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Effect of blonanserin on the proliferation and migration of glioblastoma cells

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Received August 18, 2021, accepted November 18, 2022

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Pharmazie 78: 37-41 (2023) *doi: 10.1691/ph.2023.1821*

Glioblastoma is a highly malignant and invasive brain tumor, and there is an urgent need to establish a treatment option that prevents its growth and metastasis. Blonanserin is an antipsychotic drug widely used in the treatment of schizophrenia. It has recently been reported to inhibit the growth of breast cancer cells. In this study, we investigated the effect of blonanserin on the proliferation and migration of glioblastoma cells. The anti-proliferative activity of blonanserin was evaluated in terms of cell viability, competition, and cell death pathways in glioblastoma. Cell viability studies showed that blonanserin had growth inhibitory ability regardless of the malignancy of glioblastoma cells, but at concentrations close to its IC_{50}, it only had a slight cell death–inducing effect. Blonanserin showed growth inhibitory activity without D_2 antagonism following an independent competition analysis using blonanserin and D_2 antagonists. When the anti-migration activity of U251 cells was measured, blonanserin was found to attenuate cell migration. Furthermore, treatment with blonanserin at concentrations close to its IC_{50} value inhibited extensive filament actin formation. In conclusion, blonanserin inhibited the proliferation and migration of glioblastoma cells independent of D_2 antagonism. The present study shows that blonanserin may serve as a seed compound for the discovery of new glioblastoma therapeutics to prevent the growth and metastasis of glioblastoma.

1. Introduction

Glioblastoma is a highly malignant brain tumor and one of the most disadvantageous of all carcinomas. The 5-year survival rate for affected patients is 6.9%, and even in the 15-39 age group, which has the highest survival rate, the survival rate is only 26.6% (Ostrom et al. 2022). Because of its highly invasive nature, glioblastoma spreads over a wide area of brain tissue, making surgical removal of all tumor tissue difficult, resulting in the formation of recurrent tumors from the remaining tissue (Holland 2000). The standard drug therapy for glioblastoma is temozolomide, which is an orally available alkylating agent prodrug that induces tumor cell death by methylating DNA purine bases (Zhang et al. 2012). The combination of radiotherapy and temozolomide in patients with glioblastoma showed longer survival than radiotherapy alone (Stupp et al. 2005, 2009; Wick et al. 2012); however, the median survival time of the combination therapy was only 2.5 months longer (Stupp et al. 2005). In addition, inherent resistance or tolerance to temozolomide treatment reduces its efficacy on glioblastoma chemotherapy (Weller et al. 2015). One of the major mechanisms of temozolomide resistance has been postulated to result from the increased expression of *O*(6)-methylguanine-DNA methyltransferase (MGMT) in temozolomide–resistant glioblastoma cells (Zhang et al. 2012; Lee 2016). Therefore, there is a need to discover new antitumor drugs with different pharmacological mechanisms and higher efficacy than temozolomide.

Drug repositioning is an alternative strategy for drug discovery that can reduce the time required to bring drugs to the market using clinical drugs with a known pharmacokinetic and adverse effect profile (Siegelin et al. 2021; Seliger and Hau 2018). Recently, a drug repositioning strategy against glioblastoma reported blood–brain barrier (BBB)-penetrating epileptic drugs (Hayashi et al. 2016), antidepressants (Jeon et al. 2011; Bilir et al. 2008), and schizophrenia drugs (Suzuki et al. 2016; Sanomachi et al. 2019) as potential therapeutic agents. Even when drug repositioning is difficult at the clinical dose of a drug, it is expected to become a seed compound for the discovery of new therapeutic agents by finding novel pharmacological actions (Chen et al. 2018; Huang et al. 2015).

Furthermore, pharmacological screening studies of dopamine D_2 receptor antagonists have found that several D_2 antagonists have anticancer activity against glioblastoma cells (Sachlos et al. 2012; Cheng et al. 2015). In a study on glioblastoma cells with genetic mutations in the D_2 receptor, it was reported that overexpression of DRD2 increased cell proliferation, while knockdown decreased it by over 96 h. Nonetheless, the rate of change was only approximately 10%, and the relationship between anticancer effects and D_2 antagonism remains unclear (Weissenrieder et al. 2020).

