Received: 18 September 2022 | Accepted: 12 October 2022

DOI: 10.1002/prp2.1041

INVITED REVIEW

Xanthatin suppresses proliferation and tumorigenicity of glioma cells through autophagy inhibition via activation of the PI3K-Akt–mTOR pathway

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81973337; the Key project of University Excellent Talents Support Program in Anhui, Grant/Award Number: gxyqZD2020010; the Scientific research Level promotion Project of Anhui Medical University, Grant/Award Number: 2020xkjT002 to LJF

Abstract

Glioma is the most common and aggressive primary brain tumor in adults with high morbidity and mortality. Rapid proliferation and diffuse migration are the main obstacles to successful glioma treatment. Xanthatin, a sesquiterpene lactone purified from *Xanthium strumarium* L., possesses a significant antitumor role in several malignant tumors. In this study, we report that xanthatin suppressed glioma cells proliferation and induced apoptosis in a time- and concentration-dependent manner, and was accompanied by autophagy inhibition displaying a significantly reduced LC3 punctate fluorescence and LC3II/I ratio, decreased level of Beclin 1, while increased accumulation of p62. Notably, treating glioma cells with xanthatin resulted in obvious activation of the PI3K-Akt–mTOR signaling pathway, as indicated by increased mTOR and Akt phosphorylation, decreased ULK1 phosphorylation, which is important in modulating autophagy. Furthermore, xanthatin-mediated pro-apoptosis in glioma cells was significantly reversed by autophagy inducers (rapamycin or Torin1), or PI3K-mTOR inhibitor NVP-BEZ235. Taken together, these findings indicate that anti-proliferation and pro-apoptosis effects of xanthatin in glioma are most likely by inhibiting autophagy via activation of PI3K-Akt–mTOR pathway, suggesting a potential therapeutic strategy against glioma.

KEYWORDS
autophagy, glioma, mTOR, tumorigenicity, xanthatin

Abbreviations: AKT, protein kinase B; Atg, autophagy-related proteins; AV, autophagic vesicles; Baf A1, bafilomycin A1; DMSO, dimethylsulfoxide; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; IB, Immunoblot; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; PARP, poly-(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PI3K, phosphoinositide 3-kinase; qRT-PCR, real-time quantitative polymerase chain reaction; SRB, Sulforhodamine B; TMZ, Temozolomide.

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Huaqing Chen and Tong Zhu have contributed equally to this work.

1 | INTRODUCTION

Glioma is the most common malignant primary tumor in the central nervous system, accounting for about 80% of total malignant brain tumors.[1,2] Although standardized comprehensive therapy consisting of surgical excision, radiation, and chemotherapy has been adopted, the prognosis of glioma patients is still frustrating.[1,3–5] Therefore, the identification and development of new therapeutics for glioma patients are urgently needed.

Macroautophagy (hereafter referred to as autophagy), a process by which cells form double-membraned autophagic vesicles (AV) that sequester organelles and proteins and target them for degradation in the lysosome, has been suggested to play a complex role in cancer.[6–9] There is growing evidence suggesting that, while autophagy induction may limit cancer cell survival in the earliest stages of tumorigenesis; however, in established cancers, autophagy can help cope with intracellular and environmental stresses, such as hypoxia, nutrient and growth factor deprivation, or therapeutic stresses, thereby favoring tumor progression.[7,10,11] It has been verified that autophagy disruption prevented hypoxia-associated resistance to antiangiogenic therapy and promotes cell apoptosis in glioblastoma.[12,13] In this context, it is becoming increasingly clear that agents targeting autophagy inhibition may be an effective therapeutic strategy for patients with advanced glioma.

Xanthatin is a natural bioactive sesquiterpene lactone isolated from the aerial part of *Xanthium strumarium* L. (Asteraceae), a native herb commonly used in China.[14,15] Previous research have demonstrated that xanthatin suppresses proliferation and induces apoptosis in a wide variety of tumor types.[16–22] Studies of the underlying mechanisms have shown that xanthatin has anti-proliferative, anti-angiogenesis, and pro-apoptotic effects in various cancers both in vitro and in vivo.[17,18,23–25] Our previous studies monitored the antitumor effect of xanthatin in glioma-bearing mice and glioma cells and found that xanthatin induces cell apoptosis and inhibits tumor growth via activating the endoplasmic reticulum stress-dependent CHOP pathway.[23,26] In this study, we further demonstrated that xanthatin suppresses proliferation and induces apoptosis of malignant glioma cells by inhibiting autophagic fluxes via activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway. Thus, xanthatin might be considered a promising candidate for pharmacologically interfering with autophagy that can be further optimized to be a therapeutic agent for glioma patients.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Xanthatin, obtained from Shizhou Biology Technology Co., Ltd (Nanjing, China, SZSW20180145CET), was dissolved in dimethylsulfoxide (DMSO, Sigma) at a concentration of 10 mM stock solution and stored at −20°C. Temozolomide (TMZ, chemotherapeutic agent for treating glioma) was purchased from Sigma (St. Louis, MO, USA). The following antibodies were used: rabbit anti-cleaved-caspase-3 (CST, 9654 s), rabbit anti-cleaved-PARP (CST, 5625 s), rabbit anti-Beclin1 (CST, 3495 S), rabbit anti-LC3 (Sigma, L7543), rabbit anti-p62 (Sigma, P0067), rabbit anti-Atg5 (CST, 12994 s), rabbit anti-Atg7 (Proteintech, 10088–2-AP), rabbit anti-phospho-ULK1 (CST, 4634 s), rabbit anti-phospho-mTOR (CST, 2971 s), rabbit anti-phospho-AKT (Bioss, 5193R), rabbit anti-phospho-p38 MAPK (CST, 9211 s), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Santa Cruz, 16982), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (CST, 4668), mouse anti-α-tubulin (Sigma, t6199), rabbit anti-GAPDH (Elabscience, E-AB-20059), anti-mouse secondary antibody (Sigma, GENA931), anti-rabbit secondary antibody (Sigma, GENA934), Alexa Fluor 488 labeled anti-mouse IgG (Invitrogen, A11029), Alexa Fluor 568 labeled anti-rabbit IgG (Invitrogen, A11036). The following reagents were used: autophagy inhibitor bafilomycin A1 (Baf A1)(Abcam, AB120497), autophagy inducers Torin1 (Selleckchem, S2827), and rapamycin (Rapa) (Santa Cruz, sc-3504), PI3K/mTOR inhibitor NVP-BEZ235 (MedChemExpress, HY-50673). Sulforhodamine B cell proliferation and cytotoxicity assay kit were purchased from BestBio (Shanghai, China).

2.2 | Cell culture and drug treatment

Rat glioma cell line C6 and human glioma cell line U251 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in 10% fetal bovine serum (FBS)-supplemented DMEM medium (Invitrogen, 1710802) and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. Cells were treated with xanthatin at a concentration ranging from 1 to 20 μM for varying periods of time ranging from 6 to 24 h. TMZ (100 μM) was used as a positive control. The autophagy inhibitor Baf A1 (20 nM), autophagy inducers Rapa (10 μM) or Torin 1 (20 nM), and PI3K/mTOR inhibitor NVP-BEZ235 (250 μM) were used to regulate autophagy of glioma cells.

