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Mollugin induces tumor cell apoptosis and autophagy via the PI3K/AKT/mTOR/p70S6K and ERK signaling pathways

Li Zhang, Handong Wang *, Jianhong Zhu, Jianguo Xu, Ke Ding

Department of Neurosurgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province, China

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ABSTRACT

Mollugin, a bioactive phytochemical isolated from *Rubia cordifolia* L., has shown preclinical anticancer efficacy in various cancer models. However the effects of mollugin in regulating cancer cell survival and death remains undefined. In the present study we found that mollugin exhibited cytotoxicity on various cancer models. The suppression of cell viability was due to the induction of mitochondria apoptosis. In addition, the presence of autophagic hallmarks was observed in mollugin-treated cells. Notably, blockade of autophagy by a chemical inhibitor or RNA interference enhanced the cytotoxicity of mollugin. Further experiments demonstrated that phosphatidylinositide 3-kinases/protein kinase B/mammalian target of rapamycin/p70S6 kinase (PI3K/AKT/mTOR/p70S6K) and extracellular regulated protein kinases (ERK) signaling pathways participated in mollugin-induced autophagy and apoptosis. Together, these findings support further studies of mollugin as candidate for treatment of human cancer cells.

1. Introduction

Rubia cordifolia L., also known as Madder or Indian Madder, is usually used as traditional herbal medicine to treat cough, inflammation of the joints and uteritis in Korea [1]. In addition, this plant is also used for treatment of arthritis, dysmenorrheal and hemostasis in traditional Chinese medicated diet (CMD) [2]. Mollugin is a bioactive phytochemical extracted from the roots of *R. cordifolia* L. This drug was originally developed as an anti-inflammation and neuroprotective agent [3,4]. Furthermore, mollugin has also shown preclinical anticancer efficacy in various cancer models [5–7]. The mechanisms by which mollugin kills cancer cells are still unclear. Several mechanisms such as induction of endoplasmic reticulum (ER) stress, activation of NF-E2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway and suppression of fatty acid synthase (FAS) have been proposed to account for the cytotoxicity of mollugin.

Autophagy is the process by which cells eliminate their long-lived proteins and damaged organelles [8]. Under stress situations, autophagy plays a key pro-survival role in cells by providing the nutrients to maintain the metabolism. However, extensive activation of autophagy can also lead to cell death [9]. Recent studies have shown that some agents known to induce apoptosis also activate autophagy. However the interplay between autophagy and apoptosis is intricate. Inhibition of autophagy can protect cells from undergoing apoptosis [10,11]. However, in other cases, autophagy can also kill cells by promoting apoptosis [12,13]. The discrepancies may be due to the complex and diverse interplays between autophagy and apoptosis and further studies are needed to clarify their relationship.

In the present study, we reported that mollugin induced cell death and autophagy in various cancer models. Furthermore, mollugin induced mitochondria apoptosis in human glioblastoma cells. Inhibition of autophagy significantly enhanced the apoptosis-inducing ability. We also found that the induction of apoptosis and autophagy was associated with inhibition of the PI3K/AKT/mTOR/p70S6K and ERK signaling pathways. This finding may provide an initial evidence for the combination use of mollugin and autophagy inhibitors as a novel therapeutic strategy for glioblastoma treatment.

2. Material and methods

2.1. Cell lines

Primary mouse cortical neurons were prepared as described previously [14]. U251MG, U87MG, MKN45, MCF-7, A549 and HT29 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). U251MG and U87MG were

* Corresponding author. Address: Department of Neurosurgery, Jinling Hospital, 305 East Zhongshan Road, Nanjing 210002, Jiangsu Province, China. Fax: +86 25 84817581.
E-mail address: njhdwang@hotmail.com (H. Wang).

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cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (HyClone, USA) at 37 °C and 5% CO_2 incubator. MKN45, MCF-7, A549 and HT29 were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin at 37 °C and 5% CO_2 incubator.

2.2. Reagents

Mollugin was purchased from Tauto Biotech (Shanghai, China). U0126, SP600125, SB203580, LY294002, 3-methyladenine (3-MA), bafilomycin A1 (BafA1) and acridine orange (AO) were purchased from Sigma (St. Louis, MO, USA). Z-VAD-FMK (Z-VAD) was purchased from Abcam (Cambridge, MA, USA). Anti-microtubule-associated protein 1 light chain 3 (LC3) and anti-Beclin 1 antibodies were purchased from Novus Biological (LLC, USA). Anti-p62, anti-cytochrome c and anti-Bcl-2-associated X protein (Bax) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-phosphorylated ERK, anti-ERK, anti-phosphorylated JNK, anti-JNK, anti-phosphorylated p38, anti-p38, anti-phosphorylated AKT, anti-AKT, anti-phosphorylated mTOR, anti-mTOR, anti-phosphorylated p70S6K, anti-p70S6K, anti-caspase-3, anti-caspase-8, anti-poly(ADP-ribose) polymerase (PARP), anti-autophagy-related 3 (Atg3), anti-autophagy-related 5 (Atg5), anti-autophagy-related 7 (Atg7), anti-autophagy-related 12 (Atg12), anti-B-cell lymphoma 2 (Bcl-2), anti-B-cell lymphoma-extra large (Bcl-xL), anti-β-actin and goat anti-rabbit IgG(H&L)-HRP secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.3. Cell viability assay

