Received: 31 July 2017 | Accepted: 2 February 2018
DOI: 10.1002/jcb.26781

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

AG-1031 induced autophagic cell death and apoptosis in C6 glioma cells associated with Notch-1 signaling pathway

Yan Wang[1] | **Hui Wang**[2] | **Hui Ge**[3] | **Zhuo Yang**[1]

[1] Medical School, State Key Laboratory of Medicinal Chemical Biology, Key Laboratory of Bioactive Materials for Ministry of Education, Nankai University, Tianjin, China

[2] College of Life Sciences, Nankai University, Tianjin, China

[3] AscentGene, Inc., Gaithersburg, MD

Correspondence
Yang Zhuo, College of Medicine, Nankai University, Tianjin 300071, China.
Email: zhuoyang@nankai.edu.cn; hge@ascentgene.com

Funding information
National Natural Science Foundation of China, Grant numbers: 81571804, 81771979; China Postdoctoral Science Foundation, Grant number: BS000056

Abstract

Glioma is the most common primary brain tumor with high morbidity and poor prognosis. The effect of AG-1031, which is developed as an antineoplastic drug, on C6 glioma cells is still not clear. The aim of this research was to explore the effect of AG-1031 on C6 cells, and tried to find out its potential mechanism on cytotoxicity of C6 cells. The 3-(4,5-dimethylthiazol −2-yl) −2,5- diphenyltetrazolium bromide (MTT) assay showed that AG-1031 inhibited cell viability in a concentration-dependent manner, whereas 3-methyadenine (3-MA) reduced this effect. Results from hoechst 33342 staining and Western blot assay indicated that AG-1031 induced C6 cell apoptosis. Western blot assay presented that AG-1031 notably increased the LC3-II/LC3-I ratio and decreased the expression of P62. Besides, our results showed that bafilomycin A1 increased the expression of LC3-II in cells treated with AG-1031, which indicated that AG-1031 can increase autophagy in C6 cells. Meanwhile, Western blot assay showed that AG-1031 can inhibit Notch-1 signaling by testing relative protein expressions. The expression of the intracellular domain of Notch (NICD) also decreased according to immunofluorescence assay. Additionally, the activation of Notch-1 signaling alleviated AG-1031-induced autophagic cell death and apoptosis. Furthermore, phosphorylated Akt and its downstream effector mechanistic target of rapamycin (mTOR) were reduced with AG-1031. These results suggest that AG-1031 may induce autophagic cell death through the inhibition of Akt-mTOR signaling pathway by down-regulating Notch-1 signaling pathway and activating apoptosis in C6 cells via Notch-1 signaling, which develops a new target spot to treat glioma in the future.

KEYWORDS
AG-1031, apoptosis, autophagy, C6 cells, Notch-1

1 | INTRODUCTION

Glioma is one of the most common types of primary brain tumor and has remained particularly challenging to treat.[1] It remains one of the deadliest primary brain tumors in adults despite years of basic research and clinical trials.[2] The autophagy lysosomal pathway is one such target that is being explored in multiple cancers including glioma and is a promising avenue for further therapeutic development.[3]

During autophagy, cellular components are sequestered into vesicles called autophagosomes and then delivered to

Yan Wang and Hui Wang contributed equally to this work.

lysosomes for degradation. This catabolic process generates both precursor compounds and energy supply for macromolecular synthesis and metabolic needs. As a so-called self-eating system, the products of autophagy were recycled to generate macromolecules and ATP in order to maintain cellular homeostasis.[4] From this perspective, autophagy plays a protective role especially in the response of various metabolic stresses.[5–7] However, autophagy is also known as programmed cell death type II, which means autophagy induces cell death in some cases. Therefore, exploring the role of autophagy in the process of glioma development will contribute to creating a new method for the treatment of glioma. Apoptosis, as programmed cell death type I, is considered a conventional type cell death.[8] Many studies have showed that autophagic cell death and apoptosis coexist in different chemotherapy drugs induced cancer cell death.[9,10]

Notch signaling is an evolutionarily conserved mechanism, which consists of four receptors (Notch1-4), five ligands (Jag1/2, Dll1/3/4), and downstream components in mammals.[11,12] Notch signaling controls cell communication and cell fate.[13] Research has showed that using RNA interference to reduce the expression of Notch-1, Delta-like-1, or Jagged-1 can induce cell apoptosis and inhibit proliferation in multiple glioma cell lines.[14] Besides, Notch can control glioblastoma cell proliferation and survival through Akt-dependent signaling.[15] Akt is involved in many cellular processes contributing to tumor progression, aggressiveness, and treatment resistance.[16] As a down- stream effector of Akt signaling, mTOR plays a key role in the regulation of cell proliferation, cell cycle, autophagy, and apoptosis.[17]

AG-1031 is a FDA approved small molecule drug (MW = 780 Da) for human. Using an established high throughput assay system, AG-1031 was identified as the first small molecule inhibitor of human transcriptional positive cofactor 4 (PC4). Although some preliminary experiments have proved that AG-1031 can inhibit the viability of several cancer cell lines, its effect on C6 glioma cells is still unclear. Based on these, our study was designed to investigate the influence of AG-1031 on C6 cell proliferation and to explore the possible mechanism. Our results suggest AG-1031 can induce mTOR-dependent autophagy and inhibit Notch-1 signaling, thereby providing a new view of potential application of AG-1031 as an effective antitumor medicine.

2 | MATERIALS AND METHODS

2.1 | Materials

Dulbecco's modified eagle media (DMEM) cell culture medium was purchased from GIBCO Invitrogen, Grand Island, NY. The fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT), 3-Methyladenine (3-MA), linear polyethylenimine (PEI) reagents, and hoechst 33342 dye were purchased from Sigma Chemical Co., St Louis, MO. One step TUNEL apoptosis assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Anti-LC3 IgG primary antibody (M186-3) was purchased from MBL, Nagoya, Japan. Anti-β-actin IgG primary antibody (ab8226), anti-Notch-1 ab (ab52627), anti-Hes-1 ab (ab71559), anti- Jagged-1 ab (ab7771), anti-mTOR ab (ab32028), anti-p-mTOR-ab (ab109268), anti-Bax ab (ab32503) and anti-P62 ab (ab56416) were purchased from Abcam, Cambridge, UK. Anti-Akt ab (#4691), anti-P-Akt ab (#4060), anti-Bcl-xl ab (#2764), and anti-PARP-ab (#9542) were purchased from Cell Signaling Technology, Danvers, MA. The Alexa 488-conjugated goat anti-rabbit IgG secondary antibodies were obtained from Invitrogen, San Diego, CA. The chemiluminescent HRP substrate was purchased from Millipore Corporation, Billerica, MA.

