Gypenosides inhibits the growth and induces apoptosis of glioma cells by promoting autophagy

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Abstract

Gypenosides (Gyp), a Chinese medicine and an effective ingredient extracted from *Gynostemma pentaphyllum*, plays vital roles in the progression of various cancers. However, its biological functions and mechanisms in glioma remain unclear. We evaluated the anti-cancer mechanism of Gyp in glioma to determine its potential therapeutic value. In human glioma cells, we evaluated cell viability using MTS assays and cell mobility using Transwell invasion and scratch migration assays. Additionally, we assessed apoptosis using flow cytometry and Hoechst 33342 staining, and autophagy flow was detected by Fluorescence microscope. The levels of migration, apoptosis and autophagy-related protein were examinated by Western blot. Gyp inhibited the proliferation, migration and invasion of glioma cells by down-regulating the level of matrix metalloproteinase (MMP)-2 and MMP-9. Moreover, Gyp promoted apoptosis of glioma cells by inducing Bax and cleaved-caspase-9 activation and down-regulating Bcl-2. Notably, autophagy was activated by treating with Gyp in U251 and U87 cells through down-regulation of p62 protein and up-regulation of Beclin1 and LC3 II proteins. Interestingly, treatment with autophagy flux inhibitor Baf-A1 or 3-MA blocked the inhibitory effects of Gyp in glioma cells. Combined application of Gyp with Baf-A1 or 3-MA slightly increased cell death, suggesting that induction of autophagy impaired enzyme accumulation and contributed to the growth inhibition of glioma cells. In summary, Gyp suppressed the proliferation, migration and invasion and induced apoptosis of glioma cells by promoting autophagy, suggested the potential value of Gyp in the combined treatment of glioma.

1. Introduction

Glioma is the most common intracranial malignant tumor and is characterized by high invasiveness and a low survival rate[1]{Markouli, 2021 #1}. Although recent studies have confirmed the antitumor efficacy and low toxicity of temozolomide (TMZ), chemotherapy can cause drug resistance and may therefore not be the best option for treatment in patients with glioma[2]. Accordingly, further studies on the pathogenesis of glioma and identification of new therapeutic drugs are needed.

The active ingredients of most Chinese medicines have antitumor effects[3-5]. Gypenosides (Gyp), a biologically active ingredient extracted from the natural Chinese medicine *Gynostemma pentaphyllum*, functions to lower blood lipids[6] and exerts hypoglycemic[7], pro-immunity[8], anti-aging[9], and antitumor effects[10]. For example, a previous study showed that Gyp can suppress proliferation and induce apoptosis by inhibiting the platelet-derived growth factor/AKT/p70 S6K signaling pathway in human hepatoma cancer[11]. Additionally, Gyp effectively inhibits the invasion and migration of tongue and oral cancers[12, 13]. However, the underlying mechanism of the anticancer effect of Gyp in gliomas have not been extensively investigated yet. Autophagy is one of many signal mechanisms that regulate tumor regeneration. Although in vivo and in vitro studies are widely published in various journals, there are still few new contents on autophagy regulation[14]. Thence, It is necessary to deeply analyze the mechanism of Gyp in glioma to maximize its therapeutic effect.

In the present study, we investigated the anticancer effect of Gyp on the proliferation, migration, invasion and apoptosis of glioma cells. Next, the autophagy activated by Gyp in glioma cells was also examined. More importantly, we explored whether Gyp-induced the cytotoxicity of glioma cells could be enhanced after combination with autophagy inhibitors.

2. Material And Methods

2.1 Cell lines and drugs

U251 and U87 cells were Purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modification of Eagle's medium (DMEM, Hyclone, USA) containing 10% fetal bovine serum (FBS; BI, Israel). All cells were incubated in complete medium in a constant temperature incubator containing 5% CO_2. Gyp was obtained from the Resource Platform of National Standard Material (http://www.biobw.com/, 20mg, UV≥98%, NO: D108227).

2.2 Cell viability assay

To measure the viability of glioma cells, MTS assays were performed by Promega Kit(Madison, WI, USA) according to the manufacturer's instructions. Briefly, U251 and U87 cells (3000 cells per well) were seeded into 96-well plates at a volume of 100 μL/well and cultured under different treatment conditions. After incubating for the specified time, 10 μL MTS solution was added to 90 μL DMEM per well, and the plates were incubated for 30 min. Subsequently, the absorbance of each well was recorded at 490 nm with a microplate reader (Bio-Rad, USA).

2.3 Clone formation assay

To assess the logarithmic growth of U251 and U87 cells, cells were seeded at 500 cells/well in 6-well plates. After adherence of cells to the plate, cells were left untreated (control group, 0 μg/mL) or treated with 160, 180, or 200 μg/mL Gyp (U251 cells) or 180, 200, or 220 μg/mL Gyp (U87 cells) for 14 days. Next, Using 4% paraformaldehyde and crystal violet staining solution for cell fixation and staining, respectively. Stained colonies were photographed using an HD camera, Then colonies with more than 50 cells counted by Image J.

2.4 Transwell invasion assay

Invasion experiments were performed using a transwell chamber (Millipore, Billerica, USA) containing Matrigel (BD, San Jose, CA, USA). Inoculate Gyp-treated cells into the upper chamber of serum-free medium (1.5×104 cells), and the lower chamber contains 20% FBS medium, After the cells invaded for 48 h, the cells were fixed and stained with crystal violet. The number of invaded cells in 4 randomly selected areas was counted under a light microscope with ×100 magnification.

