

## Research Article

# Ivermectin induces autophagy-mediated cell death through the AKT/mTOR signaling pathway in glioma cells

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Glioma is one of the most common types of primary brain tumors. Ivermectin (IVM), a broad-spectrum antiparasitic drug, has been identified as a novel anticancer agent due to its inhibitory effects on the proliferation of glioma cells *in vitro* and *in vivo*. However, the ability of IVM to induce autophagy and its role in glioma cell death remains unclear. The main objective of the present study was to explore autophagy induced by IVM in glioma U251 and C6 cells, and the deep underlying molecular mechanisms. In addition, we examined the effects of autophagy on apoptosis in glioma cells. In the present study, transmission electron microscopy (TEM), immunofluorescence, Western blot and immunohistochemistry were used to evaluate autophagy activated by IVM. Cell viability was measured by 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assay. The apoptosis rate was detected by flow cytometry and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Meanwhile, autophagy inhibition was achieved by using chloroquine (CQ). U251-derived xenografts were established for examination of IVM-induced autophagy on glioma *in vivo*. Taken together, the results of the present study showed that autophagy induced by IVM has a protective effect on cell apoptosis *in vitro* and *in vivo*. Mechanistically, IVM induced autophagy through AKT/mTOR signaling and induced energy impairment. Our findings show that IVM is a promising anticancer agent and may be a potential effective treatment for glioma cancers.

## Introduction

Gliomas are the most common and lethal intracranial brain malignancies, and include astrocytomas, oligodendrogliomas and ependymomas [1,2]. These cancers eventually cause a widespread invasion throughout the brain and are resistant to traditional drugs and targeted therapeutic approaches. The current standard of medical treatment consists of surgical resection to the extent feasible, followed by adjuvant radiotherapy and concomitant temozolomide (TMZ) with maintenance chemotherapy. Current treatment modalities have only modest effect on patient outcomes [3,4]. Therefore, it is urgent to explore more effective agents for glioma cancer patients in order to improve clinical outcome and quality of life.

Our previous study demonstrated that Ivermectin (IVM) is able to inhibit the viability of glioma cells *in vitro* and *in vivo* by inducing apoptosis and cell cycle arrest. We propose that IVM might be a potent and promising agent to combat glioma [5]. IVM is a derivative of avermectin and is effective in killing filariae. IVM proved useful against many parasite species via the blockade of parasite ligand-gated chloride channels [6]. The potential value of IVM in human medicine was not overlooked [7]. IVM has found a place in the clinical treatment of several other human diseases, including scabies, and has played an important role

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in the campaign to control lymphatic filariasis in humans [8]. In the recent years, IVM has been identified as a promising anticancer agent for tumor cells [9]. Previous publications have demonstrated that IVM induced cytostatic autophagy by blocking the PAK1/AKT axis in breast cancer [10]. IVM inhibited angiogenesis, growth and survival of glioblastoma [11]. Meanwhile, IVM induced cell cycle arrest and apoptosis of HeLa cells [12]. These studies suggested that IVM could be a potential new agent for cancers.

The major hallmark of cancer is the evasion of apoptosis, which shows that defective apoptosis contributes to both tumorigenesis and chemoresistance [13]. Conventional anticancer therapies primarily trigger apoptosis to promote cancer cell death [14]. Programmed cell death (PCD) describes the use of different pathways by cells for active self-destruction as reflected by different morphology: condensation prominent, type I or apoptosis; autophagy prominent, type II etc [15]. However, accumulating evidence suggested that apoptosis and autophagy could coexist in different chemotherapy drugs to induce cancer cell death [16,17]. It is also known that apoptosis and autophagy may be triggered by general upstream signaling to affect tumor cell development and therapy [18,19]. Meanwhile, apoptosis and autophagy could activate or inhibit each other, as they have many common players such as Atg5, Bcl-2 [20,21]. Since the discovery of yeast Atg-related proteins, autophagosome formation has been dissected at the molecular level. Light chain 3 (LC3) and P62 are main proteins that are extensively used for the study of autophagy [22,23]. LC3 is a key protein involved in initiating autophagy. The occurrence of autophagy was indicated by stimulating the accumulation of microtubule-associated protein 1A/1B-LC3 and increase in the LC3-II/LC3-I ratio [24,25]. P62, a well-known autophagic substrate, is incorporated in completed autophagosomes and degraded in autolysosomes [23]. Recently, AKT/mTOR pathway has been identified to play a crucial role in the progress of human cancers [26]. In malignancies, activity of the AKT/mTOR pathway can be augmented, because of the AKT/mTOR pathway together constituting one of the most prevalent classes of mutations in human tumors, which makes it an attractive target for cancer treatment [27]. The role of autophagy in cancer is complex, and this complexity is illustrated by autophagy promoting or suppressing tumorigenesis [28–30]. Therefore, inhibiting or forcing autophagic machinery would be useful in drug cancer treatment [31]. The role played by autophagy depends on the concentration and the type of cancer cells. To date, there is no literature reporting that IVM induces autophagy in glioma cells.

In the present study, IVM-induced autophagy of U251 and C6 cells was detected first *in vitro* and *in vivo*, followed by a discussion of the deep molecular mechanisms of autophagy. Moreover, we explored the relationship between apoptosis and autophagy induced by IVM.

## Materials and methods

### Reagents and antibodies

IVM was purchased from Sigma–Aldrich/Merck KGaA (Darmstadt, Germany; European Pharmacopoeia Reference Standard), purity >95%. IVM was maintained at  $-20^{\circ}\text{C}$  or dissolved by dimethyl sulfoxide (DMSO) and stored at  $-20^{\circ}\text{C}$  (used *in vitro*) as stock solutions. Antibodies used in the present study were against LC3B (CST, 2775S, U.S.A.), P62 (CST, 5114T, U.S.A.), AKT (CST, 9272S, U.S.A.), Phospho-AKT (Ser<sup>473</sup>) (Beyotime, AA329, China), mTOR (CST, 2972S, U.S.A.), Phospho-mTOR (S2448) (Boster, BM4840, China), Ki67 (Bioss, bs-23103, China),  $\beta$ -actin (Sigma–Aldrich, A1978, Germany), goat anti-rabbit immunoglobulin G (IgG) (H+L)-horseradish peroxidase (HRP; SunGene GmbH, LK2001, Germany) and goat anti-mouse IgG (H+L)-HRP (OriGene Technologies, ZB-2305, U.S.A.).