2.2 | Test drug

AG-1031 is a small molecule identified by AscentGene, Inc., Gaithersburg, MD that shows inhibitory effect on human transcription confactor PC4 and growth of different tumor cells. Before using, AG-1031 was soluble in dimethylsulfoxide (DMSO) and stored at 4°C at a concentration of 1 mg/mL. When using, it was diluted to different concentrations with DMEM medium.

2.3 | Cell culture

The rat C6 glioma cell line, which obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, was cultured with the DMEM medium contain 10% FBS and 100 U/mL penicillin and 100 U/mL streptomycin. The cells were maintained in 95% O_2 and 5% CO_2 in a sterile atmosphere.

2.4 | Cell viability assay

The cell viability was evaluated with MTT assay. In brief, cells were plated 1×10^5 per well in 96-well microtiter plates and incubated with final concentrations of AG-1031 0, 0.1, 0.3, 1, 3, 10, and 30 μM, 24 h later, MTT (20 μL, 0.5 mg/mL) was added to react with living cells for 4 h. After that, culture medium was removed, and 150 μL DMSO was added to dissolve the formazan crystal. After the mixture was dissolved completely, measure it in an ELISA reader (Elx800, Bio-TEK) with a wave length of 578 nm. The percentage of cell viability can be expressed by the formula as follows:

$$\text{cell viability}(\%) = (\text{test absorbance} - \text{blank absorbance}) / (\text{control absorbance} - \text{blank absorbance}) \times 100$$

2.5 | TUNEL assay

The cells were treated with culture medium and AG-1031 (1 μM) respectively in six-well plates. After 24 h, the cells were washed with phosphate buffer saline (PBS) once, then the cells were fixed with 4% paraformaldehyde for 1 h. After washing with PBS twice, the cells were treated with 0.1% Triton X–100 on the ice for 2 min followed by two washes with PBS. TUNEL reagent (50 μL) was added to each well and then the cells were incubated in a dark, moist environment at 37°C for 1 h. After washing three times with PBS, staining DNA with 50 μL 4′,6-diamidino-2-phenylindole (DAPI) solution (0.01 mg/mL) in each well and then the cells were incubated in the dark for 3 min followed by washing three times with PBS. Then the fluorescent signals were detect with a microscope.

2.6 | Transient transfection of plasmid

The pEGFP-Notch1-NICD-r fusion plasmid was bought from Wuhan Miaoling Bioscience & Technology Co., Ltd., Wuhan, China Cells were seeded 1×10^5 per well in six-well plates for transfection. Plasmids were transfected 4 μg/well into C6 glioma cells using linear polyethylenimine (PEI) reagents. After 4 h, the liquid medium was changed by complete medium. Then cells were cultured for 16 h before the addition of AG-1031 (1 μM). The transfection efficiency was observed by confocal microscopy (Figure S1).

2.7 | Nuclear staining analysis by Hoechst 33342

The Hoechst 33342 dye can bind to DNA in living cells, therefore, it is used to assess the changes in the nuclear morphology. The cells were seeded into six-well plates and grown on the cover slips. After treated with AG-1031 (1 μM) for 24 h, the cells were washed with phosphate buffer saline (PBS) twice, then cultured with Hoechst 33342 dye (5 μg/mL) for 30 min at a temperature of 37° in the dark. After washing cells with PBS twice, the Hoechst-stained nuclei of C6 cells were observed under microscope.

2.8 | Western blot analysis

C6 cells were seeded in the six-well plates and then treated with AG-1031 for 24 h. Using lysis buffer and 1% PMSF to lyse the cells for 15 min on ice. Cell lysates were ultra-centrifuged at 12000 rpm for 15 min at 4 °C, and then collecting the supernatant as the total cellular proteins. A BCA protein assay kit was used to determine the concentrations. Adding loading buffer to the supernatant (ratio is 4:1) and boiling it at 95-100 °C for 10 min. Samples (40 μg protein) were extracted from those cells, and were resolved to 10-13% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Blocking the membrane for 1 h subsequently and incubating it with primary antibody diluted of 1:1000 respectively during a night at 4°C. Probing the immunoreactivity by adding secondary antibody diluted to 1:5000 after washing the membrane with TBST and detecting it with chemiluminescent HRP detection kit. The protein transfer was confirmed by immunoblotting for determination of actin protein using β-actin antibody (1:1000) on the same Western blot.

2.9 | Immunofluorescence

Cells were cultured with cell culture medium and 1 μM AG-1031 for 24 h, respectively, then the cells were washed in PBS for three times and fixed by 4% paraformaldehyde for 10 min. Blocked with 10% NGS for 1 h at room temperature after the cells were permeabilized with 0.5% TritonX-100. Subsequently, cells were incubated with anti-NICD (1:500) as the primary antibody overnight at 4°C. After washing cells with PBS for three times, the cells were incubated with Alexa 488-conjugated anti-rabbit IgG (1:1000) for 1 h in the dark at room temperature. After that, the cell nuclei were stained by DAPI. Then the fluorescent signals were detect with a microscope.

2.10 | Statistical analysis

The results were expressed as mean ± SEM. The statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison post test using the SPSS (21.0) software. The significant difference was taken as $P < 0.05$.

3 | RESULTS

3.1 | AG-1031 observably inhibited the viability of C6 cells

C6 cells were treated with different concentrations (0, 0.1, 0.3, 1, 3, 10, and 30 μM) of AG-1031 for 24 h. The results were determined by the MTT assay. The cell viability of control group was defined as 100%, at the same time, other groups treated with AG-1031 were expressed as the percentage of that in the control group. As shown in Figure 1, the cell viability declined with the increased concentration of AG-1031. Specifically, the viability decreased slightly when the concentration was 0.1 μM. As the concentration increased to more than 1 μM, the viability reduced apparently. When the cells were cultivated with the concentration of 1 μM, the viability dropped to 56.57% compared to other groups. According to the results, the concentration of 1 μM was elected as the culture condition of subsequent experiments.

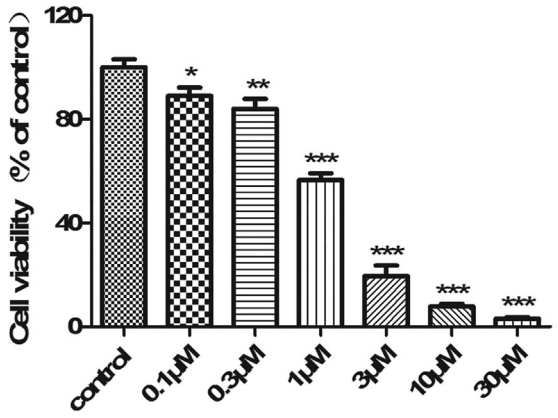

FIGURE 1 Effects of AG-1031 on the viability of C6 glioma cells detected by MTT assay Cells were treated with different concentrations (0, 0.1, 0.3, 1, 3, 10, and 30 μM) of AG-1031 for 24 h. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with that of Control group

3.2 | Morphological changes in C6 glioma cells after AG-1031 treatment

The apoptosis changes of nuclear morphology were examine by Hoechst 33342 staining. The dye can show the morphological changes of cells through bind to DNA. As shown in Figures 2A and 2B, cells without AG-1031 treatment exhibited normal nucleus structure. On the contrary, the nuclei of cells treated with AG-1031 exhibited abnormal morphology such as nuclear degradation and chromatin condensation, which are typical morphological change of apoptosis. Besides, it can be seen in Figure 2C that the percentage of apoptotic cells increased remarkably after AG-1031 treatment.

