

# $\alpha$ -hederin induces autophagic cell death in colorectal cancer cells through reactive oxygen species dependent AMPK/mTOR signaling pathway activation

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**Abstract.**  $\alpha$ -hederin, a monodesmosidic triterpenoid saponin, had previously demonstrated strong anticancer effects. In the current study, the pharmacological mechanism of autophagic cell death induced by  $\alpha$ -hederin was investigated in human colorectal cancer cells. First, through cell counting kit-8 and colony formation assays, it was demonstrated that  $\alpha$ -hederin could inhibit the proliferation of HCT116 and HCT8 cell. Results of flow cytometry using fluorescein isothiocyanate Annexin V/propidium iodide and Hoechst 33258 staining indicated that  $\alpha$ -hederin could induce apoptosis. Western blotting demonstrated that  $\alpha$ -hederin could activate mitochondrial apoptosis signal pathway. Then, using light chain 3 lentiviral and electron microscope assay, it was demonstrated that  $\alpha$ -hederin could induce autophagy in colorectal cancer cells. In addition, immunohistochemistry results from *in vivo* experiments also demonstrated that  $\alpha$ -hederin could induce autophagy. AMP-activated protein kinase (AMPK)/mechanistic target of rapamycin (mTOR) signaling was demonstrated to be activated by  $\alpha$ -hederin, which could be blocked by reactive oxygen species (ROS) inhibitor NAC. Furthermore, NAC could inhibit apoptosis and autophagy induced by  $\alpha$ -hederin. Finally, 3-MA (autophagy inhibitor) reduced the inhibition of  $\alpha$ -hederin on cell activity, but it had no significant effect on apoptosis. In conclusion,  $\alpha$ -hederin triggered apoptosis through ROS-activated mitochondrial signaling pathway and

autophagic cell death through ROS dependent AMPK/mTOR signaling pathway activation in colorectal cancer cells.

## Introduction

With ~1.4 million new cases every year worldwide, colorectal cancer is the third most common type of cancer with high incidence and mortality rates (1,2). Currently, surgery with chemotherapy is the main treatment for colorectal cancer, but the 5-year survival rate of patients with colorectal cancer remains low (3). Therefore, identifying novel anticancer drugs is necessary.

An effective treatment strategy for colorectal cancer is to induce cell death (4). A number of studies have demonstrated that cell death mainly includes four major morphologically processes: Necrosis, apoptosis, autophagy and pyroptosis (5,6). Autophagy is an important process in evolutionarily conserving cellular material and energy flow. It is a normal physiological process of metabolizing cellular material in evolved eukaryotic organisms and is one form of type II programmed cell death (7,8). Autophagy enables cells to resist metabolic stress caused by the lack of nutrition and excessive proliferation of cells, and may promote the survival of tumor cells. For example, autophagy is an important cause of drug resistance in cancer cells during chemotherapy. However, excessive autophagy may lead to autophagic cell death (9). Therefore, the degree of autophagy determines whether the cell survives or dies.

Autophagy and apoptosis are both important mechanisms determining the survival or death of cancer cells. Cross-talk of autophagy and apoptosis has been documented in the tumorigenesis and progression of cancer (10), while the interplay between the two pathways in colorectal cancer has not yet been comprehensively summarized. Although autophagy and apoptosis are significantly different in metabolic pathways and morphology, their signaling pathways are inextricably linked (11,12). According to different regulation modes, their interaction can be roughly classified into three types: Cooperative, antagonistic and promotion relationships (13).

Reactive oxygen species (ROS) are produced by aerobic cells in the process of metabolism. Previous studies have demonstrated that ROS can affect a series of intracellular

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signal pathways (14). ROS regulate the balance cell death dependent on their concentration (15). Low level ROS can activate transcription factors and promote cell proliferation, differentiation and autophagy (16). However, moderate and high concentrations of ROS can induce apoptosis and autophagic cell death (17,18). It has previously been demonstrated that ROS activate the mitochondrial apoptotic pathway (19). In addition, ROS have been demonstrated to activate autophagic death signaling pathway through AMP-activated protein kinase (AMPK)/mechanistic target of rapamycin (mTOR) signaling pathway (20). Previous studies have demonstrated that many anticancer drugs can stimulate the production of ROS in cancer cells, and then lead to cell apoptosis and autophagic cell death (21,22).

$\alpha$ -hederin, a monodesmosidic triterpenoid saponin, is a major active ingredient isolated from the leaves of ivy (*Hedera helix* L.) or *Nigella sativa*. Previous studies have demonstrated that  $\alpha$ -hederin has anticancer properties, which may be attributed to the inhibition of proliferation and motility (23,24), induction of apoptosis (25,26), membrane permeabilization (27), inhibition of epithelial to mesenchymal transition (28) and morphologic changes (27).  $\alpha$ -Hederin can activate the mitochondrial apoptotic pathway by increasing ROS (24,26). Our previous study has reported that  $\alpha$ -hederin can induce apoptosis in colorectal cancer cells (29). Based on the effects of ROS on autophagy, it was suggested that  $\alpha$ -hederin may induce autophagy through ROS.

In the present study, to determine whether  $\alpha$ -hederin can induce autophagy in colorectal cancer cells, the effects of  $\alpha$ -hederin on colorectal cancer cells were investigated. As such, its effects on autophagy and the underlying mechanisms were explored. In addition, the interplay between the apoptosis and autophagy in colorectal cancer was also explored.

## Materials and methods

**Cells and reagents.** HCT116 and HCT8 human colorectal cancer cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin at 37°C and 5% CO<sub>2</sub>.  $\alpha$ -hederin was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). ROS inhibitor N-acetyl-L-cysteine (NAC), oxidation-activated fluorescent dye DCHF-DA and Hoechst 33258 were purchased from Beyotime Institute of Biotechnology (Haimen, China). Autophagy inhibitor 3-MA was purchased from Sigma-Aldrich; Merck KGaA.