Cell viability was assessed by CCK-8 assay (Beyotime, Shanghai, China). Briefly, cells were seeded into 96-well plates at 5×10^3 cells per well and cultivated for 24 h to adhere. After treatment, 10 μl of kit reagent was added to the cells followed by incubation for 2 h. Then the OD value was read at 450 nm using a Bio-Rad ELISA microplate reader (Bio-Rad Laboratories, CA, USA). The viability rate of cells equals (the OD values of treated groups/the OD values of control group) × 100%.

2.4. Western blot analysis

After treatment, cells were harvested and lysed. An equal amount of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 10%) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dried milk for 2 h, the membrane was incubated with the primary antibodies overnight at 4 °C. Then the immunoreactive bands were visualized by enhanced chemiluminescence using HRP-conjugated IgG secondary antibodies.

2.5. Immunofluorescence

Treated cells were fixed by 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and then blocked with immunol staining blocking buffer (Beyotime, Shanghai, China). Subsequently, cells were incubated with LC3 antibody overnight at 4 °C. Following washes with phosphate-buffered saline (PBS), the coverslips were incubated with a red-labeled secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 4 h at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The staining was examined using a fluorescence microscope.

2.6. Flow cytometric (FCM) analysis of apoptosis

After treatment, cells were trypsinized, washed with PBS and suspended with 500 μl of binding buffer containing 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) (BD Biosciences, San Jose, CA, USA). After incubation for 15 min at room temperature in the dark, cells were subjected to flow cytometry assay. Flow cytometry was performed using a FACSCanto 6-color flow cytometer (BD Biosciences, San Jose, CA).

2.7. Measurement of the formation of acidic vesicular organelles (AVOs)

Treated cells were stained with 1 μg/ml AO for 15 min at 37 °C. After incubation, cells were washed three times with PBS and observed immediately under a fluorescence microscope.

2.8. Transmission electron microscopy (TEM)

For the TEM analysis, treated cells were trypsinized, rinsed twice with warm PBS (37 °C) and then fixed for 1 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 1% sucrose. After washing with PBS, the cells were postfixed in 1% osmium tetroxide (OsO_4) and embedded in Epon; 0.1 mm thin sections were stained with uranyl acetate/lead citrate (Fluka, St. Louis, MO, USA) and viewed in a JEOL JEM-1011 TEM (Tokyo, Japan).

2.9. RNA interference with shRNA

The lentivirus vectors were purchased from GenePharma Co., Ltd. (Shanghai, China). Lentiviruses vectors for expression of scrambled or Beclin-1 shRNA were diluted in medium containing 5 ug/ml polybrene. Cells were plated and transfected with lentiviruses. After 72 h, transfected cells were selected using puromycin (5 ug/ml) for 24 h. The human Beclin-1 shRNA sequence was 5′-GAA TGT CAG AAC TAC AAA CGC TGT T-3′, the scrambled shRNA sequence was 5′-TTC TCC GAA CGT GTC ACG T-3′. Western blotting analysis was performed to validate the knockdown efficiency.

2.10. Statistical analysis

The SPSS19.0 software package was used to perform all statistical analysis. Comparisons between two groups were performed using the Student's t test and between multiple groups using ANOVA analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Mollugin inhibited cell viability and induced mitochondria apoptosis in glioblastoma cells

To examine the cytotoxicity of mollugin on glioblastoma cells, U251MG and U87MG cells were treated with different concentrations of mollugin for different time points. Cell viability assays showed that mollugin induced cell death in concentration- and time-dependent manners (Fig. 1A). Importantly, mollugin alone did not affect cell viability of mouse primary neurons (Fig. 1A). In addition, mollugin showed cytotoxic effect in various cancer cell lines including MKN45 (gastric cancer cell), MCF-7 (breast cancer cell), A549 (lung cancer cell) and HT29 (colon cancer cell) (Fig. 1A).

To determine whether the reduced cell viability was due to apoptosis or necrosis, flow cytometry analysis for Annexin V/PI was used. As shown in Fig. 1B, a significant increase in the apoptotic population was observed in cells treated with mollugin.