3.3 | AG-1031 altered the expression of apoptosis-related proteins in C6 cells

The expression level of Bax and Bcl-xl were obtained from Western Blot assay. Bax and Bcl-xl are two proteins, which are involved in caspase dependent apoptotic cascade, where Bax acts as a proapoptotic protein and Bcl-xl has an antiapoptotic role.[18] Cells were treated with AG-1031(1 μM) for 24 h. It can be seen from the result that the level of Bax increased while the level of Bcl-xl decreased apparently. Bax/Bcl-xl ratio showed a clear rise in the cells cultivated with AG-1031 (Figure 3A). Meanwhile, the level of cleaved PARP was up-regulated by AG-1031 (Figure 3B). Thus, the results indicate that AG-1031 induces apoptosis of C6 glioma cells.

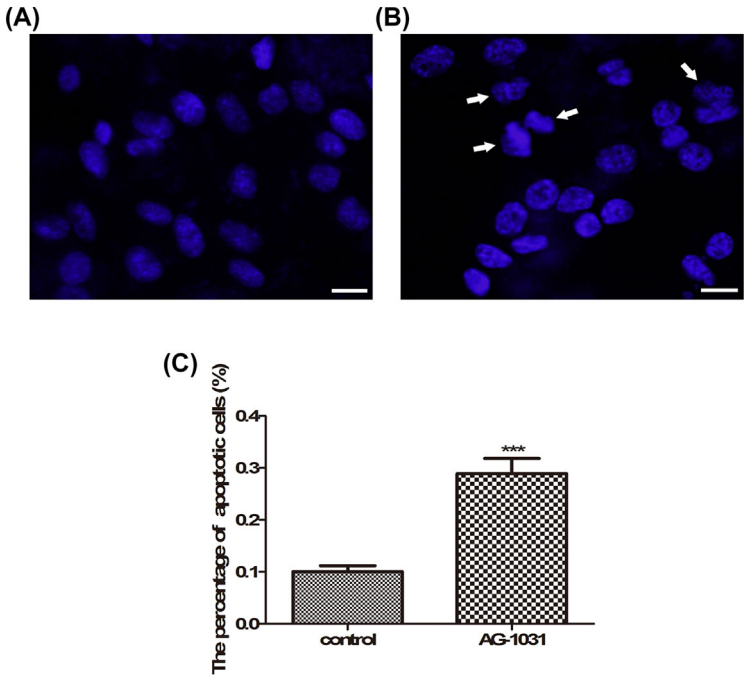

FIGURE 2 Cell morphological changes in C6 glioma cells with AG-1031 treatment for 24 h (A) control, (B) cells treated with 1 μM AG-1031. The cells were stained with Hoechst 33342 and the nuclear morphology was observed by an Olympus microscope. The white arrows showed chromatin condensation and nuclear degradation in apoptosis cells. Scale bar, 10 μm. C, percentage of apoptotic cells treated with AG-1031 $n = 3$, $^{***}P < 0.001$ as compared with that of Control group

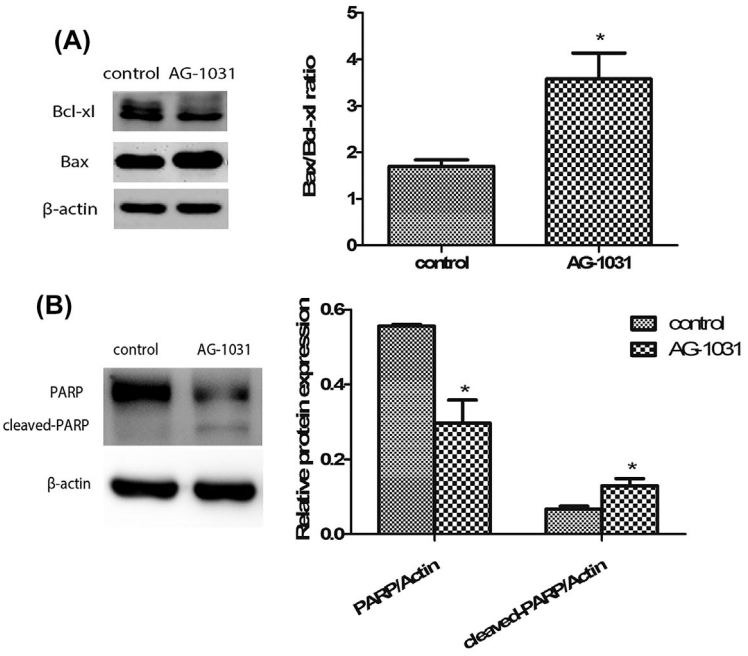

FIGURE 3 AG-1031 altered the expression of apoptosis-related proteins in C6 cells (A) AG-1031 increased the ratio of Bax/Bcl-xl. B, AG-1031 raised the expression of cleaved PARP. Cells were treated with cell medium and AG-1031 (1 μM), respectively. The expressions of Bax, Bcl-xl, and PARP were tested by Western blot assay, then was analyzed by densitometry analysis of their protein expressions from immunoblots (mean ± SEM of independent experiments, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with that of Control group)

3.4 | AG-1031 induced autophagy in C6 glioma cells

The level of autophagy was detected by Western blot assay. LC3-I was cleaved to LC3-II, while the ratio of LC3-II/LC3-I was increased during autophagy.[19] P62 is a poly-ubiquitin binding protein that binds to LC3 and is degraded by autophagy.[20] We found that the level of P62 decreased (Figure 4A) while the ratio of LC3-II/LC3-I in the cells treated with AG-1031 increased remarkably (Figure 4B) compared with that of control cells, which means AG-1031 elevated the level of autophagy of C6 cells. Furthermore, the cells were treated with bafilomycin A1 (100 nM) as lysosomotropic agents to block specific autophagosome-lysosome fusion, bafilomycin A1 treatment caused significant increase of LC3-II in both cells with AG-1031 and cells without AG-1031 (Figure 4B). These results above prove that AG-1031 increases autophagic flux in C6 glioma cells.

3.5 | AG-1031-induced cell death was altered by 3-MA

To further explore that the role of autophagy played in AG-1031-induced cell death, 3-MA as a special inhibitor of the early stages of the autophagic process was used to study the effect on cell viability by MTT assay. As shown in Figure 4C, the addition of 3-MA (5 mM) alleviated the effect of AG-1031 on C6 glioma cell viability, which suggested that autophagy may serve as a main mechanism in AG-1031-induced cell death.