Blonanserin is an antipsychotic drug widely used in the treatment of schizophrenia; it has a strong blocking effect on dopamine D_2 and serotonin 5-HT_{2A} receptors. In particular, it has a higher dopamine D_2 receptor–binding affinity than other antipsychotics and is characterized by a higher dopamine D_2 than serotonin 5-HT_{2A} receptor–binding affinity. In addition, blonanserin is postulated to cause fewer side effects following its binding to the aforementioned receptors because of its low binding affinity for other receptors, such as adrenaline α_1, histamine H_1, and muscarinic acetylcholine M_1 (Tenjin et al. 2013). The anticancer activity of blonanserin has been the only reported effect on breast cancer cells (Maeshima et al. 2021). Therefore, the purpose of this study was to determine whether blonanserin has anticancer activity against glioblastoma cells and whether this effect is associated with D_2 receptor antagonism.

2. Investigations and results

2.1. Cytotoxic effect of blonanserin on glioblastoma cells

The dose–response curve for the viability of U251 and T98G cells treated with blonanserin is shown in Fig. 1. The 50% inhibitory

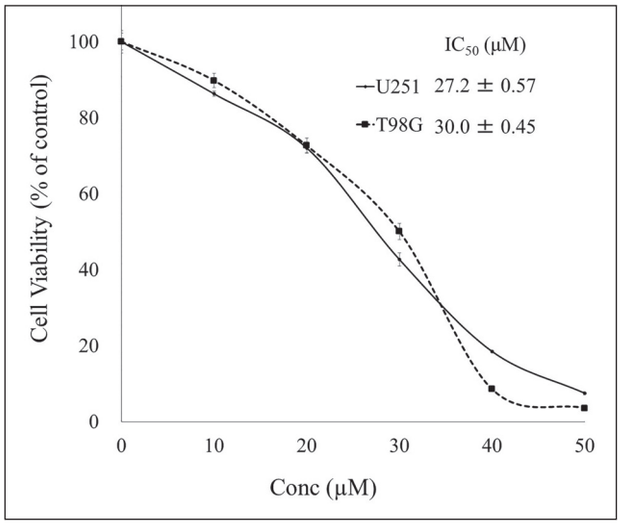

Fig. 1: Effect of blonanserin on glioblastoma cell viability. A water-soluble tetrazolium salt-8 (WST-8) assay was carried out in glioblastoma cells treated with blonanserin at the indicated concentrations for 24 h. Each point represents the mean ± SEM compared with WST-8 intensities for cells untreated with blonanserin (n = 4 independent experiments).

concentrations (IC_{50}) of blonanserin that were calculated from the dose–response of cell viability at 24 h were 27.2 μM and 30.0 μM in U251 and T98G cells, respectively.

2.2. *Effect of blonanserin on cell viability via a non-dopamine D_2 mechanism*

To investigate the role of dopamine D_2 antagonism in blonanserin–induced inhibition of cell viability, two glioblastoma cell lines were treated with both blonanserin and a D_2 agonist (dopamine, pramipexole, or bromocriptine). Treatment with a dopamine D_2 agonist did not affect blonanserin–induced inhibition of cell viability (Fig. 2).

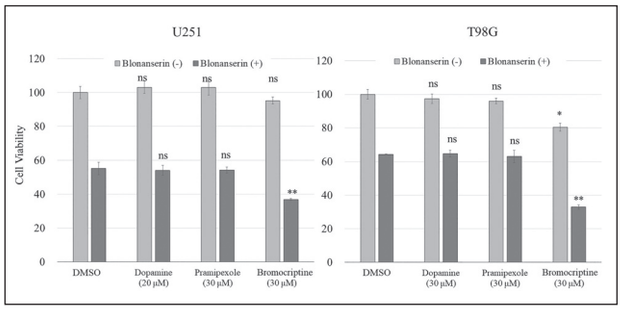

Fig. 2: Effect of blonanserin and dopamine D_2 agonists on cell viability. Glioblastoma cells were treated with both blonanserin (30 μM in U251 cells, 30 μM in T98G cells) and a dopamine D_2 agonist (dopamine, pramipexole, or bromocriptine) for 24 h. Each point represents the mean ± SEM (Control=WST-8 intensities for untreated cells); n = 4 independent experiments). Statistical analysis was performed by one-way analysis of variance and subsequent Dunnet's/hoc test in each group with and without blonanserin. The symbols' ** ($p < 0.01$) and * ($p < 0.05$) indicate significant differences between dopamine D_2 agonists and DMSO treatment by one-way analysis of variance and subsequent Dunnet's/hoc test. The symbol ns indicates not significant compared with the control. The 100% viable cells correspond to untreated cells (DMSO).

