WZY-321, a novel evodiamine analog, inhibits glioma cell growth in an autophagy-associated manner

GUAN SUN1*, CHUANG ZHANG2*, HONGMAO SONG3*, JUN GUO1, MIN LI4 and YING CAO3

1Department of Neurosurgery, The First People’s Hospital of Yancheng, Fourth Affiliated Hospital of Nantong University, Yancheng, Jiangsu 224001; 2Department of Medical Oncology, The Eighty-First Hospital of People’s Liberation Army, Nanjing, Jiangsu 210002; 3Department of Ear-Nose-Throat, Huai’an Second People’s Hospital, The Affiliated Huai’an Hospital of Xuzhou Medical University, Huai’an, Jiangsu 223002; 4Department of Neurosurgery, Jiangning Hospital Affiliated with Nanjing Medical University, Nanjing, Jiangsu 211100, P.R. China

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Abstract. Glioblastoma is one of the most aggressive types of brain tumor. The median survival rate of patients with glioblastoma (World Health Organization grade IV) is <15 months. Therefore, there is an urgent requirement for the development of novel and efficient therapeutic agents against glioma. In previous studies, WZY-321 (10-hydroxy-1-methyl-8,13b-dihydr o-5H7H-benzo[e]benzofuro[2,3;3,4]pyrido[2,1-b][1,3]oxazin-5-one), a novel evodiamine (Evo) analog, was reported to exhibit enhanced pharmacological properties and improved cytotoxicity against a number of human cancer cell lines compared with Evo. In the current study, the anti-proliferative effect of WZY-321 on SHG-44 and SWO-38 glioma cells was further studied, and its mechanism of action investigated. The results indicated that WZY-321 inhibited the proliferation of SHG-44 cells in a dose- and time-dependent manner by enhancing cellular apoptosis and inducing cell cycle arrest at the G2-M phase. Treatment of glioma cells with WZY-321 concomitantly increased the expression levels of microtubule associated protein 1 light chain 3α and Beclin1, indicating enhanced autophagy. Overall, the results of the present study revealed the anti-proliferative potential of WZY-321 in glioma cells, thus providing a possible autophagy-based therapeutic strategy for the treatment of glioblastoma.

Correspondence to: Dr Ying Cao, Department of Ear-Nose-Throat, Huai’an Second People’s Hospital, The Affiliated Huai’an Hospital of Xuzhou Medical University, 387 Fujian Road, Hua’ian, Jiangsu 223002, P.R. China
E-mail: cy20101462@163.com

Dr Min Li, Department of Neurosurgery, Jiangning Hospital Affiliated with Nanjing Medical University, 688 Wanan Road, Nanjing, Jiangsu 211100, P.R. China
E-mail: sbn133@163.com

*Contributed equally

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Introduction

The prognosis of patients with glioblastoma multiforme (GBM) and anaplastic astrocytoma remains poor, despite the current treatment modalities (1). Generally, radiation and chemotherapy are initiated following surgical treatment to improve the curative effects (2). Temozolomide (TMZ), a chemotherapeutic drug, is used as the drug of choice for treating malignant gliomas in clinical practice. The cytotoxicity of TMZ is associated with its ability to methylate guanine at the O6 position (3). Although TMZ is used as a second- and first-line treatment for astrocytoma and GBM, respectively, its effect is barely satisfactory as it extends the median survival of patients by only 2 months (4-6). Cells containing elevated levels of O6-methylguanine DNA methyltransferase repair the aberrant methylation induced by TMZ, and consequently reverse the cytotoxic effect. In addition, DNA mismatch repair deficiency makes cells tolerant to methylation and to the cytotoxic effects of TMZ (7). Therefore, the development of novel chemotherapeutic drugs for the treatment of gliomas is essential.

Evading apoptosis is a major hallmark of tumorigenesis and chemoresistance, particularly in high-grade glioblastomas and astrocytomas (8). Recently, particular attention has been paid to the crosstalk between apoptosis and autophagy. The term ‘autophagy,’ which originates from a Greek term meaning self (auto)-eating (phagy), was first coined and defined by De Duve in 1963 (9). Autophagy is the degradation process of cytoplasmic constituents in the lysosome-vacuole and is a fundamental mechanism, well conserved among eukaryotes (10). Autophagy serves a vital role in development, protein and organelle quality control, senescence, neurodegeneration and tumorigenesis. In 1999, Liang et al (11) first reported the role of Beclin1, one of the most important constituents for autophagosome formation, in the induction of autophagy and inhibition of tumorigenesis. In 2000, Kabeya et al (12) identified two forms of microtubule associated protein 1 light chain 3α (LC3), termed LC3-I and LC3-II, respectively. LC3-II serves an important role in the formation of the autophagosome membrane. Subsequent investigations in mammals used Beclin1 and
LC3 as autophagic markers. In addition, Beclin1 and LC3 are reliable markers of brain tumors.

