

Original Article

Neovibsanin B inhibits human malignant brain tumor cell line proliferation and induces apoptosis

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Abstract: The present study was designed to examine the effect of neovibsanin B on glioma cell viability, apoptosis and on the survival time in mice bearing tumor xenografts. The results demonstrated that neovibsanin B significantly reduced the cell viability of GL261-NS and GL261-AC cells in a dose-dependent manner. However the inhibition of proliferation was more significant in GL261-NS cells. The IC₅₀ value of neovibsanin B against GL261-NS and GL261-AC cells is 5 and 25 nM, respectively. The inhibitory effect of neovibsanin B on cell growth was more effective than that of vincristine (VCR) ($P < 0.05$). We also observed a significant decrease in sphere-forming ability of GL261-NS cells on treatment with neovibsanin B. The number of colonies formed by GL261-NS cells on treatment with neovibsanin B, VCR and DMSO were 3.34 ± 1.02 , 12.53 ± 3.46 and $61.34 \pm 9.89\%$ respectively after 7 days. The flow cytometry revealed a marked increase in apoptotic cell death of GL261-NS cells on treatment with neovibsanin B. The western blots showed a significant decrease in the level of activated caspase-3 on treatment with neovibsanin B after 24 h. In addition, neovibsanin B increased the median survival time of glioma-bearing mice ($P < 0.05$). Therefore, neovibsanin B effectively inhibits glioma cell viability by inducing apoptosis, and can be a potent therapeutic agent for the treatment of malignant glioma.

Keywords: Malignant glioma, neovibsanin B, apoptosis, vincristine, sphere-forming ability

Introduction

Malignant gliomas in adults account for more than 60% of all the primary brain tumors and has high rate of morbidity and mortality [1]. The molecular mechanism of malignant glioma is yet to be fully understood. Adjuvant chemotherapy in addition to radical surgery and radiation therapy has provided only a modest increase in survival. It is reported that a small population of glioma cells, glioma-initiating cells (GICs) or glioma stem cells are responsible for the initiation of glioma [2-4]. GICs are characterized by the property of initiating tumorigenesis and undergoing self-renewal. These cells are resistant to various conventional cancer therapies, thus leading to failure of traditional agents to cure human malignancy [4, 5]. Thus, the discovery of novel and efficient therapeutic strategies to target glioma stem cells is highly desired to treat malignant glioma.

Neovibsanins, novel polyfunctionalized diterpenoid compounds were isolated from the shrub *Viburnum awabuki* by Fukuyama's *et al* in 1996 [6]. These natural products were found to display neurite outgrowth activity in PC12 cells, suggesting that neovibsanin-type compounds could be promising candidates for the development of novel therapeutic agents to treat neurological diseases [7]. Williams *et al.* in 2009 reported neurite outgrowth activities of unnatural 4, 5-bi-epi-neovibsanins and their synthetic intermediates. They found minor effect of stereochemistry in the neovibsanin skeleton on biological activity [8, 9]. Imagawa *et al.* revealed the neurite outgrowth activity of racemic mixture of neovibsanins [10, 11]. The activity of neovibsanin B (**Figure 1**) in growth suppression and tumorsphere formation in glioma, particularly GICs, has not yet been elucidated. In the present study, the *in vitro* and *in vivo* effects of neovibsanin B on GL261

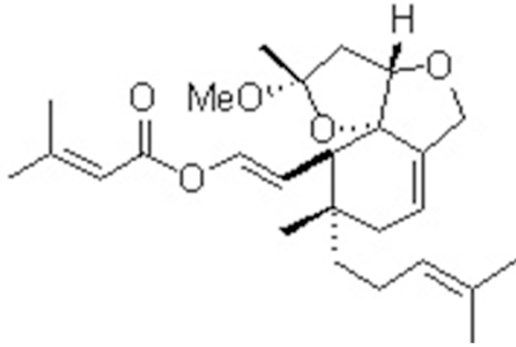


Figure 1. Structure of Neovibsanin B.

glioma cells were investigated. The results provide valuable insights into understanding the pathogenesis of GICs and offer a novel therapeutic approach for the treatment of human malignant glioma.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). The antibodies were purchased from Sigma (Sigma-aldrich, USA). Normal goat serum, 4',6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate. Anti-caspase-3 antibody (rabbit polyclonal against mouse, rat or human) and anti- β -actin antibody were obtained from Abcam Inc. (Cambridge, MA, USA) and cell counting kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan).