2.5 Wound healing assay

U87 and U251 cells were cultured in 6-well plates. when the cell density reached approximately 80–90%, a 200 μL plastic pipette tip was used to create an identical wound in the center of the well, and then treated with Gyp at a certain concentration containing 2% FBS DMEM. Cells were photographed by a microscope ×50 magnification at 0 h and 24 h.

2.6 Hoechst 33342 staining

Glioma cells were seeded on coverslips, incubated for 16 h, and treated with Gyp at a certain concentration. After cells incubated for 48 hours, fixed with 4% paraformaldehyde and stained with Hoechst 33342 (Beyotime, Shanghai, China). Then, Apoptotic cells were counted by fluorescence microscope (Leica, Germany).

2.7 Flow cytometry analysis for apoptosis

The Annexin V-FITC/PI Apoptosis Detection kit (Beyotime, Shanghai, China) was used as cell apoptosis. Briefly, U251 and U87 cells were washed with cold PBS for three times and resuspended. Staining was performed according to the manual provided by the reagent supplier, and then Flow cytometry (Beckman Coulter, Atlanta, GA, USA) analysis was carried out immediately for apoptosis.

2.8 Autophagic flux detection

A tandem green fluorescent protein GFP-mRFP-LC3 lentivirus was obtained from JiKai Biotechnology (Gene, Shanghai, China). Starvation of U251 and U87 cells transfected with GFP-mRFP-LC-3 lentivirus for 16h, replaced with complete medium. Cells were then treated with Gyp for 48 h, fixed with 4% paraformaldehyde for 30 min. Finally, the autophagic flux was detected under a fluorescence microscope at 200 ×200 magnification.

2.9 Western blotting

Protein extraction and detection were performed as described by Lv et al[15]. Anti-MMP-9, anti-MMP-2, anti-Bcl-2, anti-cleaved-caspase-9, anti-Bax, anti-Beclin1, anti-p62, and anti-LC3 antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Relative protein expression was analyzed based on the gray value of bands in western blotting, with glyceraldehyde 3-phosphate dehydrogenase as the internal reference. Each assay was repeated three times.

2.10 Statistical analysis

All statistical analysis were analyzed using GraphPad Prism 8.0 software (GraphPad, La Jolla, CA, USA), All datas were presented as means ± SD (standard deviations) from at least three independent experiments. Results with a p value less than 0.05 were considered statistically significant. The difference between the two groups was tested by independent samples, and the difference between multiple groups was analyzed by one-way analysis of variance.

3. Results

3.1 Gyp inhibited the proliferation of human glioma cells

To study the biological effects of Gyp, we first measured the viability of glioma cells following treatment with Gyp using MTS assays. The results showed that Gyp reduced the proliferation of U251 and U87 cells in a concentration- and time-dependent manner (Figure 1A). Furthermore, we explored the effects of Gyp on colony formation after culture of treated cells for 14 days. Compared with the control (0 μg/mL Gyp), treatment with a low concentration of Gyp slightly reduced colony formation, whereas treatment with a high concentration of Gyp significantly decreased colony formation rate (Figure 1B and C). These results confirmed that Gyp exhibited potent antiproliferative effects in U251 and U87 cells.

3.2 Gyp suppressed the invasion and migration of human glioma cells

To further investigate whether the invasion and migration of glioma cells were affected by Gyp, we performed Transwell and wound healing assays. Transwell assays revealed that treatment with Gyp at the half-maximal inhibitory concentration decreased cellular invasion through Matrigel membranes (Figure 2A). Additionally, wound healing assays demonstrated that treatment with Gyp decreased the migration speed of cells (Figure 2B). Moreover, the migration-related proteins MMP-2 and MMP-9 were downregulated by Gyp in U251 and U87 cells (Figure 2C). Taken together, these results suggested that Gyp may inhibit the invasion and migration of glioma cells.

3.3 Gyp promoted apoptosis in U251 and U87 cells

To detect whether Gyp induced apoptosis in glioma cells, we used Hoechst 33342 staining to observe morphological changes in cell nuclei. In untreated glioma cells, the nuclei were weakly uniform blue, whereas in Gyp-treated cells, transparent chromatin condensation and nuclear fragmentation were observed (Figure 3A). Additionally, flow cytometry analysis showed that different concentrations of Gyp increased the number of apoptotic cells (Figure 3B-C). To further explore the effects of Gyp on apoptosis in glioma cells, we verified the changes in apoptosis-related protein expression. As shown in Figure 4A-B, the expression levels of the pro-apoptotic proteins Bax and cleaved-caspase-9 increased dramatically, whereas those of the anti-apoptotic protein Bcl-2 decreased significantly, indicating that Gyp enhanced apoptosis in U251 and U87 cells. These results indicated that Gyp promoted apoptosis in glioma cells in a dose-dependent manner.