### Cell lines and cell culture

The human glioma cells U251 and mouse glioma cells C6 were obtained from the First Affiliated Hospital of Harbin Medical University. Cells were cultured in Dulbecco's minimal essential medium (DMEM; HyClone, U.S.A.) supplemented with 1% penicillin/streptomycin and 10% New-fetal bovine serum (NBS). The cells were cultured and maintained as attached cells at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ .

### Transmission electron microscopy

U251 and C6 cells were seeded in incubation bottles for 24 h. Then cells were treated with IVM for 48 h, and the untreated cells served as control group. Cells were fixed with 2.5% glutaraldehyde containing 1% tannic acid and preserved at  $4^{\circ}\text{C}$ . After washing, the cells were dehydrated using a graded series of ethanol solutions 10 min for a time, transferred to propylene oxide and embedded in epon araldite (Polysciences, Inc., Polybed 812, U.S.A.). The ultra-thin sections were observed using a JEM-100CX transmission electron microscope (Shanghai Yongming Automatic Equipments Co. Ltd., H-7650, China).

## Plasmid transfection

The GFP-LC3 plasmid was transfected to the U251 and C6 cells with Lipofectamine 2000 according to the manufacturer's protocol. Briefly,  $1 \times 10^6$  cells/well were seeded on to 6-cm dish overnight at 37°C, and transfected with the GFP-LC3 plasmid in no-serum medium for 4 h. The treated cells were visualized with an inverted fluorescence microscope.

## Western blot assay

After being treated with IVM, the concentration of the protein of cells was measured using a BCA protein assay kit. Subsequently, equal protein amounts were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, U.S.A.) and electrotransferred to a PDVF membrane (0.22  $\mu\text{m}$ ). After the transfer, the non-specific binding blots were blocked in 5% no-fat milk dissolved in TBST for 1.5 h at 37°C with gentle shaking. Then, the specific primary antibodies were incubated overnight at 4°C, followed by incubation for 1 h with the respective second antibody at room temperature. Signals were detected using MiniChemi Imager (SageCreation, Beijing, China).  $\beta$ -actin was used as the endogenous control.

## TP determination

Cellular ATP levels were determined by ATP kit following the manufacturer's protocol (Beyotime, China). In brief, after being treated with IVM for 48 h, the protein concentrations were measured by BCA Protein Assay Kit and analyzed to determine the activity of ATP, and the protein concentrations were tested by BCA Protein Assay Kit. Then, the luminescence was measured by an enzyme-linked immunosorbent assay reader (HUADONG-Y, China).

## MTT assay

Cell viability was determined by conventional 3-(4,5-dimethylthiazol2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay. C6 ( $2 \times 10^4$  cells/well) and U251 ( $1 \times 10^4$  cells/well) cells were seeded in a 96-well plate and incubated overnight. The cells were pretreated with chloroquine (CQ; 15  $\mu\text{M}$ ) for 1 h before being exposed to IVM. Next, cells were incubated with 5 mg/ml MTT reagent for another 4 h at 37°C. After the medium was carefully removed, 150  $\mu\text{l}$  of DMSO was added and agitated to dissolve the formazan crystals. The absorbance was recorded at 490 nm on an enzyme-linked immunosorbent assay reader (HUADONG-Y, China).

## Colony formation assay

Cells were seeded into a six-well plate (500 cells/well) and incubated with fresh medium in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> incubated overnight. Next the cells were treated with CQ (15  $\mu\text{M}$ ) for 1 h before being exposed to IVM and cultured in medium for 13 days. The plates were washed with PBS, fixed with 4% paraformaldehyde, stained with 0.1% Crystal Violet for 20 min, and then washed with PBS to remove the excess dye. Quantification of colony formation was also performed using ImageJ software.

## Flow cytometry

Apoptosis was analyzed *in vitro* using the Annexin V- FITC apoptosis detection kit. Cells were harvested, washed with ice-cold PBS, and then resuspended in PI/Annexin-V solution for apoptosis analysis according to the manufacturer's instructions. Apoptosis ratio was measured using a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, U.S.A.). The results were quantified using the Cell Quest software (BD Biosciences, U.S.A.), and apoptosis was calculated as percentage of early and late apoptotic cells.

## Xenograft assays in nude mice

All animal experiments were carried out in Harbin Vic Biological Technology Development Co., Ltd., Harbin, China (Experiment number: SY-2017-Mi-027). All efforts were made to minimize animal suffering and reduce the number of animals used. Five-week-old female Balb/c nude mice (Beijing Vitonlihua Experimental Animal Technology Co. Ltd, Beijing, China) were treated with U251 cells ( $2.0 \times 10^6$ ) via subcutaneous injection. All mice were randomized into four groups: (1) Control group, treated with 100  $\mu\text{l}$  saline; (2) CQ group, treated with 20 mg/kg/day CQ in 100  $\mu\text{l}$ ; (3) IVM group, treated with 20 mg/kg/day IVM in 100  $\mu\text{l}$ ; (4) IVM+CQ group, treated with 20 mg/kg/day CQ combined with 20 mg/kg/day IVM in 100  $\mu\text{l}$ . All drugs were administered via intraperitoneal injections everyday. Tumor volumes were measured with Vernier caliper and calculated as volume ( $\text{mm}^3$ ) using the equation  $V = 0.5 \times \text{length} \times \text{width}^2$ . After 24 days, the animals were humanely killed in accordance with ethical study requirements. Tumor tissues were extracted for immunostaining and weighing.

## Immunohistochemistry

Tumors excised from the nude mice were fixed in 4% paraformaldehyde, dehydrated through a graded series of ethanol solutions, embedded in paraffin, cut into 5- $\mu$ m sections, and labeled with antibody LC3, Ki67 by HRP-conjugated secondary antibody. Hematoxylin was used for background counterstaining. Images were acquired using fluorescence microscopy (Feica, Germany) and analyzed with ImageJ software (version 2.0) (National Institutes of Health).

## Terminal deoxynucleotidyl transferase dUTP nick-end labeling assays

To investigate the effect of autophagy on apoptosis in glioma cancer, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was conducted according to the manufacturer's instructions. After being deparaffinized and hydrated, slides were washed with PBS twice and incubated with proteinase K (20  $\mu$ g/ml) for 25 min at 37°C. After a second round of washes, slides were incubated with TUNEL reaction mixture prepared freshly for 1 h at 37°C in a moist chamber. Images were acquired using fluorescence microscopy (Feica, Germany) and analyzed with ImageJ software (version 2.0).

