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Research report

AG-1031 and AG-1503 improve cognitive deficits by promoting apoptosis and inhibiting autophagy in C6 glioma model rats

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ABSTRACT

High-grade gliomas (HGGs; grades III and IV) are the most common and aggressive adult primary brain tumors, and their invasive nature ranks them the fourth in the incidence of cancer death. In our previous study, we found that AG-1031 and AG-1503 showed inhibitory effects on several cancer cell lines. In this study, C6 glioma-bearing rats were treated with AG-1031 or AG-1503. Western blot results of autophagy-associated protein (LC3 II/I, Beclin-1) and apoptosis-associated proteins (caspase-3, Bcl-2, Bax) revealed that AG-1031 could activate apoptotic signal pathway via inhibiting autophagy process in cancer cells. HE staining indicated that the tumor volumes were significantly decreased in AG-1031 and AG-1503 treated rats compared to non-treated C6 glioma-bearing rats. Meanwhile, AG-1031 and AG-1503 significantly decreased the expression of VEGF, a marker of invasion ability of tumor, in tumor tissue. The novel object recognition test showed that cognitive functions in C6 glioma-bearing rats were considerably damaged, whereas AG-1031 and AG-1503 significantly impeded the cognitive impairment. AG-1031 and AG-1503 efficiently alleviated the glioma-induced impairments of long-term potentiation (LTP), which was damaged in C6 glioma-bearing rats. Furthermore, AG-1031 and AG-1503 augmented the expression of synaptophysin (SYP), which were decreased in glioma rats. In conclusion, our results suggest that AG-1031 and AG-1503 can inhibit the expansion of glioma, and improve the cognitive impairment caused by glioma in glioma-bearing rats.

1. Introduction

According to the classification of World Health Organization (WHO), anaplastic astrocytomas and glioblastoma multiformes are classified as high-grade gliomas (HGGs), while low-grade gliomas (LGGs) includes grades I and II tumors of astrocytic and grade II tumors of oligodendroglial lineage (Fang et al., 2014, Zhang et al., 2015, Kindy et al., 2016). Approximately 140,000 people were killed by malignant brain tumors worldwide per year (Towner et al., 2015). Malignant glial tumors, especially glioblastomas, can easily invade into periphery normal tissues and may not be resected entirely through medical procedures (Song et al., 2014). The vascular endothelial growth factor (VEGF) plays a crucial role in tumor angiogenesis (Folkman and Klagsbrun, 1987, Hobson et al., 2000). In addition, the continuous formation of new vessels is considered related to tumor expansion (Benjamin and Keshet, 1997). Jin Kim et al. verified that the inhibition of expression of an angiogenic factor spontaneously produced by tumor cells might suppress tumor expansion *in vivo* by treating glioma-carrying nude mice with an antibody specific for VEGF (Kim et al., 1993). Therefore, the inhibition of VEGF production or function could be an effective therapy for the inhibition of tumor expansion (Borgström et al., 1996).

Neurocognitive deficits are common in patients with brain tumors. It is said that 80% of cases were detected problems using detailed neurocognitive tests (Day et al., 2016). The majority of patients with brain tumors, especially metastases, meningiomas and malignant gliomas, are proved obvious defects in a variety of neurocognitive domains. In addition, there is no significant association between neurocognitive deficits and brain tumor location (Hoffermann et al., 2017). The presence and degree of neurocognitive deficits become increasingly important markers for the progression of brain tumors. Radiotherapy, an effective therapeutic method for various brain tumors at present, has side effects on

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quality of life and neurocognitive function in survivors (Saad and Wang, 2015). It is necessary to create new drugs and therapeutic strategies to treat neurocognitive deficits in patients with brain tumors.

The hippocampus plays a critical role in the association of information from short-term memory to long-term memory (Liu et al., 2016). It is wildly accepted that long-term potentiation (LTP), which could describe synaptic plasticity, is associated to acquisition and learning behaviors (Szu and Binder, 2016). LTP is an activity-dependent escalation in synaptic transmission between two neurons (Shapiro, 2001), which is based on cell structure as well. Synaptophysin (SYP), one of the first synaptic proteins, is demonstrated to be related to the synaptic vesicle cycle (Adams et al., 2015). Leila Tarsa et al. found that proposed irregular expression level of SYP among neurons in SYP-mutant mice might induce neurologic abnormalities (Tarsa and Goda, 2002), which indicated that SYP played a surprise role in regulating activity-dependent synapse formation. Meanwhile, postsynaptic density 95 (PSD 95), a central protein of postsynaptic signalling complexes comprising glutamate receptors, ion channels, signalling enzymes and adhesion proteins (Fernández et al., 2014), plays a significant role in synaptic transmission and synaptic plasticity (Husi et al., 2000, Grant, 2012).