3.4 Gyp induced autophagy in human glioma cells

Autophagy is a type of programmed death that can induce tumor cell death, suggesting potential applications in cancer treatment. Previously, we assessed the expression of autophagy-related proteins using western blotting. The results demonstrated that Gyp decreased the expression of p62 protein and increased the LC3-II autophagy flux and enhanced the levels of Beclin1 protein (Figure 4A and 4C). Then, to detect the status of autophagic flux, a tandem mRFP-GFP-LC3 adenovirus was used. Compared with the control group, more red puncta (GFP-mRFP-) and yellow puncta (GFP-mRFP-) were observed following

Gyp treatment of U251 and U87 cells; these changes suggested that autophagy flux was induced (Figure 5A). Next, to investigate whether Gyp-induced LC3-II was related to autophagy inhibition, Baf-A1[16] and 3-MA[17], were used to inhibit autophagic flux. As shown in Figure 5B and C, in U251 and U87 cells, pretreatment with 3-MA resulted in a decrease in LC3-II compared with that in the Gyp group. Furthermore, compared with Gyp alone, the addition of Baf-A1 to inhibit autophagy enhanced the expression of LC3-II. Overall, these results indicated that Gyp stimulated autophagy in U251 and U87 cells in a concentration-dependent manner.

3.5 Treatment with an autophagy inhibitor increased the anticancer effects of Gyp in glioma cells

To investigate whether the anticancer effects of Gyp could be enhanced by autophagy inhibitors, U251 and U87 cells were treated with 180 or 200 μg/mL Gyp, respectively, with or without autophagy inhibitors (150 nM Baf-A1 or 20 mM 3-MA) for 48 h. Cell viability was evaluated using the MTS method, and the apoptosis rate was determined by flow cytometry. Additionally, the expression levels of apoptosis-related proteins were evaluated by western blotting. The data results showed that Gyp inhibited the growth of U251 and U87 cells, and this inhibitory effect was significantly enhanced after the addition of Baf-A1 or 3-MA (Figure 6A). Moreover, flow cytometry analysis revealed that cotreatment of Gyp with Baf-A1 or 3-MA significantly increased the rate of apoptosis in glioma cells, compared with treatment using Gyp alone (Figure 6B). Similar results were observed with regard to the expression of the apoptosis-related protein cleaved-caspase-9 (Figure 6C). These observations indicated that autophagy inhibitors blocked autophagy and significantly enhanced the anticancer effects of Gyp in glioma cells.

4. Discussion

Surgery is the primary method for treating glioma; however, surgical resection is often incomplete owing to the high invasiveness of the cancer, resulting in high recurrence rates[18]. Chemotherapy is another adjuvant treatment for glioma. Although TMZ is available as a first-line chemotherapy drug for glioma, the use of this drug is limited owing to primary or secondary resistance in some patients[19]. Therefore, it is imperative to study the pathogenesis of glioma and identify novel therapeutic drugs and medicines. Gyp is a natural active ingredient extracted from *Gynostemma* and has specific anti-inflammatory and antitumor effects[7, 20]. However, the effects of Gyp on glioma have not been reported.

In this study, we found that different concentrations of Gyp could effectively inhibit the proliferation of U251 and U87 glioma cells. Consistent with these findings, a previous study showed that Gyp suppresses cell growth in various cancers, including lung cancer[21], leukemia[22], colorectal cancer[23], oral cancer[24], and hepatocellular cancers[25], through various molecular pathways. Interestingly, MMP-2 and MMP-9 have been extensively studied in gliomas, and their expression levels have been shown to increase as the degree of malignancy increases. Furthermore, MMP-2 and MMP-9 are predictors of invasive growth in glioma cells[26, 27]. In this study, we showed that cell invasion and migration were

inhibited in glioma cells treated with Gyp, consistent with previous reports of Gyp in oral cancer[13]. Additionally, we found that Gyp degraded the protease activity of MMP-2 and MMP-9 and reduced the effects of these proteins on decomposition of basement membrane components in glioma cells, thereby inhibiting the invasion and migration ability of glioma cells. We also found that apoptosis was obviously increased in a concentration-dependent manner in glioma cells treated with Gyp, and Gyp induced apoptosis in glioma cells by elevating the expression of the pro-apoptotic proteins Bax and cleaved-caspase-9 and downregulating the anti-apoptotic protein Bcl-2. These results were consistent with the results of a previous study showing that baicalin induces apoptosis in liver cancer cells[28]. Thus, our research confirmed that Gyp inhibited the proliferation, invasion, and migration of glioma cells and promoted apoptosis.

The mechanisms through which Gyp inhibits tumor growth in glioma are still unclear. Recent data indicate that autophagy plays a vital role in the occurrence and effective treatment of glioma[29]. In this study, we showed that treatment with Gyp could modulate autophagy in glioma cells. Notably, Gyp increased LC3-II autophagy flux, significantly upregulated Beclin1, and significantly downregulated p62. Furthermore, fluorescence microscopy demonstrated that autophagosome numbers were increased following treatment of glioma cells with Gyp, suggesting that Gyp activated autophagy in glioma cells. Similar results were observed in previous studies of autophagy activation in glioma[30, 31]. In addition, promotion of autophagy can induce tumor cell death, suggesting that autophagy may be a target in cancer treatment[32, 33]. Indeed, in this study, we found that inhibition of autophagy by Baf-A1 or 3-MA blocked Gyp-induced glioma cell proliferation and promoted apoptosis. These results confirmed that inhibition of autophagy enhanced the anticancer effects of Gyp in glioma cells, consistent with previous studies of cervical cancer[34].