2.3. *Effect of blonanserin on cell proliferation and cell death*

We examined whether the inhibitory effect on cell viability at the IC_{50} concentrations of blonanserin was due to cell death. As shown in Figs. 3A and 3B, treatment with 30 μM of blonanserin did not result in the formation of condensation–enriched nuclei,

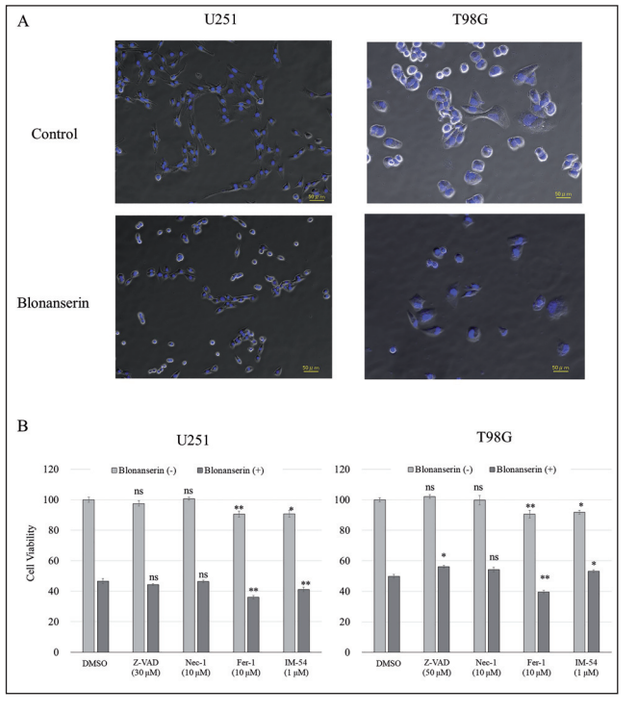

Fig. 3: Effect of blonanserin on cell proliferation and cell death. (A) Glioblastoma cells were treated with blonanserin (0 or 30 μM) for 24 h and then Hoechst 33342 staining was performed. (B) WST-8 assay was performed in untreated U251 and T98G cells and those treated with a caspase–dependent apoptosis inhibitor (Z-VAD), or a caspase–independent necrotic cell death inhibitor (Nec-1, Fer-1, IM-54), and blonanserin for 24 h. Each point represents the mean ± SEM (Control = WST-8 intensities for untreated cells; n = 4 independent experiments). Statistical analysis was performed by one-way analysis of variance and subsequent Dunnet's/hoc test in each group with and without blonanserin. The symbols' ** ($p < 0.01$) and * ($p < 0.05$) indicate significant differences between cell death inhibitors and DMSO treatment by one-way analysis of variance and subsequent Dunnet's/hoc test. The symbol ns indicates not significant compared with the control. The 100% viable cells correspond to untreated cells (DMSO).

which are characteristic of apoptosis, or disruption of the detailed membrane, which is characteristic of necrotic cell death. The pan–caspase inhibitor z-VAD-FMK slightly reduced the inhibition of viability by blonanserin in T98G cells (Fig. 3C). Furthermore, the effect of programmed necrosis inhibitors on blonanserin–induced suppression of cell viability was investigated. IM-54, an inhibitor of oxidative stress–induced necrosis, slightly decreased the effect of blonanserin–induced inhibition of T98G cell proliferation (Fig. 3C). These results indicate that blonanserin mainly inhibits cell proliferation and slightly induces cell death.

2.4. *Effect of blonanserin on U251 cell migration*

To examine the effect of blonanserin on glioblastoma migration, a scratch assay was performed. Treatment with blonanserin (20 μM) inhibited the migration of U251 cells compared to controls (Fig. 4).