TABLE 1 Primers used for qRT-PCR

Gene	Forward	Reverse
BECN1	5′-AGGAGCTGGAAGATGTGGAA-3′	5′-CAAGCGACCCAGTCTGAAAT-3′
ATG5	5′-GACCACAAGCAGCTCTGGAT-3′	5′-GGTTTCCAGCATTGGCTCTA-3′
ATG12	5′-CGGAAGATTCAGAGGTTGTG-3′	5′-GAGGCCACCAGTTTAAGGAA-3′
ATG7	5′-TGTGGAGCTGATGGTCTCTG-3′	5′-TGATGGAGCAGGGTAAGACC-3′
GAPDH	5′-CCACTCCTCCACCTTTG-3′	5′-CACCACCCTGTTGCTGT-3′

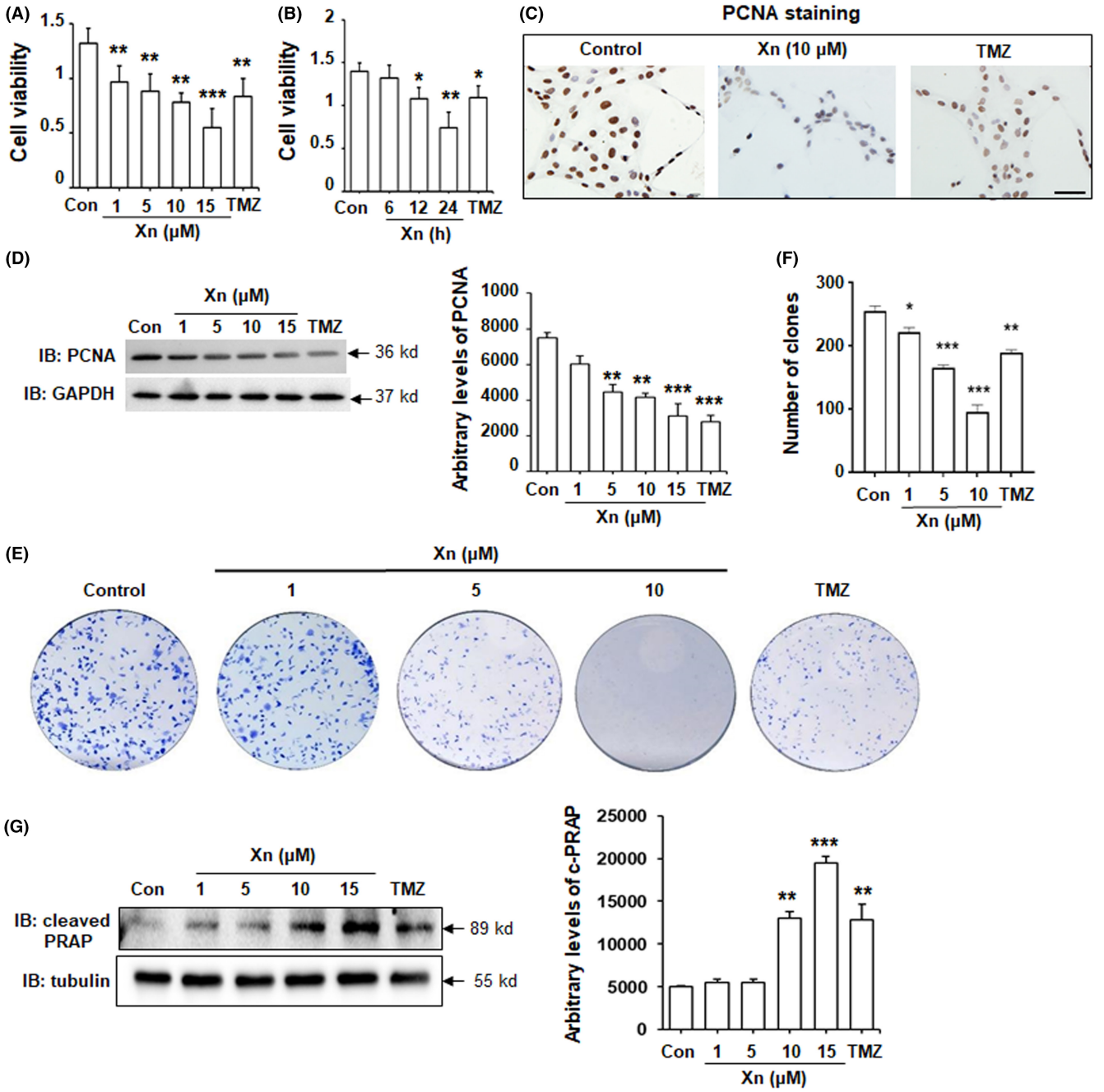


FIGURE 1 Xanthatin impairs cell proliferation and colony formation but increases apoptosis in glioma cells. (A, B) SRB assay shows the proliferation of C6 cells incubated with xanthatin at indicated concentrations for 12 h (A) or xanthatin (10 μM) for different periods of time (B). DMSO was used as vehicle control. TMZ (100 μM) was used as a positive control. (C) Representative images of DAB staining show the expression of PCNA in C6 cells treated with DMSO, xanthatin (10 μM), or TMZ (100 μM) for 12 h. (D) Left, immunoblots assay shows the protein levels of PCNA of C6 cells in the presence or absence of xanthatin. Right, quantitative analysis of PCNA protein levels. (E) Representative images of colony formation assay of C6 cells treated with DMSO, xanthatin (1, 5, 10 μM), or TMZ (100 μM) for 7 days. (F) Quantitative data showing xanthatin or TMZ treatment reduces the colony formation of C6 cells. (G) Left, immunoblots of cleaved forms of PARP (c-PARP) in C6 cells treated with DMSO, xanthatin (1, 5, 10, 15 μM), or TMZ (100 μM) for 12 h. Right, quantitative analysis of c-PARP protein levels. All the quantitative data was represented as mean ± SEM of at least 3 independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, vs. control. Scale bar = 50 μm. Con, control; Xn, xanthatin.

2.3 | Sulforhodamine B (SRB) assay

C6 and U251 cells were seeded into 96-well plates at a density of 1×10^4 per well and treated with the compounds at the indicated concentrations, Then, the cells were fixed with 10% trichloroacetic acid solution at 4°C for 1 h, washed with tap water, dried and stained with 0.4% SRB solution for 30 min. Then, the excess dye was removed by washing repeatedly with 1% acetic acid. Following this,