Autophagy (self-eating) is defined as process, in which cells degrade their own protein and organelle with lysosomes in response to normal growth requirements and adverse stress (Klionsky and Emr, 2000, Mizushima and Komatsu, 2011). An increasing number of studies in different cancers demonstrated that high level of autophagy contributed to the progression of established cancers (Rosenfeldt et al., 2013, Rao et al., 2014). Highly proliferate property of cancer cells made autophagy critical to provide energy source to meet the metabolic requirement (Greene et al., 2016). However, it has been generally accepted that apoptotic response would be aroused when stress exceeds a critical threshold (Delou et al., 2016), which leads to a regulated form of cell death (Galluzzi et al., 2015).

AG-1031 and AG-1503 are small molecule drugs that showed inhibitory activity of PC4. The two small molecules have been identified that they can be applied for oncology indication by inhibiting human transcriptional positive cofactor 4 (PC4), which is a novel factor caused cancer. It has been proved that AG-1031 and AG-1503 can inhibit the viability of several cancer cell lines. And in our previous study, AG-1031 could induce autophagic cell death and apoptosis in C6 glioma cells *in vitro* (Yan Wang et al., 2018).

Here, we aim to investigate the effects of AG-1031 and AG-1503 on C6 glioma growth in rat glioma model and their effects on the cognitive dysfunction induced by glioma. Our results suggest that AG-1031 and AG-1503 can suppress the expansion of glioma *in vivo*, and improve the cognitive impairment caused by glioma.

2. Results

2.1. AG-1031 and AG-1503 inhibited proliferation of rat glioma cell line

To test if both molecules have specific inhibitory activities on glioma cells, rat C6 cells were treated with different concentrations of AG-1031 or AG-1503. As shown in Fig. S1, either AG-1031 or AG-1503 were markedly influenced the growth of C6 cells.

2.2. Effects of AG-1031 and AG-1503 on tumor size

HE-stained sections of untreated gliomas showed heteromorphism characteristics of glioma cells, infiltration of tumor cells into normal tissues, blurred boundaries, and the formation of new blood vessels within the tumors (Fig. 1). The tumor volumes (mm^3) were defined as $ab^2/2$ (a: long diameter; b: short diameter) as previously described and the statistical analysis were shown in Fig. 1M. It showed that tumor volumes were significantly smaller in AG-1031-treated rats compared to those of untreated glioma rats (Fig. 1C and M, $P < 0.001$). The tumor volumes in AG-1503-treated rats were also decreased observably compared to C6 group rats (Fig. 1D and M; $P < 0.001$). In addition, the amount of irregular lamellar necrosis was clearly increased. In the center of the tumor necrosis, no cell structures were found; instead, large amounts of debris and shrunken glioma cells were discovered at the boundary of the necrosis and normal tumor tissues, especially in C6 group (Fig. 1F).

2.3. Effects of AG-1031 and AG-1503 on VEGF expression in glioma rats

To clarify whether AG-1031 and AG-1503 affect the expression of VEGF in glioma model rats, immunofluorescence assay was conducted. VEGF was expressed in tumor area in C6 group, AG-1031 group and AG-1503 group (Fig. 2A). The intensity of VEGF of each

