Experimental study of a novel tumstatin on C6 brain glioma *in vitro*

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Abstract. To investigate the effect of a novel tumstatin on C6 brain glioma cells, the MTT method was used to detect C6 glioma cell proliferation activity at different time periods (12, 48 and 72 h). Cell cycle distribution and apoptosis rate were detected by flow cytometry, and the acridine orange/ethidium bromide staining method was used to detect apoptosis and mitochondrial membrane potential by fluorescence microscopy. Novel tumstatin had an evident inhibitory effect on C6 glioma cells, and the most notable impact emerged after 48 h. The following were observed under the fluorescence microscope: Characteristic morphological changes of cell apoptosis were typically observed in the novel tumstatin (2,000 μg/ml) group; mitochondrial membrane potential decreased significantly ($P<0.05$); the cells in the G0/G1 phase significantly increased ($P<0.05$); and the number of cells in the S phase was reduced. There was an increase in cell apoptosis rate in the novel tumstatin (2,000 μg/ml) group compared with the novel tumstatin (1,000 μg/ml) group and the Mock group, and the data were statistically significant ($P<0.05$). Novel tumstatin may reduce the mitochondrial membrane potential, inducing cell apoptosis, and thereby exerting antitumor activity.

Introduction

Glioma is the most common intracranial primary tumor, with invasive growth and high postoperative recurrence. It is a serious threat to the life and health of patients. At present, the pathogenesis of glioma is not clear (1). Previous studies have demonstrated that the occurrence and development of glioma is associated with apoptosis (2-4). The traditional treatment principle is postoperative radiotherapy prior to chemotherapy, or either chemotherapy or radiotherapy alone, but it is difficult to improve the clinical efficacy (5); the cure rate is low. In previous years, through considerable investigation (6-8), researchers have been seeking new treatments for cerebral glioma.

At present, numerous anti-tumor drugs have been developed in domestic and foreign fields, but the majority of drugs are expensive and have untoward effects. Drug resistance is also a problem; therefore, the clinical application of these drugs is restricted (9-11). In previous years, numerous new drugs for the treatment of glioma have been developed. Carmustine, as a cell-cycle phase nonspecific alkylating antineoplastic agent commonly used in the treatment of brain glioma (12), inhibits DNA synthesis and RNA production by alkylating DNA and RNA, as well as blocking the activity of DNA polymerases. It has the most notable effect on cell cycle transition between the G1 phase and S phase, blocks S phase progression and even has an impact on cells at the G0 phase. However, long-term use of carmustine may lead to delayed myelosuppression. In the present study, carmustine was used as a positive control. New drug treatments with high efficiency, low toxicity and low price have become the focus of research in recent years. Tumstatin, which is used in the present study, is a novel tumor angiostatin from active fragments of the angiogenesis inhibition region in the thrombospondin-1 protein and angiostatin linked via a lysine. It consists of 24 amino acids, with a molecular weight of 2,707 kDa. It was developed and synthesized by GL Biochem Ltd. (Shanghai, China). The present study focuses on researching a novel tumstatin that affects C6 glioma cells, and investigating the mechanism of its inhibition effect on glioma by detection of apoptosis and mitochondrial membrane potential changes in glioma cells (13). The present study aims to provide a theoretical basis for additional research on the novel tumstatin treatment of gliomas.

Materials and methods

Cells. The C6 brain glioma cell line was purchased from the Shanghai Institute of Cellular Biology of Chinese Academy

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of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) with 10% fetal calf serum (Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, China) at 37˚C in an incubator with 5% CO_2.

Reagents. The mitochondrial membrane potential assay kit with JC-1 (catalog no. C2006), and the cell cycle and apoptosis detection kit (catalog no. C1052) were purchased from Beyotime Institute of Biotechnology (Haimen, China). Ethidium bromide (catalog no. E8751), dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich (EMD Millipore, Billerica, MA, USA). Acridine orange (catalog no. 1AB10220) was purchased from Ding Guo Changsheng Biotechnology Co., Ltd. (Beijing, China). The novel tumstatin was designed by Laboratory of Chemistry and Molecular Biology Institute of Frontier Medical Sciences, Jilin University (Changchun, China) and synthesized by GL Biochem Ltd. (Shanghai, China), with a purity of 98%. Carmustine (catalog no. 1407011) was purchased from Jinyao Pharmaceutical Co., Ltd. (Tianjin, China; serial number).

