

RESEARCH ARTICLE

Autophagy Inhibition Promotes Gambogic Acid-induced Suppression of Growth and Apoptosis in Glioblastoma Cells

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Abstract

Objective: To investigate the effects of gambogic acid (GA) on the growth of human malignant glioma cells. **Methods:** U251MG and U87MG human glioma cell lines were treated with GA and growth and proliferation were investigated by MTT and colony formation assays. Cell apoptosis was analyzed by annexin V FITC/PI flow cytometry, mitochondrial membrane potential assays and DAPI nuclear staining. Monodansylcadaverine (MDC) staining and GFP-LC3 localisation were used to detect autophagy. Western blotting was used to investigate the molecular changes that occurred in the course of GA treatment. **Results:** GA treatment significantly suppressed cell proliferation and colony formation, induced apoptosis in U251 and U87MG glioblastoma cells in a time- and dose-dependent manner. GA treatment also led to the accumulation of monodansylcadaverine (MDC) in autophagic vacuoles, upregulated expressions of Atg5, Beclin 1 and LC3-II, and the increase of punctate fluorescent signals in glioblastoma cells pre-transfected with GFP-tagged LC3 plasmid. After the combination treatment of autophagy inhibitors and GA, GA mediated growth inhibition and apoptotic cell death was further potentiated. **Conclusion:** Our results suggested that autophagic responses play roles as a self-protective mechanism in GA-treated glioblastoma cells, and autophagy inhibition could be a novel adjunctive strategy for enhancing chemotherapeutic effect of GA as an anti-malignant glioma agent.

Keywords: Gambogic acid - glioblastoma multiforme - apoptosis - autophagy

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Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive malignant central nervous system tumor in adults (Kleihues et al., 2002). Despite multimodality treatment consisting of surgical resection, followed by radiotherapy and chemotherapy, the prognosis of GBM is poor, with a median survival time of approximately only 1 year (Astner et al., 2006; Tuna-Malak and Diramali, 2008; Chen et al., 2010; Van Meir et al., 2010). Therefore, it is very important and urgent to develop effective chemotherapeutic agents for the treatment of malignant gliomas.

Gamboge, a dry resin secreted from *Garcinia hanburyi* tree, is a traditional medicine for treating diarrhea and dysentery (Chen et al., 2012). Recently, lots of studies showed that Gambogic acid (GA, C₃₈H₄₄O₈), a major active ingredient of gamboge, has potent anti-tumor effects against many different types of cancer in vivo and in vitro including lung carcinoma, hepatocarcinoma, gastric carcinoma and so on (Wu et al., 2004; Zhao et al.,

2004). An increasing number of studies tried to elucidating the molecular mechanisms of GA anti-tumor effects, and several critical signaling steps in carcinogenesis have been reported to be influenced by GA treatment (Wang and Chen, 2012). For example, GA arrested cell cycle at G₂/M phase via disturbing CDK7 mediated phosphorylation of CDC2/P34 (Yu et al., 2007); GA could interact with transferrin receptor and induce rapid apoptosis of tumor cells (Pandey et al., 2007); GA inhibited cell proliferation not only by repressing hTERT transcriptional activity but also by posttranslational modification of hTERT (Zhao et al., 2008); GA can also inhibit tumor growth by repressing angiogenesis (Lu et al., 2007). In a word, GA treatment can inhibit proliferation and induce apoptosis of many kinds of tumors via a multi-mechanism manner.

However, the anti-tumor effects and possible mechanisms of GA against human GBM have not yet been investigated. In present study, we demonstrated that GA can reduce cellular viability and induce apoptosis of GBM cells. However, we also found that GA induced autophagy in GBM cells. Numerous studies showed

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that autophagy, an evolutionary ancient process that is essential for cellular homeostasis and cell viability, can induce cell death or play a cytoprotective role in tumor cells (Maiuri et al., 2007). In most circumstances such as nutrient starvation, autophagy is induced to provide nutrients and energy for cell survival. Thus autophagy is considered as a cytoprotective process (Levine et al., 2005). While autophagy has also been linked to cell death pathways, which is called II programmed cell death or autophagic cell death (Maiuri et al., 2007). The induction of these two different types of autophagy may depend on different stimuli and cell types. Although the exact mechanisms of the two autophagy are not clear, it seems that the suppression of cytoprotective autophagy and the enhancement of cytotoxic autophagy might augment cytotoxic effects of anticancer drugs (Kanzawa et al., 2004; Takeuchi et al., 2005; Shingu et al., 2009; Pandey and Chandravati, 2012). In this study, we found that GA induced an apparent autophagy in GBM cells, and inhibition of autophagy could lead to enhancement of GA-mediated cytotoxicity through increasing apoptosis.