There were some limitations to this study. First, we demonstrated the growth inhibitory effects of Gyp in glioma cells through MTS and clone formation assays. Nevertheless, we did not study the specific cell cycle phase or mechanisms inhibited by Gyp. Additionally, although we evaluated autophagy based on the expression of autophagy-related proteins and autophagy flow experiments, these findings should still be verified using electron microscopy. Moreover, the expression of autophagy pathway proteins PI3K and mTOR were texted by Western Blot, but the regulation of those proteins are not be shown. We guess that the effect of gypenoside on glioma autophagy is not dependent. Finally, we only performed in vitro analyses; further studies are needed to confirm our findings using in vivo models.

In summary, we demonstrated that Gyp suppressed the proliferation, invasion, and migration of glioma cells in vitro and promoted apoptosis in glioma cells. In addition, Gyp induced autophagy in glioma cells. More importantly, chemical inhibition of autophagy significantly enhanced the toxicity of Gyp in glioma cells. Therefore, our findings suggested that the combination of autophagy inhibitors and Gyp could be a potentially therapeutic approach for glioma.

Abbreviations

TMZ: temozolomide; Gyp: gypenoside; FBS: fetal bovine serum; PBS: phosphate-buffered saline; PI: propidium iodide; Baf-A1: bafilomycin A1

Declarations

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Disclosure of conflict of interest

The authors declare that they have no conflicts of interest.

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Figures

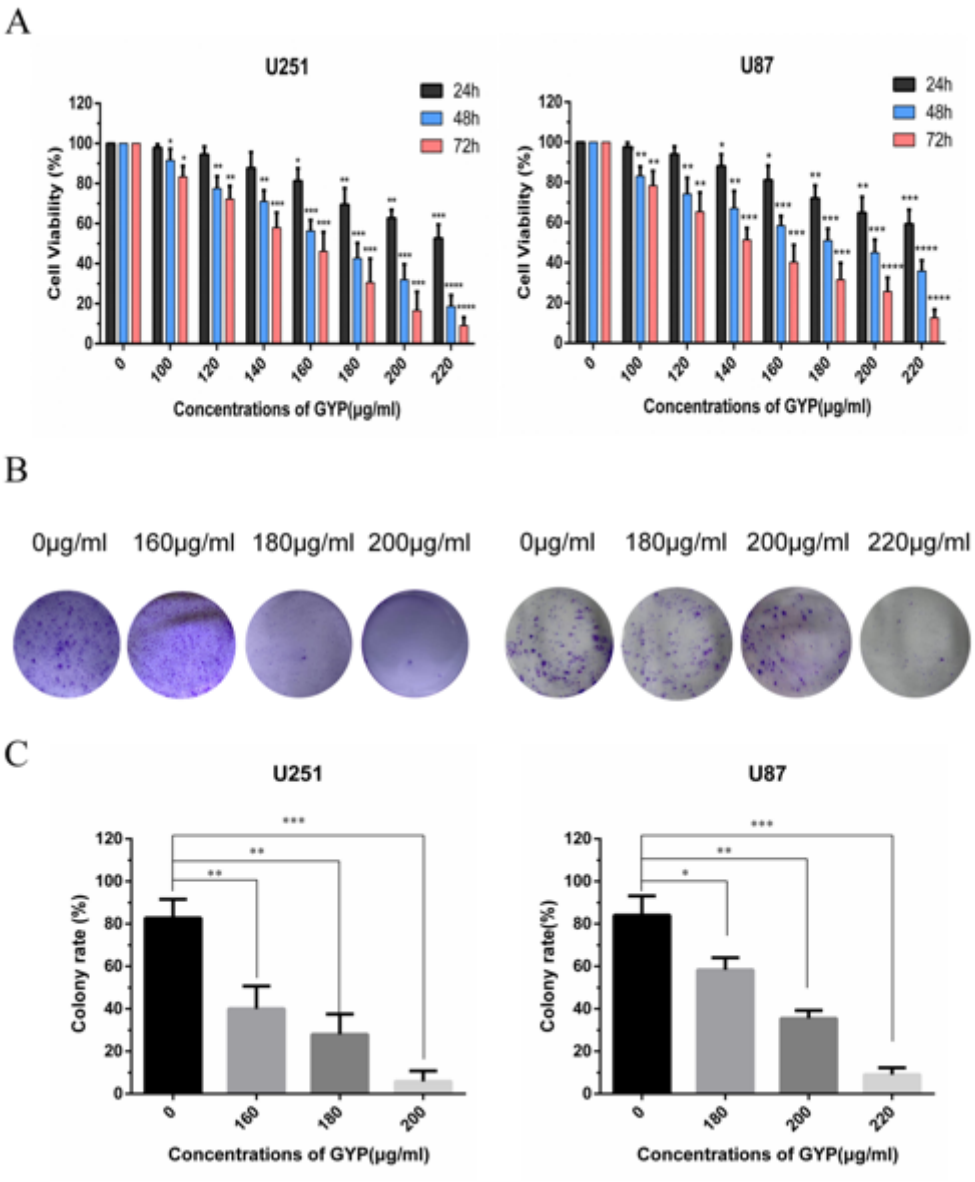

Figure 1

Gyp inhibited the proliferation of glioma cells.