3.6 | AG-1031 restrained Akt-mTOR signaling in C6 glioma cells

mTOR plays a key role in the regulation of autophagy.[21] The increased mTOR activity attenuates autophagy.[22] To find whether AG-1031 could influence Akt-mTOR signaling in C6 cells, we test p-Akt protein and p-mTOR protein expressions by Western blot assay. The results showed that the level of p-Akt protein decreased markedly with a concomitant down-regulation of p-mTOR (Figure 5). Thus, these results indicated that Ag1031 suppressed Akt-mTOR signaling pathway in C6 glioma cells.

3.7 | AG-1031 inhibited Notch signaling in C6 glioma cells

It has been proved that Notch signaling pathway activation was correlated to cyclin D1 expression and Akt activation to promote glioma cell proliferation.[23] To further investigate the role of Notch signaling in C6 cells, we examined the expression of Notch-1 and its ligand Jagged-1 by Western blot assay. The results in Figure 6A show that Notch-1 and Jagged-1 protein expressions were lowered in cells treated

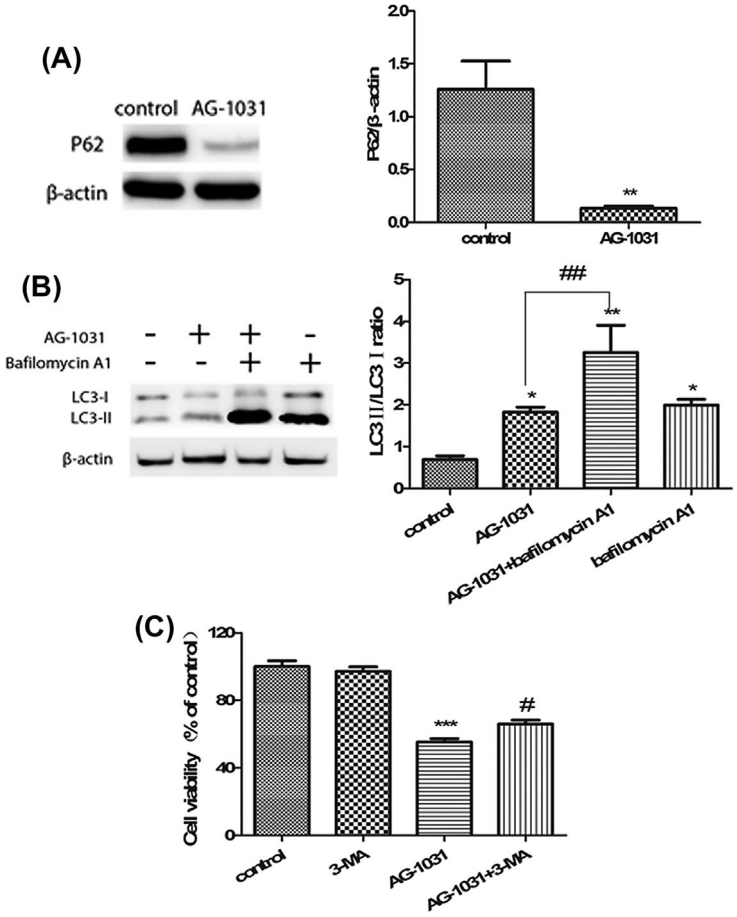

FIGURE 4 AG-1031 induced autophagic cell death in C6 glioma cells (A) AG-1031 induced degradation of p62. B, AG-1031 induced LC3 conversion. Cells were treated with cell medium, AG-1031 (1 μM), AG-1031 (1 μM) + bafilomycin A1 (100 nM), and bafilomycin A1 (100 nM), respectively. The expressions of LC3-II, LC3-I, and P62 were tested by Western blot assay, then were analyzed by densitometry analysis of their protein expressions from immunoblots. C, Effects of 3-MA on AG-1031 induced cell death tested by MTT assay. Cells were treated with cell medium, 3-MA (5 mM), AG-1031 (1 μM), and AG-1031 (1 μM) + 3-MA (5 mM), respectively. (mean ± SEM of independent experiments, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared with that of Control group, $\#P < 0.5$, $\#\#P < 0.01$ compared with 1 μM AG-1031 group)

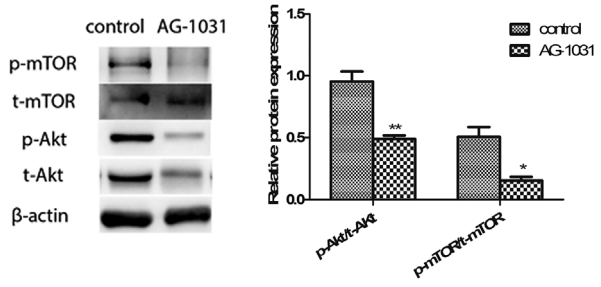

FIGURE 5 AG-1031 inhibited Akt-mTOR signaling in C6 glioma cells Cells were treated with AG-1031(1 μM) for 24 h. The expressions of p-mTOR, t-mTOR, p-Akt, t-Akt were analyzed by Western blot assay, then were measured by densitometry analysis of their protein expressions from immunoblots (mean ± SEM of independent experiments, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with that of Control group)

with AG-1031. Besides, AG-1031 reduced the expression of Hes-1, which is one of the direct targets of Notch signaling.[24]

3.8 | Expression of NICD in C6 glioma cells treated with AG-1031

Notch signaling is initiated by the interaction of a Notch receptor and Notch ligands that belongs to the Delta-Serrate-Lag2 (DSL) family on an adjacent cell. The interaction leads to the release of the intracellular domain of Notch (NICD), which translocates to the nucleus and bins to the transcriptional regulator CSL.[25,26] Immunofluorescence was used to investigate the expression of NICD in C6 glioma cells after treatment with AG-1031 for 24 h. Compared with the control group (Figure 6B), the expression of NICD in the nucleus reduced remarkably after AG-1031 treatment (Figure 6C).

