

Original Article

Betulinic acid induces autophagy-mediated apoptosis through suppression of the PI3K/AKT/mTOR signaling pathway and inhibits hepatocellular carcinoma

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Abstract: Betulinic acid (BA) is a pentacyclic triterpenoid compound that widely exists in Chinese herbal medicine, and it has remarkable biological activity. However, the involved molecular targets and mechanisms of BA are still ambiguous. Here, we aim to validate the preventive effects and molecular mechanisms of BA against hepatocellular carcinoma via related experiments. We extracted the 2D and 3D structure of BA from the PubChem database. MTT assay and colony formation assay were used to determine the anti-proliferation and cytotoxicity of BA using in vitro cell models. Hoechst 33258 staining was used to investigate the extent of apoptosis after BA treatment. Western blot and immunofluorescence experiments were used to evaluate apoptosis-related and autophagy-related proteins and molecular mechanisms. We demonstrated that BA significantly inhibited cell proliferation in HepG2 and SMMC-7721 hepatocellular carcinoma cells, but with little cytotoxicity effects on L-02 normal liver cells. We further determined that the hepatocellular carcinoma prevention effects of BA were closely correlated with apoptosis and autophagy. Furthermore, our data indicated that BA-induced autophagy has a protective effect against cancer cell proliferation and promotes cell apoptosis. Additionally, apoptosis and autophagy were induced by BA through suppression of the PI3K/AKT/mTOR signaling pathway. Collectively, our study provides experimental evidence that BA inhibits cell proliferation and induces cell apoptosis and autophagy via suppressing the PI3K/AKT/mTOR pathway. Additionally, BA is a safe and effective herbal medicine compound that can be used for the prevention of hepatocellular carcinoma growth, and may be a potential therapeutic strategy against hepatocellular carcinoma.

Keywords: Betulinic acid (BA), hepatocellular carcinoma, autophagy-mediated apoptosis, PI3K/AKT/mTOR

Introduction

Liver cancer is expected to be the sixth most common diagnosed cancer and the fourth leading cause of cancer-related death worldwide in 2018. It was estimated that new cases of liver cancer reached 841,000, with mortality as high as 782,000 annually. Hepatocellular carcinoma (HCC) alone accounts for 75-85% of all liver cancer cases [1]. Currently, HCC treatment mainly includes four types of chemotherapy [2]. Although great strides have been made in HCC diagnosis and therapy, the recurrence rate still remains high, and the 5-year survival rate is low after treatment [3]. Thus, there is an urgent need to prevent the occurrence of HCC, reduce its mortality worldwide, and improve prognosis.

Autophagy is an important physiological process that degrades intracellular components, such as unnecessary or damaged cellular organelles and protein aggregates in all living cells, and then transports them to lysosomes for recycling [4]. This process serves as an important cytoprotective mechanism that replenishes energy to maintain cellular homeostasis and viability [5]. Autophagy also plays a crucial role in tumor progression, as it fulfills a dual role of acting as a tumor promoter or as a tumor suppressor [4, 6, 7].

To date, autophagy-related proteins, including light chain 3 (LC3), p62/sequestosome 1 (SQSTM1), and beclin-1, have been identified as tumorigenesis prognostic biomarkers [8]. Upon autophagy activation, LC3 can be conjugated to

phosphatidylethanolamine (PE), and recruited to the autophagosomal membranes to form LC3B. LC3B can be degraded by lysosomes when autolysosomes form, thus acting as a marker of starvation-induced autophagy [9]. P62, the adaptor protein sequestosome 1 that shuttles ubiquitinated proteins into the autophagosome, can be degraded along with other cargo proteins upon fusion with a lysosome. Therefore, an associated accumulation of p62 reflects a process that contributes to tumorigenesis [10, 11]. Beclin-1 is a haploinsufficient tumor-suppressor gene that regulates autophagy and is involved in cell growth and proliferation, and it regulates the PtdIns3KC3-dependent generation of phosphatidylinositol 3-phosphate (PtdIns(3)P) and additional Atg proteins to orchestrate autophagosome formation [12, 13]. Moreover, autophagy-related signaling pathways have roles in many anticancer therapy processes through the inactivation of mammalian target of rapamycin (mTOR), some of which involve the PI3K/AKT, PI3K/AKT/mTOR, MEK/ERK, and AMPK pathways [4]. In some cases, autophagy may facilitate the induction of apoptosis and apoptosis-associated caspase activation [14].

Betulinic acid (BA) is a pentacyclic triterpenoid that is mainly isolated from the bark of the white birch tree (*Betula pubescens*), and it exhibits broad-spectrum anticancer activities [15-17]. The underlying mechanisms of cell death induced by BA have been demonstrated in different cancer cell types [18-20]. Accumulating evidence demonstrates that the anticancer activities of BA in lung cancer, renal carcinoma, and breast cancer cells occur via multiple signaling pathways, such as the PINK1/Parkin, AMPK, and PI3K/AKT pathways [21-23]. Several recent studies have revealed that BA induces cell death by inhibiting autophagy in multiple myeloma and microglia BA-2 cells [24, 25]. On the contrary, a few studies have shown that BA-induced autophagy is a protection mechanism in colorectal, colon, cervical, and breast cancer cells [26-28].

Currently, some research has verified that BA induces apoptosis in hepatoblastoma cells [29-31]. However, the role of BA and its underlying mechanisms involved in autophagy and apoptosis induction in HCC have still not been investigated. Herein, we sought to explore the anti-

cancer effect and mechanisms of BA, in terms of apoptosis, autophagy, and the relationship between apoptosis and autophagy, by experimental validation in vitro.