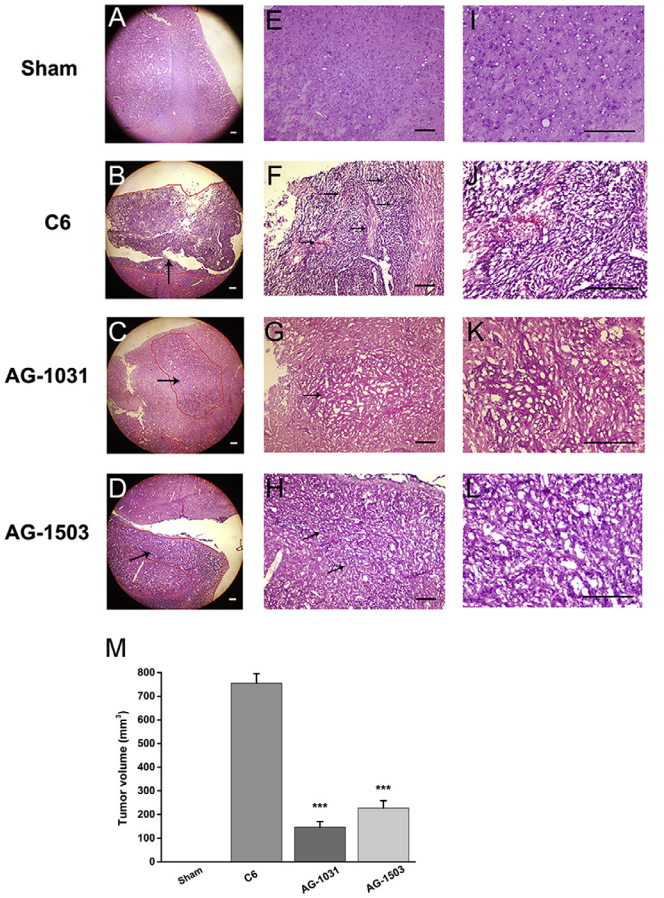

Fig. 1. Effects of AG-1031 and AG-1503 on tumor size. The brain tissue sections were prepared from Sham group, C6 group, AG-1031 group and AG-1503 group at 14–15 DPI. (A-L) HE staining histological staining of putamen session in each group at different magnifications. Bar = 1 mm. (B-D) The images with 12.5 × magnification showed the maximum cross sections of tumor in C6 group, AG-1031 group and AG-1503 group. The red circle indicated the tumor session in each group. (E-H) The images with 40 × magnification were derived from areas in B-D. (F) Black arrows indicate the necrosis and hemorrhage areas in C6 glioma rat. (I-L) The images with 100 × magnification were derived from areas in E-H. HE, hematoxylin and eosin; DPI, days post-implantation. (M) Determination of the tumor volume in each group. ***$P < 0.001$, significant difference between C6 group and AG-1031 group/AG-1503 group.

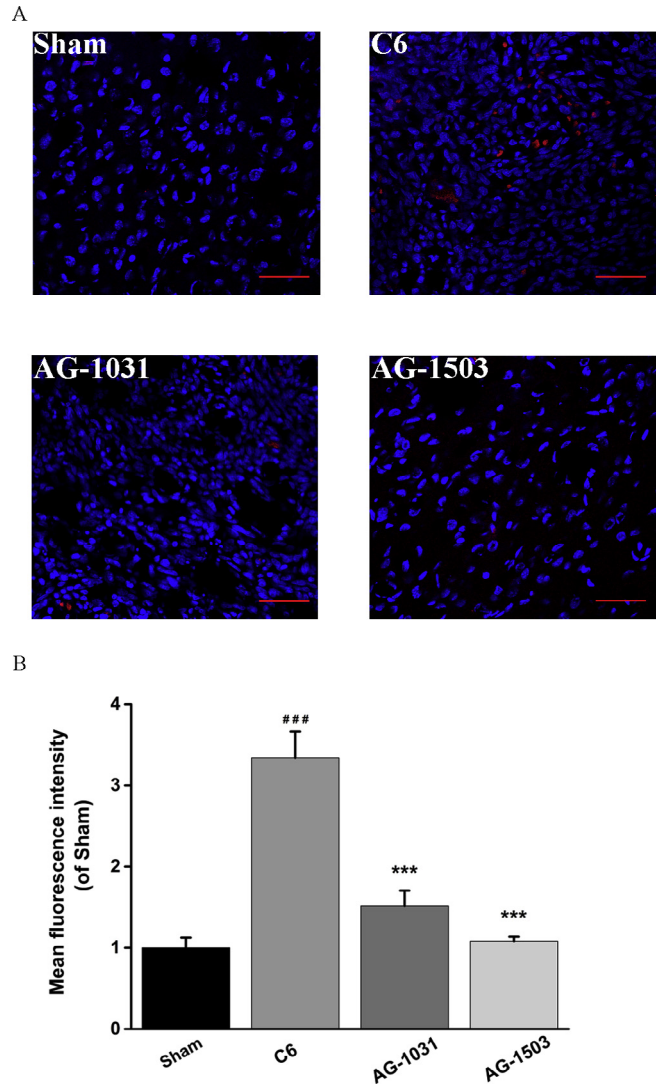

Fig. 2. Effects of AG-1031 and AG-1503 on VEGF expression in glioma rats. (A) The brain tissue sections were prepared from Sham group, C6 group, AG-1031 group and AG-1503 group at 14–15 DPI. Immunofluorescence image of C6 glioma tissue session detected by VEGF antibodies (red) indicated the expression of VEGF in each group. Nucleus was stained with DAPI (blue) (Bar = 50 μm) DPI, days post-implantation. (B) Mean fluorescence intensity of VEGF in each group. $^{***}P < 0.001$, significant difference between C6 group and AG-1031 group/AG-1503 group. $^{###}P < 0.001$, significant difference between Sham group and C6 group.

group was quantified and was shown in Fig. 2B. Notably, VEGF was abundantly expressed in C6 group compared with Sham group ($P < 0.001$), while the expressions were decreased in AG-1031 group and AG-1503 group ($P < 0.001$).

2.4. Effects of AG-1031 and AG-1503 on cognitive ability of glioma rats

The novel object recognition test was carried out to examine whether AG-1031 and AG-1503 can improve the cognitive damage caused by glioma. In the test session, which was carried out two hours after the familiarization session (Fig. 3A), the level of recognition index (RI) to the novel object was decreased in glioma rats, calculated through the formula: $RI = T2/(T1 + T2) \times 100\%$ (Mumby et al., 2002), compared to that in Sham rats (Fig. 3B, $P < 0.05$). However, the level of recognition index to the novel object was increased in AG-1031-treated and AG-1503-treated rats compared to that in glioma rats (Fig. 3B, $P < 0.05$).