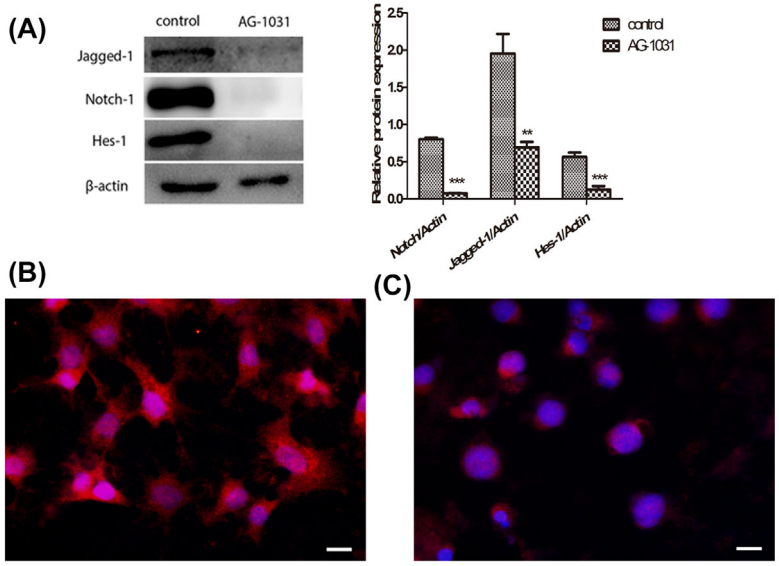

FIGURE 6 AG-1031 inhibited Notch signaling in C6 glioma cells (A) AG-1031 reduced related proteins of Notch-1 signaling. Cells were treated with cell medium and AG-1031 (1 μM) for 24 h. The expressions of Notch-1, Jagged-1, Hes-1 were tested by Western blot assay. Results from immunoblots were measured by densitometry analysis. (mean ± SEM of independent experiments, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with that of Control group). B, Expression of NICD in C6 glioma cells with cell medium. C, Expression of NICD in C6 glioma cells with AG-1031 (1 μM). Scale bar, 10 μm

3.9 | Activation of Notch-1 signaling alleviate AG-1031-induced cell death

The effect of Notch-1 signaling on AG-1031 induced cell death was assessed with MTT assay. The viability of control group was defined as 100 %, while the cell viabilities of other groups were expressed as the percentage of the viability of control group. As shown in Figure 7A, AG-1031 decreased the viability of C6 glioma cells, while the transfection of NICD mitigated AG-1031-induced cell death observably. These data imply that the inhibition of Notch-1 signaling might contribute to AG-1031-induced cell death.

3.10 | AG-1031 induced apoptosis by downregulating Notch-1 signaling

Western blot assay and TUNEL staining was used to study whether the occurrence of cell apoptosis was related to the inhibition of Notch-1 signaling. As shown in Figure 7B, AG-1031 caused elevated expression of Bax and decreased expression of Bcl-xl, which led to the elevation of Bax/Bcl-xl ratio. The ratio declined significantly with the transfection of NICD. Besides, as shown in Figure 7C, TUNEL-positive apoptotic cells (red color in TUNEL staining) can be seen in AG-1031-treated group and no obvious apoptosis cell was observed after transfecting of NICD. Results above verified that the activation of Notch-1 signaling can restore AG-1031-induced cell apoptosis.

3.11 | AG-1031 triggered autophagy via suppressing Notch-1 signaling

To further determine the relationship between autophagy and Notch-1 signaling, we performed rescue experiments by overexpressing the intracellular domain of Notch (NICD). As expected, results showed that the transfection of NICD can rescue AG-1031-induced autophagy to the control level by testing autophagy related proteins LC3 and P62 (Figure 7D). These results demonstrated that AG-1031 might trigger autophagy targeted through suppressing Notch-1 signaling.

4 | DISCUSSION

Glioblastoma (GBM) remains one of the deadliest primary brain tumors in adults in spite of years of basic research and clinical trials.[2] The current standard of medical treatment to glioblastoma consists of maximal safe resection followed by external beam radiation and temozolomide to maintain chemotherapy, while the prognosis of patients is still poor.[27,28] As patients with histologically identical tumors may have totally different outcomes, to research the distinct molecular alterations in glioma could be the best way to develop valid treatment and improve the survival rate of patients.[29] Hence, our study was try to find the effect of AG-1031 on C6 glioma cells and the molecular mechanism under it.

Our previous experiments have proved that AG-1031 can reduce the viability of several tumor cell lines such as lung