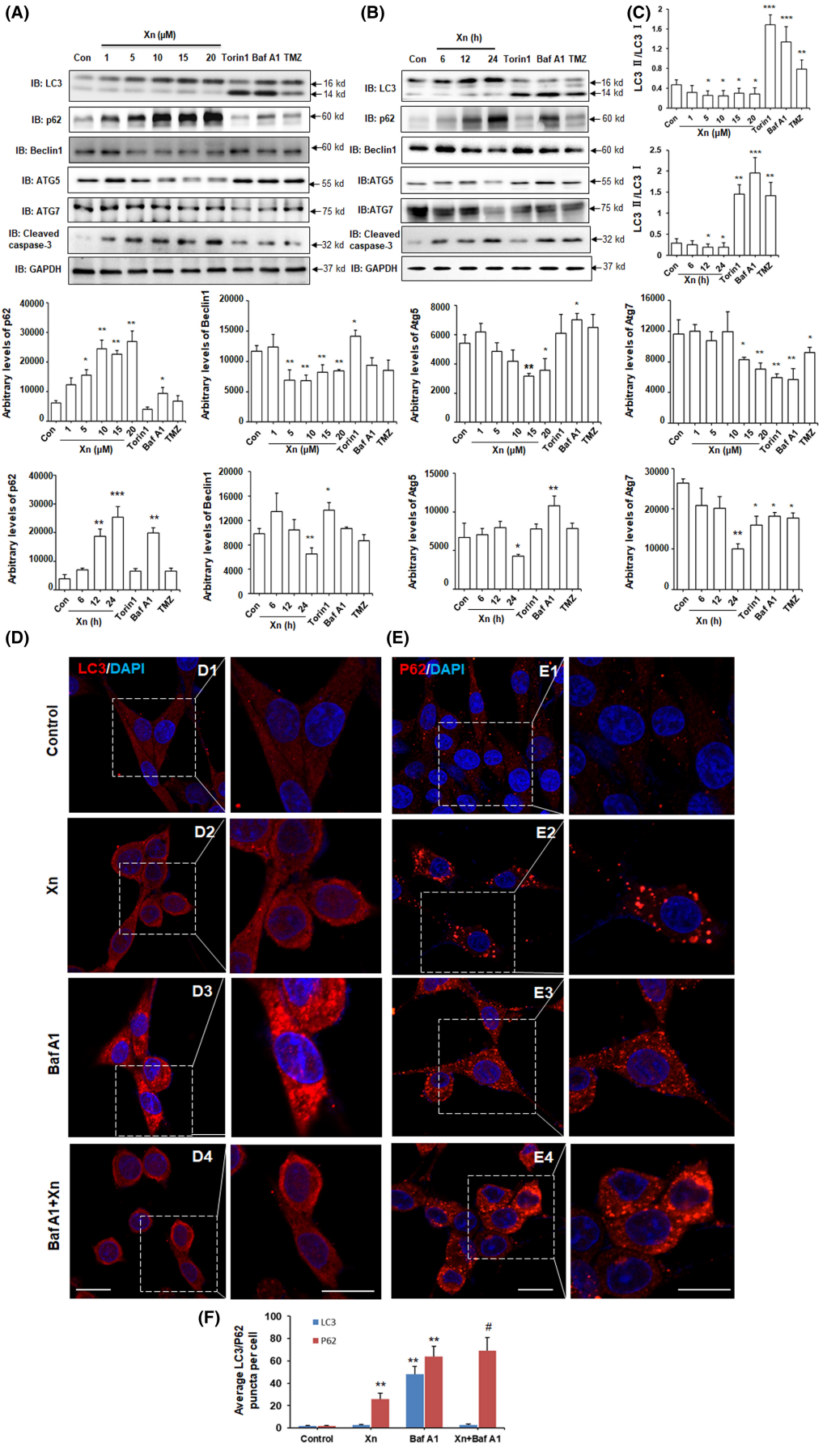

(A)
Xn (μM)
Con 1 5 10 15 20 Torin1 Baf A1 TMZ
IB: LC3
IB: p62
IB: Beclin1
IB: ATG5
IB: ATG7
IB: Cleaved caspase-3
IB: GAPDH
16 kd
14 kd
60 kd
55 kd
75 kd
32 kd
37 kd
(B)
Xn (h)
Con 6 12 24 Torin1 Baf A1 TMZ
IB:ATG5
IB:ATG7
(C)
LC3 II/LC3 I
Arbitrary levels of p62
Arbitrary levels of Beclin1
Arbitrary levels of Atg5
Arbitrary levels of Atg7
(D)
(E)
LC3/DAPI
P62/DAPI
Control
Xn
Baf A1
Baf A1+Xn
D1
D2
D3
D4
E1
E2
E3
E4
(F)
Average LC3/P62 puncts per cell
LC3
P62
Control
Xn
Baf A1
Xn+Baf A1

FIGURE 2 Xanthatin inhibits autophagosome formation in glioma cells. (A, B) Representative immunoblots against autophagy (LC3, p62, Beclin 1, ATG 5, ATG 7) and apoptosis-related proteins (cleaved caspase-3) in C6 cells incubated with indicated concentrations of xanthatin for 12 h (A) or 10 μM xanthatin for the indicated times (B). Torin 1 (20 nM, 6 h) was used to induce autophagy, and bafilomycin A1 (Baf A1, 20 nM, 6 h) was used to inhibit autophagy. TMZ (100 μM) was used as a positive control. (C) Densitometric analysis of the immunoreactive blots in A and B. (D, E) Representative confocal microscope images showing LC3 and P62 immunofluorescence in C6 cells treated with xanthatin (10 μM) for 12 h in the presence or absence of Baf A1 (20 nM). Enlarged images of the boxed region show LC3 puncta (red) (D) and p62 aggregation (red) (E). DAPI was used to stain the nucleus. (F) The graphs represent the relative density of LC3 and p62 puncta in D and E. All the quantitative data was represented as mean ± SEM of at least 3 independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, vs. control. $^{\#}p < .05$, vs. xanthatin group. Scale bars = 10 μm. Xn, xanthatin.

wells were dried and SRB dye was dissolved in 10 mM Tris base solution for OD determination at 515 nm using a multi-scan spectrum.

2.4 | Clonogenic assay

Cells were plated in 6 wells (500 cells per well) and exposed to xanthatin at indicated concentrations, TMZ (100 μM), or BEZ235 (250 μM) for continuing growing for 7 days. The drugs were then washed away and the colonies were fixed with 4% paraformaldehyde and stained with crystal violet staining. The experiments were repeated at least three times and colonies consisting of at least 3 wells were photographed with a scanner and counted under a light microscope.

2.5 | TUNEL staining

Cells were treated with different concentrations of xanthatin and processed for apoptosis assay using in situ cell death detection kit (Roche, 11684795910). DAPI was used to stain the nuclei. Fluorescence images of cells were obtained with constant parameters of acquisition. The numbers of TUNEL-positive cells (green) and the total number of nuclei (blue) were counted in 5 randomly selected fields from 4 sections of each group. The histogram shows the proportion of TUNEL-positive cells in relation to the total cell number.

2.6 | DAB staining and immunofluorescence staining

C6 cells were treated either with 10 μM xanthatin or TMZ for 12 h, and then the DAB staining for PCNA was performed as described previously.[26] C6 cells were treated either with 10 μM xanthatin or with combined xanthatin and autophagy inhibitor Baf A1 (20 nM) for 12 h and then the immunofluorescence staining for LC3 and p62 was performed as described previously.[26] The nuclei were detected with DAPI (10 ng/ml) and the images were collected with a confocal microscope (Zeiss, LSM800).

2.7 | Immunoblot (IB)

C6 and U251 cells were treated with different concentrations of xanthatin, autophagy inducer Rapa (10 μM) or Torin 1 (20 nM), or autophagy inhibitor Baf A1 (20 nM) for 12 h, respectively. Protein lysates used for IB were harvested and lysed in a sample buffer containing 2% SDS followed by SDS-PAGE. Then the proteins were transferred to PVDF membranes and probed with the indicated antibodies, which were detected using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences 34095).