## Statistical and quantitative analyses

All experiments were performed at least three times. Data were presented as the mean  $\pm$  standard deviation (SD). One-factor analysis of variance (ANOVA) test was used to assess the differences among all the experiment and control groups. The Dunnett's test was employed for post-hoc comparison of the experimental groups with the control group. GraphPad Prism Package (version 5.0; GraphPad Software, Inc., La Jolla, CA, U.S.A.) and SPSS version 20.0 statistical software (IBM Corp., Armonk, NY, U.S.A.) were used for statistical analysis.  $P < 0.05$  was considered to be indicative of a statistically significant difference.

For quantification, LC3, Ki67 staining intensity was measured from the number of positive cell nuclei in 25% fields using ImageJ software (version 2.0). All areas were chosen randomly from all sections. The intensity of bands in Western blotting was also measured by ImageJ software. The values subsequent to normalizing to the loading control in the control groups were set as 1.0.

## Results

### IVM stimulated autophagy of glioma cells *in vitro*

The effects of IVM on autophagy in U251 and C6 cells were assessed by transmission electron microscopy (TEM). TEM was utilized to observe the formation of autophagosome and ultrastructural change in nucleus. As shown in Figure 1A, TEM revealed obviously characteristic autophagosomes in U251 and C6 cells treated with IVM (15  $\mu$ M) but not in control cells (0  $\mu$ M). Arrows indicate autophagosomes containing intact and degraded cellular debris.

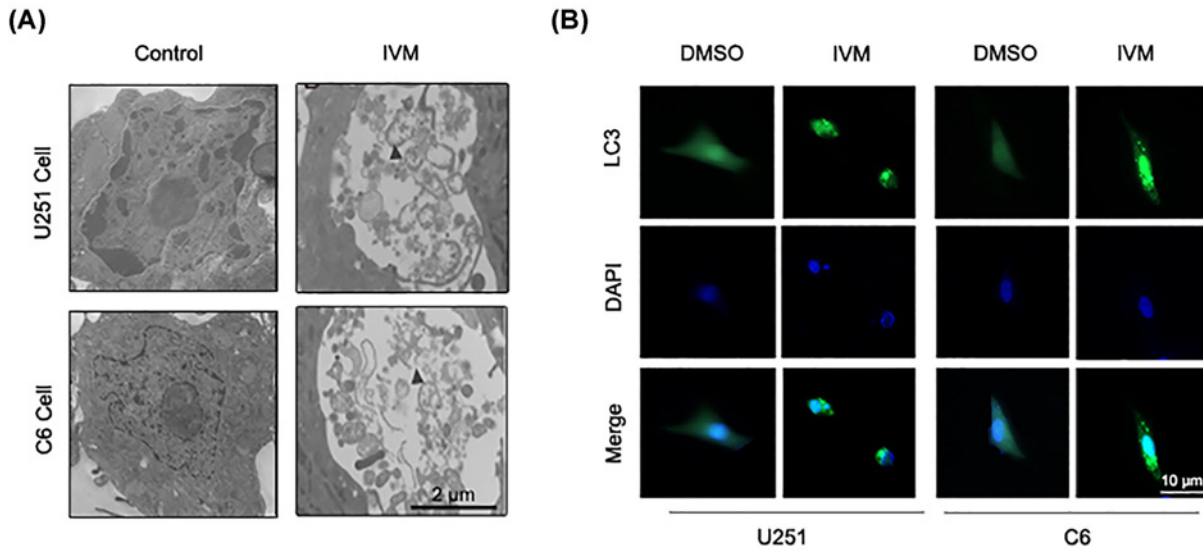
To extend these studies further, the LC3 puncta was observed by GFP-LC3 transient transfection assay. The treatment of U251 and C6 cells with IVM (0 and 15  $\mu$ M) for 48 h significantly increased the distribution and number of LC3 puncta (Figure 1B). These data indicate that IVM could stimulate autophagy in U251 and C6 cells.

### Dose-dependent effect of IVM on an autophagy-related protein LC3

Next, IVM effects were tested on autophagy-related protein in glioma cells by Western blot. It was observed that IVM enhanced the induction of autophagy in U251 and C6 cells when the cells were treated with various concentrations of IVM (0, 5, 10, and 15  $\mu$ M) for 48 h. As shown in Figure 2A,B, the levels of LC3-/LC3-I were increased, and the level of P62 was decreased following treatment with IVM in U251 and C6 cells. The observation showed that IVM significantly stimulated autophagy of U251 and C6 cells in a dose-dependent manner.

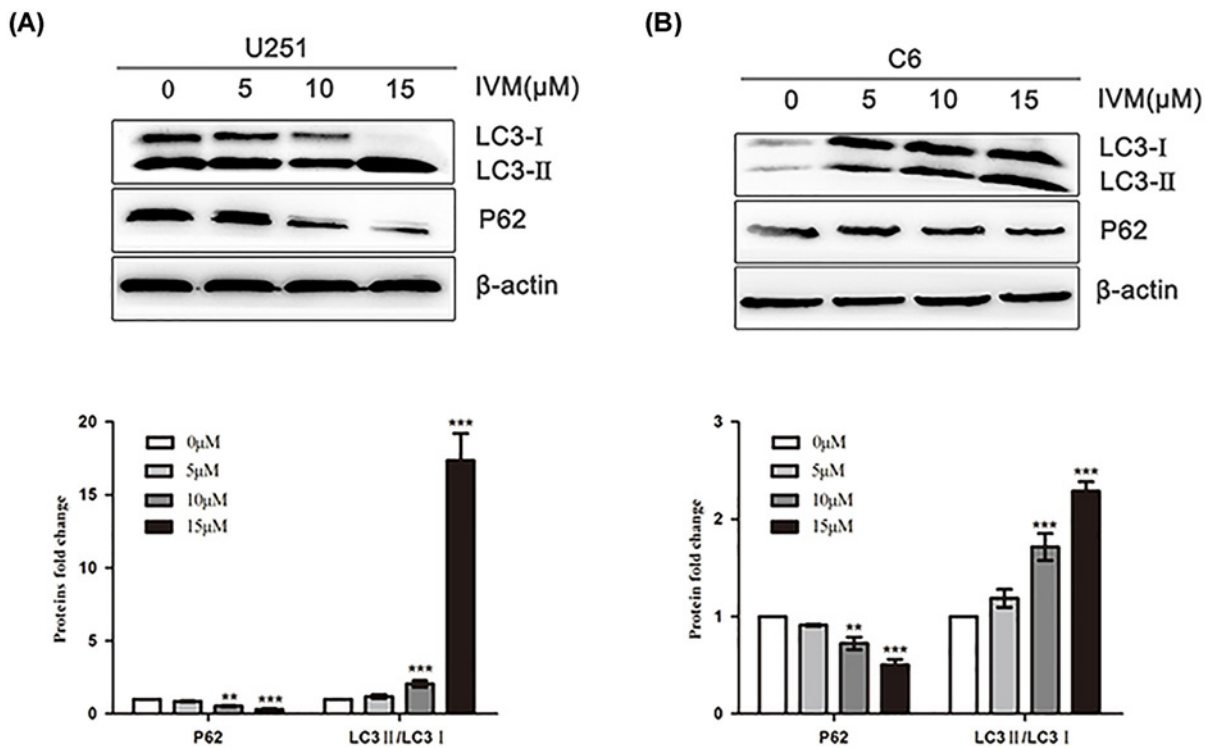
### IVM inhibited the mTOR pathway in glioma cells