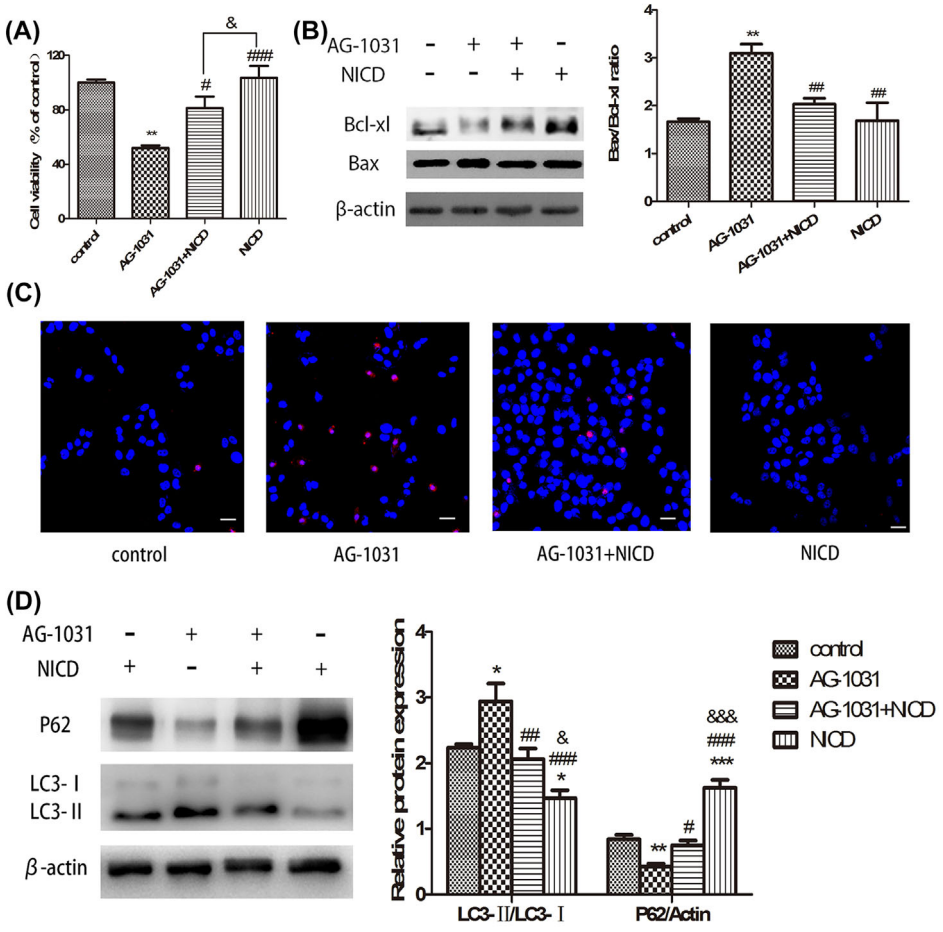

FIGURE 7 Activation of Notch-1 signaling alleviated AG-1031-induced autophagic cell death and apoptosis (A) Cells were treated with 1 μM AG-1031 for 24 h in the absence and presence of transfection of NICD. The cell viability was tested by MTT assay. $^{**}P < 0.01$ as compared with that of Control group, $\#P < 0.05$, $-\#P < 0.001$ as compared with that of AG-1031 group, $\&P < 0.5$ as compared with that of AG-1031 + NICD group. These experiments have been repeated thrice. B, Apoptosis relative protein levels of Bax and Bcl-xl were detected by Western blot in C6 cells treated with 1 μM AG-1031 for 24 h in the absence and presence of transfection of NICD. Results from immunoblots were measured by densitometry analysis. (mean ± SEM of independent experiments, $n = 3$, $^{**}P < 0.01$ as compared with that of Control group, $-P < 0.01$ as compared with that of AG-1031 group). C, TUNEL staining of C6 cells treated with 1 μM AG-1031 for 24 h containing and without transfection of NICD. Red color in TUNEL staining refers to apoptotic cells. Scale bar, 10 μm. D, Cells were with 1 μM AG-1031 for 24 h in the absence and presence of transfection of NICD. Protein levels of P62, LC3-II, and LC3-I were analyzed by Western blot assay. Results from immunoblots were measured by densitometry analysis. (mean ± SEM of independent experiments, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with that of Control group, $\#P < 0.05$, $-P < 0.01$, $-\#P < 0.001$ as compared with that of AG-1031 group, $\&P < 0.5$, $\&\&\&P < 0.001$ as compared with that of AG-1031 + NICD group)

cancer cells and leukemia cells. However, the effect of AG-1031 on C6 glioma cells is still unknown. In this study, we found that AG-1031 can inhibit the proliferation of C6 glioma cells in a concentration-dependent manner by MTT assay. Besides, alterations of the expressions of Bax, Bcl-2, PARP tested by Western blot assay, and morphological changes in C6 glioma cells observed by TUNEL assay and Hoechst 33342 staining indicated that AG-1031 can induce apoptosis in C6 glioma cells.

Autophagy, which is a process of cellular self-digestion, can degrade damaged organelles and proteins to prolong survival especially under conditions of nutrient deprivation.[30] At the same time, autophagy is also known as type II programmed cell death, and hyperactivation of autophagy can cause cell death.[31] To further investigate the level of autophagy changed by AG-1031, we tested the expressions of P62 and LC3 by Western blot assay. Results showed a down-regulation of P62 and an up-regulation of LC3II/I after treatment with AG-1031. Besides, bafilomycin A1 treatment caused an obvious increase of LC3 II in both groups. These results indicated that AG-1031 can promote the level of autophagy in C6 glioma cells.

To investigate the relationship between autophagy and cell death, we used 3-MA to inhibit autophagy. Results

showed that 3-MA can remit AG-1031 induced cell death, which indicated that AG-1031 induced autophagic cell death in C6 glioma cells. Autophagic death, which refers to the cell death mediated by autophagy, played a crucial role in physiological and pathological events. Tumorigenesis is closely related to down-regulation of autophagy.[31] Besides, many studies have showed that some antitumor materials may reduce the proliferation of glioma cells by inducing autophagy. For example, Zou et al[32] showed that oroxylin A can inhibit the proliferation of human malignant glioma including U251 cells, U118 cells and U87 cells by inducing autophagy. Chen et al[2] proved that MiR-129 had an antiproliferative capacity in U87 cells and U251 cells by inducing Beclin-1-mediated autophagy.[20] Yang et al[33] suggested that nicotinamide phosphoribosyltransferase inhibitor APO866 induced cell death in C6 glioma cells via autophagy.

The mechanistic target of rapamycin (mTOR) is an important regulator of autophagy and its inhibition can cause activation of autophagy.[34,35] mTOR can sense nutritional levels of cells and inhibit autophagy directly.[36] Studies have showed that both Akt inhibitors and mTOR inhibitors can reduce cell viability in some glioma cell lines.[37,38] As a downstream effector of PI3K, Akt plays a crucial role of mTOR activity.[39] The activated Akt induces the mTORC1-mediate signaling pathway via inhibiting tuberous sclerosis complex (TSC) 1/2 activity, which lead to the phosphorylation of ribosomal protein S6 kinase (pS6k) and eukaryotic initiation factor binding protein 1(4EBP1).[40] It has been proved that the abnormity of PI3K/Akt/mTOR signaling is distinct in various tumors and is related to tumor pathogenesis and therapy resistance.[41,42] Our research showed that AG-1031 can cause a decrease of phosphor-Akt and phosphor-mTOR, which indicated that AG-1031 inhibited Akt-mTOR signaling in C6 glioma cells, and the suppression of Akt-mTOR signaling may contribute to the activation of autophagy induced by AG-1031.

Notch signaling, which is central to cell proliferation, differentiation, apoptosis, and cancer stem cell regulation, has become a promising target for the treatment of GBM.[43] It has been proved that Notch signaling can affect cell proliferation and survival through Akt/mTOR signaling in U251 glioma cells.[17] Research has showed that Notch-1 upregulates the expression of EGFR through p53. EGFR is known to be amplified in glioma and its inhibitors have efficacy for the treatment of patients with high-grade glioma.[44,45] It has been proved that the down-regulation of Notch-1 and its ligands Delta-like-1 and Jagged-1 lead to cell apoptosis and inhibit proliferation in glioma cell lines.[14] We found that the expressions of Notch-1, Jagged-1, and Hes-1 as related protein of Notch signaling reduced after the treatment of AG-1031 for 24 h. These results indicated that the occurrence of apoptosis and the inhibition of Akt-mTOR signaling in C6 cells may associate with the suppression of Notch-1 signaling.

Apoptosis, which is known as type I programmed cell death, can be divided into two pathways including intrinsic pathway and extrinsic pathway. The former kind activates caspases after release of cytochrome C into cytosol and the latter kind is activated by Fas death receptor.[46] Both Bax and Bcl-xl belong to Bcl-2 superfamily, which controls mitochondrial integrity in intrinsic apoptotic and includes two subcategories. Bax is a part of pro-apoptotic family, while Bcl-xl is a part of anti-apoptosis family.[47] Poly (ADP-ribose) polymerase (PARP), as a vital protein involved in DNA repair, can be cleaved by caspase-3 and caspase-7 into two fragments when cell undergoes apoptosis.[48] Our results found that AG-1031 can induce both autophagic cell death and apoptosis in C6 glioma cells. Besides, there are multifarious crosstalk between apoptosis and autophagic cell death including synergy, promote antagonism, and mutual independence.[49] Autophagy and apoptosis show connections in many aspects. As an apoptotic and autophagic protein, Bcl-2 can interact with BH3-only proteins to promote apoptosis and combine with Beclin-1's domain to inhibit Beclin-1-dependent autophagy. Moreover, P53, which can trigger extrinsic apoptosis in the nucleus and intrinsic apoptosis in the cytoplasm, also shows a regulation to autophagy.[47]

Autophagy, known as a cellular self-digestion pathway, it may play a crucial part in regulating developmental pathways. A recent study has proved that autophagy can regulate Notch degradation in mammalian cells.[50] Besides, Barth et al. have found that the lack of autophagy can trigger precocious activation of Notch signaling in the somatic follicle cells.[51] Interestingly, Notch signaling was also showed a regulating effect on autophagy pathway. Lin-12 and glp-1 were two Notch genes in Nematode C. elegans. It has been found that the level of autophagy was increased in Germline-Less glp-1 Animals.[52] Furthermore, interfering Notch1 with the lentiviral vector of Notch1 shRNA elevated autophagy-related proteins in U251 cells.[53] Our study demonstrated that the activation of Notch-1 signaling significantly rescued AG-1031-induced autophagy cell death and apoptosis, which suggested that AG-1031-induced cell death may partially related to Notch-1 signaling.