Materials and Methods

Reagents

Human GBM cell lines U251 and U87MG, obtained from Wuhan University, China, were routinely maintained in DMEM with 10% fetal calf serum (FCS). Gambogic acid was purchased from Calbiochem (San Diego, CA, USA). GA was dissolved in DMSO at a concentration of 10 mM as a stock solution, and stored at -20 °C and diluted with medium before each experiment. The final DMSO concentration in medium did not exceed 0.1% (control groups were always treated with 0.1% of DMSO). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) was purchased from Roche (Roche Ltd., Nutley, NJ). PGFPc1-LC3 plasmid was a gift from Marja Jäättelä (Høyer-Hansen et al., 2007). Lipofectamine 2000 and Annexin V/PI Apoptosis Detection kit were purchased from invitrogen (Grand Island, NY, USA). JC-1 Mitochondrial Membrane Potential Assay Kit was purchased from Beyotime (Nanjing, China). 4',6-Diamidino-2-phenylindole (DAPI) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bafilomycin A1 and 3-methyl adenine were purchased from Sigma (St. Louis, MO, USA). Monodansylcadaverine (MDC) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). ECL detection kit was purchased for pierce (Pierce, Rockford, IL, USA). Antibodies were obtained or purchased from the following sources/suppliers: anti-actin antibody from Beyotime; caspase 3 antibodies from Abcam (Cambridge, MA, USA); anti-LC3, Atg 5 and Beclin 1 antibodies from Cell Signaling Technology (Beverly, MA, USA).

MTT assay

Cell viability was determined by MTT assay. Briefly, 1×10^4 U251 or U87MG cells were seeded in triplicate onto a 96-well plate and incubated for 24 h. After treatment with different concentrations of GA in the presence or absence of autophagy inhibitors, 3-MA (5 mM/L) or

bafilomycin A1 (10 nM/L), 20 μ L of MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. The medium was removed carefully and 100 μ L of DMSO was added followed by continuous shaking for 15 minutes. OD values were measured at 570 nm. Three separate experiments were performed and Cell viability was calculated according to the following formula: inhibitory rate (%) = $[1 - (\text{OD of the experimental samples} / \text{OD of the control})] \times 100\%$.

Colony formation assay

1×10^4 U251 or U87MG cells were seeded in triplicate into each well of a 6-well plate and grown in 2.5% bovine serum albumin (BSA) of DMEM medium with different concentrations of GA. The medium was replaced every two days. The cell colonies were stained and photographed on day 10 following 0.05% crystal violet staining.

DAPI staining

Morphological changes in the nucleus were determined by DAPI staining as described previously (Xi et al., 2010). Briefly, U251 and U87MG cells were treated with desired concentrations of GA for 24 h. After washing the cells with PBS, Cells were fixed for 5 min with methanol and stained with DAPI-methanol solution (1 g/mL DAPI in methanol) for 15 min at 37°C. Morphological changes of nuclei were observed and imaged using fluorescence microscope. Cells with round nuclear morphology were considered normal. Cells with Condensed/fragmented and Bright nuclei were regarded as apoptotic cells.

Annexin V-FITC/PI apoptosis assay

Apoptotic cells were analyzed using annexin V-FITC/PI double staining methods. Briefly, U251 or U87MG cells were treated with desired concentrations of GA in presence or absence of 3-MA (5 mM/L) or bafilomycin A1 (10 nM/L) for 24 h. Cells were digested into single-cell suspension with EDTA-free trypsin and stained according to the manufacturer's instruction of Annexin V/PI Apoptosis Detection kit. As soon as possible, stained cells were analyzed by flow cytometry. Early apoptotic cells show only green fluorescence and late apoptotic or necrotic cells show both green and red fluorescence.

JC-1 mitochondrial membrane potential assay

Changes in mitochondrial membrane potential were monitored by the JC-1 dye. U251 and U87MG cells were treated with different concentrations of GA for 24 h. JC-1 labeling was performed according to the manual of JC-1 mitochondrial membrane potential assay kit. Then, the cells labeled with JC-1 were detected with confocal microscopy. JC-1 can selectively enter the mitochondria and form red fluorescent "J-aggregates" in normal mitochondria with high membrane potential, whereas in mitochondria with low membrane it remains in the monomeric form in the cytosol and emits a green fluorescence.