(A) Viability of U251 and U87 cells treated with Gyp at the indicated concentrations, as determined by MTS assays at 24, 48, and 72 h. (B) Determination of colony formation after U251 and U87 cells are treated with a indicated concentration of Gyp. (C) Quantitative analysis of Cloning rates, as determined by GraphPad software. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

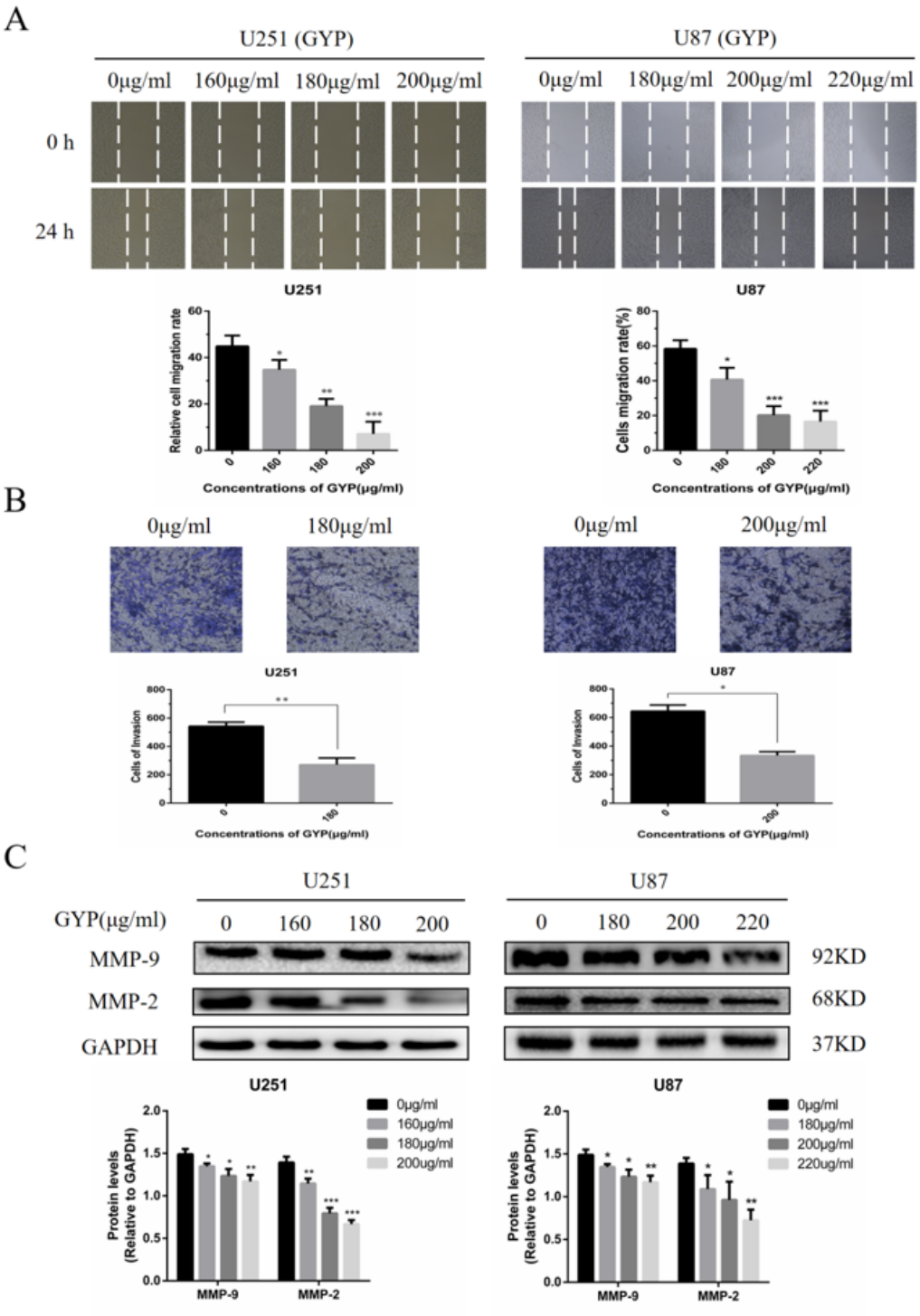

Figure 2

Gyp suppressed the invasion and migration of human glioma cells.

(A) Effects of Gyp on cell migration, as analyzed by measuring the cell migration distance in three areas of each scratch. (B) Effects of Gyp on the invasion of glioma cells. (C) Western blot analysis of MMP-2

and MMP-9 expression following treatment of U251 and U87 cells with Gyp at the Specified concentrations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