Main instruments. The Nikon ECLIPSE 80i fluorescence microscope (Nikon Corporation, Tokyo, Japan), a CO_2 incubator (model, NCO-15AC; Sanyo Manufacturing Co., Osaka, Japan), the AN YANG super clean bench (model: BSC-BOO II B2; Anyang Technology Development Co., Ltd., Suzhou, China), a flow cytometer (BD Biosciences, Franklin Lakes, CA, USA) and a microplate reader (model: 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) were all used in the present study.

Cellular viability by MTT. At the logarithmic growth phase, C6 brain glioma cells were digested with trypsin (Ameresco, Inc., Framingham, MA, USA), cells were counted, and 100 μl of cell suspension was added to each well of a 96-well plate at 37˚C in a 5% CO_2 atmosphere and incubated for 4 h. When cells attached, plates were randomly divided for the Mock group, carmustine group and novel tumstatin of different concentrations (1,000, 1,500 and 2,000 μg/ml), with a total treatment volume of 20 μl/well in 96-well plate. The Mock group was treated with culture medium at an equal volume, and each group had 8 wells. At 37˚C and in a 5% CO_2 atmosphere for 24, 48 and 72 h, each well was incubated for 4 h with MTT (20 μl). The reaction was then terminated with DMSO (150 μl/well), and cells were shocked for 10 min on the plate oscillator. An enzyme immunoassay instrument detected the absorbance, and absorption wavelength was set at 490 nm.

Acridine orange/ethidium bromide (AO/EB) double staining to detect cells apoptosis. C6 brain glioma cells in the logarithmic growth phase were digested with 0.25% trypsin. The cell suspension was adjusted to $3x10^5$ cells/ml and seeded onto 6-well plates at 37˚C in a 5% CO_2 atmosphere for 4 h. This was then randomly divided for the Mock group, the carmustine group and novel tumstatin of different concentrations (1,000, 1,500 and 2,000 μg/ml), and 2 wells were allocated to each group (50 μl/well in a total volume of 2 ml/well in 6-well plate). Coverslips were removed, covered with cells and then placed on a slide. This was followed by the dropwise addition of 5 μl of AO/EB dye to the coverslips. Slides were observed immediately by fluorescence microscopy, and images were captured (x20). The number of apoptotic cells per field was counted, and the apoptosis rate was calculated (% apoptotic cells = number of apoptotic cells/total number of cells x100%).

Detection of mitochondrial membrane potential (JC-1). C6 brain glioma cells in the logarithmic growth phase were digested with 0.25% trypsin and inoculated in 6-well plates with $3.0x10^5$ cells/ml, at 37˚C in a 5% CO_2 atmosphere for 4 h. This was randomly divided for the Mock group, the carmustine group and novel tumstatin of different concentrations (1,000, 1,500 and 2,000 μg/ml), and two wells were allocated to each group (50 μl/well with 2 ml). Carbonyl cyanide m-chlorophenylhydrazone (CCCP; according to the ratio of 1:1,000) was added to the culture medium of each cell as a positive control group. In the carmustine group and novel tumstatin of different concentrations, 0.5 ml JC-1 staining liquid (Beyotime Institute of Biotechnology, Haimen, China) was added to each well. Following incubation for 20 min at 37˚C, the supernatant was discarded, and cells were rinsed twice with JC-1 (1X) buffer in an ice bath, and 1 ml of culture medium was added to each well. This was observed under the fluorescence microscope, at the excitation wavelength of 490 nm and emission wavelength of 530 nm, and images were captured (x20).

Cell cycle detection by flow cytometry. Subsequent to treatment (method 2), cells were collected with cold PBS, percussed into a single cell, centrifuged, washed twice with PBS and resuspended with 500 μl PBS. The following components were then added: 1 mg/ml propidium iodide (PI); 10 mg/ml RNase A; and 0.01% Triton-X100, protected from light for 30 min at 37˚C. The cell cycle was detected by flow cytometry, and the percentage of each cell cycle accounted for the results.