**Antibodies.** Anti-light chain (LC)3 (12135-1-AP), anti-caspase-9 (10380-1-AP), anti-caspase-3 (19677-1-AP), anti-P62 (18420-1-AP) and anti-Beclin 1 (11306-1-AP) antibodies were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Anti-phosphorylated (p)-mTOR (#2971), anti-mTOR (#2972), anti- $\beta$ -actin (8456), anti-p-Unc-51 like autophagy activating kinase 1 (ULK1; #4634), anti-ULK1 (#4773), anti-p-P70S6K (#9205), anti-P70S6K (#9202), anti-poly(ADP-ribose) polymerase (PARP; #9532), anti-p-AMPK (#2531), anti-AMPK (#2532), anti-B cell lymphoma (Bcl)-2 (#2872),

anti-Bcl-2 associated X protein (Bax; #2772), anti-Bcl-x1 (#2762), horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin (Ig)G (#7074) and HRP-linked anti-mouse IgG (#7076) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Flow cytometry assays.** Flow cytometry assays were performed on a FACSCalibur system (Becton-Dickinson and Company, Franklin Lakes, NJ, USA). Apoptosis rate assay was performed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Cells were digested using trypsin, washed twice with cold PBS and then incubated with Annexin V-FITC and PI at room temperature for 30 min. Subsequently, the apoptosis rate was determined using flow cytometry. Intracellular ROS levels were determined through flow cytometry using DCHF-DA, an oxidation-activated fluorescent dye. Cells in dishes were loaded with PBS containing 10  $\mu$ M DCHF-DA for 20 min at 37°C. Then, cells were washed thrice with cold PBS, digested with trypsin and washed twice with cold PBS in turn. ROS assay was performed using a FACSCalibur flow cytometer (Becton-Dickinson and Company) and the data were analyzed using FlowJo software, version 7.6 (Tree Star, Inc., Ashland, OR, USA).

**Hoechst 33258 staining.** Cells were digested, dispersed and plated in a 96-well plate at a density of 1x10<sup>5</sup> cells/well. Cells were incubated with Hoechst 33258 (0.5  $\mu$ g/ml; Beyotime Institute of Biotechnology) for 3 min at room temperature. Washed 3 times with PBS, cells were observed and photographed directly under a microscope.

**Cell viability assay.** Cells were digested, dispersed and plated in a 96-well plate at a density of 1x10<sup>5</sup> cells/well. Cell viability was analyzed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. Absorbances of the wells were read at 450 nm using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Colony formation assay.** Single cells were seeded into a 35-mm cell culture dish at a density of 1,000 cells/well and cultured with  $\alpha$ -hederin at concentrations of 0, 10 and 60  $\mu$ M for 14 days. The medium was replaced every 2 days, a total of six times. Ultimately, cells were treated in turn with 4% paraformaldehyde for 20 min and crystal for 20 min, and were washed with PBS at least twice in room temperature. The colonies were counted, and images were captured. Colonies were determined as those able to form a colony consisting of  $\geq$ 50 cells.

**Western blot analysis.** Cells were lysed using cell lysis buffer (Beyotime Institute of Biotechnology) with Protease Inhibitor Cocktail (100X; Sangon Biotech Co., Ltd., Shanghai, China) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Following centrifugation at 4°C and 12,000 x g for 15 min, protein concentration in the supernatant of the lysates was determined using the Bradford Coomassie Blue G-250 method. Proteins (40  $\mu$ g per lane) were separated using 10% SDS-PAGE, and transferred onto polyvinylidene

difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) in sequence. Thereafter, PVDF membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in TBS containing 0.05% Tween-20 at room temperature for 2 h. Subsequently, PVDF membranes were incubated with primary antibodies at 1:1,000 in TBS containing 0.05% Tween-20 and 5% bovine serum albumin overnight at 4°C for 16 h. Next, PVDF membranes were incubated with corresponding HRP-conjugated secondary antibodies (1:1,000) at room temperature for 2 h. Finally, membranes were visualized using an enhanced chemiluminescence Western blot detection system (EMD Millipore). In the present study, all relative protein concentrations were normalized to that of  $\beta$ -actin.

**Lentivirus and cell fluorescence analysis.** LC3 lentivirus and mutation lentivirus were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The LC3 lentivirus consisted of the red fluorescent protein Stub-RFP, green fluorescent protein Sens-GFP, and autophagy marker protein LC3. HCT116 and HCT8 cells were infected with these lentiviruses to establish four cells line stably expressing LC3 (HCT116 LC3, HCT8 LC3) or LC3-mutation (HCT116 LC3-mu, HCT8 LC3-mu).

At first, cells were digested, dispersed and plated in a 24-well plate. When cells reached 60% confluence, the medium was replaced with infection mixture. The mixture contained RPMI-1640 medium, lentiviruses (MOI=100) and polybrene (5  $\mu$ g/ml; Shanghai GeneChem Co., Ltd.). Following incubation for 24 h, the mixture was replaced with normal medium containing FBS. These established cell lines were used to detect the occurrence of autophagy. In the absence of autophagy, the RFP-GFP-LC3 fusion protein diffuses in the cytoplasm. When autophagy is formed, it translocates into the autophagosome membrane. The fusion protein will form bright fluorescent spots, which can be observed under fluorescence microscope. Fluorescent dot aggregation was observed via confocal laser microscopy. One spot is equivalent to an autophagosome, and the activity of autophagy can be evaluated by counting. As the control, RFP-GFP-LC3-mu fusion protein could not participate in the formation of autophagosome membrane.

**RNA interference.** Small interfering (si)RNA for the human AMPK gene (Gene ID: 94557300) was synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, China). The AMPK siRNA sequence was 5'-GAUAUCAGGGAACAUGAAUdTdT-3'. The control sequence was 5'-UAAGGCUAUGAAGAGAUAC-3'. Cells were transfected with the aforementioned siRNAs at a concentration of 50 nM using Lipofectamine 3000 (Life Technologies; Thermo Fisher Scientific, Inc.) for 48 h.