Materials and methods

Reagents

BA and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China), and 3-methyladenine (3-MA) was purchased from Selleck (Shanghai, China). Fetal bovine serum (FBS), high-glucose Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and trypsin and penicillin-streptomycin solution (PS) were purchased from Gibco (Beijing, China). Radioimmunoprecipitation (RIPA) buffer and Hoechst 33258 staining solution were purchased from Beyotime (Nanjing, China). Protease and phosphatase inhibitor mixture were purchased from Roche Diagnostics (Shanghai, China). A bicinchoninic acid (BCA) assay kit was purchased from Thermo Fisher Scientific (Beijing, China). Primary antibodies including BAX, cleaved-caspase-3, LC3B, P62, beclin-1, phospho-PI3K (p-p85- α), phospho-AKT, phospho-mTOR, PI3K (p85- α), AKT, mTOR, β -actin secondary anti-rabbit, and anti-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Enhanced chemiluminescence (ECL) Advanced Reagent was purchased from Millipore (Shanghai, China).

Cell culture

The human HCC cell lines MHCC-97H and HepG2, and the normal liver cell line (L-02) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained in high-glucose DMEM supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C in a humidified incubator containing 5% CO₂.

MTT assay

To assess the effects of different treatments on cell proliferation, the MTT assay was performed. The SMMC-7721, HepG2, and L-02 cells were seeded into 96-well plates at a density of 3×10^3 cells per well. After waiting 24 h for cell attachment to occur, serial concentration gradients of BA were then added to the

wells, and they were treated for 24 h, 48 h, or 72 h, with three repeats for each concentration and each time. Cell viability analysis was then performed using MTT. Cells were incubated at 37°C for 4 h, and the absorbance was measured at a wavelength of 490 nm to detect the OD values. Independent experiments were performed in triplicate.

Colony formation assay

The colony formation assay was conducted to determine the long-term inhibitory effects of BA on both HCC cell lines. Cells were seeded into six-well plates at a density of 1×10^3 cells/well. The next day, serial concentration gradients of BA (0, 10, 20, and 40 μ M) were added to the wells, and the cells were cultured for 6 h. Cells were then cultured with fresh medium for 14 days. The resultant colonies were fixed with 4% paraformaldehyde, stained with hematoxylin-eosin, counted, and photographed. Experiments were performed in triplicate.

Western blot analysis

Western blotting was performed to determine protein abundance. Cellular protein was extracted in RIPA buffer containing a protease and phosphatase inhibitor mixture and measured with the bicinchoninic acid assay. Quantified protein samples (30 μ g) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), and blocked with 5% nonfat milk for 1 h at room temperature. Then, the membranes were probed at 4°C overnight with primary antibodies. The next day, the membranes were washed three times with Tris-buffered saline and 0.05% Tween-20 (TBST), and then incubated with the appropriate horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies for 1 h at room temperature. After washing three times with TBST, the signals were visualized by using the ECL Advance Reagent with a Bio-Rad ChemiDoc Imaging System, quantified with ImageJ software, and normalized to the corresponding bands for β -actin.

Immunofluorescence analysis

To measure LC3B expression, cells were seeded into 24-well plates at a density of 1×10^5 cells per well. After cell attachment, the cells were treated with different concentrations of BA (0, 10, 20, and 40 μ M) alone or in combina-

tion with 3-MA (5 nM) for 48 h. The cells were then fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After blocking in 5% bovine serum albumin (BSA) for 60 min, the samples were incubated with primary antibodies against LC3B at 4°C overnight and subsequently incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature. Finally, the samples were incubated with 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei for 10 min at room temperature, and were subsequently detected at a 538 nm excitation wavelength under fluorescence microscope (Leica, Germany).

Hoechst 33258 staining

In order to observe nuclear staining, nuclear chromatin, and karyorrhexis, HCC cells at 60-70% confluency per well were seeded into 24-well plates and then treated with the indicated concentration of BA or in combination with 3-MA (5 nM) for 48 h. Then, Hoechst 33258 staining was performed according to the manufacturer's protocol. The cells were fixed in 4% paraformaldehyde for 30 min, washed three times with PBS, and then incubated with Hoechst 33258 fluorescent dye (10 μ g/mL) for 10 min at room temperature. Finally, images were obtained at a 340 nm excitation wavelength with a fluorescence microscope (Leica, Germany).

The chemical ingredient's structure

The 2D and 3D chemical structure of BA was obtained from the PubChem database (<https://www.ncbi.nlm.nih.gov>).

Statistical analysis

All data were analyzed with Statistical Product and Service Solutions (SPSS) 20.0 software and are expressed as the mean \pm standard deviation (SD). Student's *t*-test or one-way analysis of variance (ANOVA) was used to determine the significance of the data between different treatment groups. A value of $P < 0.05$ was considered statistically significant.

Results

BA represses HCC cell proliferation

The 2D and 3D chemical structure of BA is shown in **Figure 1A**. To determine the direct cyto-