2.5. Effects of AG-1031 and AG-1503 on the LTP in glioma rats

In the LTP test, the stimulation of Schaffer collaterals evoked basal field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA1 region, while TBS stimulation induced LTP of the stimulated synapses for 1 h. Fig. 3C represents the time course of fEPSPs slopes, which has been normalized to the 20 min baseline period. It could be seen that the fEPSPs slopes were increased immediately after stimulation, and then gradually stabilized to a level above the baseline period. The last 15 min data were analyzed by two-way factorial AVONA. The results showed that the normalized slope of the fEPSP was significantly decreased in the glioma group compared to that of Sham group (Fig. 3D, $P < 0.001$). Meanwhile, they were augmented by AG-1031 and AG-1503 in the two groups respectively (Fig. 3D, $P < 0.001$).

2.6. Effects of AG-1031 and AG-1503 on SYP and PSD 95 expressions in glioma rats

To uncover the mechanism behind the impairment of LTP caused by AG-1031 and AG-1503, the expression levels of SYP (synaptophysin) and PSD 95 (postsynaptic density protein 95) were examined (Fig. 4A), which were associated with synaptic transmission. One-way ANOVA and LSD post-hoc analysis showed that there were significant differences in the effect of glioma and the effect of the two drugs treated on glioma rats on the SYP expression. SYP expression was decreased in glioma group compared with that of Sham group (Fig. 4B, $P < 0.05$), while that was enhanced in AG-1031 and AG-1503 group compared with that of glioma group (Fig. 4B, $P < 0.05$). However, there was no significant difference in PSD 95 expression between four groups, which indicated that effects of AG-1031 and AG-1503 on synaptic transmission had no involvement with postsynaptic density (Fig. 4C).

2.7. Effects of AG-1031 and AG-1503 on apoptosis-associated protein and autophagy-associated protein expressions in glioma rats

To uncover the mechanism that the treatment of AG-1031 and AG-1503 inhibited the glioma expansion, we assessed the protein expression level associated with autophagy and apoptosis. The reduced transformation from LC3I to LC3II is considered as the inhibition of autophagy. Western blot assay was performed to analyze the LC3 II/I and Beclin-1 expression of tumor tissue and hippocampus separately (Fig. 5A). One-way ANOVA and LSD post-hoc analysis showed a significant decrease of Beclin1 protein expression and LC3 II/I ratio in tumor tissues of AG-1031 and AG-1503 groups compared with those of glioma group (Fig. 5B, $P < 0.05$, Fig. 5C, $P < 0.01$). Meanwhile, there was no significant difference in hippocampus between each group (Fig. S2). Furthermore, we attempted to study the effects of AG-1031 and AG-1503 on cell apoptotic levels in tumor tissue. Bax, Bcl-2, caspase-3, and cleaved caspase-3 were considered to change during apoptosis (Fig. 5D). One-way ANOVA and LSD post-hoc analysis showed AG-1031 treatment contributed to an obvious increase of cleaved caspase-3 and Bax expression and a decrease of Bcl-2 expression compared to C6 group (Fig. 5E–G, $P < 0.01$). These results suggest that AG-1031 could activate the apoptotic pathway via inhibiting autophagy process in tumor cells.

3. Discussion

Malignant glioma, one of the most lethal and common types of intracranial cancer (Wang et al., 2015, Hu et al., 2016), has been receiving worldwide attention. Because of its invasive nature and specific position, the prognosis is extremely poor. Patients with