To explore the mechanism of IVM inducing autophagy in U251 and C6 cells, protein levels of AKT, mTOR and their phosphorylated forms were measured by Western blot. The signals indicated that the expression levels of p-AKT (Ser<sup>473</sup>)/AKT and p-mTOR (S24448)/mTOR were significantly and dose-dependently reduced with IVM treatment (0, 5, 10 and 15  $\mu$ M). The results revealed that IVM inhibited the AKT/mTOR pathway (Figure 3A). mTOR complexes are important in the energy generation. To verify the inhibitory effects of IVM associated with energy impairment, we detected the ATP levels after treatment with 15  $\mu$ M of IVM for 48 h. As shown in Figure 3B, the relative ATP levels were down-regulated in both U251 cells and C6 cells. These results demonstrated that IVM inhibited the AKT/mTOR pathway and induced energy impairment in glioma cancer.



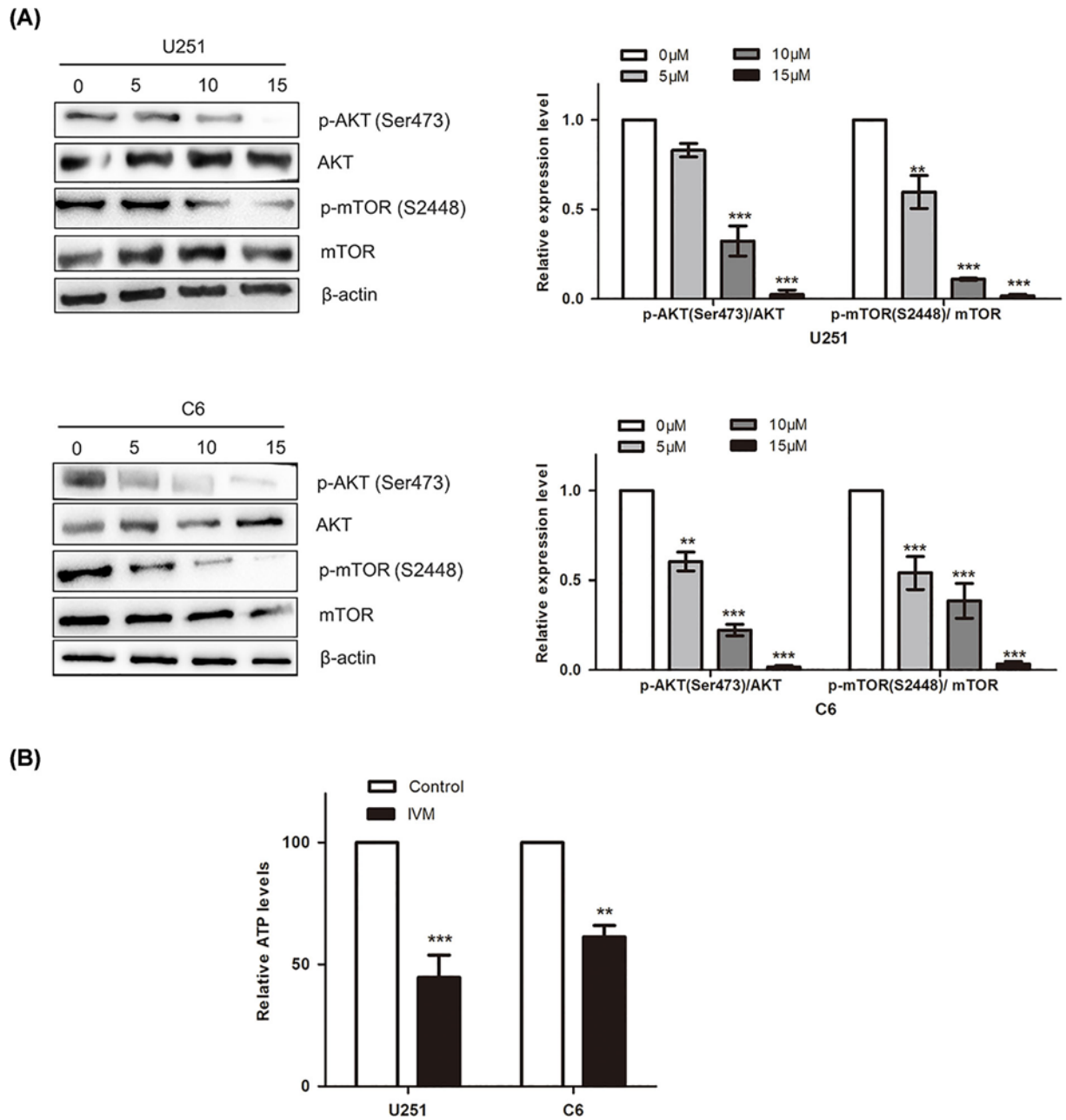
**Figure 1. IVM stimulated autophagy of glioma cells *in vitro***

(A) U251 and C6 cells were treated with IVM (0, 15 μM) for 48 h and analyzed by TEM. Scale bar, 2 μm. (B) U251 and C6 cells were transfected with GFP-LC3 plasmid, then the cells were treated with DMSO (<0.1%) or 15 μM IVM for 48 h. To determine the autophagic response, cells were inspected at 10× magnification for numbers of GFP-LC3 puncta. Scale bar, 10 μm.



**Figure 2. Dose-dependent effect of IVM on an autophagy-related protein LC3**

(A) U251 cells were exposed to different concentrations (0, 5, 10, 15 μM) of IVM for 48 h. (B) C6 cells were exposed to different concentrations (0, 5, 10, 15 μM) of IVM for 48 h, the LC3 and P62 levels were examined by Western blot. Data were presented as the means ± SD of three independent tests. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as compared with control group. β-actin was used as an internal standard.