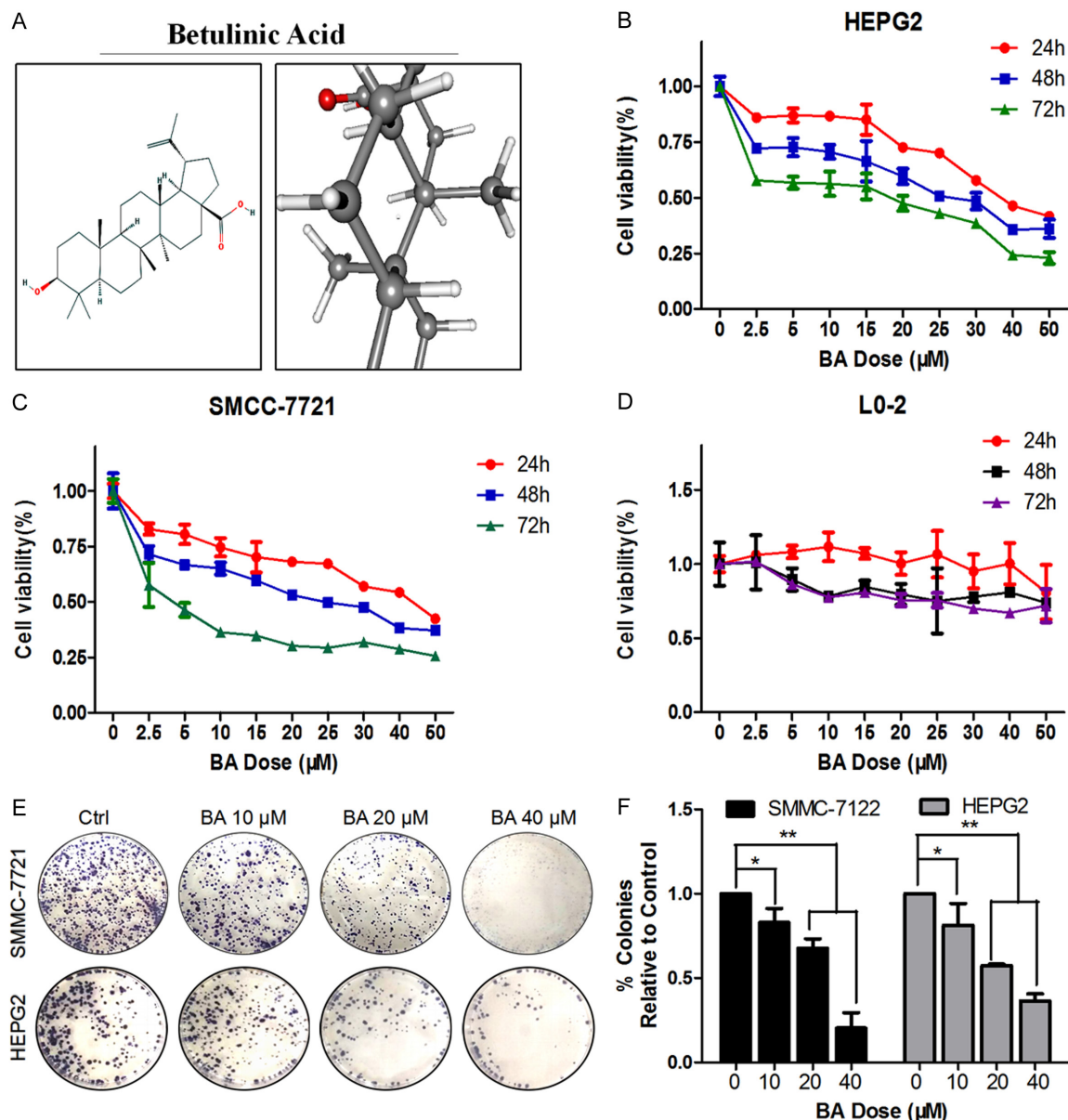


Figure 1. BA suppressed proliferation in two human HCC cell lines and showed a minimal inhibitory effect on normal liver cells. A. The 2D and 3D chemical structure of BA. B-D. Exponentially growing SMMC-7721, HepG2, and L0-2 cells were treated with BA at the indicated concentrations (0, 2.5, 5, 10, 15, 20, 25, 30, 40, and 50 μ M) for 24, 48, and 72 h. The cell viability was assessed by MTT assays. E and F. The colony formation assay was used to evaluate the long-term inhibitory effects of BA at concentrations of 0, 10, 20, and 40 μ M on HepG2 and SMMC-7721 cells, and the colony numbers were counted. (All values are expressed as the mean \pm SD, $n = 3$, $*P < 0.05$ vs. control group, $**P < 0.01$ vs. the control group).

toxicity of BA, the HCC cell lines HepG2 and SMMC-7721 were treated with incremental concentrations of BA for 24, 48, and 72 h and were analyzed with the MTT proliferation assay. We found that BA produced dose-dependent and time-dependent cytotoxicity effects on both cell lines (Figure 1B and 1C). After HepG2 and SMMC-7721 cells were treated with BA for 48 h, the half inhibitory concentration (IC₅₀)

values for BA were 24.8 μ M and 28.9 μ M, respectively. Additionally, the possible cytotoxicity of BA was also detected by MTT assay using human normal hepatocellular cells (L0-2). L0-2 cells were treated with varying concentrations of BA (0-50 μ M) for 24, 48, and 72 h, and the minimal inhibitory effect is shown on the abovementioned cell line (Figure 1D), indicating that BA exhibits a highly selective killing

trend toward HCC cells. To evaluate the long-term inhibitory effects of the BA treatment on HCC cell lines, a colony formation assay was carried out. We found that BA substantially reduced the clonogenic ability of HepG2 and SMMC-7721, further confirming the long-term cytotoxic effects of BA (**Figure 1E** and **1F**).

BA enhances apoptosis in HCC cell lines and induces morphological changes in HCC cell nuclei

Apoptosis is a key mechanism that causes cell death in cancer cells. Because BA inhibited cell proliferation in both HCC cell lines, we further investigated whether BA could increase the apoptosis effect. As expected, BA dose-dependently increased BAX and cleaved-caspase-3 protein, and downregulated Bcl-2 protein from 0 to 40 μ M in HepG2 and SMMC-7721 cells at 48 h (**Figure 2A-D**). Based on the western blotting results, Hoechst 33258 staining was utilized to observe the morphological changes of apoptotic cells by fluorescence imaging and the typical morphological characteristics of apoptosis, such as chromatin condensation, multi-lobed nuclei, and cell pyknosis. The results showed that the blue staining intensity increased, and the typical apoptotic morphological changes as mentioned above were more easily observed following BA treatment in a dose-dependent manner (**Figure 2E** and **2F**). Together, these findings indicated that BA inhibits HCC cell growth via apoptosis induction.