Autophagy is associated with drug resistance in numerous types of tumor cells. Notably, although autophagy impedes the therapeutic effects of anti-cancer drugs in some cases (13), it potentiates responses to conventional therapies in gliomas (14,15). In addition, expression levels of the autophagic proteins Beclin1 and LC3-II are much lower in higher-grade astrocytomas compared with lower-grade astrocytomas and normal brain tissue, and prognosis is positively associated with the level of autophagy (16,17). This evidence suggests that a decrease in autophagic activity may be involved in the progression of astrocytic or glioma tumors. Therefore, restoration of autophagy may inhibit tumor progression and may prove promising as a future therapeutic strategy.

Evodiamine (Evo) is a quinazolinocarboline alkaloid isolated from the fruit of *Evodiae fructus*, a traditional Chinese herb. *E. fructus* has been widely used for the treatment of gastrointestinal disorders, headache and post-partum hemorrhage (18). Evo has been reported to have various therapeutic benefits associated with the treatment of cancer, inflammation, obesity, cardiovascular diseases and pain (19-22). Studies investigating the anticancer activity of Evo have demonstrated that it inhibits the growth and metastasis of various cancer cells by regulating the cell cycle, apoptosis and autophagy (21,23). Evo induces intracellular calcium-JNK signaling-mediated autophagy and calcium-mitochondria-mediated apoptosis in glioma cells (20). However, the potential of Evo is hindered due to its limited efficiency and drug-like properties, including aqueous solubility and rapid plasma clearance (24). The development of Evo analogs with optimized drug properties is crucial for the treatment of malignant gliomas. WZY-321 is a novel Evo analog with improved drug properties. WZY-321 has demonstrated promising cytotoxic effects in cancer cells (25). The present study evaluated the regulation of autophagy and the therapeutic potential of WZY-321 in gliomas.

**Materials and methods**

**Reagents.** The Cell Counting Kit-8 (CCK-8) kit and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis staining kit and antibodies used in the study were purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA. The Annexin V-FITC binding was analyzed by flow cytometry (excitation=488 nm; emission=535 nm) using the FITC signal detector (usually FL1), and PI staining by the phycoerythrin emission signal detector (usually FL2), using FlowJo 8.8 software (Tree Star, Inc., Ashland, OR, USA).

**Cell culture.** SHG-44 glioma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The human glioma cell line SWO-38 was established by the Department of Neurosurgery at the Jiangning Hospital (Nanjing, China) (26). The cells were cultured at 37°C in DMEM supplemented with 10% FBS, 200 mM L-glutamine, 100 U·ml penicillin, 100 µg·ml streptomycin, 100 mM sodium pyruvate and 1% non-essential amino acids. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂. The medium was changed every 2 days and the cells were passaged every 3 days. The density of the seeded cells in different biological replicates was the same.

**Assessment of cell viability.** SHG-44 and SWO-38 cells (5×10⁴ cells·100 µl) were seeded into each well of a 96-well plate and pre-incubated for 24 h in a humidified incubator (37°C; 5% CO₂). Total volumes of 10 µl of various concentrations of WZY-321 (0, 5, 10, 30 and 50 µM) were added to the cells and incubated for 24 or 48 h. Following incubation, 10 µl CCK-8 solution was added to each well and incubated for 1-4 h. Cell viability was proportional to the optical density and was quantitatively measured by spectrophotometry (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm. The viability of cells in the control group incubated for 48 h was defined as 100% viable.