2.5. *Effect of blonanserin on filamentous actin morphology*

Fluvoxamine, which has growth inhibitory effects on glioblastoma cells, has been reported to disrupt stress fibers and focal adhesions (Hayashi et al. 2016). Therefore, the effect of blonanserin on the F-actin cytoskeleton was examined using phalloidin staining. Formation of the F-actin cytoskeleton was induced in control cells (Figure 5A), but not in blonanserin–treated cells (Fig. 5B).

3. Discussion

In the present study, we hypothesized that blonanserin, a dopamine D_2 antagonist, may be an effective drug option for treating glioblastoma. To support this hypothesis, blonanserin showed inhibi-

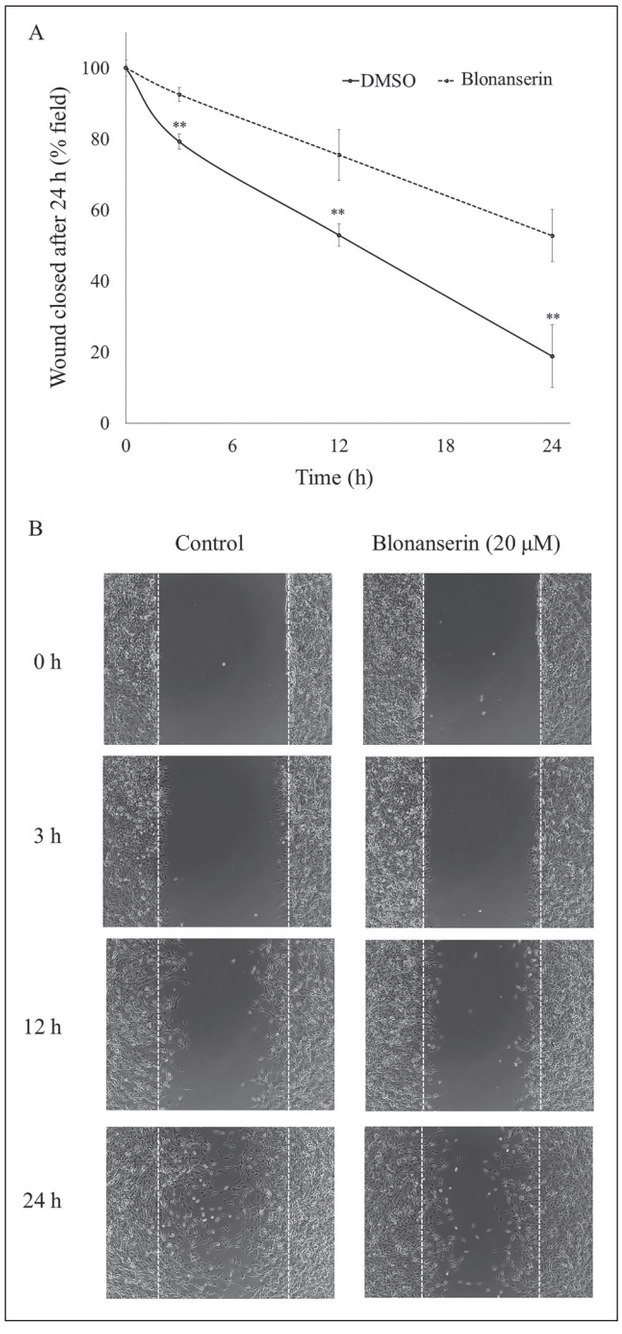

Fig. 4: Effect of blonanserin on cell migration in U251 cells. U251 cells were treated with blonanserin (20 μM) for 0, 3, 12, and 24 hours to examine cell migration into the scratch area (average of 5 independent points). (A) U251 cells were untreated or treated with blonanserin at 0, 3, 12, and 24 hours after scratching. (B) Scratch residuals were analyzed at 3, 12, and 24 hr after scratching. The symbol ** ($p < 0.01$) indicates significant differences between blonanserin-treated (dotted line) and untreated (solid line) cells by two-tail t-test.

tory effects on glioblastoma cell growth. The inhibitory activity of the compound was also effective against temozolomide–resistant glioblastoma cells (Fig. 1).