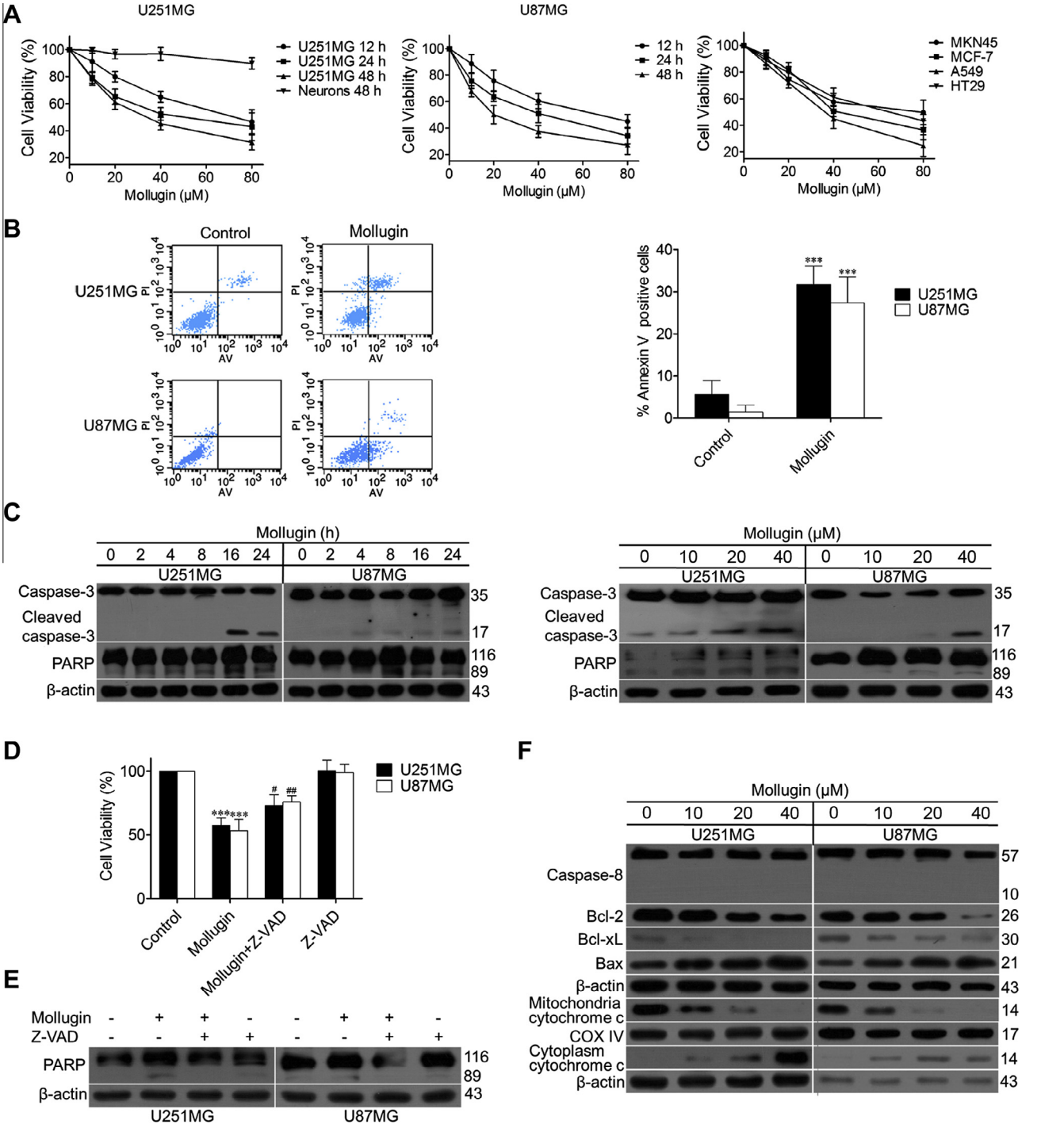

Fig. 1. Mollugin inhibited cell viability and induced mitochondria apoptosis in cancer cells. (A) Left and middle: U251MG, U87MG cells and mouse primary astrocytes were treated with mollugin at the indicated concentrations for different time points, and the cell viability was analyzed by CCK-8 assay. Right: human cancer cell lines MKN45, MCF-7, A549 and HT29 were treated with mollugin at the indicated concentrations for 48 h, and the cell viability was analyzed by CCK-8 assay. All values represent the means ± SD of three independent experiments. (B) Cells were treated with DMSO (control) or 40 μM mollugin for 24 h, and cell death was determined by flow cytometry followed by Annexin V/PI staining. Apoptotic: Annexin V-positive cells. The data are presented as mean ± SD from three independent experiments. $^{***}P < 0.001$ vs. control cells. (C) Cells were exposed to mollugin for various periods at a concentration of 40 μM or for 24 h at various concentrations. After treatment, the expression levels of caspase-3 and PARP were detected by western blotting. (D) Cells were pre-treated with 25 μM Z-VAD for 1 h followed by the treatment of 40 μM mollugin for another 24 h. The cell viability was analyzed. Columns: mean of three independent experiments; bars: SD. $^{***}P < 0.001$ vs. control cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. cells treated with mollugin alone. (E) Cells were treated was described in (D). The cleavage of PARP was analyzed. (F) Cells were treated with mollugin for 24 h at increasing concentrations and the protein levels of caspase-8, Bcl-xL, Bcl-2, Bax and cytochrome c were measured. COX IV was used as a loading control for mitochondria extracts. β-actin was used as a loading control for cytoplasmic and whole cell extracts.