Determining BA's ability to stimulate autophagy in HCC cell lines

BA can stimulate autophagy in HCC cell lines, and thus, we next investigated whether BA could promote the autophagy effect on both HepG2 and SMMC-7721 cells. Western blotting analysis was used to investigate the three key indicators for autophagy, LC3B-II, beclin-1, and P62. The results demonstrated that BA stimulated the autophagy process, as evidenced by the increased expression of LC3B-II and beclin-1, and the decreased level of P62 in a significant dose-dependent manner (**Figure 3A-D**), indicating that BA could induce autophagy in HCC cell lines. In addition, immunofluorescence assay was also carried out to observe the red fluorescence staining intensity of LC3B expression. It also revealed that the autophagy-induced protein LC3B significantly increased in

a dose-dependent manner following BA treatment (**Figure 3E** and **3F**). The above results implied that BA could induce autophagy in the two HCC cell lines.

BA augments autophagy-induced apoptosis in HCC cells

We continued to validate whether the apoptosis activity of BA was autophagy-dependent. Immunofluorescence assay was carried out to examine the LC3B expression to confirm that 3-MA, an autophagy inhibitor, could reduce the autophagy of both HepG2 and SMMC-7721 cells by BA. Furthermore, 3-MA combined with BA reduced LC3B expression compared with BA alone (**Figure 4A** and **4B**). Then, western blot analysis was also used to examine the apoptosis proteins. The results revealed that 3-MA treatment resulted in the significant suppression of apoptosis by BA, as shown by the decreased expression of cleaved-caspase-3 and BAX and increased Bcl-2 expression (**Figure 4C-E**). Next, Hoechst 33258 staining was also performed to demonstrate that the staining intensity and typical morphological characteristics of apoptosis strengthen after 20 μ M BA treatment. It was observed that 3-MA combined with BA reduced the blue staining intensity, and typical apoptotic morphological changes were not easily observed compare with BA alone (**Figure 4F**). All the above findings suggested that BA might promote autophagy to induce HCC cellular apoptosis, and thereby suppress HCC cell proliferation.

BA stimulates autophagy and apoptosis via suppressing PI3K/AKT/mTOR signaling

Based on the above results, we confirmed the cytotoxic effects on HepG2 and SMMC-7721 cells. However, the mechanism BA used to mediate its bioactivity remained unclear. To further investigate the underlying molecular mechanisms as to how BA regulates PI3K/AKT/mTOR signaling in HCC cells, western blot analysis was carried out. As labeling indicates (**Figure 5A-D**), we found that BA administration remarkably downregulated the protein expression ratio of p-mTOR/mTOR, p-PI3K/PI3K, and p-AKT/AKT in a dose-dependent manner, in both cell lines at 48 h. These data indicated that the stimulation of autophagy and apoptosis by BA to generate the highlighted anticancer effects may require the suppression of the PI3K/AKT/mTOR pathway.