The inhibitory activity of blonanserin on cell proliferation was not attenuated by treatment with the D_2 agonists, dopamine, pramipexole, or bromocriptine (Fig. 2). These results may suggest that the growth inhibitory activity of blonanserin is not due to D_2 antagonism. In a study in which D_2 antagonists were applied to glioblastoma cells with forced expression or knockdown of D_2 receptors, the difference in growth inhibitory capacity was approximately 10% (Weissenrieder et al. 2020). Haloperidol has dopamine D_2 antagonist activity, but its antagonist activity is only about one-tenth that of blonanserin. Haloperidol was recently

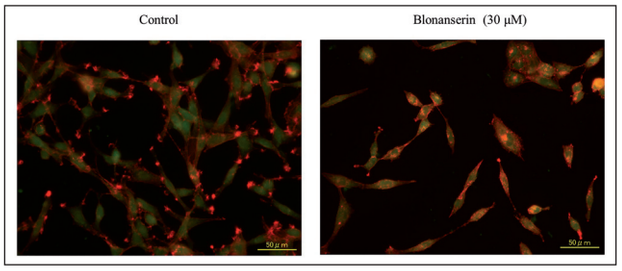

Fig. 5: Effect of blonanserin on filamentous actin (F-actin) cytoskeleton. Immunofluorescence staining with rhodamine X conjugated phalloidin (red, F-actin) and Alexa Fluor 488-conjugated DNase I (green, G-actin) showed that the F-actin cytoskeleton was decreased in the blonanserin (30 μM) group compared with the control group after 24 h.

found to inhibit glioblastoma cell proliferation, and its IC_{50} value was comparable to that of blonanserin in this study (Papadopoulos et al. 2020). The present results may be consistent with previous reports that D_2 antagonism does not affect glioblastoma cell growth inhibition.

Although blonanserin has been reported to inhibit cell proliferation in breast cancer cells, the underlying mechanism is yet to be clarified (Maeshima et al. 2021). The anticancer effect mechanism of drugs on glioblastoma or neuroblastoma has been reported to involve not only apoptosis but also caspase–independent cell death for example, necroptosis (Jiang et al. 2011), ferroptosis (Yee et al. 2020), and oxidative stress–induced necrosis (Alshangiti et al. 2019). Morphological observation of glioblastoma cells stimulated with blonanserin at concentrations close to the IC_{50} value showed little formation of apoptotic bodies or disruption of cell membranes. There was also no marked difference in the inhibitory effect on cell proliferation when blonanserin was combined with caspase inhibitors and various caspase–independent cell death inhibitors. These results suggest that the anticancer effect of blonanserin is not related to the induction of cell death.

In the scratch assay, the ability of cell migration was suppressed by blonanserin stimulation compared with that observed in the controls (Fig. 4). Furthermore, antibody staining of U251 cells showed that F-actin, which plays an important role in cytoskeleton formation, was reduced by blonanserin stimulation at concentrations close to the IC_{50} (Fig. 5). The inhibition of glioblastoma cell migration by inhibiting F-actin polymerization has been reported using the antidepressant fluvoxamine (Hayashi et al. 2016). In addition, a comprehensive DNA methylation study of neuroblastoma cells showed that stimulation with blonanserin increased the methylation of CpG sites of DNA for cell adhesion and morphogenesis, which are related to F-actin (Murata et al. 2014). These results suggest that blonanserin inhibits the proliferation and migration of human glioblastoma cells by inducing a defect in F-actin formation.

We have shown that the effective concentration of blonanserin required to inhibit glioblastoma cell migration and proliferation *in vitro* is approximately 20-30 μM (Figs. 1 and 4). However, at clinical doses of blonanserin (8-24 mg/day), the maximum blood concentration of blonanserin is about 2 nM, requiring approximately 1,000-fold higher doses to exert its anticancer effects. Therefore, the clinical significance of the results of this study is not considered high. However, blonanserin has an 8-membered carbon ring, a benzene ring, and a piperazine moiety, and each substructure can be chemically converted. Therefore, blonanserin may be a promising seed compound for the discovery of new glioblastoma therapeutics.

In conclusion, this study demonstrated that blonanserin inhibits cell proliferation and migration of human glioblastoma cells. Further studies are required to provide additional data on the anticancer effects and underlying inhibitory mechanisms of blonanserin. Our results suggest that blonanserin may serve as a seed compound for the discovery of new glioblastoma therapeutics.