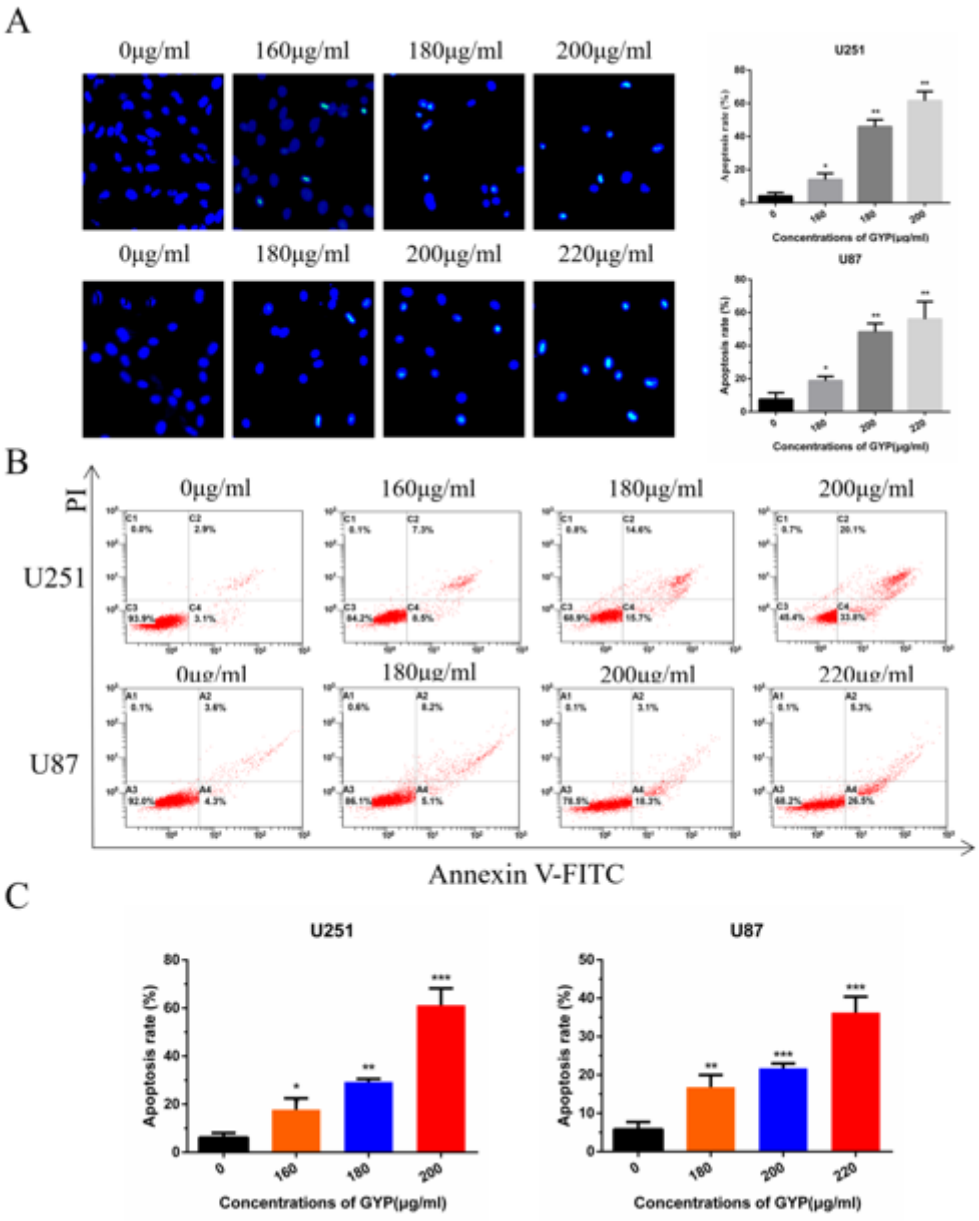

Figure 3

Gyp promoted apoptosis in U251 and U87 cells.

(A) Effects of Gyp on U251 and U87 cell apoptotic bodies, as observed by fluorescence microscopy with Hoechst 33342 staining (10×). (B) Flow cytometry was used to detect the rates of apoptotic cells following treatment with different concentrations of Gyp. (C) Quantitative analysis of apoptosis rates, as determined by flow cytometry. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