Based on the researches mentioned above and the results of our study, we raised a hypothesis that the autophagic cell death and apoptosis induced by AG-1031 may associate with down-regulation of Notch-1 signaling (Figure 8). Although we have found a possible mechanism of AG-1031 in C6 glioma cells, further understandings of regulatory factors between Notch-1 signaling and Akt-mTOR signaling and potential upstream signaling of Notch-1 signaling still remain unclear.

In conclusion, our study showed that AG-1031 can induce autophagic cell death of C6 glioma cells and lead to occurrence of apoptosis as an anticancer drug. Besides, AG-1031 can improve the level of mTOR-dependent autophagy and restrain Notch-1 signaling in C6 glioma cells. We also found that AG-1031 induced autophagic cell death

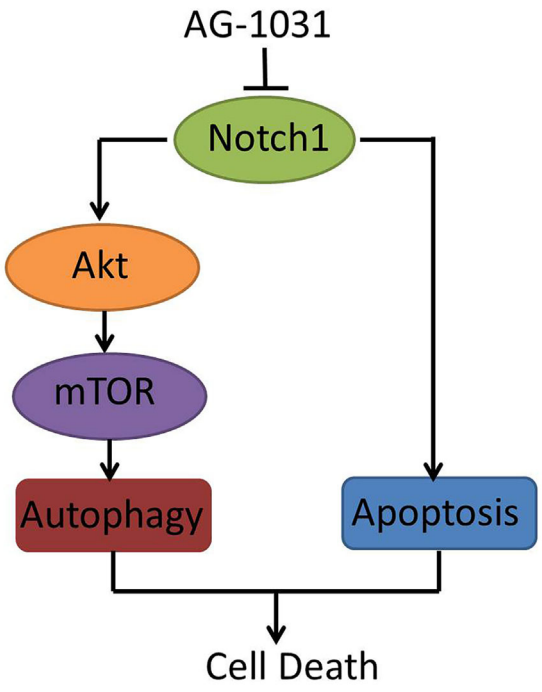

FIGURE 8 Suggested pathway induced by AG-1031 leading to autophagy and apoptosis in C6 glioma cells AG-1031 can inhibit Akt-mTOR signaling pathway by down-regulating Notch-1 signaling pathway and induce apoptosis and mTOR-triggered autophagy in C6 glioma cells

and apoptosis was associated to the activation of Notch-1 signaling. These findings may provide a new prospect for the application of AG-1031 and a novel targeted therapy for the treatment of gliomas.

ACKNOWLEDGMENTS

This work was supported by grant from the National Natural Science Foundation of China (81571804, 81771979) and China Postdoctoral Science Foundation (BS000056).

CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

ORCID

Zhuo Yang http://orcid.org/0000-0001-5212-0053

REFERENCES

1. Bush NAO, Chang SM, Berger MS. Current and future strategies for treatment of glioma. *Neurosurg Rev*. 2017;40:1–14.
2. Chen R, Cohen AL, Colman H. Targeted Therapeutics in Patients With High-Grade Gliomas: Past, Present, and Future. *Curr Treat Options Oncol*. 2016;17:1–11.
3. Niroop K, Latika K, Roth KA. Autophagy in brain tumors: a new target for therapeutic intervention. *Brain Pathol*. 2012;22:89–98.
4. Levine B, Klionsky DJ. Development by self-Digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 2004;6:463–477.
5. Wang H, Gao N, Li Z, Yang Z, Zhang T. Autophagy alleviates melamine-Induced cell death in PC12 cells via decreasing ROS level. *Mol Neurobiol*. 2016;53:1718–1729.
6. Zhang X, Yin H, Li Z, Zhang T, Yang Z. Nano-TiO2 induces autophagy to protect against cell death through antioxidative mechanism in podocytes. *Cell Biol Toxicol*. 2016;32:513–527.
7. Fu J, Wang H, Gao J, Yu M, Wang R, Yang Z, Zhang T. Rapamycin effectively impedes melamine-Induced impairments of cognition and synaptic plasticity in wistar rats. *Mol Neurobiol*. 2017;54:819–832.
8. Galluzzi L, Vicencio JM, Kepp O, Tasdemir E, Maiuri MC, Kroemer G. To die or not to die: that is the autophagic question. *Curr Mol Med*. 2008;8:78–91.
9. Hao H, Zhang D, Shi J, Wang Y, Chen L, Guo Y, Ma J, Jiang X, Jiang H. Sorafenib induces autophagic cell death and apoptosis in hepatic stellate cell through the JNK and Akt signaling pathways. *Anticancer Drugs*. 2016;27:192.
10. Wang N, Feng Y, Zhu M, Tsang CM, Man K, Tong Y, Tsao SW. Berberine induces autophagic cell death and mitochondrial apoptosis in liver cancer cells: the cellular mechanism. *J Cell Biochem*. 2010;111:1426–1436.
11. Artavanis-Tsakonas S, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999;284:770–776.
12. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*. 2009;137:216.
13. Lai EC. Notch signaling: control of cell communication and cell fate. *Development*. 1991;131:965.
14. Purow BW, Haque RMNoel MW, Su Q, Burdick MJ, Lee J, Sundaresan T, Pastorino S, Park JK, Mikolaenko I, Maric D. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res*. 2005;65:2353.
15. Moutal A, Honnorat J, Massoma P, Désormeaux P, Bertrand C, Malleval C, Watrin C, Chounlamountri N, Mayeur ME, Besançon R. CRMP5 controls glioblastoma cell proliferation and survival through Notch-Dependent signaling. *Cancer Res*. 2015;75:3519.
16. Chautard E, Ouédraogo ZG, Biau J, Verrelle P. Role of Akt in human malignant glioma: from oncogenesis to tumor aggressiveness. *J Neurooncol*. 2014;117:205–215.
17. Zhao N, Guo Y, Zhang M, Lin L, Zheng Z. Akt-mTOR signaling is involved in Notch-1-mediated glioma cell survival and proliferation. *Oncol Rep*. 2010;23:1443–1447.
18. Kamalinia G, Khodagholi F, Atyabi F, Amini M, Shaerzadeh F, Sharifzadeh M, Dinarvand R. Enhanced brain delivery of deferasirox-lactoferrin conjugates for iron chelation therapy in neurodegenerative disorders: in vitro and in vivo studies. *Mol Pharm*. 2013;10:4418–4431.
19. Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to human. *Autophagy*. 2007;3:181–206.
20. Xiong C, Zhang Y, Shi Y, Lian H, Tu H, Song H, Yin J, Peng B, Zhou B, He X. MiR-129 triggers autophagic flux by regulating a novel Notch-1/ E2F7/Beclin-1 axis to impair the viability of human malignant glioma cells. *Oncotarget*. 2011;7:9222–9235.
21. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. MTOR regulation of autophagy. *FEBS Lett*. 2010;584:1287.