**Electron microscopy.** After being treated with 10  $\mu$ M  $\alpha$ -hederin for 24 h, HCT116 and HCT8 cells were digested with trypsin and washed twice with cold PBS. Subsequently, these cells were fixed overnight using 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C and post-fixed in 1% OsO<sub>4</sub> buffer for 1 h at 4°C. After being dehydrated in a graded series of ethanol, the cells were embedded in spur resin at 56°C overnight. The spur resins were cut into ultrathin sections (60 nm) and stained with saturated solutions of uranyl acetate and lead citrate at

room temperature for 10 min. Finally, the autophagosomes in these cells were observed using an electron microscope.

**In vivo tumor xenograft model.** A total of 10 male nude mice (BALB/c nu/nu; age, 5 weeks) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The initial body weight of all nude mice was ~18 g. Nude mice were bred in specific pathogen-free (SPF) conditions at the Laboratory Animals Breeding and Research Centre, Shanghai University of Traditional Chinese Medicine (Shanghai, China). The housing conditions for mice were as follows: Room temperature, 20-26°C and 12-h light/dark cycle. The mice received 5 g food and 100 ml water per 100 g body weight per day. Two million HCT116 cells in 0.1 ml PBS were injected into subcutaneous tissues of each mouse. After 2 weeks, mice were weighed and randomly divided into control group and  $\alpha$ -hederin group (n=5/group).  $\alpha$ -Hederin group mice were injected intraperitoneally with  $\alpha$ -hederin (2 mg/kg body weight) and the control group were injected intraperitoneally with the same volume of PBS (control) every 3 days for 3 weeks. Finally, the tumor-bearing mice were sacrificed and the tumors were excised and weighed. The protocol was approved by the Ethics Committee of Shuguang Hospital affiliated with Shanghai University of Traditional Chinese Medicine.

**Immunohistochemistry assay.** All tumors from tumor xenograft model were formalin-fixed for 24 h at room temperature, embedded in paraffin, sectioned serially to 5- $\mu$ m thickness and mounted on glass slides. Subsequently, immunohistochemical staining of LC3 was carried out using a standard protocol (30). Anti-LC3 antibody (18725-1-AP; 1:50) for immunohistochemistry was purchased from ProteinTech Group, Inc. HRP-labeled goat anti-human IgG (A0201; 1:50) was purchased from Beyotime Institute of Biotechnology. Anti-LC3 antibody and HRP-labeled goat anti-human IgG were all diluted in PBS containing 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA). Meanwhile, serial sections were stained with hematoxylin and eosin (H&E).

**Statistical analysis.** All data were subjected to a single-factor analysis of variance with post hoc Fisher's least significant different test, or Student's t-test using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean  $\pm$  standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

**$\alpha$ -hederin inhibits cell viability and proliferation of colorectal cancer cells.** Previous studies have suggested that  $\alpha$ -hederin has anticancer properties against different cancers (24,27,29,31-36), including colorectal cancer (29). To evaluate the effect of  $\alpha$ -hederin on the proliferation of colorectal cancer cells, its effect on the viability of two colorectal cancer cell lines (HCT116 and HCT8) was tested. Furthermore, colony formation assay was performed to determine cell proliferation.

In cell viability assay, HCT116 and HCT8 wells were divided into seven groups according to  $\alpha$ -hederin



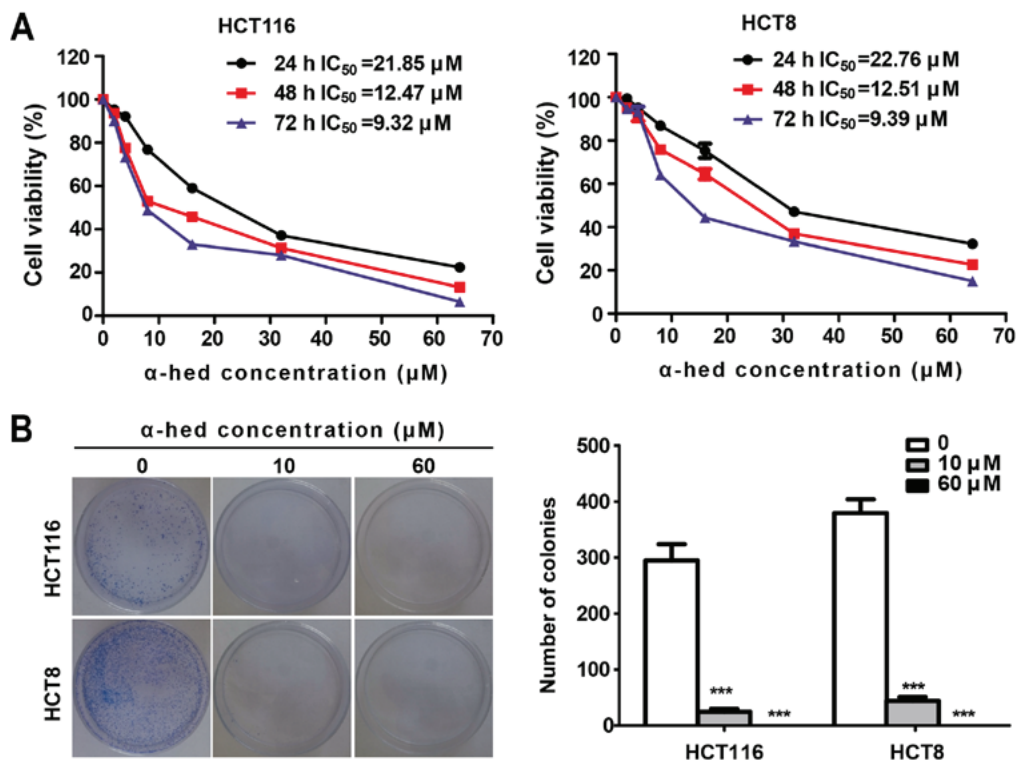


Figure 1.  $\alpha$ -hederin inhibits colorectal cancer cell proliferation. (A)  $\alpha$ -hederin inhibits cell viability of colorectal cancer cells. Colorectal cancer cell lines HCT8 and HCT116 were incubated with increasing doses of  $\alpha$ -hederin (0–64  $\mu$ M) for 24, 48 and 72 h to analyze cell proliferation using cell counting kit-8 assay. (B)  $\alpha$ -hederin inhibits colony formation of colorectal cancer cells. HCT8 and HCT116 were incubated with  $\alpha$ -hederin at 0, 10 and 60  $\mu$ M for 10 days. Representative colony images are presented. \*\*\* $P$ <0.001 vs. 0  $\mu$ M.  $\alpha$ -hed,  $\alpha$ -hederin; IC<sub>50</sub>, half maximal inhibitory concentration.