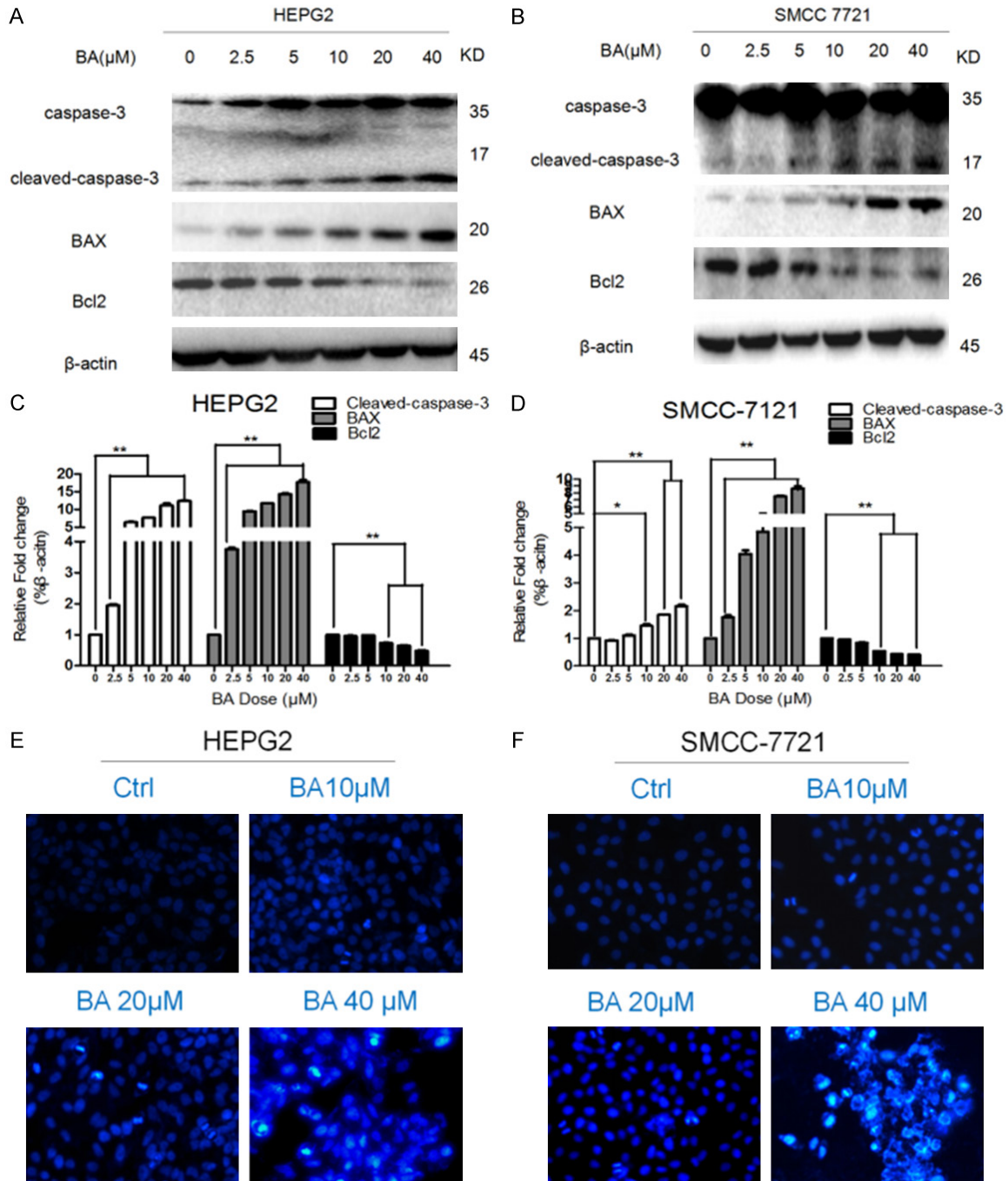


Figure 2. BA induced cytotoxicity via apoptosis induction in HCC cell lines. A-D. The protein levels of BAX, Bcl-2, caspase-3, and cleaved-caspase-3 of the two hepatocellular carcinoma cell lines following treatment with different BA concentrations (0, 2.5, 5, 10, 20, and 40 μ M) were determined by western blotting analysis. E, F. Hoechst 33258 staining showed typical apoptotic morphology changes in HepG2 and SMMC-7721 cells after treatment with 0, 10, 20, and 40 μ M of BA for 48 h. (200 \times , scale bars: 100 μ m). (All data are represented as the mean \pm SD, n = 3, * P < 0.05 vs. the control group, ** P < 0.01 vs. the control group).

Discussion

According to the estimation of World Health Organization (WHO), patient mortality from liver

cancer will exceed more than 1 million in 2030 [32]. The increasing mortality rate due to hepatocellular carcinoma is a growing concern worldwide [33]. Because hepatocellular carci-

The anticancer effect of betulinic acid

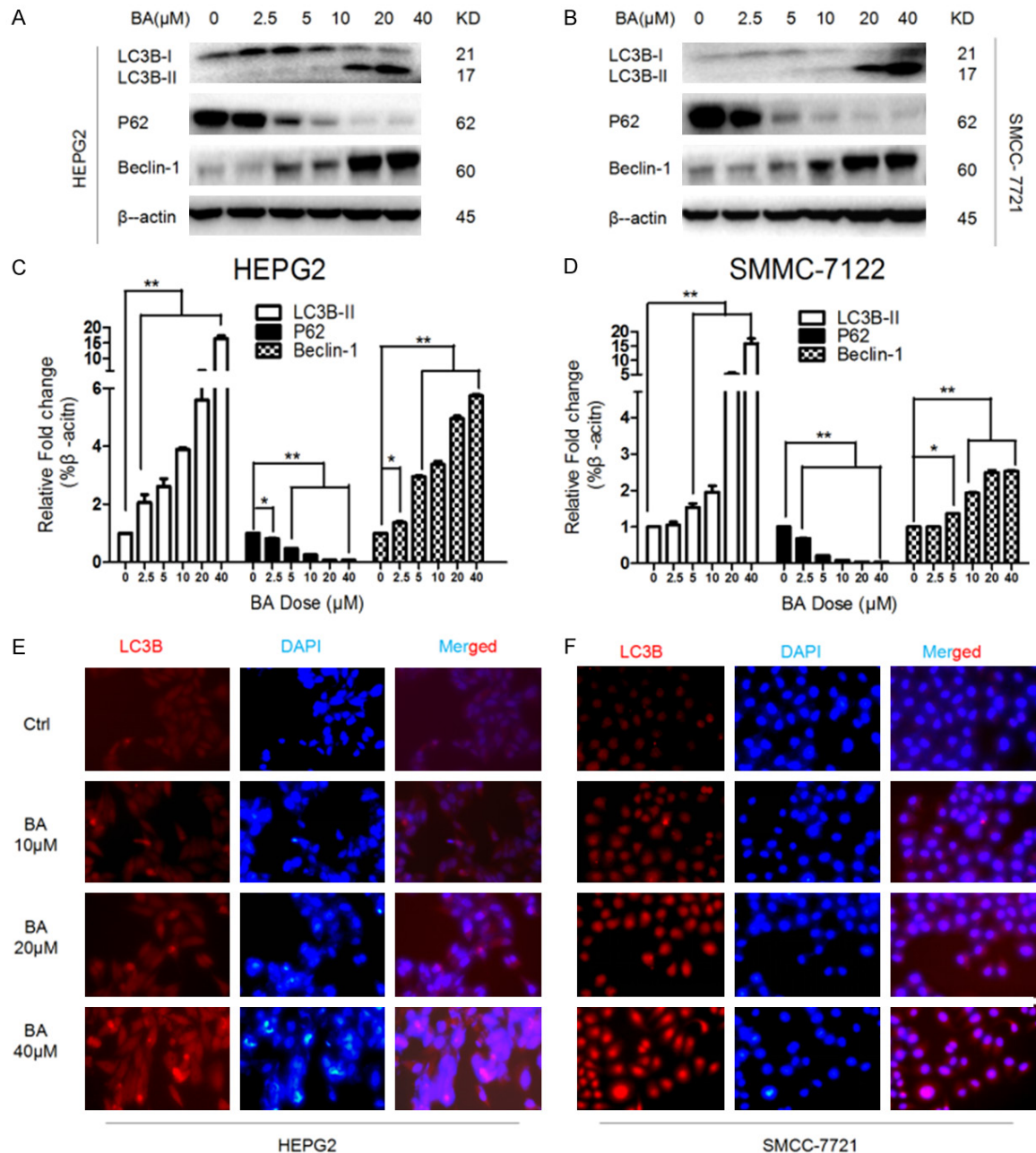


Figure 3. BA induced cytotoxicity by autophagy in HCC cell lines. A-D. Total cellular extract of HepG2 and SMMC-7721 cells treated with different concentrations (0, 2.5, 5, 10, 20, and 40 μ M) of BA for 48 h was prepared and subjected to western blot to examine the protein expression of LC3B-II, P62, and beclin-1. E, F. The immunofluorescence assay was performed to examine the LC3B levels after BA treatment (0, 10, 20, and 40 μ M) of HepG2 and SMMC-7721 cells (200 \times , scale bars: 100 μ m). (All data are represented as the mean \pm SD, n = 3, * P < 0.05 vs. the control group, ** P < 0.01 vs. the control group).

noma is a complex disease, a multidisciplinary approach in specialized hospitals is required to maximally influence the course of the disease and its outcome. Although surgical therapies, tumor ablation, transarterial therapies, and

systemic therapies have been demonstrated to improve patient outcomes [34], therapeutic breakthroughs are still emerging. Thus, novel therapies remain an unmet medical need in hepatocellular carcinoma treatment.