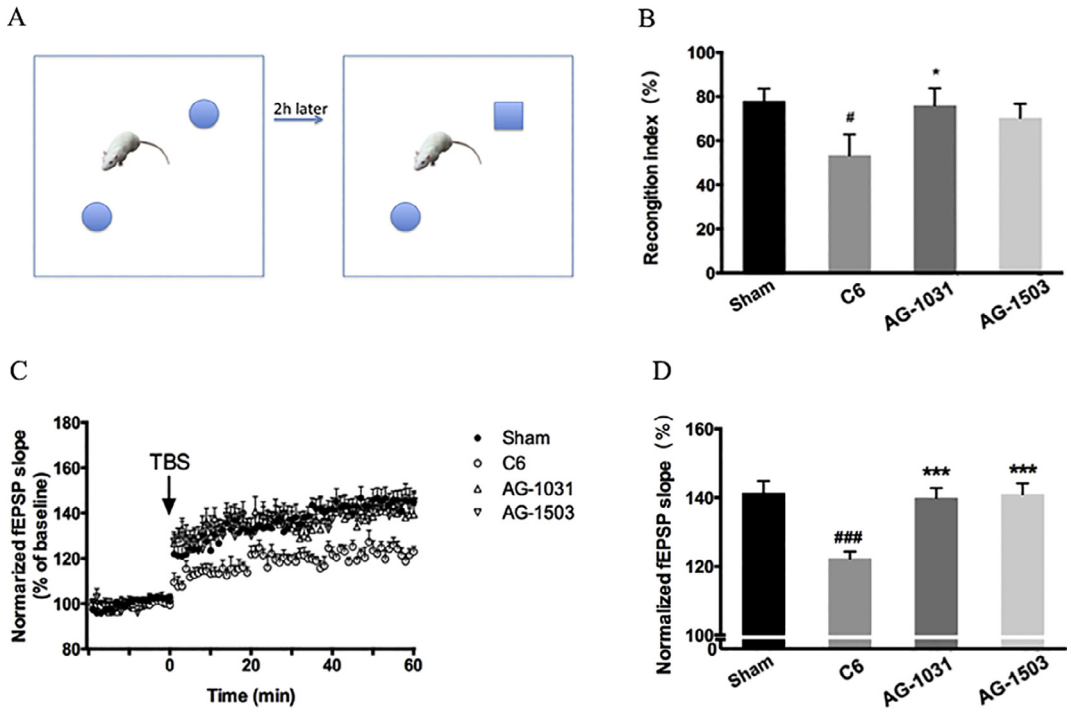

Fig. 3. Effects of AG-1031 and AG-1503 on cognitive behavior and the LTP in glioma rats. (A) Schematic diagram of novel object recognition. The test session (10 min) was carried out two hours after the familiarization session (5 min). (B) Mean recognition index (RI) to the novel object of rats in each group, calculated through the formula: RI = T2/(T1 + T2)×100% in test session. (C) Time course changes of LTP in the CA3 region. Each point represents the average of four consecutive evoked responses. TBS was used to induce LTP. (D) Mean fEPSP slope of LTP was determined as responses between 45 and 60 min after TBS. Data represent mean ± SEM. Error bars indicate SEM. (n = 7 in each group). $^{*}P < 0.05$, $^{***}P < 0.001$, significant difference between C6 group and AG-1031 group/AG-1503 group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, significant difference between Sham group and C6 group.

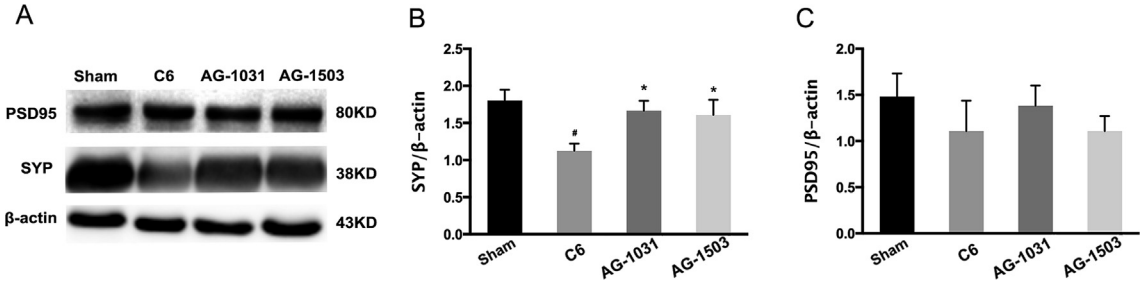

Fig. 4. Effects of AG-1031 and AG-1503 on SYP and PSD 95 expression in glioma rats. (A) SYP (38KD) and PSD 95 (80KD) protein expression level in each group, as determined by the western blot analysis of whole hippocampal lysates. (B and C) Corresponding histogram of SYP protein and PSD 95 protein expression. The expression value of SYP (B) and PSD 95(C) were normalized with β-actin. Data represent mean ± SEM. Error bars indicate SEM. (n = 4 in each group). $^{*}P < 0.05$, significant difference between C6 group and AG-1031 group/AG-1503 group. $^{\#}P < 0.05$, significant difference between Sham group and C6 group.

malignant glioma have an average survival time of 1–2 years even with the available therapies that include surgery, radiation and chemotherapy (Stupp et al., 2005).

The C6 glioma model is a classic malignant brain tumor model, which possesses a number of histopathological characters with human GBM (glioblastoma) (Wang et al., 2015). In our study, C6 gliomas were efficaciously cultivated in putamen region of SD rats. After the injection of 1.0×10^6 C6 glioma cells, the C6 glioma model was established successfully.