**Measurement of apoptosis.** SHG-44 cells (1×10⁵ cells-well) were cultured in complete medium in 6-well plates for 24 h, and treated in triplicate with different concentrations of WZY-321 (10, 30 and 50 µM) for 24 h. The control cells were treated with vehicle [1% dimethyl sulfoxide (DMSO) in complete medium]. Cell morphology was observed under optical microscope (magnification, x20). The cells were subsequently harvested, washed, and stained with PI and FITC-Annexin V in the dark at 25°C for 15 min using the Annexin V-FITC apoptosis staining kit, according to the manufacturer’s protocols. The percentage of apoptotic cells was determined by flow cytometry using an FC500 cytometer (Beckman Coulter, Inc., Brea, CA, USA). Annexin V-FITC binding was analyzed by flow cytometry using a FC500 cytometer (Beckman Coulter, Inc., Brea, CA, USA). The percentages of cells in different phases of cell cycle were analyzed by ModFit 4.1 software (Verity Software House, Topsham, ME, USA).
Western blot analysis. SHG-44 cells were incubated at 37°C in the presence or absence of WZY-321 at a concentration of 0, 10, 30 and 50 µM for 24 h. 3-MA was used as an autophagy inhibitor and cells were pretreated with 2.5 mM 3-MA at 37°C for 12 h prior to treatment with WZY-321. Following incubation, cells were collected, centrifuged at 13,000 x g at 4°C for 15 min and washed twice with ice cold PBS. The pellets were re-suspended in lysis buffer. The cells were lysed on ice for 20 min and the lysates were centrifuged at 13,000 x g at 4°C for 15 min. The protein concentration in the supernatant was determined using bicinchoninic acid protein assay reagents. Equal amounts of protein (20 µg-well) were separated via SDS-PAGE on a 10% gel and transferred to a polyvinylidene fluoride Hybond-P membrane. Membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies against: Beclin1 (cat. no. sc-48341); LC3 (cat. no. sc-271625); apoptosis regulator Bcl-2 (Bcl-2; cat. no. sc-509); cyclin-dependent kinase 1 (cdc2; cat no. sc-54); cyclin D1 (cat. no. sc-70899); poly (ADP-ribose) polymerase (cat. no. sc-136208); and β-actin (cat. no. sc-58673) or GAPDH (cat. no. sc-293335) (1:1,000 dilution; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) by gentle rotation overnight at 4°C. β-actin and GAPDH were used as loading controls. The membranes were subsequently washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:20,000 dilution; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature and visualized using enhanced chemiluminescent reagent (EMD Millipore, Billerica, MA, USA). The densitometric analysis was performed using Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Values are expressed as the mean ± standard error of the mean calculated from three independent experiments. Statistical analysis was performed using Student’s t-test (for two groups) or one-way analysis of variance followed by Duncan’s multiple-range test (for three or more groups) Analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

WZY-321 induces cytostatic effects in SHG-44 and SWO-38 glioma cells. SHG-44 and SWO-38 glioma (26) cells were pretreated with various concentrations of WZY-321 (5, 10, 30 or 50 µM) for 24 or 48 h. The number of viable cells was determined using the CCK-8 assay. As presented in Fig. 2, WZY-321 significantly reduced the viability of SHG-44 and SWO-38 cells in a dose-dependent manner. Treatment with 10 µM WZY-321 for 48 h reduced the cell count to ~30% compared with the control group. In addition, incubation with WZY-321 for 24 h significantly decreased the viability of SHG-44 glioma cells. Prolonged incubation of cells with WZY-321 for up to 48 h displayed little benefit. SWO-38 cells were less sensitive to WZY-321 compared with SHG-44 cells. The histological subtypes of SHG-44 and SWO-38 cells may explain this sensitivity (27). Therefore, in the subsequent studies, SHG-44 glioma cells were incubated with WZY-321 for 24 h prior to processing the samples for subsequent pharmacological tests.