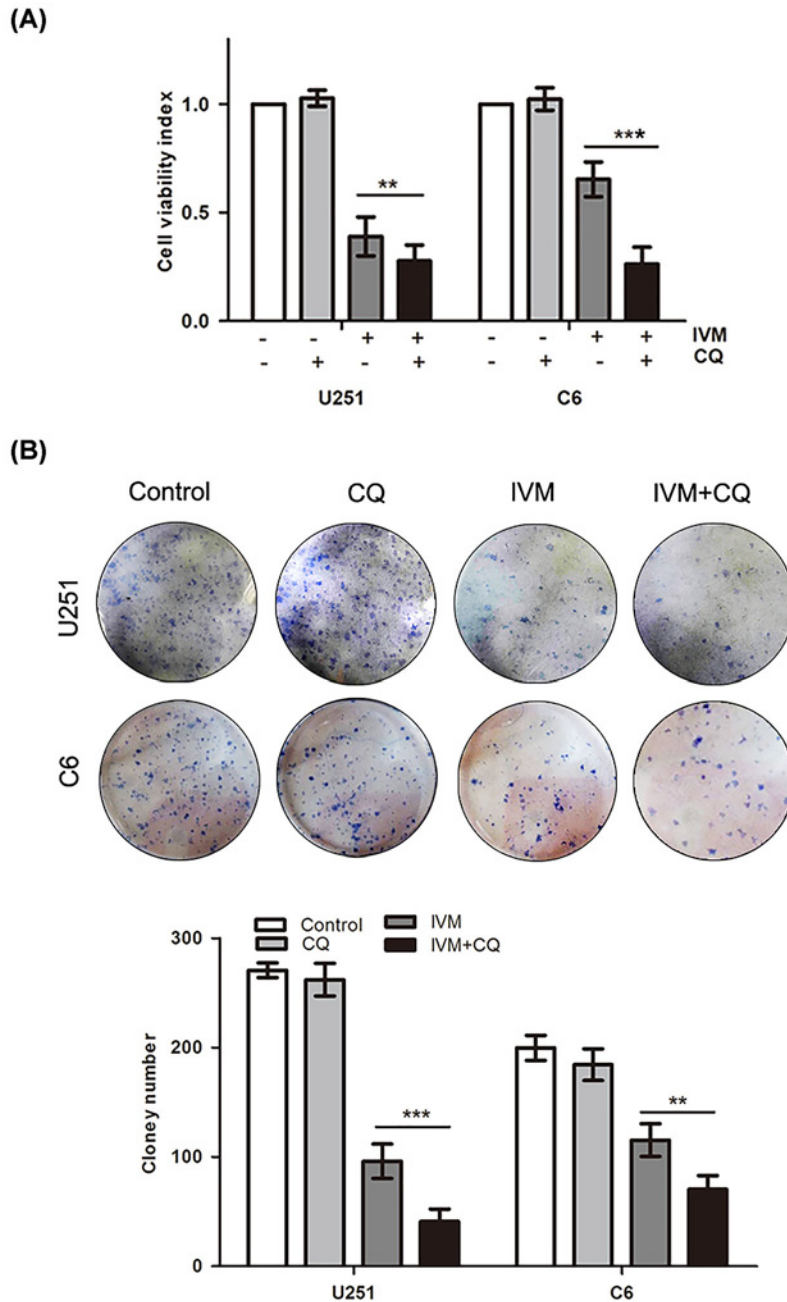


**Figure 3. IVM inhibited the mTOR pathway in glioma cells**

(A) U251 and C6 cells were incubated with IVM at different concentrations (0, 5, 10, 15  $\mu\text{M}$ ) for 48 h, the effects of IVM on the levels of AKT, p-AKT (Ser<sup>473</sup>), mTOR, p-mTOR (S2448) were examined by Western blot. (B) U251 and C6 cells were treated with 15  $\mu\text{M}$  IVM (0, 15  $\mu\text{M}$ ) for 48 h. The relative ATP levels were measured. Data were presented as the means  $\pm$  SD of three independent tests. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as compared with control group.  $\beta$ -actin was used as an internal standard.

## Effects of autophagy on cell growth ability

To confirm the anticancer effects of autophagy on glioma cells, MTT assay and colony formation analyses were conducted to assess the growth viability of glioma cells. We evaluated the combined effects of IVM and CQ on cell proliferation using U251 and C6 cells. As shown in Figure 4A, IVM co-treatment with CQ decreased the cell growth ability in U251 and C6 cell cells, and notably CQ exhibited a lesser effect on glioma cells. As shown in Figure 4B, we found that IVM co-treatment with CQ significantly inhibited colony formation and induced significant decrease in the colony formation ratio. These results indicated that autophagy increased cell growth ability in U251 and C6 cells.