Moreover, mollugin obviously induced cleavage of caspase-3 and PARP (Fig. 1C), confirming the induction of apoptosis. To further demonstrate that mollugin induced caspase-dependent apoptosis, cells were co-treated with mollugin and the pan-caspase inhibitor Z-VAD. Results showed that Z-VAD treatment suppressed not only cell death but also cleavage of caspase-3 and PARP triggered by mollugin (Fig. 1D and E). Depending on various cell death stimuli, apoptosis can be divided into mitochondrial death pathway and

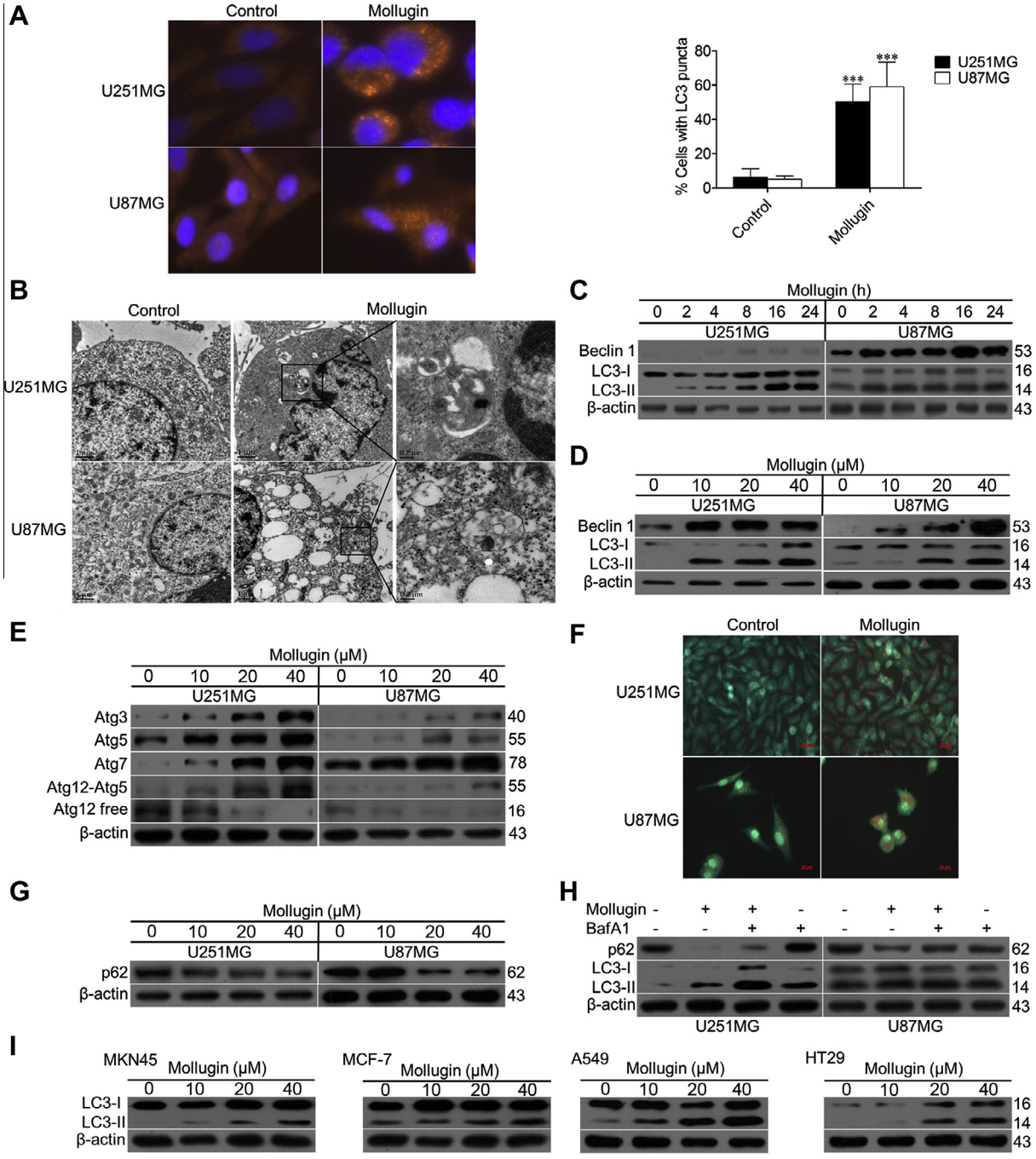

Fig. 2. Mollugin induced autophagy in human glioblastoma cells. (A) Cells were treated with DMSO (control) or 40 μM mollugin for 24 h. After treatment, cells were stained with LC3 antibody and appropriate secondary antibody and observed under a fluorescence microscope, the nuclear was stained with DAPI. The percentage of cells presenting typical LC3 puncta (>5 puncta per cell) was depicted. Data were means ± SD from three independent experiments (at least 100 cells were counted for each experiment; $^{***}P < 0.001$ vs. control cells). (B) Cells were treated as described above, then cells were harvested and subjected to transmission electron microscopy as described in Section 2. (C and D) Cells were treated with mollugin for various periods at a concentration of 40 μM (C) or exposed to various concentrations of mollugin for 24 h (D), then western blotting analysis was performed to detect the expression of Beclin 1 and LC3. (E) Cells were treated with mollugin for the indicated concentrations at 24 h and subjected to western blotting. (F) Cells were treated as described in (A). AVOs induced by mollugin were stained with AO. Bar, 20 μm. (G) Cells were treated with mollugin for various concentrations in 24 h, the expression of p62 was detected by western blotting. (H) Cells were treated with 40 μM mollugin for 24 h in the presence or absence of 10 nM BafA1. Protein levels of p62 and LC3 were detected. (I) MKN45, MCF-7, A549 and HT29 cells were treated with mollugin for the indicated concentrations at 24 h. The expression of LC3 was determined by western blotting. β-actin was used as loading control.

death receptor pathway. In order to investigate which pathway was involved in mollugin-induced apoptosis, we evaluated several apoptosis indicators such as Bcl-xL, Bcl-2, Bax, cytochrome c and caspase-8. As shown in Fig. 1F, mollugin down-regulated Bcl-2 and Bcl-xL, up-regulated Bax and released cytochrome c from mitochondria without affecting caspase-8, suggesting that mitochondrial apoptosis was activated. Collectively, these data indicated mollugin induced cell death in human glioblastoma cells through the mitochondrial apoptosis pathway.

3.2. Mollugin induced autophagy in glioblastoma cells

We next determined whether mollugin affected the autophagy pathway. To achieve this, we first used immunofluorescence.