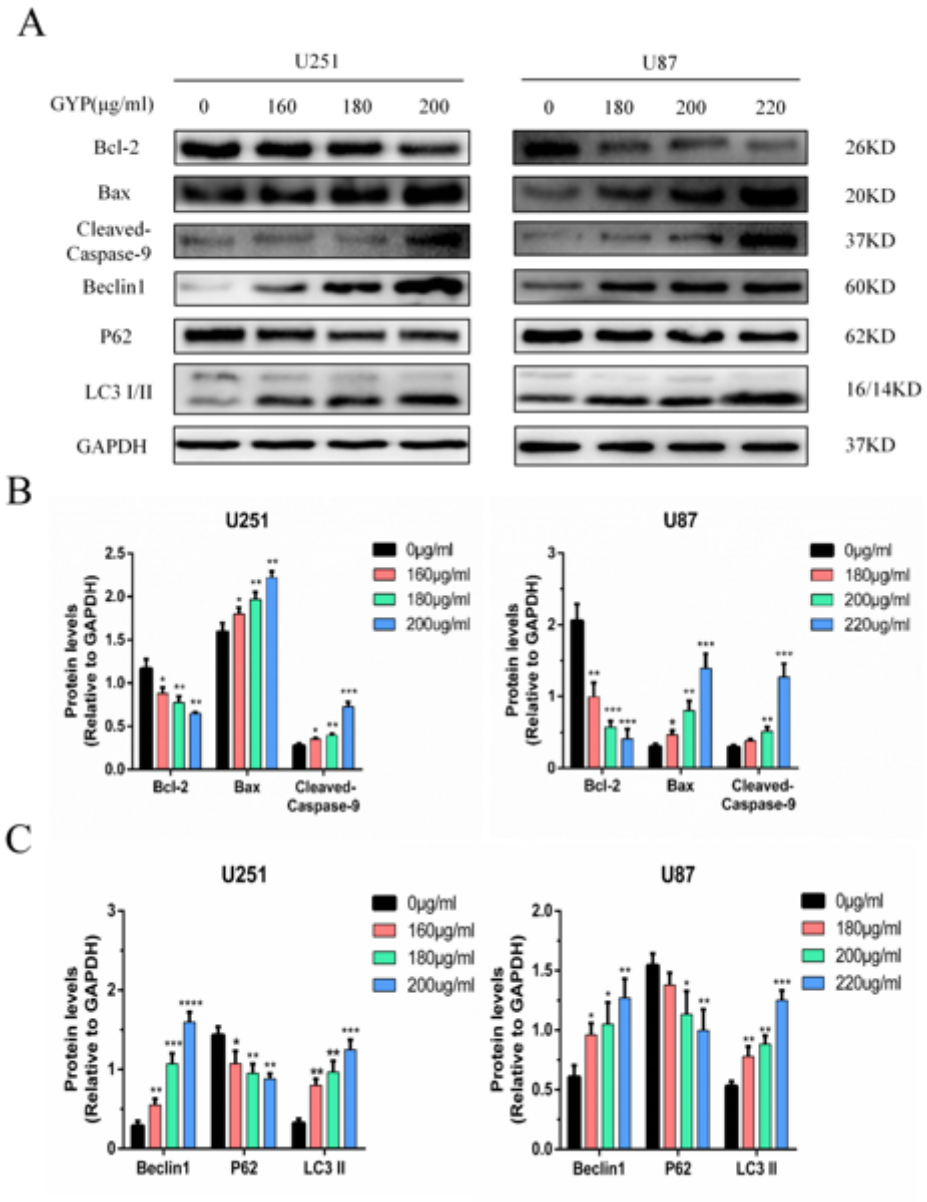

Figure 4

Gyp affected the expression of apoptosis- and autophagy-related proteins in glioma cells.

(A) Glioma cells were treated with Gyp at the specified concentration for 48 h, and protein levels of Bax, Bcl-2, cleave-caspase-9, Beclin1, p62, and LC3-II were assessed by western blotting. (B) Quantitative analysis of apoptosis-related protein expression. (C) Quantitative analysis of autophagy-related protein expression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

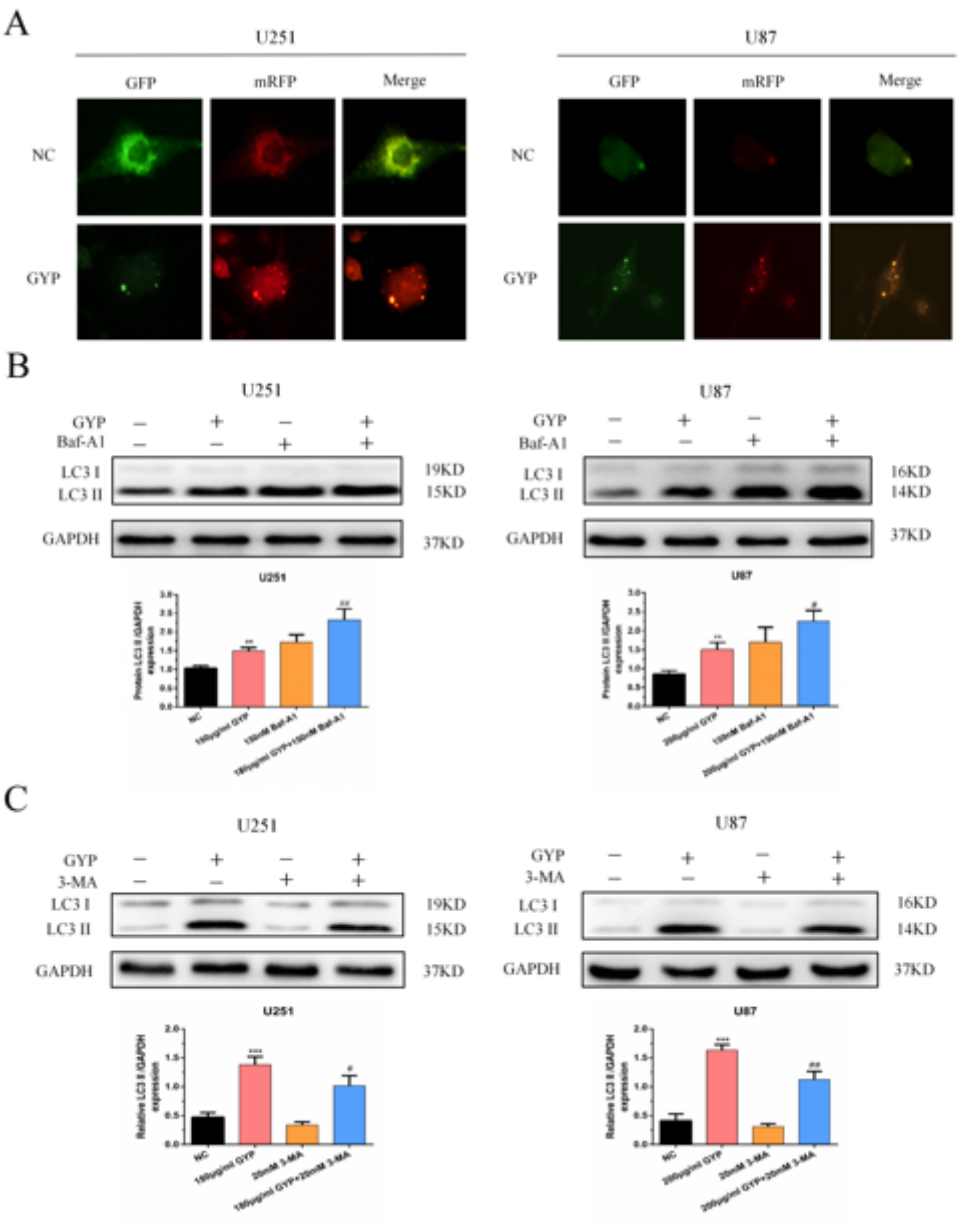

Figure 5

Gyp induced autophagy in human glioma cells.