Cell culture

GL261 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM/F12 culture medium containing 10% FBS. Cells were maintained at 37°C in a humidified environment containing 5% CO₂.

Immunocytochemistry analysis

On the poly-L-lysine (Sigma) coated coverslips cell spheres were fixed with 4% paraformaldehyde. After blocking with normal goat serum, cells were incubated with primary antibodies. Washing of cells with PBS was followed by staining with Cy3-conjugated goat anti-rabbit or

FITC-conjugated goat anti-rabbit secondary antibody for 45 min. counterstaining of cell samples with 100 mg/ml DAPI for 20 min was followed by analysis with a confocal laser scanning microscope (Leica, Mannheim, Germany).

Determination of cell viability

Onto 96-well-plates 2.5 × 10⁵ cells/ml were seeded and treated with different concentrations of neovibsanin B (1, 2, 3, 4, 5, 6, 8, 10 nM) after 24 h. Dimethyl sulfoxide (DMSO) was used as a negative control. After 5 days of incubation, 10 μ l of thawed CCK-8 solution was added to each well. Plates were incubated for 4 h at 37°C and the absorbance was read at 450 nm with a reference wavelength of 600 nm using the Thermo Scientific™ Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Colony formation assay

GL261-NS cells were seeded at a density of 2.5 × 10⁵ cells/ml onto 96-well plates and incubated with 5 nM neovibsanin B, 10 μ M vincristine (VCR) dissolved in DMSO or DMSO as a negative control. After 7 days of incubation, an Olympus CX22 microscope (Olympus Corp., Inc., Tokyo, Japan) was used to calculate the number of colonies.

Flow cytometric analysis

For determination of apoptosis of the cells flow cytometry using the Annexin V-conjugated Alexa Fluor488 (Alexa488) Apoptosis Detection kit was employed. The cells after overnight serum starvation were treated with neovibsanin B for 96 h. The cells were harvested and washed in PBS. Then the cells were incubated with Alexa488 and propidium iodide in the dark. FACS Calibur instrument (BD Biosciences) equipped with the Cell Quest 3.3 software was used for analysis of stained cells.

Western blot analysis

The cells after treatment were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM sodium orthovanadate and 1 mM NaF), and using dye-binding method (Bio-Rad)

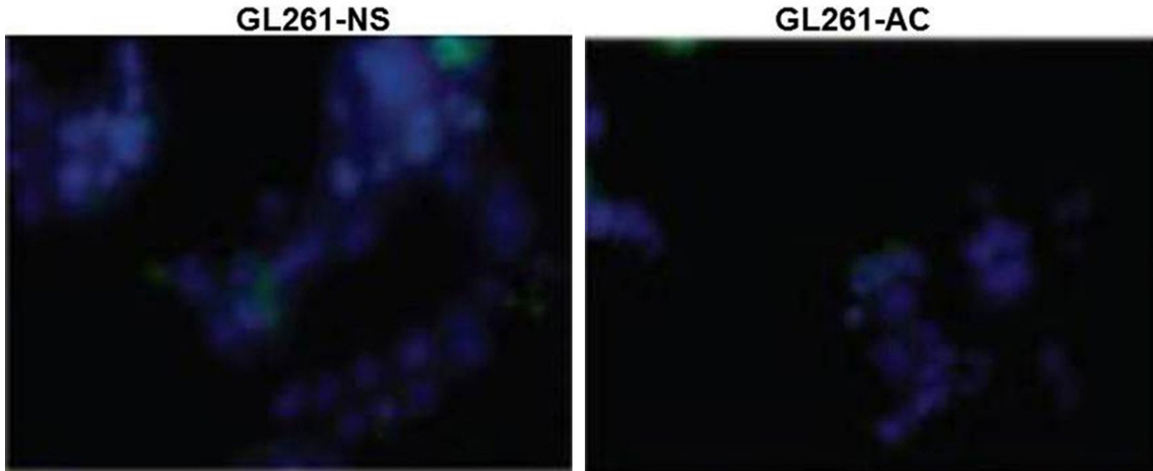


Figure 2. Neurosphere-like clones formed by GL261-NS and GL261-AC cells.