VEGF is generally overexpressed and augmented in gliomas (Plate et al., 1992, Plate et al., 1993), which contributes to the angiogenesis of glioma cells and unrestrained proliferation of tumor (Ferrara et al., 2003, Hicklin and Ellis, 2005). It is widely accepted that angiogenesis inhibitors could decrease vascular permeability in conjunction between tumor and normal tissues, finally augmenting the perfusion of cytotoxic agents to intratumoral sites (Teicher et al., 2010). Therefore, the levels of VEGF changed by AG-1031 and AG-1503 in gliomas rats were investigated. The results of our study indicated that AG-1031 and AG-1503 could effectively inhibit the expression of VEGF in gliomas resulting in an increasing apoptosis of the glioma cells. Meanwhile, we found that the volumes of tumor were significantly reduced in AG-1031 and AG-1503 group than those in C6 group from the results of HE staining. Therefore, AG-1031 and AG-1503 could inhibit the expansion of intracranial gliomas, and one possible mechanism may be that AG-1031 and AG-1503 could shrink the expression of VEGF.

Apoptosis, programmed cell death (PCD), which regulates the homeostasis between cell proliferation and cell death, can be activated in response to physiologic stresses or as a result of anticancer agents (Fesik, 2005, Hanahan and Weinberg, 2011). Our observation of elevated Bax expression and cleaved caspase-3 expression in AG-1031 group may suggest the treatment triggered apoptotic pathway as well. Furthermore, a deeper study of the primary

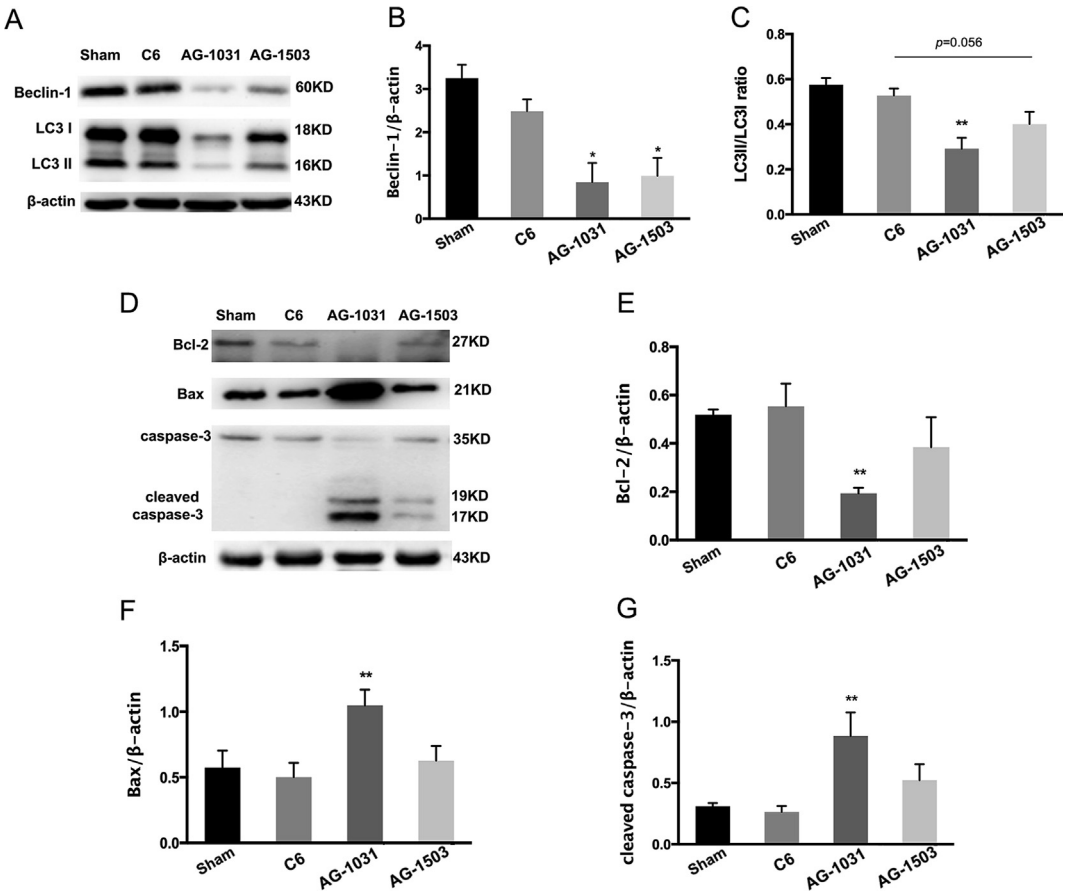

Fig. 5. Effects of AG-1031 and AG-1503 on apoptosis-associated and autophagy-associated protein expressions in glioma rats. (A) Beclin-1 (60KD) and LC3 II/I (18/16KD) protein expression level in each group, as determined by the western blot analysis of tumor tissue lysates. (B and C) Corresponding histogram of Beclin-1 protein and LC3 II/I protein expression. The expression value of Beclin-1 (B) was normalized with β-actin. Data represent mean ± SEM. Error bars indicate SEM. (n = 4 in each group). (D) Bcl-2 (27KD), Bax (21KD) and caspase-3/cleaved caspase-3 (35/19/17KD) protein expression level in each group, as determined by the western blot analysis of tumor tissue lysates. (E-G) Corresponding histogram of Bcl-2, Bax and cleaved caspase-3 protein expression. The expression value of Bcl-2 (E), Bax (F) and cleaved caspase-3 (G) was normalized with β-actin. Data represent mean ± SEM. Error bars indicate SEM. (n = 4 in each group). $^{**}P < 0.01$, significant difference between C6 group and AG-1031 group.