Analysis of apoptosis by Annexin V-fluorescein isothiocyanate (FITC)/PI. Following treatment (method 2), cells were collected with cold PBS, resuspended with 195 μl Annexin V-FITC and incubated for 10 min away from light. PI (10 μl) was then added, and cells were mixed gently in an ice bath (protected from light) and detected by flow cytometry. The results were expressed as apoptosis rate.

Statistical analysis. Statistical analysis was performed by SPSS 13 statistical software (SPSS, Inc., Chicago, IL, USA). The data were expressed as the mean ± standard deviation. Statistical significance was compared between the treatment and the control groups by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cellular viability by MTT. The results demonstrated the effect of different dose models of tumstatin on C6 glioma cells. At 24 h, cells were spindle-shaped, which was observed in each experimental group under a fluorescence microscope, and no significant difference was observed between each group ($P>0.05$). At 48 h, novel tumstatin significantly inhibited C6 glioma cell growth ($P<0.05$), whereas growth of the Mock

Table I. Effect of novel tumstatin on the activity of C6 cells.

Group	OD value		
	24 h	48 h	72 h
Mock group	0.201±0.034	0.247±0.037	0.250±0.043
Carmustine group (100 μg/ml)	0.194±0.030	0.193±0.034[a]	0.221±0.039
Novel tumstatin group (1,000 μg/ml)	0.201±0.025	0.231±0.063	0.218±0.032
Novel tumstatin group (1,500 μg/ml)	0.199±0.037	0.194±0.036[a]	0.212±0.025
Novel tumstatin group (2,000 μg/ml)	0.200±0.028	0.183±0.035[a,b]	0.215±0.023

Data are expressed as the mean ± standard deviation; n=40; [a]P<0.05 compared with the Mock group and the novel tumstatin group; [b]P>0.05 compared with the carmustine group. OD, optical density.

group markedly increased. Significant differences were visible in the novel tumstatin (2,000 μg/ml) group compared with the Mock group and novel tumstatin (1,000 μg/ml; P<0.05). No significant difference was observed in the novel tumstatin (2,000 μg/ml) group compared with the carmustine group (P>0.05). At 72 h, each experimental group showed accelerated and spiral cell growth. Among these groups, cells in the carmustine group and the novel tumstatin group grew more slowly compared with the Mock group. At 72 h, the novel tumstatin group had no inhibitory effects on C6 cell growth (Table I and Fig. 1).

AO/EB double staining immunofluorescence and morphological observation of cell apoptosis. C6 glioma cells were treated with novel tumstatin for 48 h, and using AO/EB staining, apoptosis was observed under a fluorescence microscope. The results showed a large number of cells in the Mock group were stained green, and the cells were spindle-shaped with a whole nucleus and clear demarcation. With the increase in drug concentration, the number of normal cells was gradually reduced, while the number of apoptotic cells, which emitted red fluorescence in the cytoplasm and nucleus, increased. Significant differences were visible in the novel tumstatin (2,000 μg/ml) group compared with the Mock group and novel tumstatin (1,000 μg/ml; P<0.05). No significant difference was observed in the novel tumstatin (2,000 μg/ml) group compared with the carmustine group (P>0.05; Table II and Fig. 2A).

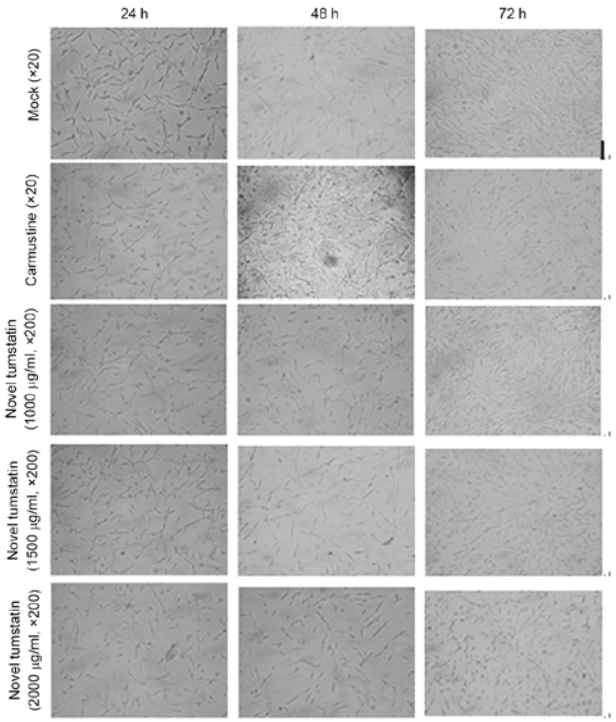

Figure 1. Inhibitory effects of different doses (0, 1,000, 1,500 and 2,000 μg/ml) of novel tumstatin on C6 glioma cells at 24, 48 and 72 h. The effects of novel tumstatin were determined by the MTT assay.