The glioma xenograft model received xanthatin (10, 20, 40 mg/kg) or TMZ (5 mg/kg) treatment as described in our previous studies.[23,26] Protein extraction from xenograft glioma tissue and IB analysis was performed as described previously.[26]

2.8 | Real-time quantitative polymerase chain reaction (qRT-PCR)

Total RNA in glioma cells was extracted using TRIzol reagent (Invitrogen, 15596018) followed by reverse transcription to synthesize cDNA. All gene transcripts were quantified by quantitative PCR with SYBR Green QPCR Master Mix (Toyobo, Osaka, Japan) and an ABI 7500 Fast real-time PCR system (Applied Biosystems, USA). The relative expression levels of gene transcripts were evaluated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control. The primers used in this study are listed in Table 1.

2.9 | Statistical analysis

The results are presented as the mean ± SEM. The one-way ANOVA followed by Dunnett's test was used to perform the statistical comparison. *p*-values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Xanthatin suppresses cell proliferation, colony formation, and induces apoptosis of glioma cells

In vitro cytotoxicity of xanthatin on glioma cells was evaluated by sulforhodamine B (SRB) colorimetric assay. As shown in Figure 1, xanthatin inhibited glioma cell proliferation in a dose- and time-dependent manner both in C6 and U251 cells (Figure 1A,B; Figure S1). Decreased tumor cell proliferation induced by xanthatin was also measured by proliferating cell nuclear antigen (PCNA),

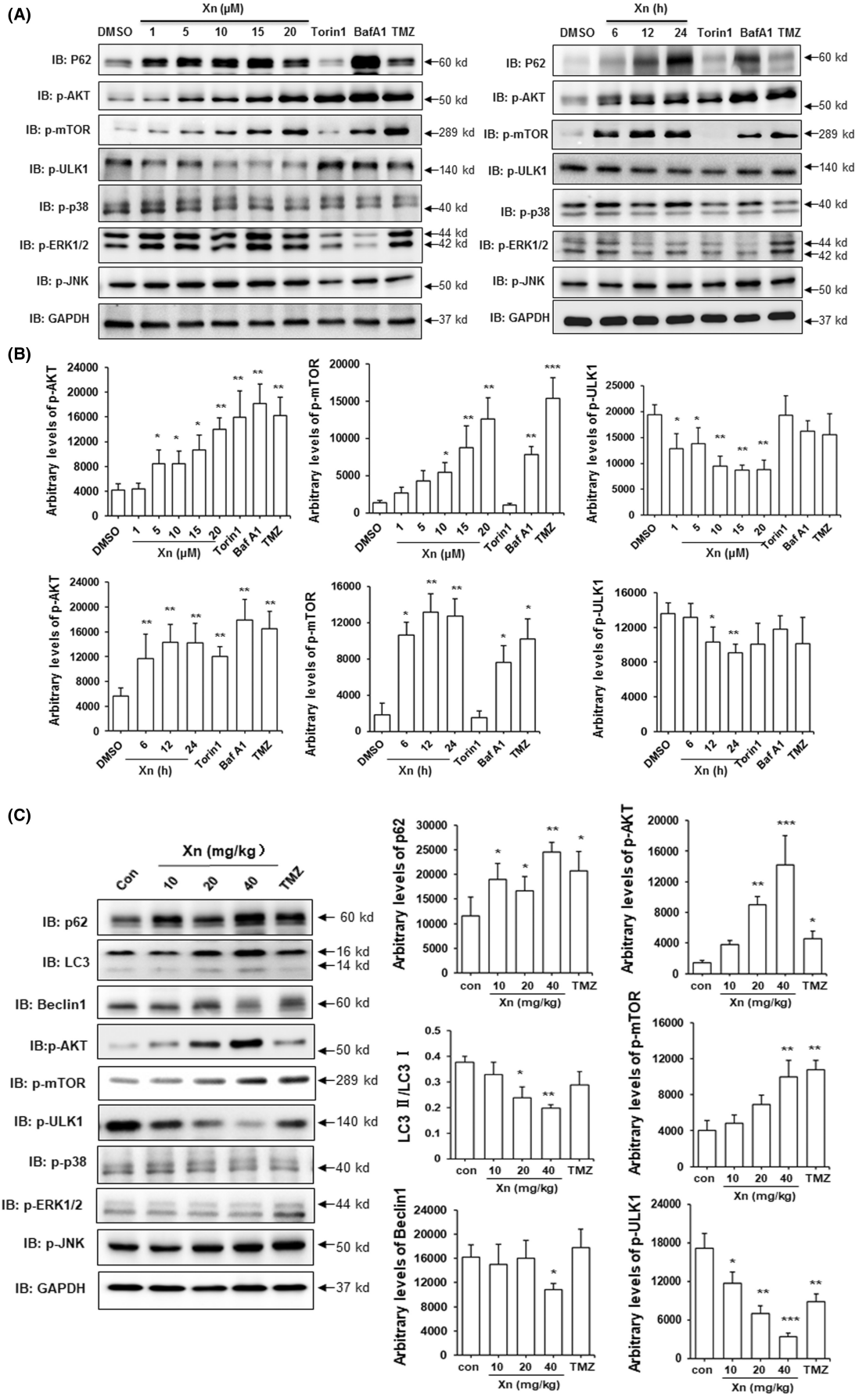

(A)
Xn (μM)
DMSO 1 5 10 15 20 Torin1 BafA1 TMZ
IB: P62
IB: p-AKT
IB: p-mTOR
IB: p-ULK1
IB: p-p38
IB: p-ERK1/2
IB: p-JNK
IB: GAPDH
60 kd
50 kd
289 kd
140 kd
40 kd
44 kd
42 kd
37 kd
Xn (h)
DMSO 6 12 24 Torin1 BafA1 TMZ
(B)
Arbitrary levels of p-AKT
Arbitrary levels of p-mTOR
Arbitrary levels of p-ULK1
Xn (μM)
Xn (h)
(C)
Xn (mg/kg)
Con 10 20 40 TMZ
IB: p62
IB: LC3
IB: Beclin1
IB:p-AKT
IB: p-mTOR
IB: p-ULK1
IB: p-p38
IB: p-ERK1/2
IB: p-JNK
IB: GAPDH
16 kd
14 kd
Arbitrary levels of p62
Arbitrary levels of p-AKT
LC3 II /LC3 I
Arbitrary levels of p-mTOR
Arbitrary levels of Beclin1
Arbitrary levels of p-ULK1
con 10 20 40 TMZ
Xn (mg/kg)

FIGURE 3 Xanthatin inhibits autophagy via PI3K/Akt/mTOR pathway in glioma (A) Immunoblots shows the phosphorylated protein levels of AKT, mTOR, ULK1, p38, ERK1/2, and JNK in C6 cells incubated with indicated concentrations of xanthatin for 12 h (Left) or 10 μM xanthatin for different times (Right). P62 levels were used as an indicator of autophagy inhibition. (B) The bar graphs represent a densitometric analysis of the immunoreactive blots in A. (C) Left, representative images of immunoblots for indicated proteins in xenograft tumors of nude mice treated with xanthatin (10, 20, 40 mg/kg) or TMZ (5 mg/kg). Right, quantitative analysis of p62, LC3, Beclin 1, phospho-AKT, phospho-mTOR, phospho-ULK1, phospho-p38, phospho-ERK1/2, and phospho-JNK in left panel. All the quantitative data was represented as mean ± SEM of at least 3 independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, vs. control. Abbreviation: Xn, xanthatin. Con, control.