mechanisms of AG-1031-induced apoptosis is critical for its clinical application in glioma treatment and prevention. Bi et al. indicated that suppression of autophagy at a late stage by chloroquine (CQ) could reduce the proliferation in C6 glioma cells and enhance the anti-glioma efficiency of quercetin *in vivo* (Bi et al., 2016). The glioma initiation and growth in glioblastoma mouse model were specifically suppressed by inhibition autophagy via RNAi against autophagy-associated genes (Atg7, Atg13 or Ulk1) (Gammoh et al., 2016). In addition, blocking autophagy by inhibiting the Akt/mTOR pathway and activating ERK1/2 pathway could improve the efficacy of temozolomide/curcumin on the treatment for glioblastoma (Shingu et al., 2009). These results proved that autophagy is crucial for the formation and growth of glioma. LC3, a homologue of yeast Atg8, is essential for autophagy. In the dynamic process of autophagosome formation, LC3-I, localized in the cytoplasm, was translated into LC3-II localized to preautophagosomes and autophagosomes. Thus, LC3 II/I ratio is widely used to monitor autophagy flux (Tanida et al., 2004). In addition, Beclin-1 is another essential mediator for autophagy. Beclin-1 recruits many important molecules to form a multimeric complex to participate in the formation of autophagosome (Zhu et al., 2009). The results of LC3 II/I ratio and Beclin-1 in our studies suggested that AG-1031 and AG-1503 inhibited autophagy process. The deficiency of autophagy in cancer cells would results in multiple metabolic problems. The inhibition of autophagy means deprivation of energy resources to cancer cells when they encounter stress, resulting in the activation of apoptosis and cell death.

In addition to threatening the physical health of patients, malignant glioma can cause neurocognitive deficits (Amanda et al., 2015), which negatively affect patients' health-related quality of life (HRQOL) (Klein, 2012, Martin et al., 2012). Even minor cognitive dysfunction may deprive an individual's chance to return to his work or other activities (Gehring et al., 2008). Therefore, the target of new treatment in these patients should thus not only aim at survival rates, but also pay attention to functional outcome such as the improvement of cognitive deficits. The neurocognitive performance was evaluated by novel object recognition test. Results showed the significant deficits of neurocognition in glioma rats. And the impairments were improved by the treatment with AG-1031 and AG-1503. As the basic functional indicator of synaptic plasticity, LTP is considered the cellular basis of learning and memory (Mcnaughton et al., 1978). Our results of electrophysiological recording indicated that LTP was damaged in glioma rats, and the impairment was improved by AG-1031 and AG-1503.

Furthermore, synapse-associated proteins expression levels were detected by Western blot assay. The expression level of reference protein (β-actin) was relatively constant in each group (Fig. 4). Moreover, the amount of protein (40 μg) in each group was equal, and the results of Western blot assay were calibrated with β-actin. So, the Western blot data in our study could accurately reflect the protein expression levels. As we all known, SYP, a presynaptic protein located on synaptic vesicle membrane, is a maker of synaptic plasticity. Previous studies have demonstrated that neurotransmitter can be inhibited when the SYP function

was interfered by anti-SYP (Alder et al., 1992). Therefore, the absence of the protein may cause changes in structure and finally lead to dysfunctions of synaptic plasticity and cognition. Results showed the up-regulation of SYP after the treatment with AG-1031 and AG-1503. However, the postsynaptic protein, PSD 95 was not notably altered comparable in the four groups. It indicated that the presynaptic mechanism rather than postsynaptic mechanism might participate in the changes of synaptic plasticity.

4. Conclusion

In this study, we demonstrate that AG-1031 and AG-1503 can significantly inhibit C6 tumor expansion, a possible mechanism could be that the treatment triggers apoptosis via the suppression of autophagy process and results in a down-regulating expression of other factors involved in angiogenesis, such as the VEGF in tumor tissues. Meanwhile, AG-1031 and AG-1503 may improve the deficits of synaptic plasticity and cognitive function by enhancing the expression of SYP.

5. Methods and materials

5.1. Test drugs

AG-1031 and AG-1503 are small molecules identified by AscentGene, Inc.(USA) that show inhibitory effects on the growth of different tumor cells. The compounds were dissolved in DMSO at 5 mg/ml and stored at 4 °C. Before using, they were diluted to the concentration of 1 mg/mL with PBS.