Effects of the novel tumstatin on C6 brain glioma cell mitochondrial membrane potential. Under the fluorescence microscope, in the CCCP positive control group, mitochondrial membrane potential of C6 glioma cells was lost completely following treatment with novel tumstatin (10 μm) for 20 min, and JC-1 staining showed green fluorescence. In the Mock group, the majority of cells were stained red, and cells had slender protuberance. Following treatment with the novel tumstatin (2,000 μg/ml) group on C6 glioma cells for 48 h, the majority of cells showed green fluorescence, and no cell had protrusions or appeared rounded, suggesting that cells were apoptotic; red fluorescent cells were spindle-shaped. However, the protuberance was short compared with the normal control group, the novel tumstatin (1,000 μg/ml) group and the novel tumstatin (1,500 μg/ml) group. Significant differences were visible in the novel tumstatin (2,000 μg/ml) group compared with the Mock group and the novel tumstatin (1,000 μg/ml) group (P<0.05). No significant difference was observed in the novel tumstatin (2,000 μg/ml) group compared with the carmustine group (P>0.05; Table II and Fig. 2B).

Effects of the novel tumstatin on brain glioma cell cycle and apoptosis. The present study showed that at 48 h, novel tumstatin altered the cell cycle. In the novel tumstatin (2,000 μg/ml) group, the G0/G1 phase rate increased significantly, while the number of cells in the S phase decreased significantly (P<0.05). Significant differences were visible in the novel tumstatin (2,000 μg/ml) group compared with the Mock group and the novel tumstatin (1,000 μg/ml) group (P<0.05). No significant difference was observed in the novel

Table II. Apoptotic effect of novel tumstatin on C6 glioma cells after 48 h.

Group	48 h	
	Mitochondrial membrane potential	AO/ED double staining
CCCP positive control group	74.80±17.52	
Mock group	20.00±6.20	10.00±2.00
Carmustine group (100 μg/ml)	39.85±4.38[a]	44.20±11.43[a]
Novel tumstatin group (1,000 μg/ml)	18.60±5.02	18.40±4.92
Novel tumstatin group (1,500 μg/ml)	29.80±3.42	35.80±8.28[a]
Novel tumstatin group (2,000 μg/ml)	40.20±6.83[a,b]	43.80±5.01[a,b]

Data are expressed as the mean ± standard deviation; n=30; [a]P<0.05 compared with the Mock group and the novel tumstatin group; [b]P>0.05 compared with the carmustine group. CCCP, carbonyl cyanide m-chlorophenylhydrazone; AO/ED, acridine orange/ethidium bromide.