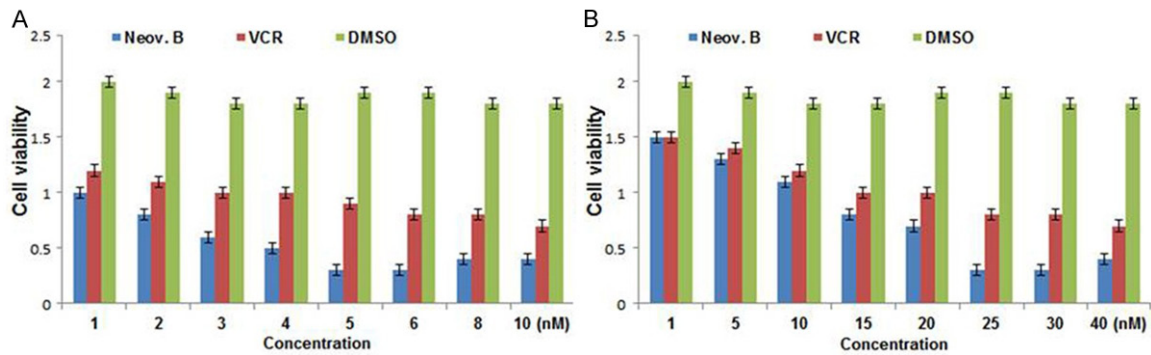


Figure 3. Neovibsanin B reduces the viability of GL261-NS and GL261-AC cells. GL261-NS and GL261-AC cells cultured in a 96-well plate were treated with different concentrations of neovibsanin B or VCR. DMSO was used as a negative control.

protein concentration was determined. The proteins were resolved on 15% SDS-PAGE and transferred to nitrocellulose membranes. Digtonin-based subcellular fractionation technique was used for cytosolic and mitochondrial fractions. Onto DS-PAGE equal volumes of cytosolic and mitochondrial fractions were resolved and transferred to nitrocellulose membranes. The membranes after incubation with primary antibody were washed, and then incubated with horseradish peroxidase anti-mouse or horseradish peroxidase antirabbit antibodies. Enhanced chemiluminescence system was used for visualization of immunoreactive bands. Gel imaging analysis system (Kodak ID, Kodak, Rochester, NY, USA) was used to analyse band images and densitometric analysis for quantification of intensities of the immunoreactive bands.

Preparation of a tumor-bearing animal model

Sixty mice (8 week-old) weighing 30 ± 5 g were obtained from the Experimental Animal Center of Shandong University (Jinan, China). The experimental procedures were approved by the ethics review committee for animal studies at Qilu Hospital, Shandong University and according to animal welfare. The mice were randomly assigned to four groups of 15 each: Sham-surgery, sham-surgery plus neovibsanin B, tumor plus placebo and tumor plus neovibsanin B. The mice in tumor groups were orthotopic transplanted GL261-NS cells. Briefly, mice anesthetized with 400 mg/kg chloral hydrate were injected 5 μ l of cell suspension (2×10^6 cells/ml) into the right caudate nucleus. Mice were placed on a stereotaxic instrument and micro-electrical drill (RWD Life Science, Inc., Shenzhen, China) was used to make a hole of 3