WZY-321 triggers apoptosis in SHG-44 glioma cells. SHG-44 cells treated with WZY-321 for 24 h displayed marked alterations in cell morphology, exhibiting chromatin condensation, which suggested the cytostatic effect of WZY-321 may be associated with cell apoptosis (Data not shown). To confirm whether WZY-321 is able to induce apoptosis in SHG-44 cells, vehicle- or WZY-321-treated SHG-44 cells were stained with Annexin V-PI for flow cytometry analysis. Externalization of phosphatidylinerse (PS) from the inner leaflet to the outer leaflet of the plasma membrane is a distinct phenomenon observed in early apoptotic cells. Annexin V has high affinity for PS, and fluorochrome-labeled Annexin V may be used for the detection of apoptotic cells. By contrast, PI is used to detect necrotic cells due to its ability to permeate the damaged cell membrane. In the present study, flow cytometric analysis identified four groups of cells: i) Viable cells (Annexin V−, PI−); ii) early apoptotic cells (Annexin V+, PI−); iii) late apoptotic cells (Annexin V+, PI+); and iv) necrotic cells (Annexin V−, PI+). The percentage of apoptotic SHG-44 cells detected by Annexin V-FITC and PI double staining following incubation with various concentrations of WZY-321 (0, 10, 30 or 50 µM) for 24 h is presented in Fig. 3. The number of early-stage apoptotic SHG-44 cells was increased in the 50 µM WYZ-321-treated group (11.3%) compared with that in the control group (1.6%), in a dose-dependent manner. The upper left quadrants representing the necrotic cells were comparable in each group. However, the increase in the number of advanced stage apoptotic cells following treatment with WZY-321 was not significant. These results demonstrated that WZY-321 may dose-dependently induce apoptosis in SHG-44 cancer cells.

WZY-321 induces cell cycle arrest at the G2-M phase. One of the mechanisms by which chemical antitumor agents inhibit cell proliferation is through the induction of cell cycle arrest (28). To determine this mechanism involved in the suppression of cell growth by WZY-321, the DNA content of the cell nuclei was detected by flow cytometry. SHG-44 cells were treated with varying concentrations of WZY-321 (0, 10, 30 and 50 µM). As presented in Fig. 4A and B, the number of cells which arrested at the G2-M phase increased from 45.9 to 51.6% in a dose-dependent manner, along with a concomitant decrease in the percentage of cells in the S and G1 phases. Western blot analysis was performed to determine the expression levels of cell cycle regulatory proteins. As displayed in Fig. 4C, treatment of SHG-44 cells with WZY-321 resulted in a decrease in the protein expression levels of cdc2 and...
cyclin D1, in a dose-dependent manner. These results suggest an association between WZY-321-induced apoptosis and the accelerated entry of cells into G2-M arrest.

WZY-321 enhances autophagy in SHG-44 glioma cells. The cytotoxicity of certain anti-tumor agents is induced by autophagy (29-31). To assess the association of autophagy with the
pro-apoptotic effects of WZY-321, a number of autophagic markers were analyzed. Beclin1, a novel Bcl-2-interacting coiled-coil protein has structural similarity to yeast autophagy-related protein 6. The proven importance of Beclin1 in tumor suppression triggered research into the role of autophagy in tumor suppression. LC3 is the mammalian homolog of yeast autophagy-related gene 8. LC3-II is derived from LC3-I following cleavage from the C-terminal and serves an important role in the formation of autophagosomes, as it localizes to the outer and inner membranes. Beclin1 and LC3 are frequently-used markers for the evaluation of tumorigenesis in brain tumors. In the present study, SHG-44 glioma cells were incubated with varying concentrations of WZY-321 for 24 h and the expression levels of Beclin1 and LC3 were evaluated. As presented in Fig. 5, incubation with various concentrations of WZY-321 increased the expression levels of Beclin1 and LC3-II. Accordingly, statistical analysis of the immunoblotting confirmed the above findings (Fig. 5). Notably, while expression levels of Beclin1 and LC3-II in cells incubated with 30 µM WZY-321 were comparable with those treated with 10 µM WZY-321, levels in the 50 µM group were slightly higher compared with the untreated group for LC3-II-LC3-I (*P<0.05), and significantly higher for Beclin-1 (**P<0.01), indicating a dose-dependent induction of autophagy by WZY-321 in SHG-44 cells.

Treatment with an autophagy inhibitor inhibits the apoptotic effects of WZY-321. A complex association exists between autophagy and apoptosis. The activation of autophagy may potentiate cytotoxicity, or impede autophagic cell death. In order to probe whether enhanced autophagy contributed to the anti-cancer potential of WZY-321, or impeded the therapeutic effects of WZY-321, 3-MA was used as an autophagy inhibitor and the expression levels of Bcl-2 and Beclin1 were analyzed (Fig. 6). In WZY-321-treated SHG-44 cells, a marked decrease was observed in the protein expression of Bcl-2, confirming the apoptotic effect of WZY-321. However, this apoptotic effect was blocked when cells were pretreated with 3-MA prior to treatment with WZY-321. As presented in Fig. 6B, the expression level of Beclin1 was lower in 3-MA-pretreated SHG-44 cells treated with WZY-321 compared with cells treated with WZY-321 alone. In addition, the expression levels of Bcl-2 were higher in 3-MA-pretreated SHG-44 cells treated with WZY-321 compared with cells treated with WZY-321 alone.