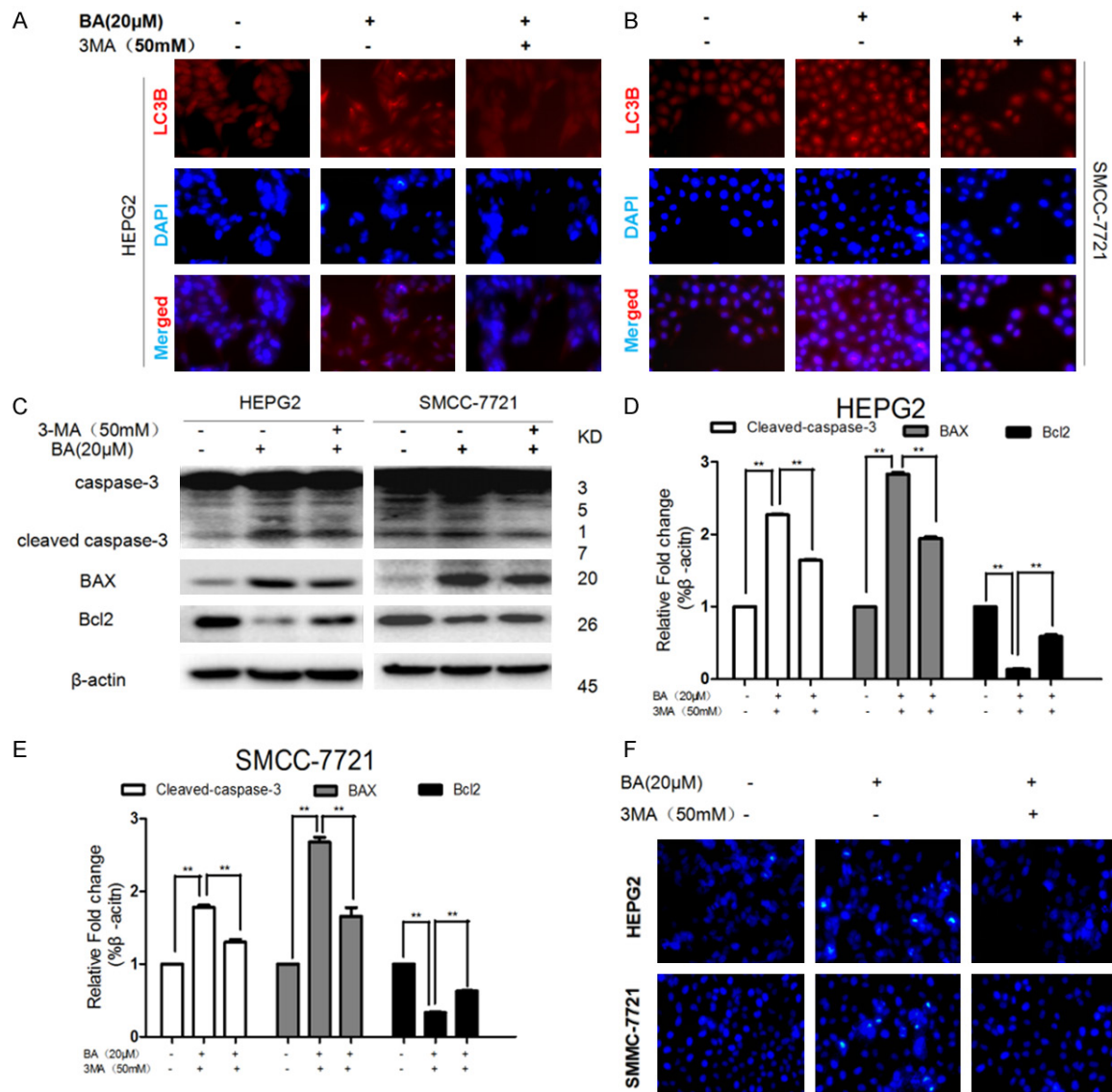


Figure 4. BA augments autophagy-induced apoptosis in HCC cells. A and B. The fluorescent images of both HCC cell lines treated with BA (20 μ M) alone or combined with 3-MA (5 mM) for 48 h from immunofluorescence assay to demonstrate whether 3-MA could reduce BA-induced autophagy (200 \times , scale bars: 100 μ m). C and D. HepG2 and SMMC-7721 cells were treated with BA (20 μ M) alone or in combination with 3-MA (5 mM) for 48 h. Western blotting analysis was used to examine the apoptosis-related protein expression of cleaved-caspase-3 BAX and Bcl-2 after the indicated treatment. E and F. The fluorescent images of HepG2 and SMMC-7721 cell nuclei treated with BA (20 μ M) alone or combined with 3-MA (5 mM) from Hoechst 33258 staining to demonstrate whether 3-MA could reduce BA-induced apoptosis (200 \times , scale bars: 100 μ m). (All values are represented as the mean \pm SD, $n = 3$, $**P < 0.01$ vs. the control group or BA treatment group).

In recent years, because there has been increasing interest in multi-target strategies for hepatocellular carcinoma treatment, natural and chemically modified natural products have assumed new and important roles at different treatment stages. Hence, it is necessary to develop more efficient and highly selective molecules that inhibit hepatocellular carcinoma

cell growth. Specifically, Llovet et al. claimed that two major molecular subtypes of HCC have been proposed: a proliferation class and non-proliferation class, 50% each [35]. HCCs of the proliferation class are characterized by activation of cell proliferation and survival-related signaling pathways, such as the PI3K-AKT-mTOR, RAS-MAPK, and MET cascades [36-38].