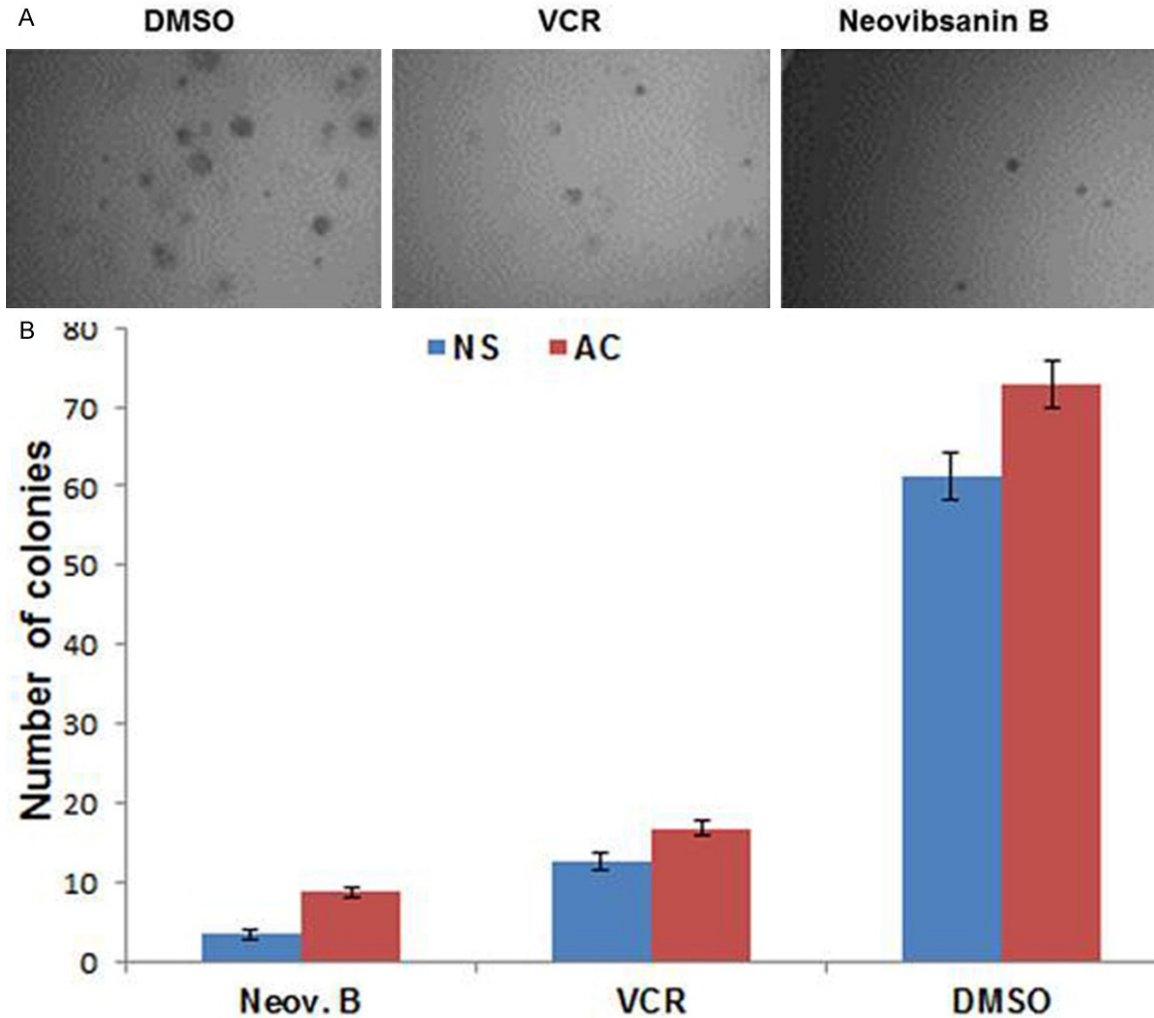


Figure 4. Neovibsanin B suppresses the colony formation of GL261-NS. Cells were cultured in 5 nM neovibsanin B, or 10 μ M VCR for 7 days. A. Morphology of colonies after drug treatment in GL261-NS cells. B. Number of colonies in each well of a 96-well plate was counted for GL261-NS and GL261-AC cells.

mm diameter on the skull 1.4 mm anterior to the anterior fontanel and 2.0 mm lateral to the sagittal suture. From the bregma and the dura mater stereotaxic coordinates were obtained according to a mouse brain atlas (12). Normal saline was given to the mice of sham surgery group, whereas neovibsanin B was injected to the mice of neovibsanin B treatment group after 24 h. This treatment was continued for 15 days. Upto 2 months after surgery the animal survival time was assessed.

Statistical analysis

For statistical analyses the SPSS 13.0 statistical software package (SPSS, Inc., Chicago, IL, USA) was used and the data are presented as the mean \pm S.D. One-way analysis of variance

with Fisher's least significant difference test was used for analysis of the results. The differences were considered statistically significant at $P < 0.05$.

Results

GL261-NS and GL261-AC cell identification and characterization

GL261-NS and GL261-AC cells when cultured in serum-free culture medium supplemented with bFGF, EGF, L-glutamine and insulin formed neurosphere-like clones (**Figure 2**). GL261-NS cells in these spheres showed higher expression of the stem cell marker, CD133 and very low level of GFAP expression, a biomarker for differentiated glial cells.