Immunoblotting assay

After treatment with different concentrations of GA in presence or absence of 3-MA (5 mM/L) or bafilomycin

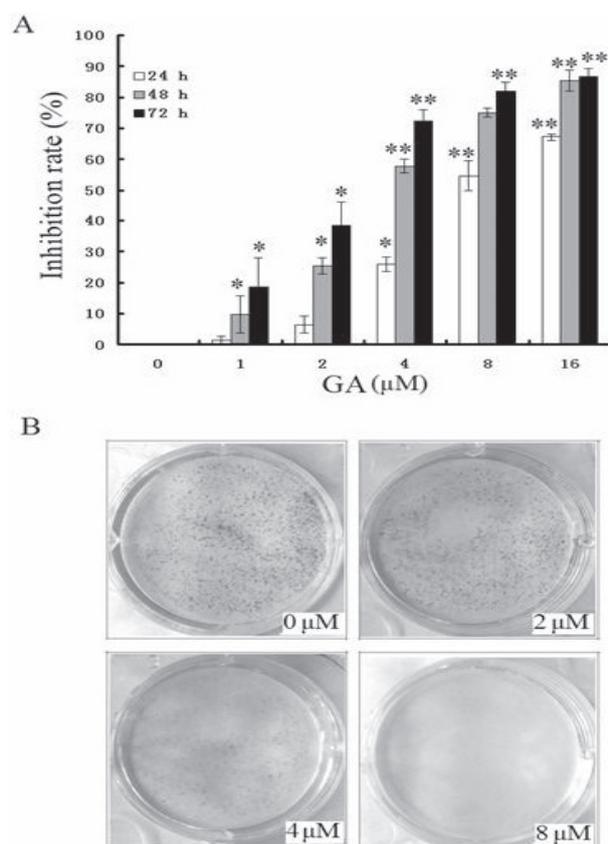


Figure 1. Effects of GA Treatment on the Growth and Proliferation of Glioblastoma Cells. (A) The viability of U251 cells was assessed by the MTT assay. The values were expressed as mean inhibitory rate \pm SD, $n = 3$ (*means $P < 0.05$ and **means $P < 0.01$). (B) Cell colony formation assay. U251 cells were treated with different concentrations of GA and the resulting colonies were visualized by crystal violet staining

A1 (10 nM/L) for 24 h, cells were lysed with lysis buffer (50 mM/L Tris-HCl, 2% w/v SDS, 10% glycerol, 10 mM/L dithiothreitol, pH 6.8), supplemented with protease inhibitor mix (Thermo Fisher). Equal amounts of the protein samples were electrophoresed by SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked with in a 5% non-fat milk-TBST solution for 1 h at room temperature and then incubated with desired primary antibodies against caspase 3, LC3, Atg 5, Beclin 1 and β -actin at 4°C overnight. After washing with TBST, Membranes were incubated with appropriate secondary antibodies for 2 h at room temperature and detected with ECL detection kit.

Labeling of autophagic vacuoles with monodansylcadaverine (MDC)

MDC is also a specific *in vivo* marker for autophagic vacuoles as the same as LC3. To further confirm that GA treatment can induce autophagy, U251 and U87MG cells were treated with different concentrations of GA for 12 h. The autophagic vacuoles were labeled with MDC by incubating with 0.05 mM/L MDC in PBS at 37°C for 2 h. Then, cells were washed three times with cold PBS buffer and immediately observed using confocal microscopy.