concentration (0, 2, 4, 8, 16, 32 and 64  $\mu$ M). Each group contained six wells. As presented in Fig. 1A, the cell viability of the two cell lines decreased significantly with the increase of  $\alpha$ -hederin concentration. The half maximal inhibitory concentration (IC<sub>50</sub>) of  $\alpha$ -hederin in HCT116 was 21.85, 12.47 and 9.32  $\mu$ M for 24, 48 and 72 h, respectively. Similarly, IC<sub>50</sub> values of  $\alpha$ -hederin in HCT8 cells treated for 24, 48 and 72 h were 22.76, 12.51 and 9.39  $\mu$ M, respectively. Therefore,  $\alpha$ -hederin decreased the viability of both colorectal cancer cell lines in a dose- and time-dependent manner. Colony formation assay using the adhesive culture system was used to analyze the antiproliferation effects of  $\alpha$ -hederin. It was demonstrated that  $\alpha$ -hederin treatment resulted in a dose-dependent decrease in the efficiency of colony formation (Fig. 1B).  $\alpha$ -Hederin concentrations at 10  $\mu$ M caused a significant decrease in the number of colonies in both colorectal cancer cell lines ( $P$ <0.001). Furthermore, after increasing  $\alpha$ -hederin concentrations to 60  $\mu$ M, no colonies were observed.

*$\alpha$ -hederin induces apoptosis in colorectal cancer cells.* Previous studies have demonstrated that  $\alpha$ -hederin may induce cell apoptosis (27,37). In the present study,  $\alpha$ -hederin-induced apoptosis rates of colorectal cancer cells were determined using flow cytometry with Annexin V/PI (Fig. 2A) and microscopy using Hoechst 33258 staining (Fig. 2B). Flow cytometry results indicated that the  $\alpha$ -hederin-induced (0, 10 and 60  $\mu$ M) apoptosis rates were  $5.51\pm 1.18$ ,  $8.97\pm 1.69$  and  $21.75\pm 2.85\%$  in HCT116 cells. Apoptosis rates in HCT8 cells were  $3.54\pm 1.3$ ,  $7.66\pm 1.89$  and  $15.66\pm 2.36\%$  (Fig. 2A). Compared with control, 10  $\mu$ M  $\alpha$ -hederin induced higher levels of apoptosis in HCT116

and HCT8 cells ( $P$ <0.01), with 60  $\mu$ M  $\alpha$ -hederin inducing even greater levels of apoptosis ( $P$ <0.001). In addition, Hoechst 33258 staining exhibited results similar to those for flow cytometry (Fig. 2B).

The mitochondrial signaling pathway is one of the major pathways involved in apoptosis (38). To observe the effect of  $\alpha$ -hederin on the mitochondrial signal pathway, the expression of mitochondrial apoptosis-related proteins was measured in HCT116 cells. As presented in Fig. 2C, compared with the control, the expression of activated caspase-9 (cleaved caspase-9), activated caspase-3 (cleaved caspase-3), PARP degradation (cleaved PARP), and Bax were all increased in HCT116 cells treated with  $\alpha$ -hederin. Furthermore,  $\alpha$ -hederin increased these proteins expression of HCT116 cells in a time-dependent manner. Conversely,  $\alpha$ -hederin decreased the expression of Bcl-2 and Bcl-x1 in a time-dependent manner.

*$\alpha$ -hederin induces autophagy in colorectal cancer cells.* Analysis of Figs. 1A and 2A indicated that the apoptosis rate did not match the inhibition rate of  $\alpha$ -hederin on colorectal cancer cell viability. This suggested that  $\alpha$ -hederin-induced cell death may have involved other mechanisms. Autophagy rates in HCT116 and HCT8 cells were determined using LC3 lentivirus (39). As a negative control, mutant LC3 (LC3-mu) was used as it could not bind to autophagosomes and form bright fluorescent spots. As presented in Fig. 3A,  $\alpha$ -hederin induced LC3 but not LC3-mu aggregation in HCT116 and HCT8 cells. Compared with controls, 10  $\mu$ M  $\alpha$ -hederin exhibited significantly higher autophagic rate in HCT116 and HCT8 cells ( $P$ <0.001). The presence of autophagosomes in