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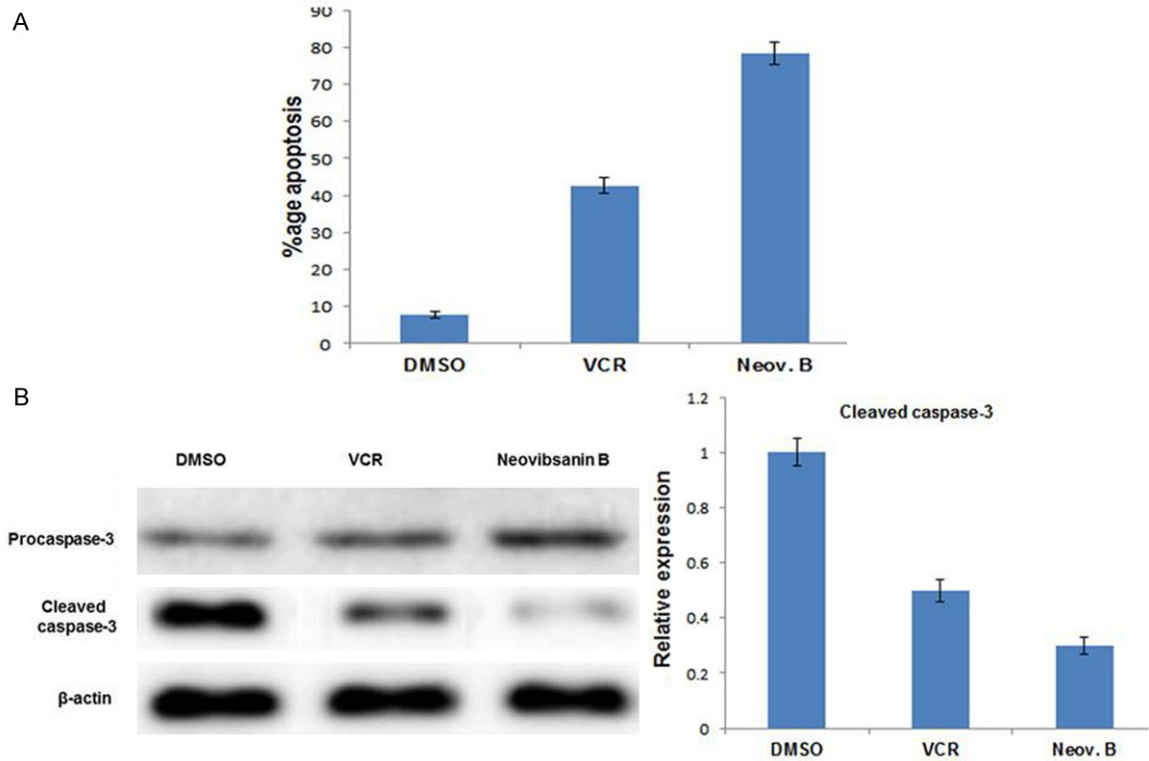


Figure 5. Neovibsanin B promotes the apoptosis of GL261-neurosphere cells. A. Cell apoptosis was evaluated by flow cytometry using Annexin V-fluorescein isothiocyanate/propidium iodide double staining. B. Protein expression of pro-caspase-3 and cleaved caspase-3 was examined by western blot analysis. B-actin was used as an internal control.

Effect of neovibsanin B on cell viability of GL261-NS and GL261-AC cells

We used CCK-8 kit to determine the effects of neovibsanin B on the viability of GL261-NS and GL261-AC cells. The commonly used anti-cancer agent, VCR was also used as positive control. The results revealed a significant decrease in the viability of GL261-NS and GL261-AC cells on treatment with neovibsanin B in a dose dependent manner. The decrease in viability was more in GL261-NS cells compared to that in GL261-AC cells. The IC_{50} value of neovibsanin B against GL261-NS and GL261-AC cells is 5 and 25 nM, respectively (**Figure 3**). Also the inhibition of cell viability due to neovibsanin B at the IC_{50} concentration was more compared to VCR ($P < 0.05$).