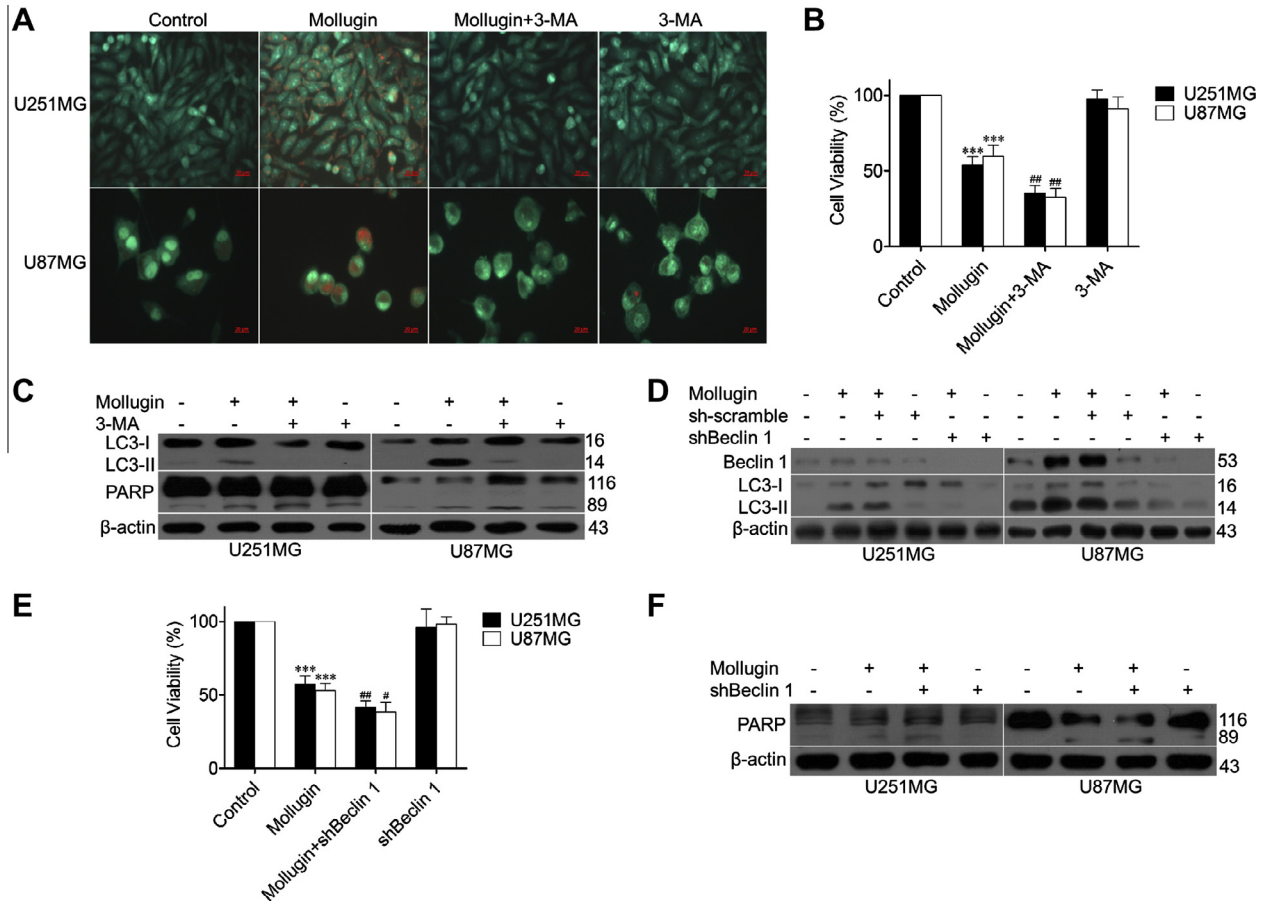

Fig. 3. Inhibition of autophagy enhanced the anticancer effect of mollugin. (A) Cells were exposed to 40 μM mollugin in the presence or absence of 3 mM 3-MA for 24 h. Then cells were stained with AO and observed under a fluorescence microscope. Bar, 20 μm. (B and C) Cells were treated as described above, the cell viability (B) and the expression of LC3 and PARP (C) were analyzed. (D) Cells were transfected with either Beclin-1 shRNA or a non-target control shRNA for 72 h and then co-treated with 40 μM mollugin for another 24 h, the expression of Beclin 1 and LC3 was verified. (E and F) After transfection, cells were treated with 40 μM mollugin for another 24 h, and the cell viability (E) and the cleavage of PARP (F) were analyzed. Columns: mean of three independent experiments; bars: SD. $^{***}P < 0.001$ vs. control cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. cells treated with mollugin alone. β-actin was used as an internal control.

Results of the fluorescence microscopy revealed that mollugin-treated cells exhibited a significant increase in the number of cells with LC3 punctate dots compared to control cells (Fig. 2A). In addition, TEM analysis showing autophagic vacuoles containing cellular material or membranous structures in mollugin-treated cells further confirmed the induction of autophagy (Fig. 2B). Meanwhile, mollugin treatment increased the expression of Beclin 1, LC3-II and some other autophagy-related proteins (Fig. 2C–E).

Induction of autophagy includes not only an increase of autophagosome, but also an up-regulation of autophagic flux. To examine the autophagic flux, we first analyzed the formation of AVOs by AO staining. Fig. 2F showed that mollugin treatment significantly induced the formation of red fluorescent AVOs compared to control cells. During autophagy, p62 is wrapped into autophagosome and degraded in autolysosome. Then, we determined the