4. Experimental

4.1. Materials

Blonanserin (Tokyo Chemical Industry (TCI), Tokyo, Japan), dopamine (3-hydroxytyramine hydrochloride, TCI, Tokyo, Japan), pramipexole (TCI, Tokyo, Japan) or bromocriptine mesylate (FUJIFILM Wako Pure Chemical, Osaka, Japan) Z-VAD-Fmk (Peptide Institute, Osaka, Japan) were purchased from commercial source. Necrostatin-1, ferrostatin-1, and IM-54 were prepared by according to reported literature (Degterev et al. 2005; Dixon et al. 2012; Dodo et al. 2005).

4.2. Cell culture

Human glioblastoma cell lines (U251 and T98G cells) were obtained from Dr. T. Sasayama (Kobe University, Japan). U251 and T98G cells were cultured in Dulbecco's Modified Eagle Medium (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (Biosera, Kansas, USA) without antibiotics. Cells were grown in an incubator at 37 °C under a 5% CO_2 atmosphere and seeded at a density of 80% confluence using 0.05 w/v% Trypsin-0.53 mmol/L EDTA-4Na Solution with Phenol Red (FUJIFILM Wako Pure Chemical, Osaka, Japan).

4.3. Measurement of cell viability and proliferation

Test compounds were prepared by diluting a DMSO solution of blonanserin 1000-fold in medium to final concentrations of 0, 10, 20, 30, 40, and 50 μM. U251 and T98G cells were seeded at a density of 3,000 cells/well in 96-well plates and incubated at 37 °C for 24 h. Next, the cells were treated with the test compound in medium solution, and incubated at 37 °C for 24 h. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

4.4. Measurement of cell migration in vitro (wound healing assay)

U251 cells were seeded at a density of 200,000 cells in a 3.5 cm dish, incubated at 37 °C for 48 h to 100% confluence, and then scratched with a 200 μL pipette tip (Liang et al. 2007). The medium was changed to remove floating cells and replaced with a 10% serum medium containing blonanserin. After the initial scratching, the scratch area was measured at 5 points in each dish, and the U251 cells were incubated at 37 °C in a CO_2 atmosphere for 1, 3, 12, and 24 h. For each of the measurement times, the rates of wound closure were measured. To assess the ability of cells to migrate, the area of cells at the initial wound was compared to the area after each measurement time (1, 3, 12, and 24 h of incubation).

4.5. Fluorescence imaging

U251 cells were seeded at a density of 80,000 cells/well in a 3.5 cm glass–based dish and incubated at 37 °C for 24 h. The cells were treated with blonanserin in DMSO (final concentrations of 0 and 30 μM) and incubated at 37 °C for 24 h. Cells were stained with Hoechst 33342 (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. The fluorescent signal was detected using a fluorescence microscope. The cytoskeleton was dyed with Phalloidin–Rhodamine X conjugated (FUJIFILM Wako Pure Chemical, Osaka, Japan) and deoxyribonuclease I, Alexa Fluor™ 488 Conjugate (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. Briefly, the excess media were removed and the cells were washed with PBS. Next, 4% formaldehyde in PBS (Nacalai tesque, Kyoto, Japan) was added to the cells at ambient temperature for 1 h and further washed with PBS. The cells were incubated with 1.0% (v/v) Triton X-100 in PBS for 10 min three times. Lastly, the cells were incubated with phalloidin–rhodamine X (1:2000) and deoxyribonuclease I, Alexa Fluor™ 488 Conjugate (1:250) in 0.03% (v/v) Triton X-100 in PBS at 4 °C for 12 h, washed with PBS, and then observed under a fluorescence microscope.

4.6. Statistical analysis

Data are presented as means ± standard error (S.E.) (n = number of observations). We performed at least three independent experiments on different days to confirm their reproducibility. Data were analyzed statistically using the Student's t-test or one-way analysis of variance and subsequent Dunnet's/hoc test for comparison with the control group (JMP Pro® 15, SAS Institute).

Funding: This work was supported in part by JSPS KAKENHI, Grant Numbers 21K09142 (to T.S.).

Acknowledgements: We would like to thank Dr. Takashi Sasayama for providing glioblastoma cells. We would like to thank Editage (www.editage.com) for English language editing.

Conflict of interest: The authors declare no conflict of interest.

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