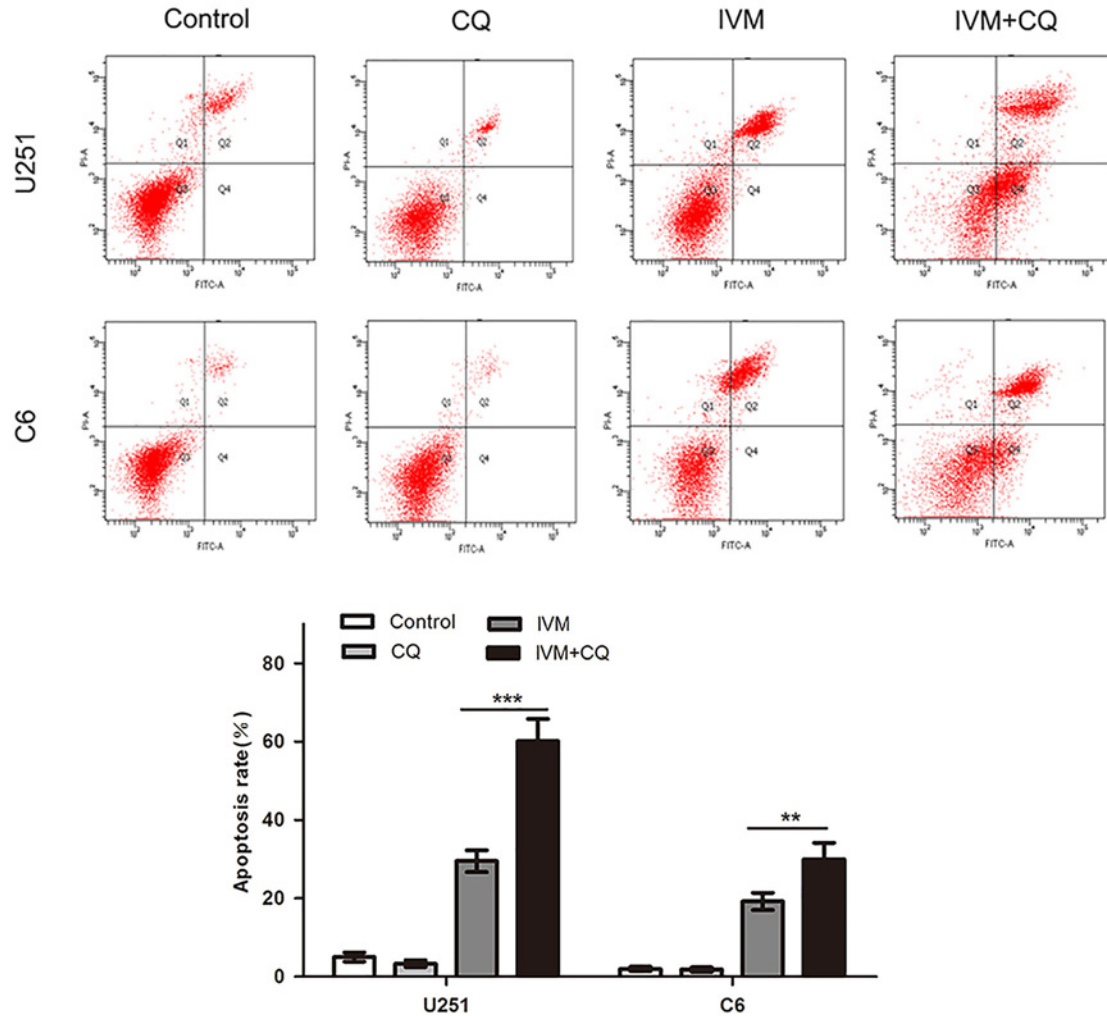


**Figure 4. Effects of autophagy on cell growth ability**

U251 cells and C6 cells were pretreated with CQ (15  $\mu$ M) for 1 h before being exposed to IVM (15  $\mu$ M). Cell viability was measured by MTT (A) and colony formation analysis (B). Data were presented as the means  $\pm$  SD of three independent tests. \*\* $P$ <0.01, \*\*\* $P$ <0.001 as compared with IVM group.

## Effects of autophagy on IVM-induced apoptosis

Autophagy is a mediator regulating cell apoptosis. We investigated the effects of autophagy on IVM-induced apoptosis by flow cytometry analysis. The percentage of apoptotic cells was enhanced by CQ in IVM-treated cells compared with those treated with IVM alone (Figure 5). Specifically, the fraction of apoptotic cells was increased from  $29.53 \pm 2.16\%$  in IVM-treated U251 cells to  $60.10 \pm 1.24\%$  in co-treated cells, and the apoptotic cell percentage increased from



**Figure 5. Effects of autophagy on IVM-induced apoptosis**

U251 cells and C6 cells were pretreated with CQ (15  $\mu$ M) for 1 h before being exposed to 15  $\mu$ M IVM. The percentage of apoptotic cell was evaluated by flow cytometry after cells were incubated with IVM (15  $\mu$ M) in the presence or absence of CQ (15  $\mu$ M). Q2 plus Q4 areas were calculated as the apoptosis ratio. Data were presented as the means  $\pm$  SD of three independent tests. \*\* $P$ <0.01, \*\*\* $P$ <0.001, as compared with IVM group.

19.20  $\pm$  3.28% to 30.42  $\pm$  1.12% in C6 cells. Together, these findings showed that autophagy reduced IVM-induced apoptosis in glioma cells.

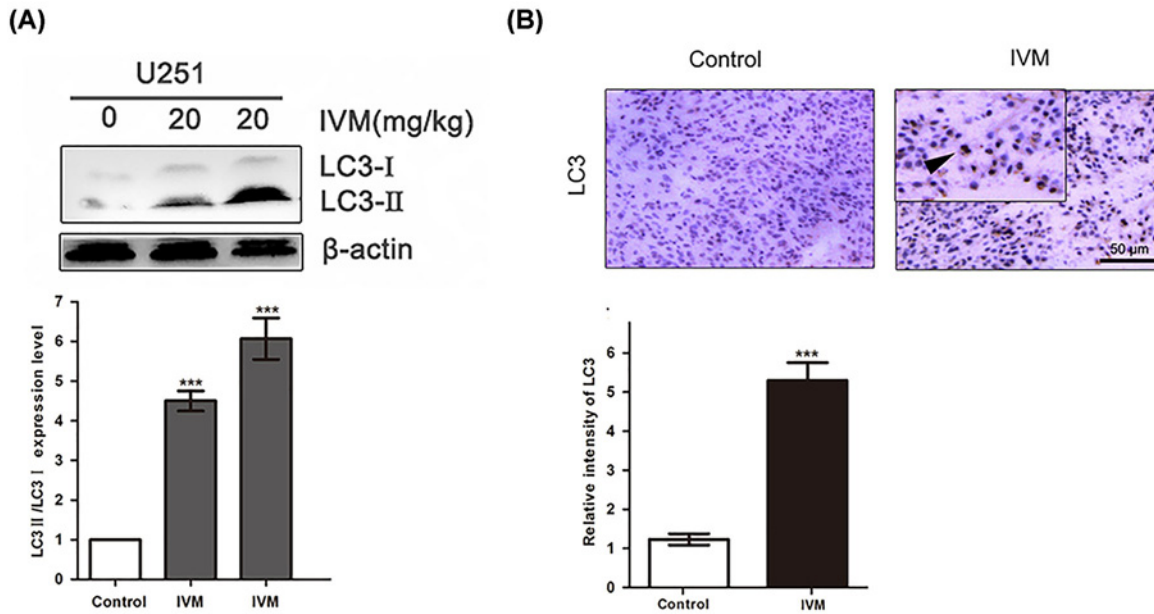
### IVM stimulated autophagy of glioma cells *in vivo*

To conform whether IVM stimulated tumor autophagy *in vivo*, U251 cells were injected subcutaneously into athymic nude mice. As shown in Figure 6A, we observed LC3-II expression was more pronounced in IVM-treated tumors compared with the control group. Meanwhile, as shown in Figure 6B, mouse xenografts stained with LC3 showed stronger LC3 staining from IVM-treated xenografts compared with the control group. These results indicated that IVM stimulated autophagy of glioma cells *in vivo*.