Autophagy detection by GFP-LC3 expression

Exponential U251 or U87MG cells in 6-well plates

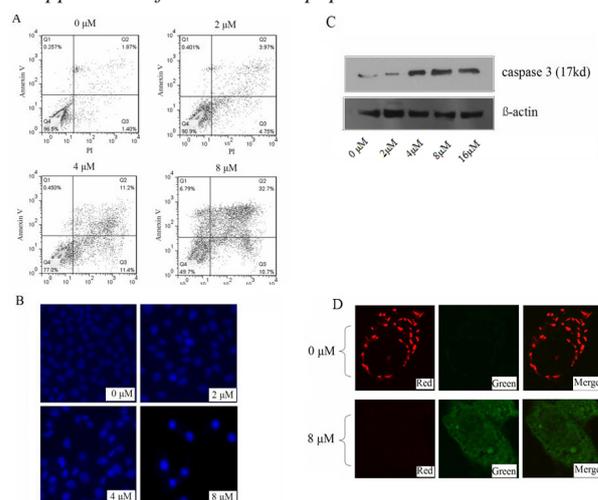


Figure 2. GA Induces Cell Apoptosis of Glioblastoma Cells. (A) U251 cells were treated with different concentrations of GA and apoptotic cells were analyzed by annexin V-FITC/PI double staining. (B) U251 cells were treated with different concentrations of GA for 24h and nuclei were stained by DAPI. (C) U251 cells were treated with different concentrations of GA for 24h, and the expression levels of indicated proteins were detected by western blotting. (D) U251 cells were treated with indicated concentrations of GA and mitochondrial membrane potential was monitored by the JC-1 dye. Red fluorescence represents the “J-aggregates” form and green represents the monomeric form of JC-1

were transfected with pGFPc1-LC3 plasmid using the Lipofectamine 2000. After 24 h transfection, cells were treated with different concentrations of GA in presence or absence of autophagy inhibitors for 12 h. The patterns of GFP-LC3 in transfected cells were examined and imaged by confocal microscopy.

Statistical analysis

Data from three independent experiments were expressed as mean \pm SD. Student's t test was used to assess variables in this study. $P < 0.05$ was considered a significant difference.

Results

GA inhibited proliferation and induced apoptosis of GBM cells

MTT cell proliferation assay showed that GA treatment significantly inhibited proliferation of U251 cells in a dose- and time-dependent manner (Figure 1A). Figure 1B showed that GA also significantly suppressed the colony formation ability of U251 in a dose-dependent manner. In addition, the same results were obtained in U87MG cells (data not shown).

In order to determine whether the cell growth inhibition caused by GA treatment was accompanied with apoptosis, apoptosis parameters were analyzed by annexin V-FITC/PI flow cytometry, DAPI staining and Western-blot assay. As shown in Figure 2A, GA caused significant increases in apoptotic cell number with Annexin V-FITC staining in a time- and dose-dependent manner. Consistent with flow cytometry analysis, DAPI staining also showed that the number of apoptotic cells with condensed and fragmented

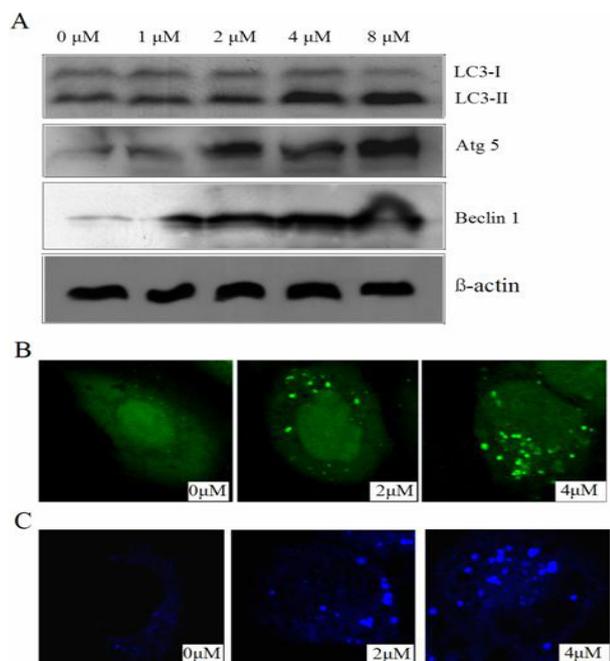


Figure 3. GA Treatment Induces Autophagy in Glioblastoma cells. (A) U251 cells were treated with indicated concentrations of GA for 24h and the expressions of autophagy-related proteins were detected by western blotting. (B) Confocal microscopy for analysis of punctate pattern of LC3 localization in U251 cells transiently transfected with the GFP-LC3-vector and treated with vehicle (control) or GA for 12 h. (C) The autophagic vacuoles were observed with MDC

DNA was significantly increased following GA treatment (Figure 2B). Caspase 3, a member of the interleukin-1 beta-converting enzyme family, is thought to be associated with apoptosis induction. The inactive 32 kD caspase 3 is processed during apoptosis generating an activated 17 kD fragment, which can induced apoptosis. Figure 2C showed that the p17 fragment of the active caspase 3 in U251 cells was significantly increased in a dose-dependent manner upon GA treatment.