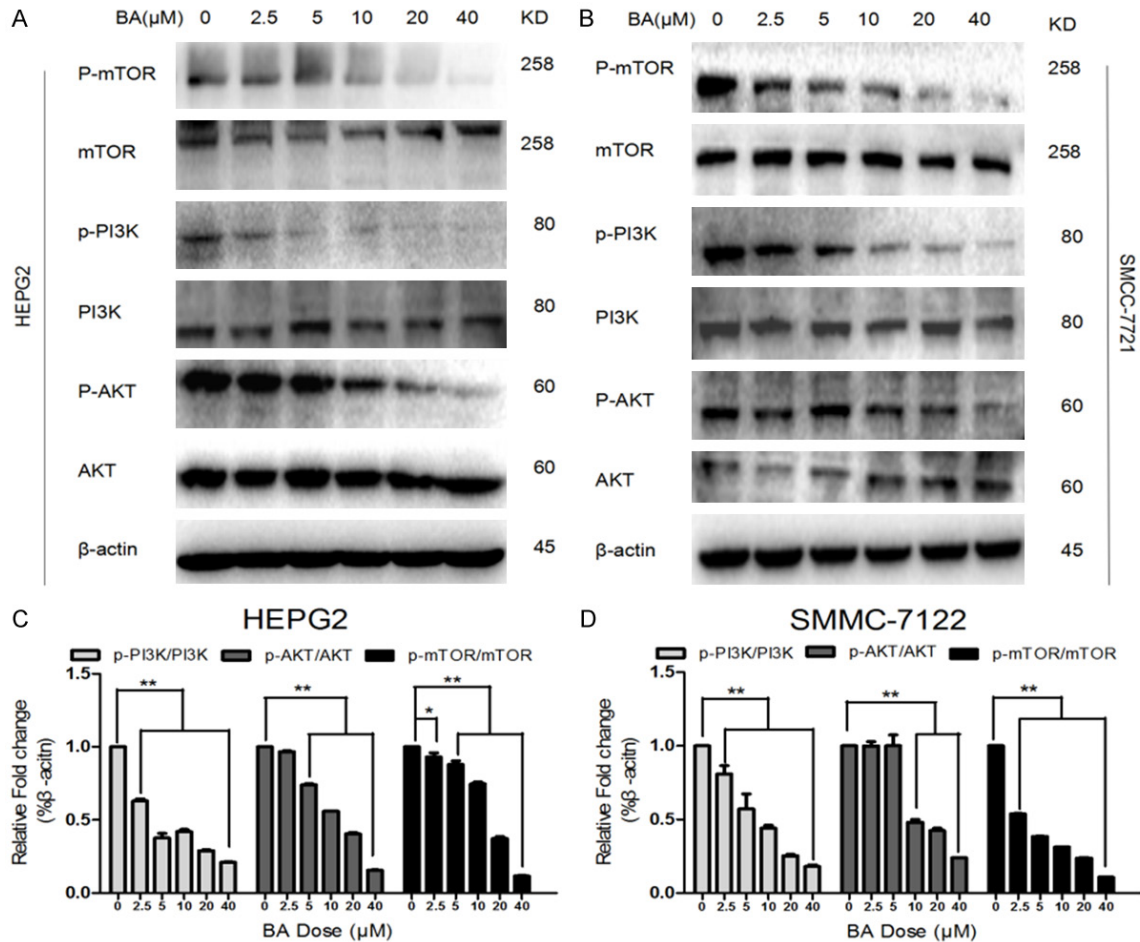


Figure 5. BA promoted autophagy and apoptosis via suppressing the PI3K/AKT/mTOR pathway in HCC cells. A-D. The protein expression of p-mTOR, p-PI3K, p-AKT, PI3K, AKT, and mTOR was detected by western blot analysis after treating the two HCC cell lines with different BA concentrations (0, 2.5, 5, 10, 20, and 40 μ M). (All values are represented as the mean \pm SD, $n = 3$, * $P < 0.05$ vs. the control group, ** $P < 0.01$ vs. control group).

In the present study, we found that BA, a novel natural compound, displays potent antiproliferative activity in HepG2 and SMMC-7721 cells by inducing cell death in vitro. Apoptosis is considered as type I programmed cell death, while autophagy is known as type II programmed cell death [39]. Mechanically, we initially discovered that BA induces apoptosis and elicits autophagy. Furthermore, for the first time, we have confirmed that promotion at early-stage autophagy can enhance BA-induced apoptosis. In particular, we found that BA caused no cytotoxic effects on normal liver cells. Our findings further implicate a possible side effect of BA with good safety regarding liver function. Hence, it is of great value to investigate the effects and mechanisms of BA in hepatocellular carcinoma.

Cancer cells avert apoptosis, and the molecular mechanisms of apoptosis have been confirmed and divided into intrinsic and extrinsic apoptotic pathways. The extrinsic pathway is activated when death-inducing ligands bind to cell-surface death receptors, while cell-intrinsic apoptosis stimulation includes DNA damage, growth factor deprivation, and oxidative stress [40]. Herein, we observed that BA induced cell apoptosis by downregulating the protein expression levels of cleaved-caspase-3 and BAX, and upregulating the protein expression levels of Bcl-2, in addition to displaying dose-dependent apoptosis induction in HCC cells. Once an apoptotic signal occurs, Bax translocates from the cytosol to the mitochondria, and then increases cytochrome c release, which was previously inhibited by Bcl-2. Next, cytochrome c releases