### Effects of autophagy on IVM-induced tumor growth inhibition *in vivo*

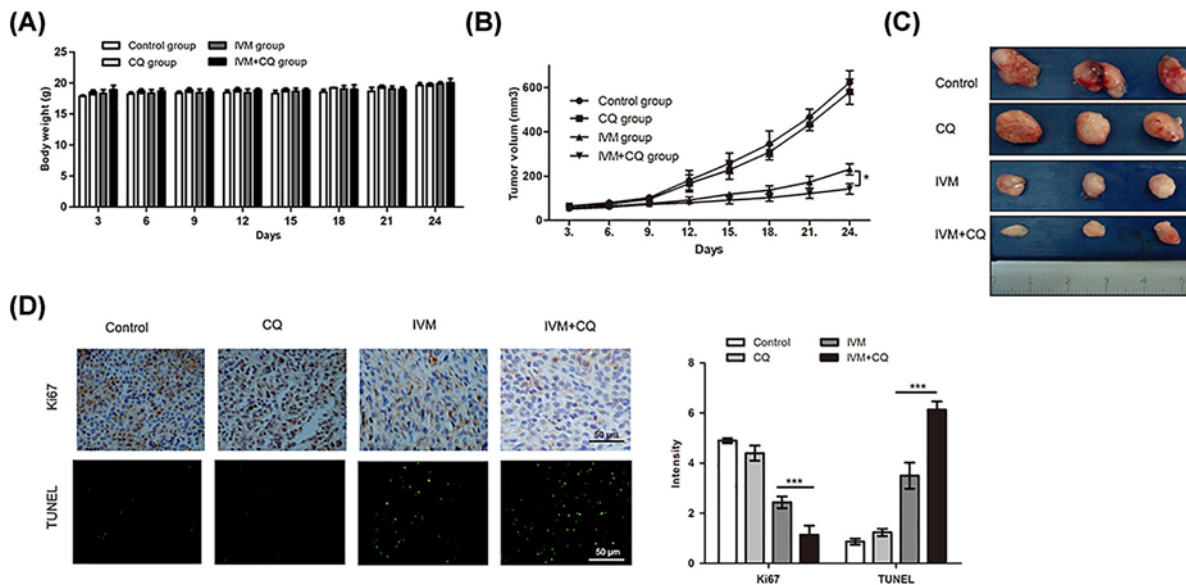
Considering the promising *in vitro* results on the autophagy effects of apoptosis in glioma cells, we investigated whether autophagy could reduce IVM-induced tumor apoptosis in a nude mouse model. As shown in Figure 7A,





**Figure 6. IVM stimulated autophagy of glioma cells *in vivo***

(A) Orthotopic xenograft tissues were extracted to assess the levels of LC3-II by Western blot analysis. (B) LC3 expression in orthotopic xenografts was examined by immunohistochemistry. Arrows indicate positive cells which stained brown. Scale bar, 50  $\mu$ m. Data were presented as the means  $\pm$  SD of three independent tests.  $\beta$ -actin was used as an internal standard. \*\*\* $P$ <0.001, as compared with IVM group.



**Figure 7. Effects of autophagy on IVM-induced tumor growth inhibition *in vivo***

(A) Charting of mouse weight with time. Mice were killed days after the indicated treatments, the tumor volume (B,C) were measured. (D) Immunohistochemistry staining result of Ki67 and TUNEL assay on tumor sections. Scale bar, 50  $\mu$ m. Data were presented as the means  $\pm$  SD of three independent tests. \* $P$ <0.05. \*\*\* $P$ <0.001, as compared with IVM group.

there was no observed sign of toxicity between the test group and the control group on all measured days. In addition, xenografts treated with IVM+CQ grew at a slower rate compared with those treated with IVM alone (Figure 7B). Notably, tumor growth curves in mice treated with IVM+CQ had a relatively slow trend compared with the IVM group (Figure 7C). Apoptosis was assessed by TUNEL staining and immunohistochemistry in tumor sections, to characterize the molecular mechanisms underlying the observed effects in the xenograft model. As shown in Figure 7D, the apoptotic status of tumor tissues was assessed by TUNEL assay showed that apoptotic cells was increased by IVM+CQ treated cells compared with treated with IVM alone. The expression levels of Ki67 were examined by immunohistochemistry showed that IVM+CQ reduced the levels of Ki67 compared with IVM-treated tissues. TUNEL staining and immunohistochemistry demonstrated more dead cells and an evident increase in apoptotic proportions in IVM+CQ-treated tumor tissues. In generally, these data recapitulated the observations made *in vitro* and showed that autophagy reduced the apoptotic effects of IVM on U251 *in vivo*.

## Discussion

IVM, a semi-synthetic macrocyclic lactone derivative of the avermectin family, has recently been characterized as a potential anticancer agent due to observed antitumor effects [32]. IVM inhibited the proliferation and increased apoptosis of various human cancer types. Our previous studies have suggested that IVM inhibited the growth of glioma cells by inducing apoptosis [5]. Accumulating evidences suggested that apoptosis and autophagy have been activated as consequences of chemotherapy or radiotherapy to induce cancer cell death [2,13]. The role of autophagy in cancer progression is varied. The precise mechanisms of autophagy effects, particularly on glioma cancer, have not been clear. In the present study, the effects of IVM on the autophagy ability of glioma cancer U251 and C6 cells were investigated. Additionally, the potential underlying molecular mechanisms on autophagy induction, and the relationship between autophagy and apoptosis was explored.

In the present study, autophagy was significantly induced by IVM treatment in U251 and C6 cells, as demonstrated using the TEM, GFP-LC3 transient transfection assay and Western blot. Autophagy can be regulated by mediating the phosphorylation of mTOR and inhibition of the AKT/mTOR signaling pathway with different drugs can play the role resembling a double-edged sword. Our research showed that IVM can cause a decrease in p-AKT (Ser<sup>473</sup>)/AKT and p-mTOR (S2448)/mTOR, which indicated that IVM inhibited AKT/mTOR signaling in U251 and C6 glioma cells, and the suppression of AKT/mTOR signaling may contribute to the activation of autophagy induced by IVM. Additionally, studies have shown that inducing a non-protective autophagy by reducing ATP levels offered a three-pronged approach to facilitate cancer cell death. Meanwhile, the mTOR complexes played important roles in the regulation of nutrition and energy generation, and it could be inhibited during the nutrition depletion. Our data demonstrated that IVM inhibited the AKT/mTOR pathway and induced energy impairment in glioma cells. Other studies have shown that IVM induces cytostatic autophagy through the blocking PAK1/Akt/mTOR axis in breast cancer [10]. Autophagy can be regulated by mediating the phosphorylation of mTOR, and inhibition of the AKT/mTOR signaling pathway in different cancer cells can play the role: it can inhibit apoptosis or promote apoptosis. Although the two studies had similar mechanisms in autophagy, the role of autophagy in cell apoptosis was opposite.