Effect of neovibsanin B on colony formation in glioma cell lines

We also examined the effect of neovibsanin B on colony formation in GL261-NS and GL261-AC cells. The results showed a significant

decrease in sphere-forming ability of GL261-NS and GL261-AC cells on treatment with neovibsanin B at a concentration of 5 and 25 nM. The number of colonies formed by GL261-NS cells on treatment with neovibsanin B, VCR and DMSO were 3.34 ± 1.02 , 12.53 ± 3.46 and $61.34 \pm 9.89\%$ respectively after 7 days (**Figure 4**). Similarly in GL261-AC cells, the number of colonies formed was 8.67 ± 2.21 , 16.78 ± 4.34 and $72.69 \pm 10.34\%$, respectively by neovibsanin B, VCR and DMSO. These results clearly suggest that neovibsanin B is significantly more effective at reducing colony formation than standard chemotherapeutic agent VCR.

Effect of neovibsanin B on apoptosis of GL261-NS cells

Apoptotic cell death was measured using the Annexin V-FITC/propidium iodide Apoptosis Detection kit. The results from flow cytometry revealed a marked increase in apoptotic cell death of GL261-NS cells on treatment with neovibsanin B (**Figure 5A**). The percentage of apoptotic cells 78.5 ± 9.3 , 42.7 ± 6.5 and $7.8 \pm$

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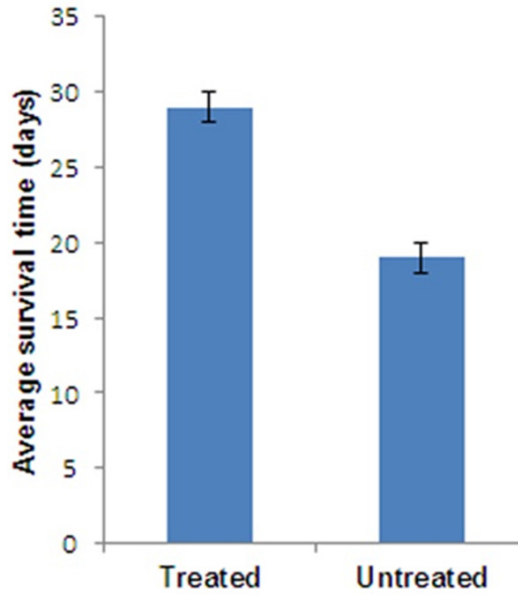


Figure 6. Average survival time in neovibsanin B treated and untreated mice after 65 days.

2.4 respectively in neovibsanin B, VCR and DMO treated cells (**Figure 5A**). The western blots showed a significant decrease in the level of activated caspase-3 on treatment with neovibsanin B after 24 h (**Figure 5B**). Thus, neovibsanin B promotes apoptosis of GL261-NS cells.

Neovibsanin B prolongs the median survival time of tumor-bearing mice

We also examined the effect of neovibsanin B on the survival time in mice bearing tumor xenografts. The mice bearing tumor xenografts on treatment with neovibsanin B had longer survival time compared to untreated mice. The survival time of treated and untreated mice was 29 and 19 days, respectively ($P < 0.05$, **Figure 6**). All animals in the sham-surgery and sham-surgery plus neovibsanin B groups survived for the entire study period.

Discussion

A small population of glioma cells, glioma-initiating cells (GICs) or glioma stem cells are shown to be responsible for the initiation of glioma [2-4]. These GICs are characterized by the property of initiating tumorigenesis and undergoing self-renewal. The present study demonstrates that GL261 glioma cells form NS-like clones and express CD133 in higher

proportion. In addition, GL261-NS cells in these spheres showed higher expression of the stem cell marker, CD133 and very low level of GFAP expression, a biomarker for differentiated glial cells. When GL261-AC cells were induced to differentiate in culture medium containing FBS the most of the differentiated cells became adherent and expressed the glial cell marker GFAP. These results were in agreement with the earlier reported results from Tunan et al. [13].

The most commonly used chemotherapeutic agents for the treatment of malignant glioma are temozolomide and nitrosourea derivatives like ACNU. However, there is development of chemoresistance in the patients which is a major hindrance to the application of these alkylating agents [14]. Similarly, for vincristine and vinblastine it is reported that patients develop vincristine and vinblastine. Tumor stem cells have the characteristic property of regenerating from the eradicated original tumor, thus leading to tumor recurrence [15]. Therefore, the discovery of a novel molecule for targeting tumor stem cells is needed to develop more effective treatment strategies for glioma.