a scaffolding protein involved in DNA replication (Figure 1C,D). Consistently, colony formation assay showed that, compared with the control group, C6 glioma cells treated with xanthatin (5 and 10 μM) formed significantly smaller and fewer colonies, suggesting that xanthatin remarkably inhibited cell growth and proliferation (Figure 1E,F). Similar results were assessed in U251 cells (Figure S1). Meanwhile, we found that the levels of cleaved poly-(ADP-ribose) polymerase (PARP) which is known to induce apoptosis were dramatically increased after xanthatin treatment in a dose-dependent manner (Figure 1G; Figure S1). Taken together, these results indicate that xanthatin has anti-proliferation and pro-apoptosis activity in glioma.

3.2 | Xanthatin inhibits autophagic flux by decreasing autophagosome formation in gliomas

Several reports have shown that autophagy was involved in the pro-cell death effect of some anticancer drugs. Here, to verify whether xanthatin was capable of regulating autophagy, we examined the expression of autophagy-related proteins in glioma cells. Treatment of glioma cells with xanthatin caused a dose- and time-dependent accumulation of LC3-I, whereas no significant increase of LC3-II, which was shown as the decrease in the LC3-II/LC3-I ratio (Figure 2A–C; Figure S2). As we know, relatively stable levels of LC3-II could be due to either inhibited conversion of LC3-I to LC3-II, an essential step in autophagosome formation, or increased LC3-II degradation. To answer this question, glioma cells were treated with xanthatin in the presence of the lysosomal inhibitor Baf A1, a vacuolar-type ATPase inhibitor that blocks autophagic degradation, followed by assessment of LC3 puncta which could visualize the formation of LC3-II. Figure 2D,F showed that cells treated with xanthatin did not show an appreciable change in LC3 puncta in cells (D2) compared with control groups (D1). In contrast, the number of LC3 puncta per cell in Baf A1 treated cells was significantly higher, as evidenced by the clear presence of red dots in cells (Figure 2D3,F). Interestingly, combining xanthatin and Baf A1 treatment resulted in less LC3 dots (Figure 2D4,F) compared with cells treated with Baf A1 alone (Figure 2D3,F). These results confirm that xanthatin inhibits autophagosome formation in C6 cells.

To further evaluate autophagy flux change, the turnover of autophagic substrate p62 (also known as SQSTM1) was detected in glioma cells. As we expected, gradually increased p62 levels were observed with regard to various xanthatin doses at different treatment times (Figure 2A–C; Figure S2). Consistently, the pronounced formation of p62 puncta was also observed in cells exposed to xanthatin in the presence or absence of Baf A1 (Figure 2E2,E4,F), which was similar to that in cells treated with Baf A1 alone (Figure 2E3,F), indicating a lack of autophagic degradation function in xanthatin-treated glioma cells.

To evaluate the inhibition effect of xanthatin on autophagy in vivo, we detected the expression tendency of LC3 and p62 in athymic mice bearing glioma xenografts received the intraperitoneal (i.p.) injection of xanthatin for 14 consecutive days. As shown in Figure 3C, xanthatin decreased the LC3-II to LC3-I ratio and increased p62 expression which is consistence with the findings in glioma cell lines. Thus, these data clearly demonstrate that xanthatin may act as an autophagy inhibitor, which suppresses autophagosome formation and decreases autophagic flux.

3.3 | Xanthatin suppresses the formation of autophagosome through down-regulating Beclin 1 and ATGs expressions

As we know, phagophore, autophagosome, and autophagolysosome formation are finely regulated by at least 30 autophagy-related proteins (Atg). Except lipidation of LC3, Atg5, Atg7, Atg12, and Beclin 1 (mammalian homolog of Atg 6) also participate in the early stages of this process. Immunoblots analysis revealed that the levels of Beclin-1 were decreased in glioma cell lines and athymic mouse xenografts treated with xanthatin (Figures 2A–C, 3C; Figure S2). Moreover, ATG5 and ATG7 protein levels were decreased in C6 cells (Figure 2A–C). Consistent with these, mRNA expressions of *BECN1*, *ATG5*, *ATG7*, and *ATG12* were also decreased in U251 cells exposed to xanthatin (Figure S2). Taken together, xanthatin inhibits autophagosome formation in glioma cells through downregulating Beclin 1 and ATGs.

3.4 | Xanthatin suppresses autophagy by activating PI3K/Akt/mTOR pathway in gliomas

The PI3K/Akt pathway is a major upstream activator of mTOR and has been implicated in the regulation of autophagy. As shown in Figure 3, xanthatin increased the phosphorylation of Akt (ser473), a downstream target of PI3K, then directly activates mTORC1 via the phosphorylation of mTOR at Ser2448 in (A, B). ULK1, like its