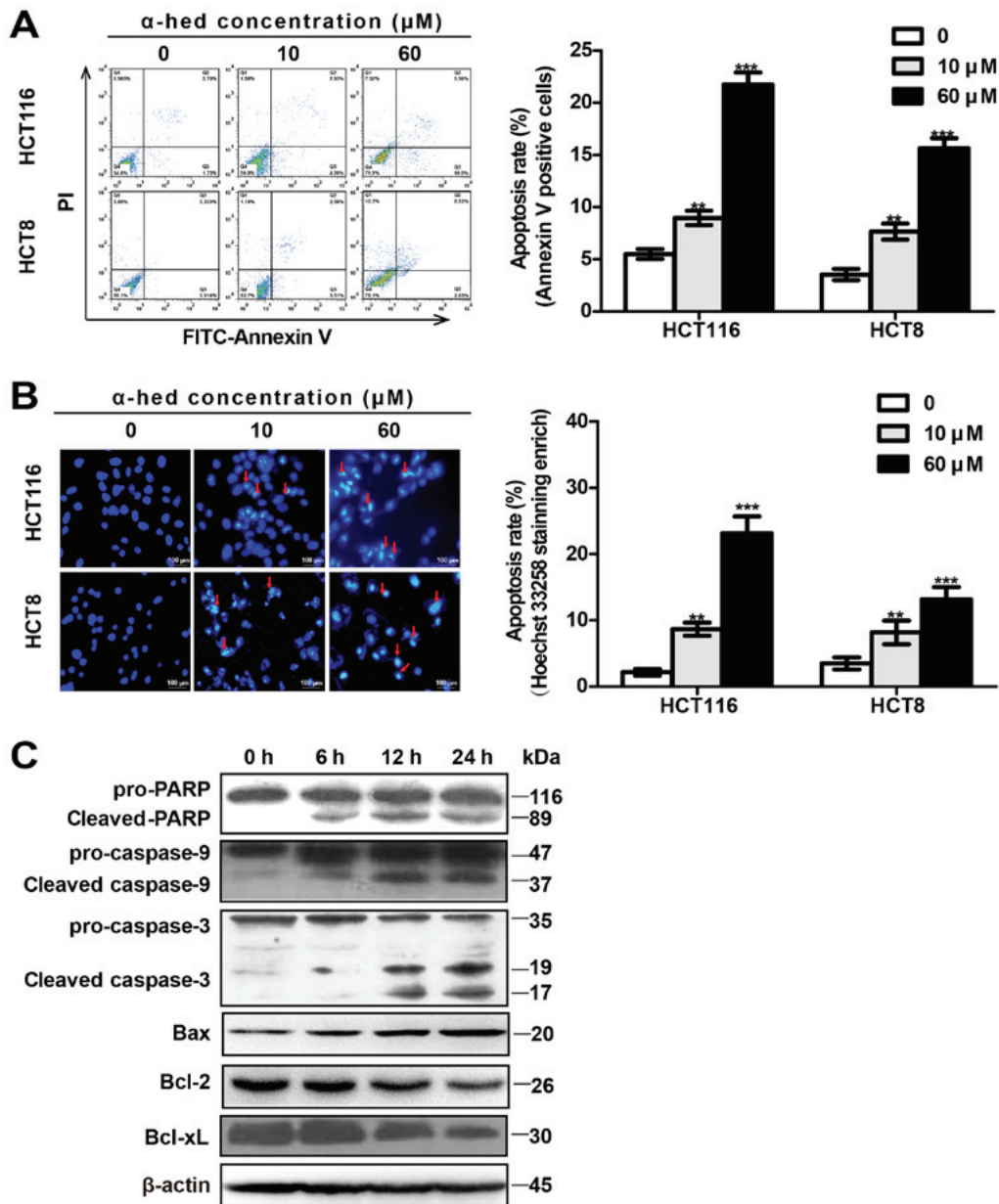


Figure 2.  $\alpha$ -hederin induces colorectal cancer cell apoptosis. (A) Apoptosis analysis of  $\alpha$ -hederin-treated cells through flow cytometry using Annexin V/PI. HCT8 and HCT116 cells were treated with 0, 10 and 60  $\mu$ M  $\alpha$ -hederin for 24 h. (B) Apoptosis analysis of  $\alpha$ -hederin-treated cells through microscopy using Hoechst 33258 staining (magnification, x200). Following treatment with  $\alpha$ -hederin for 24 h, Hoechst 33258 staining was used to identify apoptotic cells (red arrows). Histograms on the right represent apoptosis rates following Hoechst 33258 staining enrichment. (C) HCT116 cells were treated with 10  $\mu$ M  $\alpha$ -hederin for 6, 12 and 24 h, after which their lysates were analyzed by western blotting for mitochondrial apoptosis pathway protein levels using anti-PARP, anti-caspase-9, anti-caspase-3, anti-Bax, anti-Bcl-2, and anti-Bcl-xL antibody. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. 0  $\mu$ M. PI, propidium iodide;  $\alpha$ -hed,  $\alpha$ -hederin; FITC, fluorescein isothiocyanate; PARP, poly (ADP-ribose) polymerase; Bcl, B cell lymphoma; Bax, Bcl associated X protein.

HCT116 cells was also evaluated using an electron microscope. As presented in Fig. 3B, autophagosomes were present in  $\alpha$ -hederin-treated HCT116 and HCT8 cells. This result further supported that  $\alpha$ -hederin induced autophagy in colorectal cancer cells.

*Effect of  $\alpha$ -hederin on LC3 expression in vivo.* *In vitro* results had demonstrated that  $\alpha$ -hederin could induce autophagy in colorectal cancer cells. To investigate the inducing autophagy effect of  $\alpha$ -hederin *in vivo*, a subcutaneous xenograft model of HCT116 cells in nude mice was used. As presented in Fig. 4A,  $\alpha$ -hederin significantly inhibited tumor growth compared with the control. According to the results of H&E staining (Fig. 4B),

tumors treated with  $\alpha$ -hederin exhibited marked necrosis. LC3 puncta was assessed using immunohistochemistry to evaluate the effect of  $\alpha$ -hederin on autophagy *in vivo*. As presented in Fig. 4B, the presence of LC3 puncta was observed in samples treated with  $\alpha$ -hederin. In addition, the necrotic area also exhibited highly aggregated LC3 puncta. While, the control exhibited significant diffuse cytoplasmic staining without puncta. These results suggested that  $\alpha$ -hederin could inhibit tumorigenicity through promoting autophagy of colorectal cancer cells *in vivo*.