expression of p62 in mollugin-treated cells. Results of western blotting analysis revealed that mollugin reduced the amount of p62 in a concentration-dependent manner (Fig. 2G). We further examined the autophagic flux by LC3 turnover assay. Treatment with BafA1, which blocks autolysosome formation, led to an increase in LC3-II levels. The BafA1-induced increase of LC3-II was augmented when combined with mollugin (Fig. 2H). Coincident with the enhanced LC3 turnover, the mollugin-induced reduction of p62 was prevented by BafA1 (Fig. 2H). Together, these data illustrated that mollugin induced autophagy in glioblastoma cells.

3.3. Mollugin induces autophagy in various types of cancer cells

Mollugin has been shown to induce apoptosis in various cancer cell lines [5–7], indicating that the apoptosis-inducing ability of mollugin was not cell-specific. Then to exclude the possibility that mollugin-induced autophagy was specific to glioblastoma cells, we treated different cancer cell lines (MKN45, MCF-7, A549 and HT29) with various concentrations of mollugin. Results revealed that mollugin also induced conversion of LC3B in all the cell lines we tested (Fig. 2I).

3.4. Autophagy was not required for mollugin-induced apoptosis

We then seek to understand the role of autophagy played in mollugin-induced apoptosis. To this end, we first used autophagy inhibitor 3-MA. As shown in Fig. 3A and C, 3-MA evidently reversed autophagy induction by mollugin. Conversely, 3-MA enhanced the cell death and apoptosis caused by mollugin (Fig. 3B and C), suggesting that autophagy served as a protective role in this situation. To exclude the off-target effects of 3-MA, we used shRNA to specifically knock down Beclin 1. Fig. 3D shows that the levels of Beclin 1 and LC3-II were apparently decreased in Beclin 1 shRNA-treated cells. In line with 3-MA, knockdown of Beclin 1 promoted the cell death and cleavage of PARP (Fig. 3E and F). Collectively, these data