(A) Representative images of GFP-mRFP-LC3-transfected U251 cells treated with 180 μg/mL Gyp and U87 cells treated with 200 μg/mL Gyp for 48 h under a fluorescence microscope (40×). (B) Protein expression of LC3-II in glioma cells treated with Gyp plus Baf-A1 for 48 h. (C) Protein expression of LC3-II in glioma cells treated with Gyp plus 3-MA for 48 h. Data are presented as means ± standard deviations. *$p < 0.05$ compared with the control group; #$p < 0.05$ compared with the Gyp group. Each set of experiments was carried out three times.

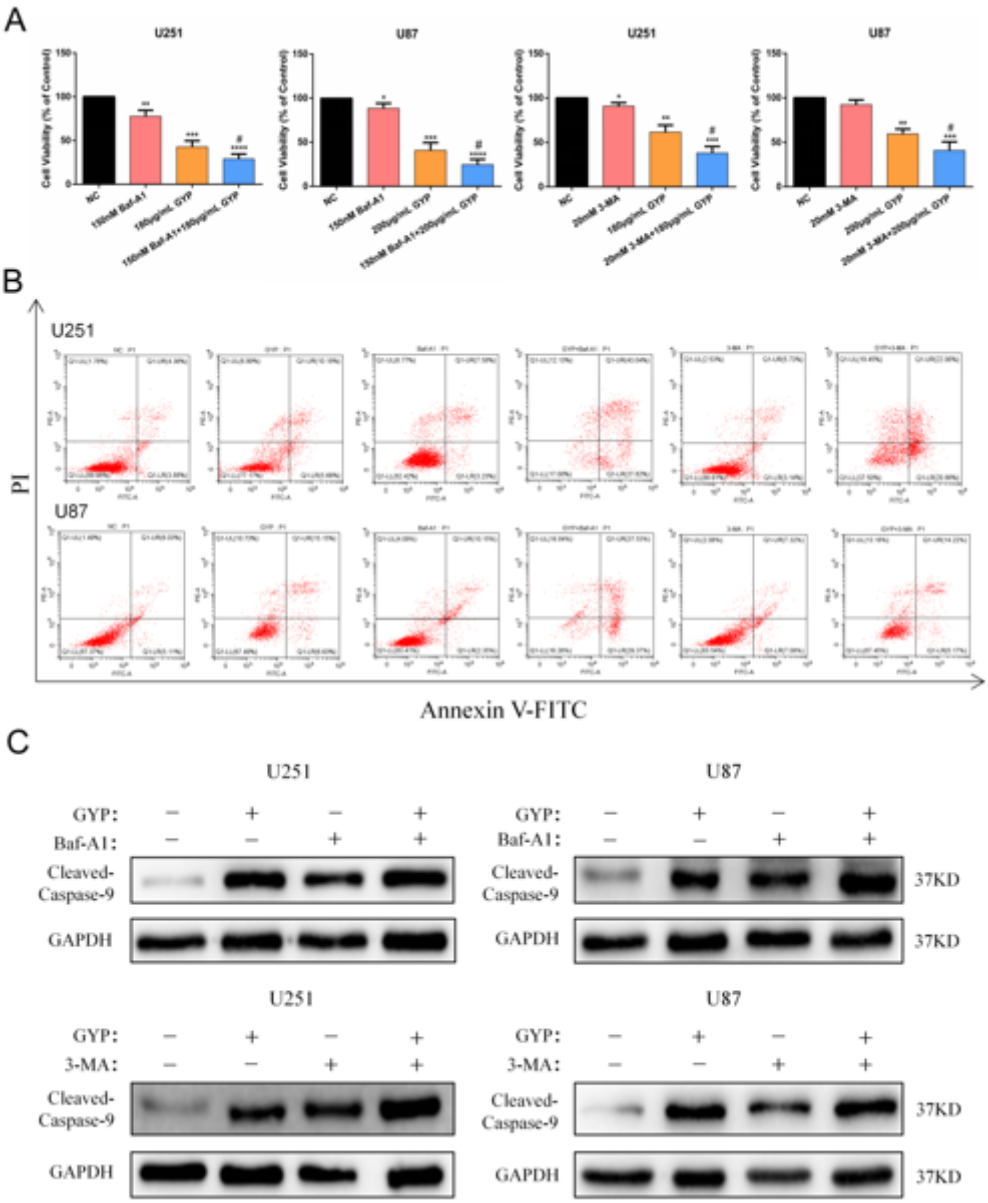

Figure 6

The autophagy inhibitors Baf-A1 and 3-MA increased the anticancer effects of Gyp in glioma cells in vitro.

(A–C) U251 cells were treated with 150 nM Baf-A1, 20 mM 3-MA, 180 μg/mL Gyp, or 20 mM 3-MA plus 180 μg/mL Gyp for 48 h, and U87 cells were treated with 150 nM Baf-A1, 20 mM 3-MA, 200 μg/mL Gyp, or 20 mM 3-MA plus 200 μg/mL Gyp for 48 h. Cell viability was measured by MTS assays, and flow cytometry was used to detect the rates of apoptosis in glioma cells. Western blotting was used to detect the expression levels of cleaved-caspase-9. Data are expressed as means ± standard deviations. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group; #p < 0.05 compared with the Gyp group.

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