, Schleicher and Schuell). Membrane was blocked for 1 h at the room temperature with blocking buffer (TBS containing 0.1% Tween 20 (Sigma, USA) and 5% milk). Primary antibodies (applied for 1 h at room temperature, or overnight at 4 °C) were: anti-Bcl-2 (mouse monoclonal Ab-1, Oncogene), anti-Bax (rabbit polyclonal N-20, Santa Cruz), and anti-actin (mouse monoclonal C-2, Santa Cruz). They were diluted 1:1000, except: anti-actin (1:500). Thereafter, membranes were incubated for 1 h with HRP-labeled secondary antibodies (Amersham Pharmacia Biotech, Sweden): sheep anti-mouse (diluted 1:2500) and donkey anti-rabbit (diluted 1:5000), and then developed by an ECL system according to the manufacturer's instructions (Amersham).
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