Now, we appreciate that autophagy serves dual roles during tumorigenesis [33]. On the one hand, autophagy is also known as type II programmed cell death, and hyperactivation of autophagy can cause cell death [34]. On the other hand, autophagy may serve as a key mechanism of cell survival [35]. Therefore, autophagy may have different effects at different stages of cancer. Further studies are necessary to understand the precise role of autophagy in cancer cells [36]. Our previous experiments proved that IVM could induce apoptosis and cell cycle arrest of glioma cells *in vitro* and *in vivo* [5]. The result showed that IVM inhibited Bcl-2 expression and induced Bax expression. A high ratio of Bax to Bcl-2 can cause the release of Cytochrome-*c*. In addition, in the cytosol, Cytochrome-*c* resulted in the activation of caspase-3 and caspase-9. And the activated caspase-9 was cleaved and thereby induced the activation of other caspases, such as caspase-3, which in turn may influence mitochondrial function, and subsequently contributed to apoptotic cell death [5]. Additionally, autophagy is mediated by activation of a series of ATG proteins. Beclin 1 included a BH3-like domain, which binds to the anti-apoptotic Bcl-2 proteins family such as Bcl-2, so the relationship between autophagy and apoptosis in cancers is complex. The relationship between autophagy and cell death reduced by IVM on glioma cells was still unknown. The autophagy flux of glioma was inhibited by CQ which could decrease lysosomal function [37]. In our study, the results indicated that the effect of autophagy inhibitor involved in autophagy and apoptosis. The inhibition of autophagy reduced the cell ability measured by MTT and colony formation assay, and increased apoptosis measured by flow cytometry in glioma cells. Autophagy and apoptosis played different roles in IVM-induced death of glioma cells. Apoptosis was an important cause of death of U251 and C6 cells, while autophagy protected cells from death in this paper.

To examine whether IVM is important for autophagy induction *in vivo*, nude mice were injected subcutaneously with U251 cells and treated with IVM. The selected dose of the IVM injections was 20 mg/kg, and IVM could reduce the tumor mass of U251 xenografts [5]. *In vivo*, compared with control group, IVM treatment was significantly more effective in suppressing the tumor growth without affecting body weight, and in regulating the amount of LC3 in tumor tissues. Our data indicated that IVM induced autophagy *in vivo*.

Furthermore, to support and verify our experimental results *in vitro* with more reliable evidence, we tested the effect of IVM combined with CQ in a xenograft nude mouse model. In clinical trials, the most commonly used treatment to inhibit autophagy is CQ which arrests the latter step of autophagy, fusion and degradation of autolysosome [38,39]. CQ, approved for treatment of malaria, is already tested in several clinical trials in combination with various anticancer treatments [40]. In follow-up experiments, the dose of CQ injections was 15 mg/kg, and it did not exert a significant inhibitory effect on tumor growth [7]. We found that combined treatment with IVM and CQ resulted in inhibitory effects on tumor growth compared with treatment with IVM alone. These data indicated that the combination of IVM and CQ had anti-glioma cancer more effects both *in vitro* and *in vivo*. The results of the present study *in vivo* were in agreement with those of some previous studies *in vitro*. Interestingly, the tumor volume change in IVM+CQ is not drastic compare to the IVM ( $*P < 0.05$ ). These findings were not consistent with the previous studies *in vitro* ( $***P < 0.001$ ). On the one hand, this discrepancy may be attributed to the difference between *in vivo* and *in vitro*. In nude mice, all drugs were administered via intraperitoneal injection, so the availability of the drug for absorption may be not completely. On the other hand, dose probably matters. It was found that the intraperitoneal injection of IVM at doses of 20 mg/kg prominently inhibited the growth of glioma cells *in vivo*. In our study, the concentration of IVM is no less than 20 mg/kg, consistent with the dose of IVM in previous studies. The dose of CQ injections was 15 mg/kg. Taken together, the effect of IVM and CQ on tumor growth is likely dependent on cell type and concentration. Thus, our study supports that combination therapy with IVM and an autophagy inhibitor enhances the anti-glioma activities of IVM.

IVM is widely used for the management of scabies and lymphatic filariasis in humans [8]. Recently, IVM has been indicated to be beneficial in treatment of cancer, such as breast cancer, HeLa cells [10,12]. In our study, these data suggested that combination therapy of IVM and the CQ had low levels of organ-related toxicity and enhances anti-glioma activity *in vivo*, and provide the groundwork for the potential application of IVM in anticancer therapy. Therefore, IVM may be a relatively effective and safe agent in glioma patients. Further studies in glioma animal models as well as in human clinical trials are necessary.

## Conclusion

In conclusion, we found that IVM was an effective agent for inducing autophagy of glioma cancer cells *in vitro* and *in vivo*. Autophagy is mediated by the activation of the AKT/mTOR signaling pathway, suggesting the vital role of autophagy in protection of glioma cells subjected to IVM condition. These findings indicated that IVM may have potential therapeutic applications for the treatment of glioma cells.

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## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## Author Contribution

Aili Gao performed conception and design, and was responsible for experiments performance. Jingjing Liu contributed to experiments performance. Chen Chen, Xiaoxing Wang, Faling Qu and Haiyang Wang performed data analysis. Kongbin Yang, Qing Wang, Ning Zhao and Jing Meng contributed to conception and design, data analysis, manuscript writing. Hongsheng Liang contributed to financial support and final approval of manuscript.

## Ethical Approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional and/or national research committee (Harbin Vic Biological Technology Development Co., Ltd., Harbin, China). The protocol was approved

by the management and welfare of experimental animal ethics committee, Animal Experiment of Harbin Vic Biological Technology Development Co., Ltd., Harbin, China.

## Abbreviations

CQ, chloroquine; DMSO, dimethyl sulfoxide; HRP, horseradish peroxidase; IgG, immunoglobulin G; IVM, Ivermectin; LC3, light chain 3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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