JC-1 staining can be used to distinguish between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. As show in figure 2D, in control cells, JC-1 accumulates and aggregates in the mitochondria, giving off a bright red fluorescence, whereas in the GA-treated cells, JC-1 remains in the cytoplasm in its monomer form, fluorescing green, which suggested that GA treatment altered mitochondrial transmembrane potential and caused apoptosis of U251 cells.

As a conclusion, our results suggested that GA treatment significantly suppressed cell proliferation and colony formation, induced apoptosis in GBM cells in a time- and dose-dependent manner, which suggested GA might be a promising chemotherapeutic agent for GBM.

GA triggers autophagy in GBM cells

Interesting, a number of blebbing or large vacuoles in GA-treated cells were observed by light microscopy in this study, which suggested that GA might activated an autophagic response. Microtubule-associated protein 1 light chain 3 (LC3) is a reliable marker of autophagosomes. LC3 exists two forms in cells, LC3-I and LC3-II.

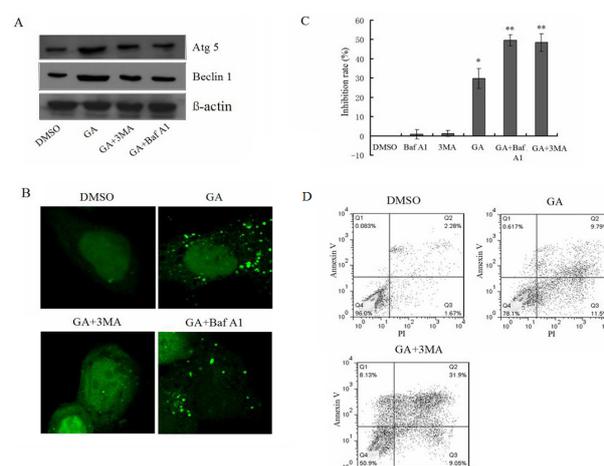


Figure 4. Autophagy Inhibitors Enhance the Cytotoxicity Induced by GA in Glioblastoma Cells. (A) Western blot analyses of Beclin 1 and Atg 5 expressions in cells treated with 4 μ M GA in the absence or presence of 3-MA or bafilomycin A1 (pretreated for 2 h). (B) Confocal microscopy for analysis of punctate pattern of LC3 localization in U251 cells treated with 4 μ M GA for 12 h in the absence or presence of 3-MA or bafilomycin A1 (pretreated for 2 h). (C) Cell viability was determined by MTT assay after treatment with 4 μ M of GA for 24 h in the absence or presence of 3-MA or bafilomycin A1 (pretreated for 2 h). (D) U251 cells were treated with 4 μ M of GA for 24 h in the absence or presence of 3-MA (pretreated for 2 h) and apoptosis was determined by flow cytometry for Annexin V-FITC and PI double labeling

LC3-I is a diffuse cytosolic form. During autophagy, LC3-I is processed to LC3-II that is recruited to the autophagosomes and shows a punctate staining pattern by confocal microscopy. To determine whether treatment of cells with GA results in induction of autophagy in GBM cells, we investigated the change in the expression levels of LC3-II, a specific marker of autophagosomes, in GA-treated U251 and U87MG cells (Kabeya et al., 2000). As shown in Figure 3A, the expression levels of LC3-II were significantly increased in U251 cells treated with GA in a dose-dependent manner. Noteworthy, this concentration range of GA also induced apoptosis in U251 cells. In addition, Atg 5 and Beclin 1 are also required for autophagosome-formation (Kihara et al., 2001; Bommareddy et al., 2009). Therefore, the expression levels of these two proteins were investigated in GA-treated cells. The expression levels of ATG5 and Beclin 1 proteins in the cells exposed to GA were also increased in a dose-dependent manner (Figure 3A). Furthermore, we investigated the pattern and localization of LC3 in U251 (Figure 3B) and U87MG (data not shown) cells by confocal laser microscopy. The cells with only 0.1% DMSO treatment exhibited diffuse and weak green fluorescence, whereas, the cells exposed to GA exhibited a characterized punctate pattern of LC3-II.

To further confirm that GA could indeed induce autophagosomes, autophagic vacuoles formation was investigated by MDC labeling. As shown in Figure 3C, the result showed that the formation of autophagic vacuoles was significantly increased in U251 cells with GA treatment in a dose-dependent manner, whereas only few of vesicles were observed in 0.1% DMSO-treated cells.