Discussion

In the present study, it was hypothesized that there is an association between autophagy and the anti-tumorigenic potential of WZY-321. Treatment with WZY-321 inhibited the growth of glioma cells in a dose-dependent manner. This is the first time, to the best of our knowledge, that the association of the anti-tumorigenesis potential of WZY-321 with increased autophagic levels in glioma has been demonstrated.

Recent studies have reported that Evo inhibits the proliferation of a variety of cancer cells by inducing apoptosis (20,32). However, the moderate anti-tumor potency, sub-optimal physicochemical properties and patent ineligibility of Evo have impeded its direct clinical application. Structural modification of natural products may increase their potential to be used as drugs, in addition to the likelihood of obtaining patents. WZY-321 is a novel analog of...
Evo designed using a scaffold-hopping-based structural modification strategy. In the present study, the antiproliferative effect of WZY-321 on the growth of SHG-44 and SWO-38 cells was assessed using the CCK-8 assay. The results indicated that the viability of SHG-44 cells treated with WZY-321 for 24 h was significantly decreased. This indicated the potent cytotoxicity of WZY-321, which merited further investigation for the development of novel potential anti-tumor agents. SHG-44 cells treated with WZY-321 for 24 h displayed marked alterations in cell morphology, exhibiting chromatin condensation, which indicated cell apoptosis. The apoptosis-associated protein Bcl-2 was also detected in order to verify apoptosis. However, there are limitations to the present study; cells were only exposed to the drug for 24 or 48 h, whereas exposure for 72 h may have enhanced the apoptotic potential of WZY-321. Furthermore, there is absence of data relating to additional apoptosis-associated proteins, including caspase 3, 7 and 9.

There is a complex association between autophagy and apoptosis. In the treatment of GBM and anaplastic astrocytoma, autophagy mediates cell survival against anti-cancer therapies, while its excessive activation potentiates cytotoxicity and induces autophagic cell death. The inhibition of autophagy has been reported to synergize with the effect of erlotinib in inducing GBM cell death (33). By contrast, in malignant glioma cell lines, arsenic trioxide has been reported to inhibit glioma growth through the induction of autophagic cell death (34). Autophagy contributes to gefitinib-induced growth inhibition in glioma cells (35). These seemingly controversial observations result from the complex tumor microenvironment and diverse therapeutic mechanisms of anti-cancer drugs (36), all of which adds complexity to the study of autophagy-associated cancer therapies.

A previous report indicated the induction of intracellular calcium-JNK signaling-mediated autophagy and calcium-mitochondria-mediated apoptosis by Evo in glioma cells (37). These reports confirm the findings from the current study that the therapeutic potential of WZY-321 in glioma cells was associated with autophagy. However, these results require careful interpretation; the present results indicated that reduced autophagy impeded the apoptotic effects of WZY-321. However, the association between apoptosis and autophagy in SHG-44 glioma cells treated with WYZ-321 requires further investigation. Furthermore, the exact role...
of autophagy in the anti-cancer action of WZY-321 requires further pharmacological evaluation. In addition, topoisomerase 1 was previously identified as one of the targets of Evo (38). As an analog of Evo, future studies on the potential targets of WZY-321 and its mechanisms of action are required.

In conclusion, the application of WZY-321, an analog of Evo, in targeting glioma cell survival was identified. WZY-321 decreased the proliferation of SHG-44 glioma cells in a dose-dependent manner by enhancing apoptosis and inducing cell cycle arrest at the G2-M phase. Treatment with WZY-321 significantly increased the expression of two autophagy-associated proteins, LC3 and Beclin1, in a dose-dependent manner in SHG-44 glioma cells, and this may be associated with its therapeutic potential. The results from the present study provide a possible insight into the development of novel autophagy-based therapeutic strategies for the treatment of glioblastoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GS, ML and YC conceived and designed the study; GS and JG were responsible for the development and methodology of the study; GS, CZ and HS acquired the data; GS and CZ analyzed the data; GS wrote the manuscript; YC and ML supervised the study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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