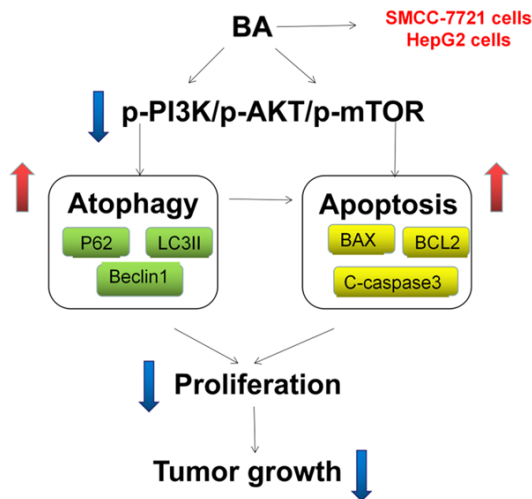


Figure 6. Working model. Diagram illustrating BA-induced autophagy and apoptosis against hepatocellular carcinoma via suppressing PI3K/AKT/mTOR signaling.

into the cytosol, and forms a complex with Apaf-1 and procaspase-9, which becomes an intracellular 'apoptosome'. This leads to caspase-9 activation, which then activates downstream caspases such as caspase-3. Finally, the terminal events of apoptosis are inevitable [41], and cell death occurs through apoptosis. In this study, BA enhanced the Bax/Bcl-2 ratio and appeared to possess a caspase-dependent tendency. Moreover, the loss of Bcl-2 family protein expression modulation affected mitochondrial function, suggesting that BA induced mitochondria-mediated apoptosis in HCC cells. The above results were related to one of the antiproliferation mechanisms of BA.

Morphologically, cell death underlies three broad classifications: apoptosis, autophagic cell death, and necrosis. Autophagy, the catabolic pathway centered on lysosomal degradation of bulk or selected cargo, is also considered as an important regulator and, in some cases, even an executor of cell death [42]. Several molecules have been implicated in the regulation of autophagy, such as LC3, P62, and beclin-1 [43]. LC3-I is converted to LC3-II via lipidation by ubiquitin-like conjugation, which has been widely used as an autophagic marker [44], and the number of LC3-II puncta is related to the number of 'autophagosomes'. Furthermore, the downregulation of beclin-1 partially prevents cell death and inhibits the formation of LC3 puncta, while the inhibition of autophagy and apoptosis further inhibits cell death [42].

p62 is a protein between ubiquitination protein aggregates and autophagosomes, and the accumulation of p62 is due to the damage of the autophagosomal machinery. Additionally, p62 is an important factor that mediates selective autophagy, especially aggregation autophagy [45]. Thus, the protein expression of LC3-II, p62, and beclin-1 is associated with autophagy. The current results showed that BA treatment induced autophagy in HCC as evidenced by LC3 conversion, beclin-1 accumulation, and p62/SQSTM1 downregulation, suggesting that autophagy occurred in HCC cells. These findings revealed that BA treatment promoted HCC cell death through autophagy. As previously described, BA may trigger both apoptotic and autophagic cell death in hepatocellular carcinoma.

Typical autophagy-related proteins can embellish apoptotic signaling during cancer therapy. One of the most obvious interactions is the Bcl-2-beclin-1 complex. It has been reported that Bcl-2 binds beclin-1 directly via its homology domain 1 (BH1) and Bcl-2 homology domain 3 (BH3), inhibiting the autophagy activity of beclin-1. Moreover, Bcl-2 was also shown to inhibit autophagy induction through its interaction with mitochondrial ambra 1 [46]. p62 also makes a molecular connection between autophagic and apoptotic signaling, and can enhance caspase-8 activity and contribute to the autoproteolytic release of caspase-8 into the cytosol [46], which finally results in the activation of the proapoptotic caspase-8→caspase-3 cascade that subsequently triggers apoptosis and then cell death [47]. Beclin-1 contains two caspase cleavage sites that lead to the binding of pro-apoptotic protein with anti-apoptotic protein to stimulate cytochrome c release, caspase activation, and eventually apoptosis induction [42].

In the present study, BA not only induced apoptosis but also autophagy. 3-MA, which can inhibit the formation of autophagosomes, was chosen to inhibit autophagy formation at an early stage. The fluorescence staining results showed that the two HCC cell types treated with early autophagy inhibitor 3-MA plus BA exhibited a greater decrease in LC3-II accumulation, compared with BA treatment alone. Then, we elucidated the link between autophagy and mitochondrial apoptosis induced by BA. The western blot data showed that cotreatment with BA and 3-MA decreased the apoptosis

rate, Bax/Bcl-2 ratio, and the levels of cleaved-caspase-3 compared with BA alone in HCC cells. Similarly, the Hoechst 33258 staining also revealed that cotreatment with BA and 3-MA decreased the staining intensity and typical morphological characteristics of apoptosis, further suggesting that BA plus 3-MA inhibited BA-induced apoptosis. Therefore, it is likely that BA induced apoptosis in HCC cells upon coincubation with the early-stage autophagy inhibitor 3-MA. Collectively, the above results indicate that 3-MA potentially decreased the apoptotic activity of BA via inhibiting autophagosome formation.

Novel molecules selectively inhibit the proliferation of cancer cells via the PI3K/AKT/mTOR signaling pathway, which regulates cell apoptosis and autophagy [48, 49]. Phosphoinositide 3-kinase (PI3K) can activate the serine/threonine kinase AKT, following AKT phosphorylation and activation, and then activate the serine/threonine kinase mTOR through a series of reactions [50]. In this study, a significant decrease in p-mTOR/p-PI3K/p-AKT with a concomitant increase in autophagy and apoptosis pathway markers was observed, indicating that BA inhibits hepatocellular carcinoma by stimulating autophagy-associated cell death via suppressing PI3K/AKT/mTOR signaling (**Figure 6**).

Taken together, our findings elucidate how BA inhibits hepatocellular carcinoma cells by stimulating autophagy-associated cell death, thus uncovering a novel mechanism underlying the potent antitumor effects of the natural targeting agent BA in hepatocellular carcinoma. However, due to some limitations in our study, future studies are required to demonstrate that BA can offer therapeutic potential for the treatment of cancer.

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Disclosure of conflict of interest

None.

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