Taken together, these data indicated that GA was able to induce autophagosome formation in U251 and U87MG cells.

Inhibition of autophagy enhances the growth inhibition and pro-apoptotic effect of GA

As mention above, autophagy can be divided into two types: protective autophagy and autophagic cell death. To evaluate the roles of autophagy in GA-induced apoptosis, we investigated the effects of autophagy specific inhibitors (3-MA and bafilomycin A1) on GA-induced apoptosis. 3-MA exerts its autophagy-inhibiting effect before the formation of autophagosome by inhibiting III PI3K activity (Tassa et al., 2003). Bafilomycin A1 blocks the fusion of autophagosomes with lysosomes by inhibiting vacuolar H⁺ ATPase at a late step of autophagosome formation (Yamamoto et al., 1998). After pretreatment with autophagy inhibitor 3-MA (5 mmol/L) and bafilomycin A1 (10 nmol/L) for 2 h, U251 and U87MG cells were treated with 4 μM of GA and 3-MA or bafilomycin A1 for an additional 24 h. As shown in Figure 4A and 4B, 3-MA and bafilomycin A1 effectively inhibited the GA-induced upregulated expressions of Beclin 1 and Atg 5 and the accumulation of GFP-LC3-II in autophagic vacuoles. Consequently, MTT assay showed that 3-MA pretreatment markedly enhanced the growth inhibition induced by GA treatment (Figure 4C). The number of apoptotic cells was also markedly increased in the cells pretreatment with 3-MA (Figure 4D). Consistent with the results from 3-MA, pretreatment of bafilomycin A1 also markedly increased GA-induced cell growth inhibition (Figure 4C). Collectively, these results suggest that inhibition of autophagy sensitized the U251 cells to GA-induced growth inhibition and apoptosis.

Discussion

The anticancer effects of Gambogic acid have been widely investigated in several types of cancer, including lung carcinoma, hepatocarcinoma, gastric carcinoma and so on (Wu et al., 2004; Zhao et al., 2004). GBM is the most common and aggressive primary brain cancer (Kleihues et al., 2002). GBM patients generally have a poor prognosis with a median survival time of 10-12 months (Astner et al., 2006; Van Meir et al., 2010; Chen et al., 2011). In the present research, we evaluated the anti-GBM effect of GA. Our results showed that GA efficiently inhibited the proliferation and induced apoptosis of U251 and U87MG cells in a time- and dose-dependent manner.

Several important anticancer agents, including arsenic trioxide, 5-FU, tamoxifen, imatinib, paclitaxel and cisplatin, has been shown to induce autophagy in a few types of cancer cells (Bursch et al., 1996; Paglin et al., 2001; Carew et al., 2007; Shingu et al., 2009; Goussetis et al., 2010; Liu et al., 2011; Xi et al., 2011; Yang et al., 2011). In the present study, we have demonstrated that autophagy is also induced in glioblastoma cells during the course of GA treatment. GA treatment increased the accumulation of MDC in autophagic vacuoles, converted LC3-I into LC3-II and unregulated the expressions of several important autophagosome-regulatory genes such

as Atg5 and beclin 1 in GBM cells.

Prior studies showed conflicting views of the role of autophagy in cancer chemotherapy. According these results, the autophagy induced by anti-cancer agents was mainly divided into two types: one is the protective autophagy that serve as a protective mechanism against apoptosis; the other is called autophagic cell death that can result in cell death. There is emerging evidence that protective autophagy plays an important role in cellular resistance to chemotherapy (Herman-Antosiewicz et al., 2006; Li et al., 2010). In our study, we investigated the contributions of autophagy to the survival of GBM cells during GA-induced apoptotic cell death. As expected, we found that, pretreatment of autophagy inhibitors (3-MA and bafilomycin A1) in GBM cells efficiently inhibited GA-induced autophagy and improved GA-induced cell growth inhibition and apoptotic cell death. These findings suggest that GA-induced autophagy might provide a self-defense mechanism for GBM cell survival, and inhibition of autophagy could improve the therapeutic effect of GA in the treatment of malignant glioma.

In conclusion, our data showed that GA could significantly inhibit the growth and proliferation, induce the apoptotic cell death along with the induction of autophagy in GBM cells, and inhibition of autophagy could enhance the growth inhibition and proapoptotic ability of GA. Our findings indicated that appropriate modulation of autophagy is necessary for sensitizing tumor cells to anti-GBM therapy of GA.

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