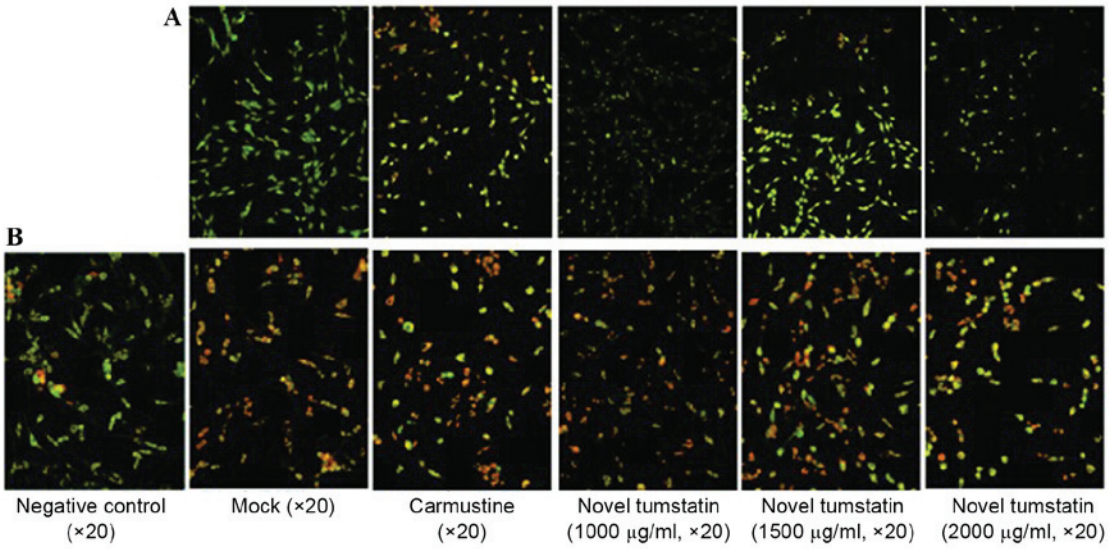

Figure 2. Apoptotic effects of novel tumstatin on the C6 cells after 48 h. (A) C6 glioma cells were treated by novel tumstatin for 48 h, using acridine orange/ethidium bromide staining for observation of cell apoptosis. (B) Mitochondrial membrane potential assay determined the apoptotic effect.

tumstatin (2,000 μg/ml) group compared with the carmustine group (Table III and Fig. 3). Results showed that the novel tumstatin (2,000 μg/ml) group evidently promoted apoptosis of C6 glioma cells.

Discussion

In previous years, the incidence rate of malignant tumors has been gradually rising. In brain tumors, the incidence of malignant gliomas is the most evident (12), which accounts for ~35-60% of intracranial tumors (14). The pathogenesis of the disease remains unclear, and there has been no major breakthrough in treatment of the disease. At present, surgery combined with chemotherapy and radiotherapy reduces the recurrence and metastasis of tumors; however, the cure rate is low and there has been no treatment breakthrough. It is the focus of clinical research on effective glioma treatment.

According to studies in the literature, numerous antitumor drugs have been investigated, but the majority of the drugs are expensive and have adverse reactions. There are also problems of drug resistance; therefore, the clinical application of these drugs is restricted (9-11). Therefore, current studies are focused on searching for efficiency, low toxicity, efficacy and stability of antitumor drugs, and an ideal peptide drug will possess these advantages precisely. In the present study, the effects of tumstatin were observed on neurogliocytoma through an *in vitro* experiment using C6 glioma cells.

The development of tumors is a complex process and is associated with cell apoptosis. The imbalance between cell proliferation and apoptosis was associated with tumorigenesis and development. Therefore, inhibition of tumor cell proliferation and inducing the apoptosis of tumor cells have become important methods for the treatment of cancer (15-18). The present study used the MTT method and light microscopy to assess the inhibition of proliferation and induction of apoptosis. The results revealed that novel tumstatin has an inhibitory effect on the proliferation of C6 glioma cells, and the most notable function appears at 48 h; the novel tumstatin (2,000 μg/ml) group showed significant inhibition of cell proliferation compared with the Mock group and the novel

Table III. Novel tumstatin impact on the cell cycle and apoptosis of C6 glioma cells.

Group	G0/G1-phase	S-phase	G2/M-phase	Apoptosis rate, %
Mock group	46.41±8.57	49.67±9.94	3.92±4.00	4.88±0. 81
Carmustine group (100 μg/ml)	62.68±2.98	36.23±3.49	1.08±0.55	36.78±8.33
Novel tumstatin group (1,000 μg/ml)	52.88±2.71	44.36±0.34	2.78±2.63	10.90±0.97
Novel tumstatin group (1,500 μl/ml)	58.82±1.70	40.09±2.32	1.10±0.65	30.96±4.16
Novel tumstatin group (2,000 μl/ml)	67.75±3.27[a,b]	28.88±2.32[a]	2.77±2.09	45.96±1.71

Data are expressed as the mean ± standard deviation; n=15; [a]P<0.05 compared with the Mock group and the novel tumstatin group; [b]P>0.05 compared with the carmustine group.