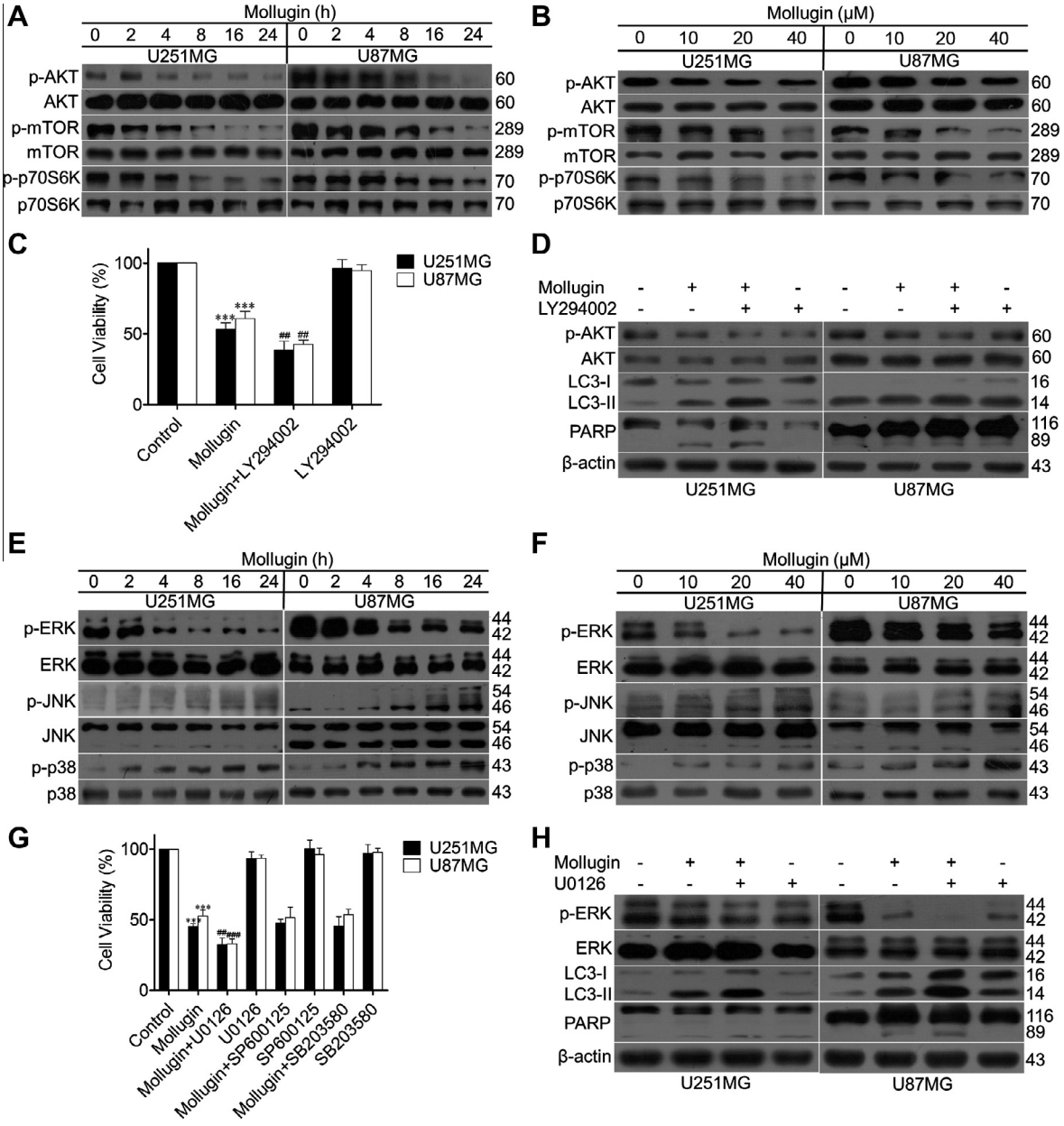

Fig. 4. Effects of mollugin on the PI3K/AKT/mTOR/p70S6K and MAPK signaling pathways in glioblastoma cells. (A and B) Cells were exposed to 40 μM mollugin for various durations (A) or various concentrations of mollugin for 24 h (B), the expression of phospho-AKT, AKT, phospho-mTOR, mTOR, phospho-p70S6K and p70S6K was detected. (C and D) Cells were pretreated with 10 μM LY294002 for 1 h followed by the treatment of 40 μM mollugin for 24 h. The cell viability (C) and the expression of p-AKT, AKT, LC3 and PARP (D) were determined. (E and F) Cells were treated as describe in (A and B), the expression of phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38 was detected. (G) Cells were pretreated with 10 μM U0126, 10 μM SP600125 and 10 μM SB203580 for 1 h followed by the treatment of 40 μM mollugin for 24 h. The cell viability was measured. Columns: mean of three independent experiments; bars: SD. $^{***}P < 0.001$ vs. control cells. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. cells treated with mollugin alone. (H) Cells were pretreated with 10 μM U0126 for 1 h followed by the treatment of 40 μM mollugin for 24 h. The protein levels of p-ERK, ERK, LC3 and PARP were determined.

strongly suggested that mollugin-induced autophagy played a protective role against the cytotoxic effect of mollugin.

3.5. Mollugin inhibits PI3K/AKT/mTOR/p70S6K signaling pathway in glioblastoma cells

The PI3K/AKT/mTOR/p70S6K signaling pathway is one of the major pathways regulating apoptosis and autophagy. Thus, we determined whether mollugin affected this pathway. As shown in Fig. 4A and B, mollugin treatment decreased phosphorylation of AKT, mTOR and p70S6K. To further determine the role of this pathway in the cytotoxic effect of mollugin, we used the PI3K kinase inhibitor LY294002. Pretreatment of LY294002 further promoted the cell death, conversion of LC3 and cleavage of PARP caused by mollugin (Fig. 4C and D), demonstrating that mollugin-induced apoptosis and autophagy was partly mediated by the PI3K/AKT/mTOR/p70S6K signaling pathway.