5.2. Cell culture and proliferation assay

C6 rat glioma cells that originated from rat brain glioma were obtained from the American Type Culture Collection (ATCC, USA). The system of cell culture and the proliferation assay detected the cytotoxicity of AG-1031 and AG-1503 were specified described in the Supplementary material and methods.

5.3. Animals

Adult, male Sprague-Dawley (SD) rats (180–220 g) were purchased from the Laboratory Animal Center of Academy of Military Medical Science of People's Liberation Army, P.R. China. Animals were kept in controlled temperature and humidity conditions with a light/dark cycle of 12hrs, under veterinary surveillance for animal health and comfort. The animal care and experimental protocol were approved by the Ethical Commission at Nankai University, China.

5.4. C6 glioma model and treatment

Rats were randomly divided into four groups, Sham group, C6 group, AG-1031 group, and AG-1503 group (n = 7 for each group). The establishment of C6 glioma rat model was described in the Supplementary material and methods as described previously in the literature with minor modifications (Plate et al., 1993).

Glioma rats were randomly assigned into three groups after the surgery: C6 group, AG-1031 group and AG-1503 group. AG-1031 and AG-1503 were intended for intravenous injection only with the dose of 6 mg/m^2 body surface area in clinical treatment. Then, they were switched into the equivalent dosage in rats according to HED (human equivalent dose) guidance suggested by the Food and Drug Administration (FDA). Rats in AG-1031 group and AG-1503 group were treated with AG-1031 and AG-1503 with a dose of 1 mg/kg respectively for 5 days by tail vein injection. Animals of Sham and C6 groups were intravenously injected with the equal volume of PBS during the five days.

5.5. Novel object recognition (NOR)

The NOR test was performed according to the guidelines of Leger *et al* (Leger et al., 2013) with minor modifications. The experimental details of NOR test were given in Supplementary material and methods.

5.6. Electrophysiological test

After behavioral test, LTP (long-term potentiation) between the hippocampal Schaffer collaterals and CA1 pyramidal neurons was evaluated by *in vivo* electrophysiological techniques. The protocol was adopted and modified on the basis of our previous study (Fu et al., 2016). The methods were shown particularly in the Supplementary material and methods.

5.7. Western blot assay

After electrophysiological test, rats were sacrificed and brains were removed for biochemical analysis. Tissues that including hippocampus, the C6 gliomas and the normal brain tissues were detached and the protein expressions were analyzed by Western blot assay. Three repeated measurements were performed in each animal (n = 4 for each group). More details were provided in the Supplementary material and methods.

5.8. Hematoxylin and eosin (HE) staining

The rat brains were harvested and fixed in 4% paraformaldehyde, and preserved in optimal cutting temperature compound and stored at -80 °C. The brain tissues were sliced in 10 μm sections and stained with HE for the histological studies.

5.9. Immunochemical staining

The expression of vascular endothelial growth factor (VEGF) was analyzed by immunohistochemical staining. The details were introduced in Supplementary material and methods. The reproducibility of the staining was confirmed by re-immunostaining through the same method in multiple, randomly selected specimens. Sections were analyzed using an Olympus FV1000 laser-scanning confocal microscope (Olympus, Japan).

5.10. Statistical analysis

To determine the appropriate sample size in each group, the standard deviation of the LTP level in C6 glioma rats was 9.1% in previous study. For our power calculation, we assumed standard deviation was equal in each group. We wanted to show a difference of 15% in the LTP level between groups. With $\alpha = 0.05$, one-sided and a power of 99%, we needed at least six rats per group. So, we employed 28 rats in this study. For all animal experiments, rats were randomly divided into four groups. And double blinding method was used for group assignment and outcome assessment. All experiments were repeated at least three times. All of the statistical analyses were performed using IBM SPSS Statistics 22 software (Chicago, IL, United States). All data were presented as mean ± SEM. In this study, we want to show the difference between groups caused by single variable so we choose one-way ANOVA to analyze the data obtained from the behavior test, electrophysiological experiments and Western blot assay. All data were analyzed by homogeneity test of variance to ensure the variance of difference was equal. To detect significant differences between

groups, ANOVAs were supported by post hoc LSD test. P-values less than or equal to 0.05 were considered statistically significance.

6. Authors' contributions

Zhuo Yang and Hui Ge directed the experiments and revised the manuscript. Shuang Hao performed the experiments, analyzed the data and wrote the manuscript. Jing Gao contributed to the experiments, and was a major contributor in writing the manuscript. Hui Wang contributed to the C6 rat glioma cells culture experiments. Yan Zhang and Andrey Pavlov performed the identification of AG-1031 and AG-1503. All authors read and approved the final manuscript. The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.brainres.2018.06.026.

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