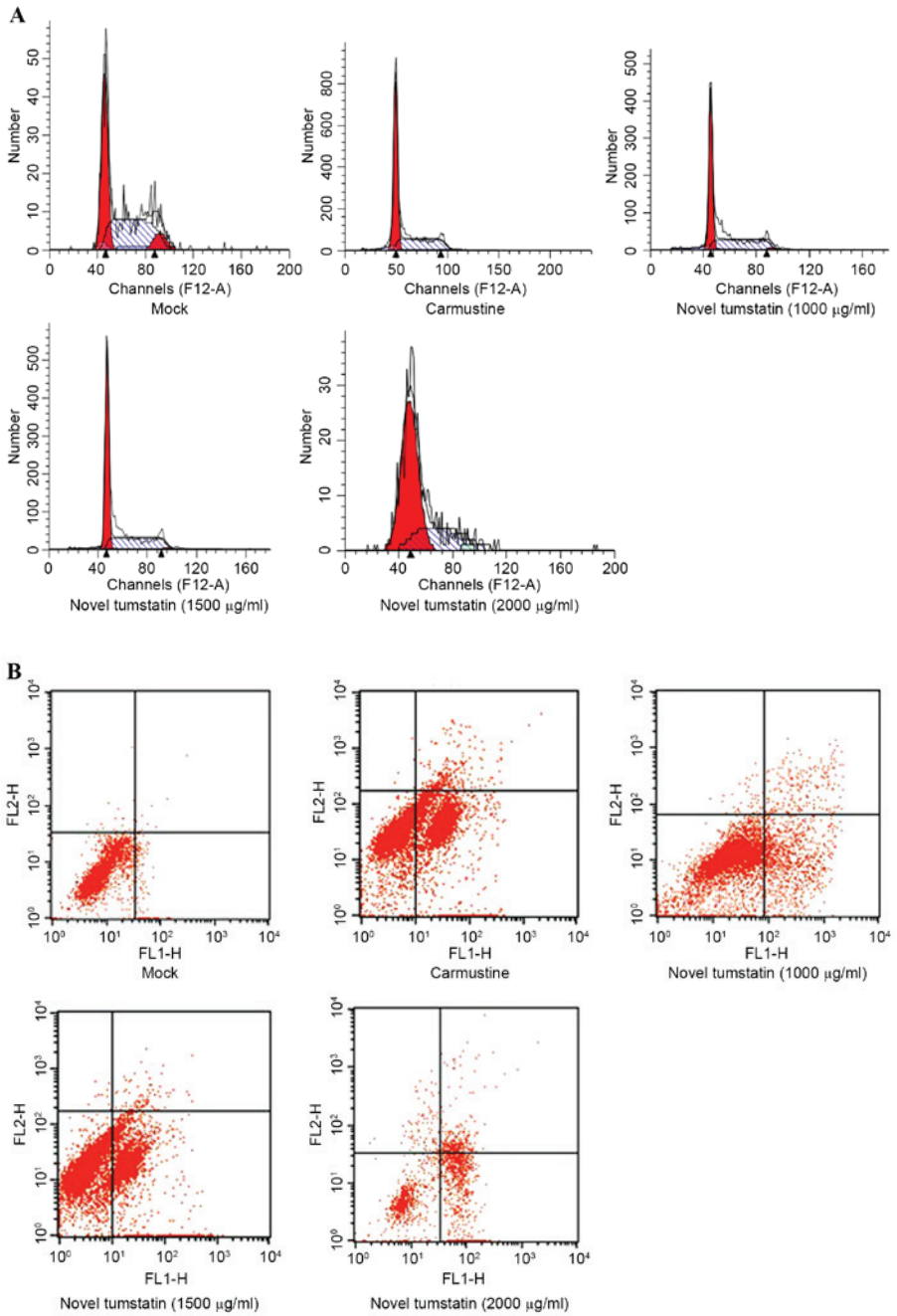

Figure 3. (A) Effects of different doses of novel tumstatin on the cell cycle of C6 glioma cells after 48 h. Cells were treated with different doses (0, 1,000, 1,500 and 2,000 μg/ml) of novel tumstatin and carmustine for 48 h, then analyzed by flow cytometry. (B) Effects of different doses of novel tumstatin on the cell apoptosis of C6 glioma cells after 48 h. Cells were treated with different doses (0, 1,000, 1,500 and 2,000 μg/ml) of novel tumstatin and carmustine for 48 h, and were then double labeled with Annexin V-fluorescein isothiocyanate/propidium iodide and analyzed by flow cytometry.

tumstatin (1,000 μg/ml) group (P<0.05). Under microscopy, the Mock group cells were in good condition, the novel tumstatin (2,000 μg/ml) group had marked effects on inhibition on cell growth, and the number of cells with decreased growth state was low.