3.6. Mollugin induced autophagy through inhibition of the ERK signaling pathway in glioblastoma cells

It has been well documented that the MAPK signaling pathways played an important role in regulating apoptosis and autophagy, thus the effects of mollugin on this pathway were studied. Mollugin treatment increased the phosphorylation of JNK and p38 while decreased the phosphorylation of ERK (Fig. 4E and F). Then to delineate which pathway was involved in the cytotoxicity of mollugin, we assessed the effects of a panel of pharmacologic

inhibitors, including those of MEK1/2 kinase (U0126), JNK kinase (SP600125) and p38 kinase (SB203580) on affecting cell death induced by mollugin. Of these inhibitors, only U0126 exhibited the ability to promote the cytotoxicity of mollugin, whereas others had no appreciable effects on mollugin-mediated suppression of cell viability (Fig. 4G). Furthermore, western blotting confirmed that ERK signaling pathway was involved in the autophagy and apoptosis induced by mollugin (Fig. 4H). Collectively, these data indicated that mollugin-induced apoptosis and autophagy were partly due to its inhibition of ERK signaling pathway.

4. Discussion

There are now a growing number of studies showing a potential therapeutic effect of mollugin in treatment of cancers, although the underlying mechanisms remain to be explained. Our current study demonstrated for the first time that mollugin is a potent toxic agent toward various cancer cells, including glioblastoma, gastric cancer, breast cancer, lung cancer and colon cancer. Then to further clarify the cytotoxicity of mollugin, we used two glioblastoma cell lines, U251MG and U87MG. We found that mollugin displayed significant mitochondria apoptotic features in glioblastoma cells. Additionally, the mollugin-induced cell death and apoptosis was reversed by Z-VAD, further confirming the induction of cell death dependent of the caspase-mediated apoptotic pathways.

In addition to the induction of apoptosis in glioblastoma cells, we proposed another role of mollugin, the induction of autophagy. Firstly, TEM examination displaying typical ultrastructural features of autophagosomes confirmed the induction of autophagy by mollugin. Furthermore, mollugin markedly translocated LC3 to autophagosomes as reflected by the formation of LC3 puncta. In addition, mollugin increased the expression of autophagy-associated proteins such as Beclin 1 and LC3-II. Finally, mollugin induced p62 degradation and LC3 turnover, indicating the ability of mollugin to promote autophagic flux. More importantly, the autophagy induction ability of mollugin was further verified in other cancer cells, indicating that the autophagy induced by mollugin was not cell type-specific.

Having documented that mollugin induced apoptosis and autophagy, we then investigated the role of autophagy played in apoptosis. Generally, the current methods to study the relationship between apoptosis and autophagy are commonly relied on (i) the presence of autophagic features in apoptotic cells and (ii) influence of cell apoptosis via suppression of autophagy. Our study found that inhibition of autophagy aggravated both the cytotoxicity and apoptosis induced by mollugin, suggesting the pro-survival role of autophagy. Indeed, inhibition of autophagy has been shown to promote the anti-cancer effects of β-Elemene [10], Interferon-β [11] and FTY720 [15]. Consist with these observations, our data suggested that the autophagy may serve as an additional target for adjuvant anticancer therapy.

The PI3K/AKT/mTOR/p70S6K is an important signaling pathway in regulating autophagy in eukaryotic cells. This pathway also plays a variety of physiological roles, such as regulation of cell growth and cell survival [16,17]. MAPKs are serine/threonine protein kinases belonging to the CDK/MAPK/GSK3/CLK (CMGC) kinase group. They are involved in many cellular processes, including cell growth, autophagy and apoptosis [18,19]. In our study, we found that mollugin inhibited AKT, mTOR, p70S6K and ERK while activated JNK and p38. The PI3K kinase inhibitor LY294002 further promoted mollugin-induced autophagy and apoptosis. However, of the three MAPKs inhibitors, only the ERK inhibitor U0126 influenced the apoptosis and autophagy. These data suggest that the inhibition of PI3K/AKT/mTOR/p70S6K and ERK signaling pathways by mollugin resulted in two opposite results: on one hand, it induced mitochondria apoptosis, which resulted in cell death; on the other hand, it activated a protective autophagy to protect cells from death.

Taken together, our study provides the first evidence that mollugin induced mitochondria apoptosis and autophagy via inhibition of the PI3K/AKT/mTOR/p70S6K and ERK signaling pathways in glioblastoma cells. These results make mollugin an attractive therapeutic agent for developing alternative treatment protocols, and possibly, for combining with other anticancer agents to overcome drug resistance and achieve better outcomes.

Conflict of interest statement

The authors declare no potential conflicts of interest.

Acknowledgments

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