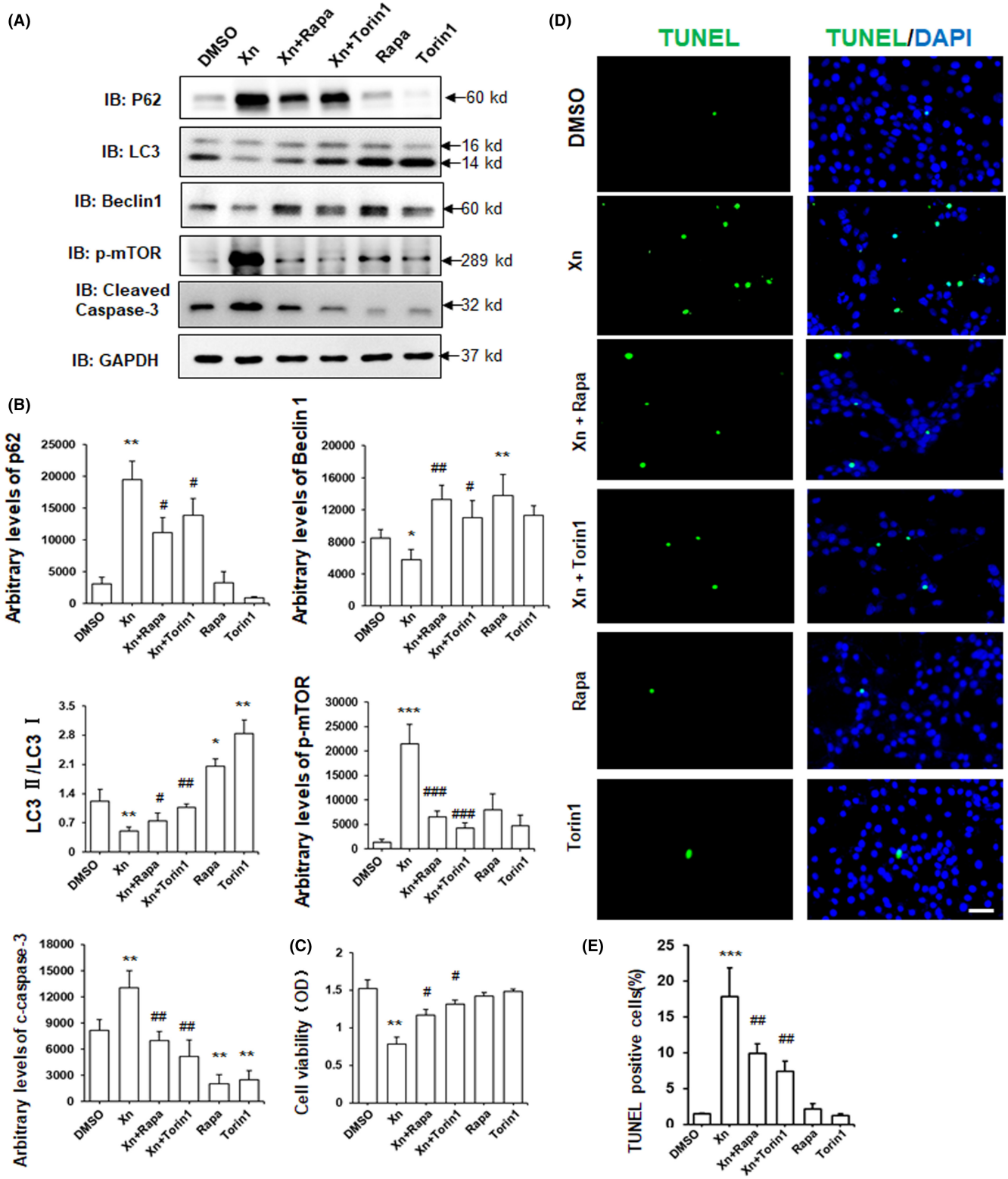


FIGURE 4 Autophagy induction partially reverses xanthatin-induced glioma cell death. (A) Representative images of immunoblots for selected proteins involved in autophagy (Beclin 1, p62, LC3, phosphor-mTOR) and cell apoptosis (cleaved caspase-3) in C6 cells incubated with 10 μM xanthatin for 12 h in the presence or absence of autophagy inducer Rapa (10 μM) or Torin1 (20 nM). GAPDH was used as a loading control. (B) Relative density of p62, LC3, Beclin 1, p-mTOR, and cleaved caspase-3 were determined by densitometry of the blots. (C) SRB assay shows the viability of C6 cells treated with xanthatin (15 μM), Rapa (10 μM), or Torin1 (20 nM) as indicated for 12 h. (D) TUNEL staining was used to show apoptosis of C6 cells treated with xanthatin (15 μM) for 12 h, with or without Rapa (10 μM), Torin1 (20 nM). (E) Quantitative analysis of TUNEL positive C6 cells in D. All the quantitative data was represented as mean ± SEM of three independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, vs. control. $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$, vs. xanthatin group. Scale bars = 50 μm. Con, control; Xn, xanthatin.

yeast homolog Atg1, is a key initiator of autophagy that is negatively regulated by the mTOR kinase. Our results showed that xanthatin treatment reduced phospho-ULK1 levels in a dose- and time-dependent manner (Figure 3A,B). Consistently, xanthatin significantly upregulated the levels of phospho-Akt and phospho-mTOR, but decreased the expression of phospho-ULK1 in athymic mice bearing glioma xenografts (Figure 3C). To gain insight into the signaling pathway involving the autophagy modulation, we also assessed the effects of xanthatin on MAPK signaling pathway. Interestingly, no significant changes in the downstream molecules expression of the MAPK pathway including p-ERK1/2 (thr202/tyr204), p-JNK (thr183/tyr188), and p-p38 (thr180/tyr182) were observed in xanthatin-treated C6 cells and implanted gliomas (Figure 3A–C). These results suggested that the PI3K/Akt/mTOR signaling pathway plays a central role in regulating the autophagy process promoted by xanthatin.

3.5 | Autophagy induction reverses xanthatin-induced glioma cells apoptosis

We tested the glioma cells' viability in the presence of autophagy inducers, Rapa or Torin 1 to assess the involvement of autophagy in the anti-proliferative and pro-apoptosis effect of xanthatin. As expected, treatment with xanthatin combined with autophagy inducers Rapa and Torin1 engendered increased LC3-II and decreased p62 expression compared to xanthatin alone (Figure 4A,B). Meanwhile, combined treatment with Rapa or Torin1 increased cell proliferation as monitored by SRB assay compared with xanthatin alone (Figure 4C). The increased cell apoptosis evidenced by elevated cleaved caspase-3 level and the number of TUNEL-positive cells induced by xanthatin was also partially reversed by Rapa or Torin1 treatment (Figure 4A,B,D,E). These results suggested the anti-proliferation and pro-apoptosis activity of xanthatin against glioma cells depends largely on its ability to inhibit autophagy.

3.6 | The anti-proliferation and pro-apoptosis effect of xanthatin was partially abolished by the dual PI3K and mTOR inhibitor NVP-BEZ235

Dual PI3K/mTOR inhibitor NVP-BEZ235 was applied to further confirm PI3K/Akt/mTOR pathway is mainly responsible for the pro-apoptotic and anti-tumor effect of xanthatin. Compared with xanthatin alone, treatment with xanthatin plus NVP-BEZ235 increased cell proliferation as monitored by SRB assay and colonial cellular density (Figure 5A,B). Moreover, xanthatin combined with NVP-BEZ235 decreases TUNEL-positive apoptotic cells compared with xanthatin alone (Figure 5C). However, NVP-BEZ235 itself had no effect on glioma cell proliferation and apoptosis although it induced autophagy to some extent (Figure 5). Taken together, NVP-BEZ235 induced autophagy activation could partially reverse the tumor-suppressive effect of xanthatin.

4 | DISCUSSION

Although xanthatin has been implicated as an antitumor therapy in glioma, the molecular mechanism underlying its antitumor effect has remained obscure. Here, we provide strong evidence that xanthatin inhibits glioma cells proliferation and promotes apoptosis through autophagy inhibition via activating the PI3K/Akt/mTOR pathway (Figure 6). Therefore, utilizing xanthatin may be accounted as an alternative therapy against glioma.

Autophagy has been proposed as a "double-edged sword" in tumors: enhancing or blocking tumor survival, depending on the stages of the tumor and various tumor tissues.[6,7] Basal autophagy has been harnessed by some tumor cells as a survival mechanism to protect against ischemia and signals that induce apoptosis. Accumulating evidence shows that the induction of autophagy is crucial for glioma initiation and growth.[27,28] In this study, we found that xanthatin obviously suppressed autophagy which was determined by the increased P62 level coincided with the decreased Beclin-1 and LC3-II to LC3-I ratio. Interestingly, xanthatin induced an increase in LC3-I while LC3-II is relatively stable, suggesting that the transition from LC3-I to LC3-II was hampered, which was further verified by the absence of LC3 fluorescent puncta in cells combined with xanthatin and lysosome inhibitor Baf A1 treatment. But Geng's group showed that xanthatin triggers autophagy as evidenced by autophagosome accumulation in human colon cancer cells.[29] The reason for the different phenomena in tumor cells treated with xanthatin remains obscure. We suspect that it may depend on the stage of tumor type, the degree of tumor progression, oncogenic drivers, and the intensity of the activating signal.