Neovibsanins, was found to display neurite outgrowth activity in PC12 cells, suggesting that neovibsanin-type compounds could be promising candidates for the development of novel therapeutic agents to treat neurological diseases [16]. However, the present study for the first time demonstrates that neovibsanin B significantly reduces the cell viability of GL261-NS and GL261-AC in a dose-dependent manner. The inhibition of cell proliferation was more significant in GL261-NS cells. Neovibsanin B caused cell death in GL261-NS cells by inducing apoptosis. The neovibsanin B induced cell apoptosis of GL261-NS cells was found to be related to caspase-3 activity. The effect of neovibsanin B on the survival time in mice bearing tumor xenografts revealed longer survival time of the treatment group compared to untreated. All animals in the sham-surgery and sham-surgery plus neovibsanin B groups survived for the entire study period. Thus suggesting that neovibsanin B may be an effective therapy for targeting GICs in glioma, reducing tumor burden and extending survival.

In conclusion, the present study demonstrated that neovibsanin B significantly reduces cell viability of GL261-NS and GL261-AC cells,

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induces apoptotic cell death prolongs the median survival time of glioma-bearing mice. Therefore, neovibsanin B can be a promising therapeutic agent for the treatment of malignant glioma.

Disclosure of conflict of interest

None.

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References

- [1] Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med* 2008; 359: 492-507.
- [2] Stiles CD, Rowitch DH. Glioma stem cells: a midterm exam. *Neuron* 2008; 58: 832-46.
- [3] Vescovi AL, Galli R, Reynolds BA. Brain tumour stem cells. *Nat Rev Cancer* 2006; 6: 425-36.
- [4] Natsume A, Kinjo S, Yuki K, Kato T, Ohno M, Motomura K, Iwami K, Wakabayashi T. Glioma-initiating cells and molecular pathology: implications for therapy. *Brain Tumor Pathol* 2011; 28: 1-12.
- [5] Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, Pilozzi E, Larocca LM, Peschle C, De Maria R. Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* 2006; 13: 1238-41.
- [6] Fukuyama Y, Minami H, Takeuchi K, Kodama M, Kawazu K. Neovibsanines A and B, Unprecedented Diterpenes from *Viburnum awabuki*. *Tetrahedron Lett* 1996; 37: 6767-70.
- [7] Fukuyama Y, Kubo M, Minami H, Yuasa H, Matsuo A, Fujii T, Morisaki M, Harada K. Rearranged Vibsanin-Type Diterpenes from *Viburnum awabuki* and Photochemical Reaction of Vibsanin B. *Chem Pharm Bull (Tokyo)* 2005; 53: 72-80.
- [8] Gallen MJ, Williams CM. Total Synthesis of (\pm)-5,14-bis-epi-Spirovibsanin A. *Org Lett* 2008; 10: 713-15.
- [9] Chen AP, Williams CM. Total Synthesis of (\pm)-2-O-Methylneovibsanin H. *Org Lett* 2008; 10: 3441-43.
- [10] Imagawa H, Saijo H, Kurisaki T, Yamamoto H, Kubo M, Fukuyama Y, Nishizawa M. Total Synthesis of (\pm)-Neovibsanin B. *Org Lett* 2009; 11: 1253-55.
- [11] Esumi T, Mori T, Zhao M, Toyota M, Fukuyama Y. First Enantiocontrolled Formal Synthesis of (+)-Neovibsanin B, A Neurotrophic Diterpenoid. *Org Lett* 2010; 12: 888-91.
- [12] San Diego. Franklin KB, Paxinos G, editors. *The Mouse Brain in Stereotaxic Coordinates*. 2nd edition. Academic Press; 1997. pp. 49-95.
- [13] Tunan C, Liang Y, Fei L, Rong H, Shengli H, Yi Y, Chuan L, Zhao Li, Chuhua F, Liu C, Zhi C, Jishu X, Hua Feng. Salinomycin inhibits the tumor growth of glioma stem cells by selectively suppressing glioma-initiating cells. *Mol Med Reports* 2015; 11: 2407-12.
- [14] Sarkaria JN, Kitange GJ, James CD. Mechanisms of chemoresistance to alkylating agents in malignant glioma. *Clin Cancer Res* 2008; 14: 2900-08.
- [15] Singh A, Settleman J. EMT, cancerstem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* 2010; 29: 4741-51.
- [16] Kim KY, Yu SN, Lee SY, Chun SS, Choi YL, Park YM, Song CS, Chatterjee B, Ahn SC. Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization. *Biochem Biophys Res Commun* 2011; 413: 80-6.