*$\alpha$ -hederin induces autophagy of colorectal cancer cells through the AMPK/mTOR pathway.* Given that dephosphorylation of

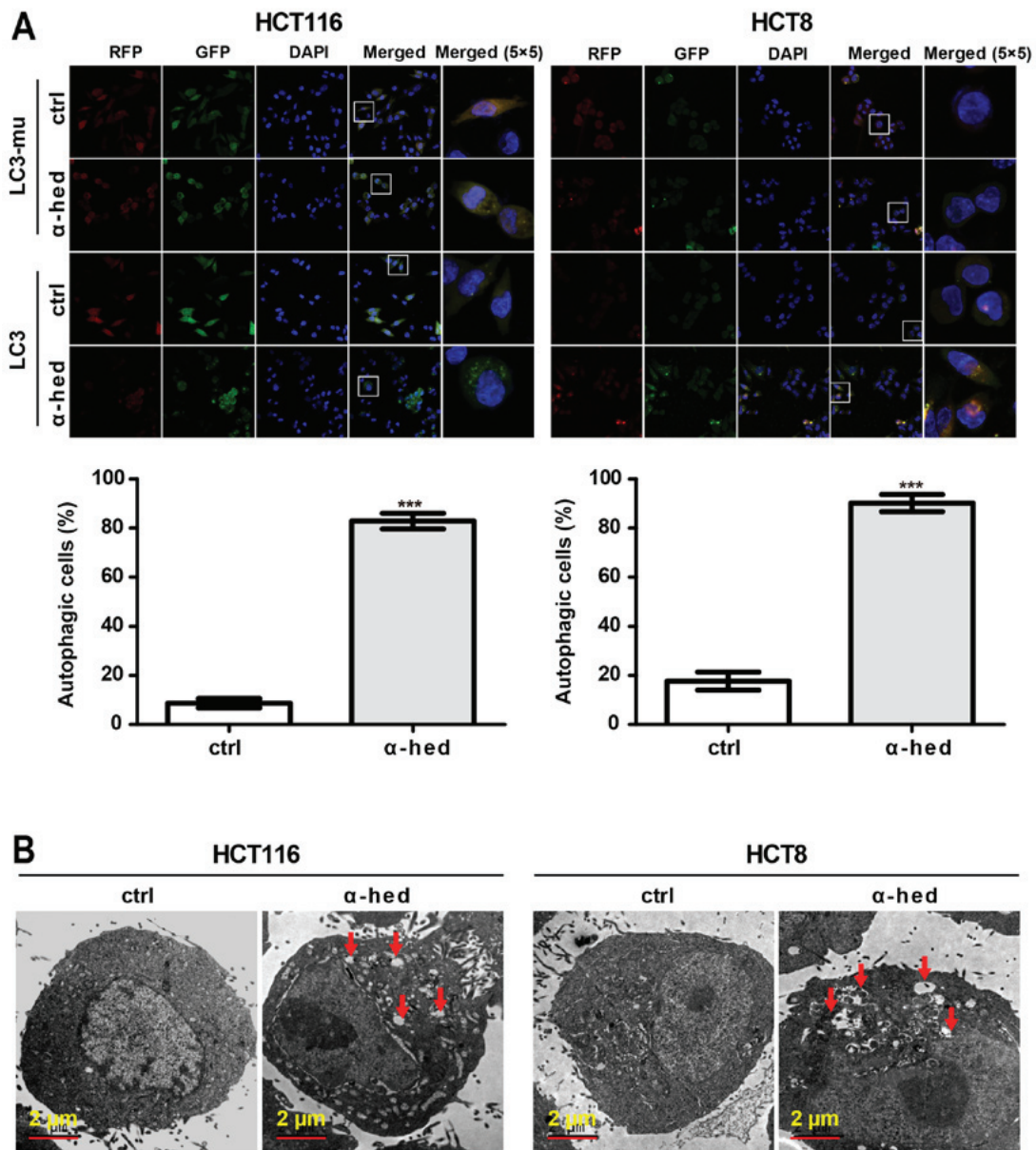


Figure 3.  $\alpha$ -hederin induces autophagy in colorectal cancer cells. (A) HCT116 and HCT8 cells were infected with LC3 and LC3-mu. After these cells were treated with  $10 \mu\text{M}$   $\alpha$ -hederin for 24 h, autophagy rate assay was then performed. Images were captured using laser confocal microscopy. Magnification of (RFP, GFP, DAPI and Merge magnification,  $\times 400$ ; Merge  $5\times 5$  magnification,  $\times 10,000$ ). Histograms present autophagy rates according to the proportion of highlighted cells. (B) Normal and autophagic HCT116 and HCT8 cells were photographed using an electron microscope. Red arrows in the graphs represent autophagosomes. \*\*\* $P < 0.001$  vs. ctrl. LC3, light chain 3 lentivirus; LC3-mu, mutation light chain 3 lentivirus;  $\alpha$ -hed,  $\alpha$ -hederin; ctrl, control.

p-mTOR and degradation of LC3 I to LC3 II are the major mechanisms involved in autophagy (40), LC3 II protein levels were used to determine the extent of cell autophagy (41). After treating HCT116 cells with  $\alpha$ -hederin for 24 h, cell lysates were used to detect p-mTOR and LC3 II protein levels. As presented in Fig. 5A, an increase in  $\alpha$ -hederin concentration resulted in a gradual increase in LC3 II levels but a gradual decrease in p-mTOR protein levels. HCT116 cells were also treated with  $10 \mu\text{M}$   $\alpha$ -hederin for 6, 12 and 24 h. The results demonstrated that, over time,  $\alpha$ -hederin caused a gradual decrease in p-mTOR, p-ULK1, p-P70S6K and P62 protein levels but a gradual increase in p-AMPK and beclin-1 protein levels (Fig. 5B).

AMPK/mTOR is a major signaling pathway involved in autophagy (42). In this signaling pathway, AMPK serves

as the activator of autophagy. AMPK activation induces dephosphorylation of mTOR, which separates it from the ULK1 complex. The subsequent dephosphorylation of ULK1 then initiates autophagy (43). To verify the role of the AMPK/mTOR pathway in  $\alpha$ -hederin-induced autophagy, the expression of autophagy-related signals was detected in HCT116 cells treated with AMPK siRNA. It was demonstrated that AMPK siRNA restored the expression of p-mTOR, p-P70S6K and p-ULK1, which had been decreased by  $\alpha$ -hederin (Fig. 5C). Results for p-AMPK indicated that although  $\alpha$ -hederin increased LC3 II, AMPK knockdown did not restore LC3 II.