There are many pathways involved in autophagy in cancer cells. Especially, the PI3K/AKT/mTOR pathway has attracted extensive attention as the modulator of autophagy.[30] In many cellular settings, triggering of autophagy relies on the inhibition of the mammalian target of rapamycin complex 1 (mTORC1), an event that promotes the activation of several autophagy proteins (Atgs) involved in the initial phase of membrane isolation.[6,31] Enlargement of this complex to form the autophagosome requires the participation of 2 ubiquitin-like conjugation systems. One involves the conjugation of ATG12 to ATG5 and the other of phosphatidylethanolamine to LC3.[6,31] ATG7 is an autophagy-related E1-like enzyme that is essential for two ubiquitination-like reactions, ATG12-conjugation and LC3-lipidation.[31,32] The ATG12-ATG5 conjugate forms a complex with ATG16 to act as an E3-like enzyme for the formation of LC3-PE, which binds to the autophagosome membrane.[32] Reduced expression of ATG5 and ATG7 in xanthatin-treated glioma cells possibly explains the reasons why xanthatin significantly inhibits the autophagosome formation as evidenced by increased LC3-I and unregulated LC3-II expression.

We further proved that the anti-proliferation effects of xanthatin against glioma are associated with PI3K/AKT/mTOR-mediated autophagy. A combination of autophagy inducer Rapa or torin1 renders glioma cells resistant to xanthatin antitumor action to a certain extent, accompanied by an increased expression level of LC3-II, while

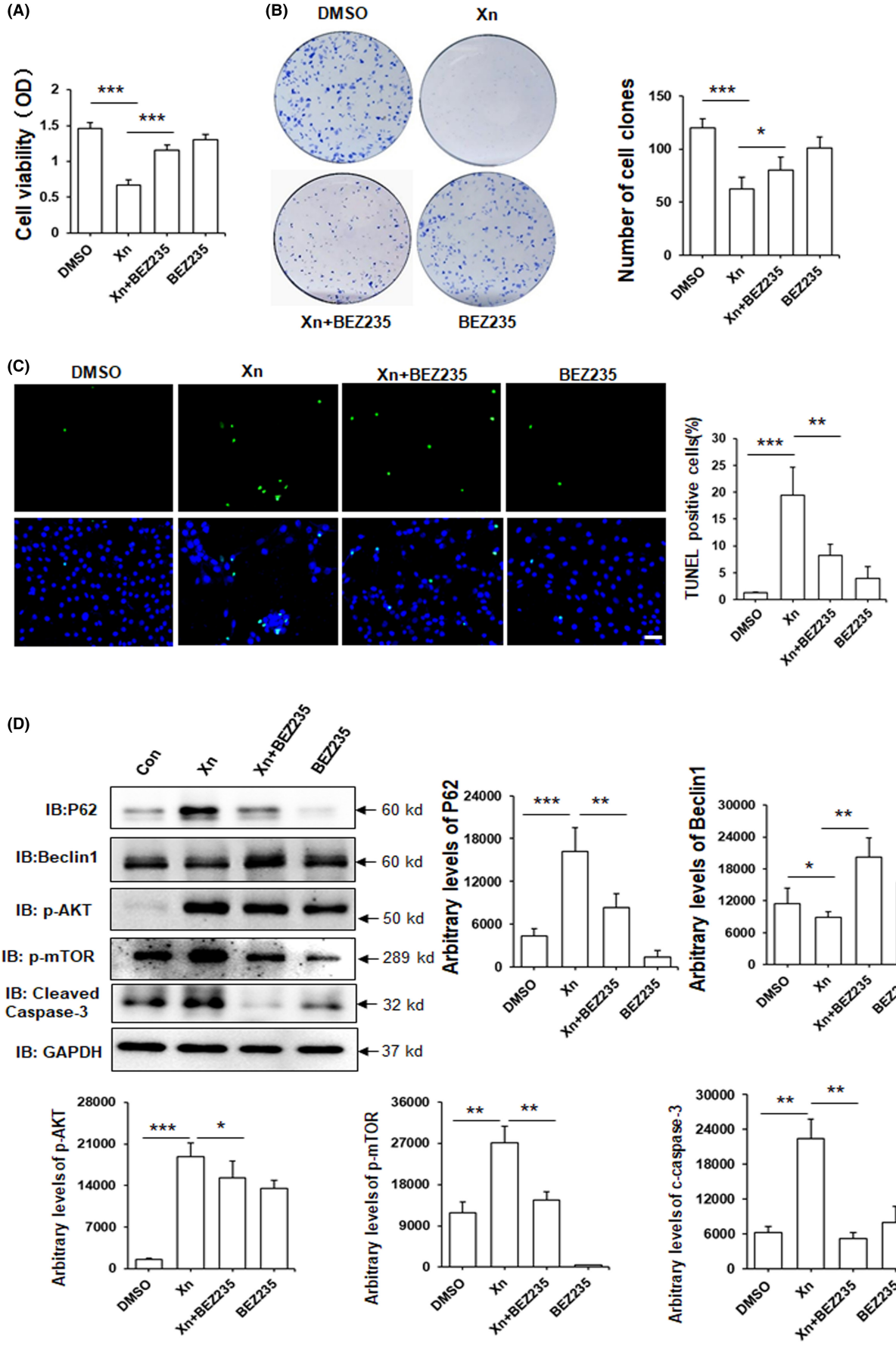
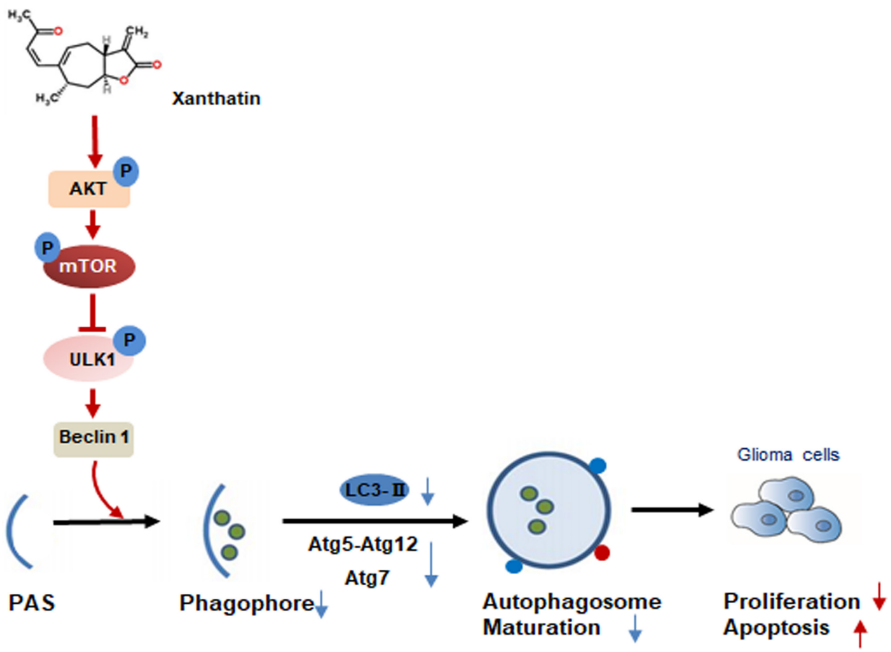