22. Yu L, Mcphee CK, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F. Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature*. 2010;465:942.
23. Rand MD, Grimm LM, Artavanistsakonas S, Patriub V, Blacklow SC, Sklar J, Aster JC. Calcium depletion dissociates and activates heterodimeric Notch receptors. *Mol Cell Biol*. 2000;20:1825–1835.
24. Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol*. 2003;194:237–255.
25. Lasky JL, Wu H. Notch signaling, brain development, and human disease. *Pediatr Res*. 2005;57:104R–109R.
26. South AP, Cho RJ, Aster JC. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol*. 2012;23:458–464.
27. Stupp R, Mason WP, Van dB, Martin J, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352:987–996.
28. Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med*. 2008;359:492–507.
29. Grant R, Kolb L, Moliterno J. Molecular and genetic pathways in gliomas: the future of personalized therapeutics. *CNS oncol*. 2014;3:123.
30. Sharma K, Le N, Alotaibi M, Gewirtz DA. Cytotoxic autophagy in cancer therapy. *Int J Mol Sci*. 2014;15:10034–10051.
31. Shimizu S, Yoshida T, Tsujioka M, Arakawa S. Autophagic cell death and cancer. *Int J Mol Sci*. 2014;15:3145–3153.
32. Zou M, Hu C, You Q, Zhang A, Wang X, Guo Q. Oroxylin A induces autophagy in human malignant glioma cells via the mTOR-STAT3-Notch signaling pathway. *Mol Carcinog*. 2014;54:1363.
33. Yang P, Zhang L, Shi QJ, Lu YB, Wu M, Wei EQ, Zhang WP. Nicotinamide phosphoribosyltransferase inhibitor APO866 induces C6 glioblastoma cell death via autophagy. *Pharmazie*. 2015;70:650.
34. Desai BN, Myers BR, Schreiber SL. FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction. *Proc Natl Acad Sci*. 2002;99:4319–4324.
35. Shacka JJ, Roth KA, Zhang J. The autophagy-lysosomal degradation pathway: role in neurodegenerative disease and therapy. *Front Biosci*. 2008;13:718.
36. Hale AN, Ledbetter DJ, Gawriluk TR. Autophagy: regulation and role in development. *Autophagy*. 2013;9:951–972.
37. Fujiwara K, Iwado E, Mills GB, Sawaya R, Kondo S, Kondo Y. Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. *Int J Oncol*. 2007;31:261–261.
38. Annovazzi L, Mellai M, Caldera V, Valente G, Tessitore L, Schiffer D. MTOR, S6 and AKT expression in relation to proliferation and apoptosis/autophagy in glioma. *Anticancer Res*. 2009;29:3087.
39. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev*. 2004;18:1926–1945.
40. Li X, Wu C, Chen N, Gu H, Yen A, Cao L, Wang E, Wang L. PI3K/Akt/mTOR signaling pathway and targeted therapy for glioblastoma. *Oncotarget*. 2016;7:33440–33450.
41. Jhanwar-Uniyal M, Jeevan D, Neil J, Shannon C, Albert L, Murali R. Deconstructing mTOR complexes in regulation of Glioblastoma Multiforme and its stem cells. *Adv Biol Regul*. 2013;53:202–210.
42. Duzgun Z, Eroglu Z, Biray AC. Role of mTOR in glioblastoma. *Gene*. 2015;575:187–190.
43. Jean-Louis B, Adrian M, Maria L. Notch signaling in glioblastoma: a developmental drug target? *BMC Med*. 2010;8:72.
44. Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, Lu KV, Yoshimoto K, Huang JH, Chute DJ. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med*. 2005;353:2012–2024.
45. Purow BW, Sundaresan TK, Burdick MJ, Kefas BA, Comeau LD, Hawkinson MP, Su Q, Kotliarov Y, Lee J, Zhang W. Notch-1 regulates transcription of the epidermal growth factor receptor through p53. *Carcinogenesis*. 2008;29:918–925.
46. Kiraz Y, Adan A, Kartal YM, Baran Y. Major apoptotic mechanisms and genes involved in apoptosis. *Tumor Biol*. 2016;37:1–16.
47. Mukhopadhyay S, Panda PK, Sinha N, Das DN, Bhutia SK. Autophagy and apoptosis: where do they meet? *Apoptosis*. 2014;19:555–566.
48. Jubin T, Kadam A, Jariwala M, Bhatt S, Sutariya S, Gani AR, Gautam S, Begum R. The PARP family: insights into functional aspects of poly (ADP-ribose) polymerase-1 in cell growth and survival. *Cell Prolif*. 2016;49:421–437.
49. Bhutia SK, Mukhopadhyay S, Sinha N, Das DN, Panda PK, Patra SK, Maiti TK, Mandal M, Dent P, Wang XY. Autophagy: cancer's friend or foe? *Adv Cancer Res*. 2013;118:61.
50. Wu X, Fleming A, Ricketts T, Pavel M, Virgin H, Menzies FM, Rubinsztein DC. Autophagy regulates Notch degradation and modulates stem cell development and neurogenesis. *Nat Commun*. 2016;7:10533.
51. Barth JM, Hafen E, Kohler K. The lack of autophagy triggers precocious activation of Notch signaling during Drosophila oogenesis. *BMC Dev Biol*. 2012;12:35.
52. Lapierre LR, Gelino S, Melendez A, Hansen M. Autophagy and lipid metabolism coordinately modulate life span in germline-less C. elegans. *Curr Biol*. 2011;21:1507–1514.
53. Yao J, Zheng K, Li C, Liu H, Shan X. Interference of Notch1 inhibits the growth of glioma cancer cells by inducing cell autophagy and down-regulation of Notch1-Hes-1 signaling pathway. *Med Oncol*. 2015;32:610.

SUPPORTING INFORMATION

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How to cite this article: Wang Y, Wang H, Ge H, Yang Z. AG-1031 induced autophagic cell death and apoptosis in C6 glioma cells associated with Notch-1 signaling pathway. *J Cell Biochem*. 2018;1–11. https://doi.org/10.1002/jcb.26781