*ROS-dependent AMPK activation by  $\alpha$ -hederin.* Previous studies have demonstrated that ROS is a major factor in



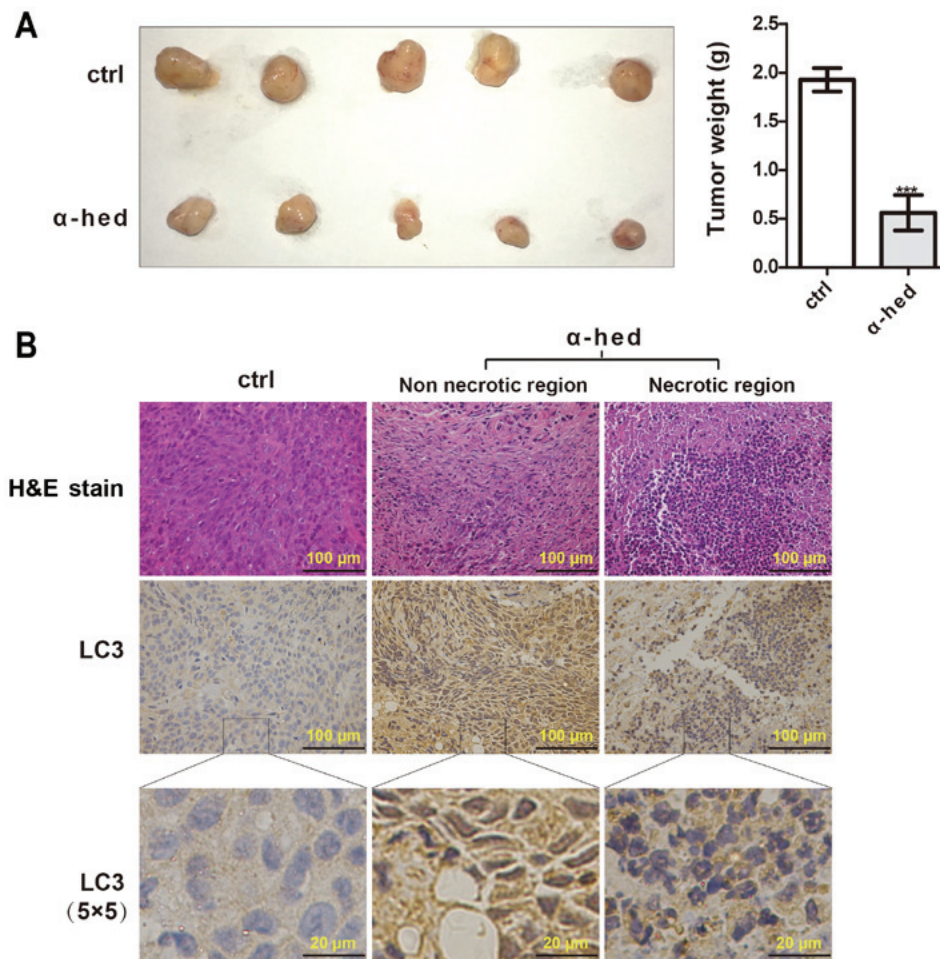


Figure 4.  $\alpha$ -hederin inhibits the proliferation and promotes the production of LC3 II in colorectal cancer cells *in vivo*. A subcutaneous xenograft model of HCT116 cells was treated with  $\alpha$ -hederin for 3 weeks. (A) Tumors were photographed and weighed. (B) H&E staining was used to evaluate the differences of tissue morphology. Immunohistochemistry was performed to evaluate the expression of autophagic marker LC3. \*\*\* $P < 0.001$  vs. ctrl. LC3, light chain 3; H&E, hematoxylin and eosin;  $\alpha$ -hed,  $\alpha$ -hederin; ctrl, control.

$\alpha$ -hederin-induced apoptosis (37,44). ROS production is also important for AMPK/mTOR pathway activation. To verify the role of ROS in  $\alpha$ -hederin-induced AMPK/mTOR pathway activation, oxidation-activated fluorescent dye DCHF-DA was used to detect intracellular ROS using flow cytometry. HCT116 cells were treated with 10  $\mu$ M each of  $\alpha$ -hederin and ROS inhibitor NAC for 24 h at 37°C then incubated in RPMI-1640 medium supplemented with 5  $\mu$ mol/ml DCHF-DA for 30 min at room temperature. Fig. 6A demonstrated that although  $\alpha$ -hederin increased ROS levels, ROS inhibitor NAC reversed this effect. Furthermore, NAC inhibited  $\alpha$ -hederin-induced AMPK/mTOR pathway activation (Fig. 6B). Subsequently, the effects of NAC and  $\alpha$ -hederin on apoptosis were detected by flow cytometry. As presented in Fig. 6C, NAC alone did not affect the apoptosis rate but NAC reduced the apoptosis rate treated by  $\alpha$ -hederin in HCT116 cells ( $P < 0.001$ ). Additional, results from confocal microscopy demonstrated that NAC could inhibit  $\alpha$ -hederin-induced autophagy (Fig. 6D).