Research demonstrates that there are numerous factors inducing cells to be apoptotic; the internal factors are associated with the regulation of certain genes and mitochondrial membrane potential, while the external factors consist of γ-ray, hypoxia and anti-tumor drugs (19). The level of apoptosis examined by flow cytometry was intuitive and accurate (20-22). In the present study, the apoptosis rate of cells in response to the novel tumstatin was investigated. Tumstatin (2,000 μg/ml) significantly increased the apoptosis rate of C6 glioma cells at 48 h.

The results showed that novel tumstatin and carmustine induce C6 glioma cell apoptosis, thereby increasing the percentage of G0/G1 phase cells; however, no significant difference was observed between the tumstatin (2,000 μg/ml) and carmustine groups (P>0.05). Following treatment with 2,000 and 1,000 μg/ml tumstatin, a significant difference was observed in the percentage of G0/G1 phase cells (P<0.05). Additionally, a more significant decrease was observed in the percentage of S phase cells, DNA synthesis and cell proliferation in the tumstatin (2,000 μg/ml) group compared with the carmustine groups (P<0.05). Therefore, it was speculated that novel tumstatin arrests the growth of C6 tumor cells through the G0/G1 phase and induces cell apoptosis, and this effect is time and dose-dependent.

JC-1 is an ideal probe, which is widely used for the detection of mitochondrial membrane potential. When mitochondrial membrane potential is high, JC-1 gathers in the mitochondrial matrix, which forms a polymer with red fluorescence. However, when the mitochondrial membrane potential is low, JC-1 cannot gather in the mitochondrial matrix and becomes a single state, emitting green fluorescence. Detection of the mitochondrial membrane potential by light color may be convenient. The decrease of mitochondrial membrane potential is a hallmark of early apoptotic events (23,24). This research adopts the JC-1 detection model to observe the effect of novel tumstatin on mitochondrial transmembrane potential of C6 brain glioma cells. The results show that at 48 h, the mitochondrial membrane potential of the novel tumstatin (2,000 μg/ml) group was decreased. Significant differences were visible in the novel tumstatin (2,000 μg/ml) group compared with the Mock group and the novel tumstatin (1,000 μg/ml; P<0.05). No significant difference was observed in the novel tumstatin (2,000 μg/ml) group compared with the carmustine group (P>0.05). This indicated that novel tumstatin may act directly on the mitochondria of C6 cells, and the mitochondrial membrane permeability increased, causing a decrease in mitochondrial transmembrane potential and thereby inducing cell apoptosis.

AO/EB staining was used to detect the apoptosis of C6 cells. The results revealed that novel tumstatin acted on C6 glioma cells after 48 h. A large number of cells were dyed green in the Mock group, and the cells were spindle-shaped with a visible nucleus and clear demarcation. With an increase in drug concentration, the number of normal cells gradually reduced, while the number of apoptotic cells increased, which emitted red fluorescence in the cytoplasm or nucleus. Significant differences in the apoptosis rate were visible in the novel tumstatin (2,000 μg/ml) group compared with the Mock group and the novel tumstatin (1,000 μg/ml) group (P<0.05). The proportion of apoptotic cells increased gradually with the increase in drug concentration. The results were consistent with the cell cycle and mitochondrial membrane potential. Furthermore, a previous study demonstrated that tumstatin elicits an evident change in the cell cycle by down-regulating cyclinD1 expression (not shown) (25); it causes a significant increase in the number of cells in the G0/G1 phase, but decreases the number of cells in the S phase. Additionally, tumstatin can notably inhibit proliferation, but triggers apoptosis in C6 glioma cells, which is possibly associated with a decline in mitochondrial membrane potential. These results may provide pharmacological evidence for tumstatin as a new anti-tumor drug in the treatment of brain glioma.

In summary, novel tumstatin significantly inhibits the proliferation of C6 glioma cells, inducing cell apoptosis. The possible molecular mechanism of apoptosis is associated with the decrease in mitochondrial membrane potential. On this basis, additional studies will explore the impact of novel tumstatin on the protein expression and cell signal transduction.

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