(A)
Cell viability (OD)
DMSO
Xn
Xn+BEZ235
BEZ235
(B)
DMSO
Xn
Xn+BEZ235
BEZ235
Number of cell clones
(C)
DMSO
Xn
Xn+BEZ235
BEZ235
TUNEL positive cells(%)
(D)
Con
Xn
Xn+BEZ235
BEZ235
IB:P62
IB:Beclin1
IB: p-AKT
IB: p-mTOR
IB: Cleaved Caspase-3
IB: GAPDH
60 kd
60 kd
50 kd
289 kd
32 kd
37 kd
Arbitrary levels of P62
Arbitrary levels of Beclin1
Arbitrary levels of p-AKT
Arbitrary levels of p-mTOR
Arbitrary levels of c-caspase-3

FIGURE 5 Combined PI3K and mTOR inhibitor NVP-BEZ235 partially ameliorated the anti-proliferation effect of xanthatin against glioma cells. (A) SRB assay shows the proliferation of C6 cells treated with xanthatin (10 μM), NVP-BEZ235 (250 μM), or a combination of xanthatin and NVP-BEZ235 for 48 h. (B) Representative images showing the synergistic effects of NVP-BEZ235 (250 μM) and xanthatin (10 μM) on colony formation of C6 cells, and quantification of colony numbers ($n = 3$). (C) TUNEL staining of C6 cells treated with xanthatin and/or NVP-BEZ235 as indicated concentrations for 12 h. Quantitative analysis of TUNEL-positive cells is also exhibited. (D) Immunoblots result shows the levels of p62, Beclin 1, phospho-mTOR, phospho-AKT, and cleaved caspase-3 in C6 cells incubated with xanthatin (10 μM) and/or NVP-BEZ235 (250 μM) for 12 h. The bar graphs represent a densitometric analysis of the immunoreactive blots. All the quantitative data was represented as mean ± SEM of at least 3 independent experiments. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$. Scale bar = 50 μm. Abbreviation: Xn, xanthatin. Con, control.

FIGURE 6 Schematic diagram of xanthatin suppresses glioma cells proliferation and tumorigenicity via inhibiting autophagy. Xanthatin activates the PI3K/Akt/mTOR signaling pathway which exerts an inhibitory effect on the formation of autophagosome by dysregulating Beclin 1 and ATGs. Autophagy induction with the autophagy inducer or dual PI3K and mTOR inhibitor partially ameliorates the antitumor effects of xanthatin. PAS, pre-autophagosomal structure.



the decreased expression level of p62. Also, a similar phenomenon was observed for C6 glioma cells treated with PI3K and mTOR dual inhibitor NVP-BEZ235, suggesting that PI3K/Akt/mTOR-mediated autophagy is at least partially responsible for the anti-proliferation and anti-tumorigenic effects of xanthatin in glioma.

mTOR signaling pathway has been identified as a key driver of carcinogenesis in several cancer types. Targeting the PI3K/AKT/mTOR signaling pathway is an important therapeutic strategy for a variety of tumors.[30,33] In this study, we found xanthatin suppress glioma cells proliferation through autophagy inhibition via activation of the PI3K-Akt–mTOR pathway. These findings seem to be paradoxical to the common paradigm that the growth and progression of tumor are linked to the activation of mTOR. In fact, mTORC1 could directly control cell growth and proliferation by modulating its downstream targets 40S ribosomal S6 kinases (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), which is not dependent on autophagy induction.[34] The relationship between the mTOR signaling pathway and tumor development in autophagy is still complex. A recent study also found that loss of mTOR, most likely mTORC1, promotes liver tumorigenesis in autophagy-deficient mice, which indicated that both hyper- and hypo-activation of mTOR are detrimental to the normal physiological functions resulting in the development of tumors.[35] Therefore, the relationship between glioma cell proliferation and autophagy regulated by PI3K/Akt/mTOR may be interdependent and interactive, which needs to be further confirmed.

Taken together, our study uncovers a more detailed mechanism by which xanthatin inhibits the proliferation and tumorigenicity of malignant glioma which might offer a possible molecular basis for the further development of xanthatin as a promising drug for glioma treatment. However, our study also has certain limitations. First, this study is mainly performed in cell lines or cell line-based xenograft models. Although cell lines have been used successfully for target discovery and mechanistic studies, they have limitations in mimicking the complex responses of drugs in vivo. To overcome these limitations, better animal models can be further utilized, such as patient-derived xenograft (PDX) models. Second, regarding the complex role of autophagy in the tumor, additional studies are needed to confirm the effect of xanthatin on autophagy at various stages and types of glioma. The roles of xanthatin in the different tumor microenvironment have important guiding significance for clinical intervention. Third, although hyperactivation of PI3K/Akt/mTOR signaling is commonly observed in several cancers, the non-kinase functions of this signaling were not been fully explored yet. If other PI3K/Akt/mTOR

signaling components exert critical roles in tumorigenesis and if this function is influenced by xanthatin remains a challenging question to answer. Nevertheless, the data now gathered in this study might pave do way to evolve for future studies in more complex systems.

5 | CONCLUSION

The present study demonstrates that xanthatin effectively inhibits the proliferation and tumorigenicity of malignant glioma cells mainly by suppressing autophagy through activating the PI3K/Akt/mTOR pathway. This prompts a new mechanistic pathway for the further development of xanthatin as a promising drug for glioma treatment, alone or combined with other conventional chemotherapy drugs.

AUTHOR CONTRIBUTIONS

Conceived and designed the research: Lijie, Yuxian, and Yongqiang. Conducted experiments: Huaqing, Tong, Wenshuang, Xiaojie, Zemin, Yuyang, and Min. Performed data analysis: Tong, Xiaojie, Sixing, and Yujun. Wrote or contributed to the writing of the manuscript: Lijie and Min.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

FUNDING STATEMENT

This work was supported by the National Natural Science Foundation of China (81973337 to LJF); the Scientific research Level promotion Project of Anhui Medical University (2020\u00D7kjT002 to LJF); and the Key Project of University Excellent Talents Support Program in Anhui (gxyqZD2020010 to LJF).

DISCLOSURE

The authors declare that they have no conflicts of interest with the contents of this article.

ETHICS STATEMENT

This study was approved by the Institutional Animal Care and Use Committee at Anhui Medical University, Hefei Province, China.

SIGNIFICANCE STATEMENT

The purpose of this study is to better understand the underlying mechanisms of xanthatin, a natural sesquiterpene lactone from xanthium strumarium L., on glioma therapy. Our findings found that xanthatin significantly suppressed glioma cell proliferation and induced apoptosis by inhibiting autophagy via activation of the PI3K-Akt–mTOR pathway. It is a powerful complement to the current mechanisms of the anti-tumor effect of xanthatin and highlights the importance of targeting autophagy in new therapeutics for glioma patients.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Chen H, Zhu T, Huang X, et al. Xanthatin suppresses proliferation and tumorigenicity of glioma cells through autophagy inhibition via activation of the PI3K-Akt–mTOR pathway. *Pharmacol Res Perspect*. 2023;11:e01041. doi:10.1002/prp2.1041