*Autophagy and apoptosis comprise the major mechanisms by which  $\alpha$ -hederin induces colorectal cancer cell death. To clarify the interplay between apoptosis and autophagy induced by  $\alpha$ -hederin in colorectal cancer, the effects of autophagy*

inhibitor 3-MA on apoptosis and autophagy were investigated. HCT116 cells were treated with 2 mM 3-MA for 48 h to evaluate the effect of 3-MA alone on cell viability. The results indicated that 2 mM 3-MA alone had no significant effect on cell viability of HCT116 cells (Fig. 7A). Then, HCT116 cells were treated with  $\alpha$ -hederin (2, 4, 8, 16, 32 and 64  $\mu$ M) or  $\alpha$ -hederin plus 2 mM 3-MA for 48 h, after which cell viability was tested. As presented in Fig. 7B, 2 mM 3-MA significantly reduced the effect of  $\alpha$ -hederin on cell viability ( $P < 0.05$ ). Furthermore, HCT116 cells were treated with 2 mM 3-MA, 10 mM  $\alpha$ -hederin and 2 mM 3-MA with 10 mM  $\alpha$ -hederin for 48 h. As presented in Fig. 7C, 3-MA had no significant effect on  $\alpha$ -hederin-induced apoptosis. Finally, apoptosis rate was detected by flow cytometry. It was also demonstrated that 3-MA blocked the  $\alpha$ -hederin-induced autophagosome formation (Fig. 7D).

## Discussion

$\alpha$ -Hederin, a novel type of drug derived from the leaves of ivy or *Nigella sativa*, had exhibited strong anticancer activities in recent studies. Previous studies had demonstrated that  $\alpha$ -hederin could induce cell apoptosis (23,26,27,37). In the present study, it was demonstrated that  $\alpha$ -hederin activated the

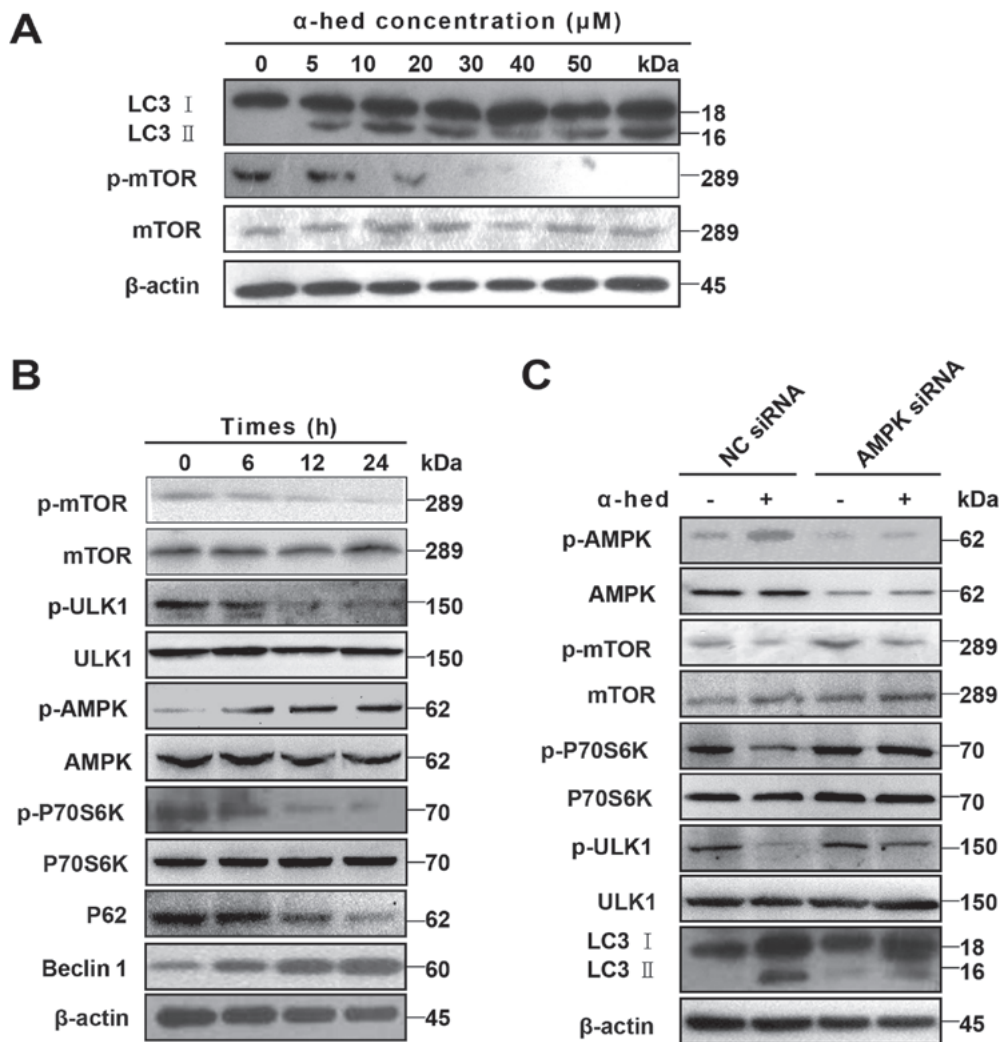


Figure 5. AMPK/mTOR pathway participated in  $\alpha$ -hederin-induced autophagy. (A)  $\alpha$ -hederin upregulated LC3 II levels and inhibited p-mTOR in a dose-dependent manner. (B) After HCT116 cells were treated with  $10 \mu$ M  $\alpha$ -hederin for 6, 12 and 24 h, expression levels of p-mTOR, mTOR, p-ULK1, ULK1, p-AMPK, AMPK, p-P70S6K, P70S6K, P62 and beclin1 were determined using specific antibodies. (C) HCT116 cells were treated with AMPK siRNA and NC siRNA for 3 days, with  $\alpha$ -hederin being added during the last 2 days. The expression levels of p-AMPK, AMPK, p-mTOR, mTOR, p-ULK1, ULK1, p-P70S6K, P70S6K and LC3 were then evaluated using western blotting. AMPK, AMP-activated protein kinase; mTOR, mechanistic target of rapamycin; LC3, light chain 3; p, phosphorylated; ULK1, Unc-51 like autophagy activating kinase 1; siRNA, small interfering RNA; NC, normal control;  $\alpha$ -hed,  $\alpha$ -hederin.

mitochondrial apoptosis signal pathway in colorectal cancer cells. However, the results also demonstrated that the rate of apoptosis induced by  $\alpha$ -hederin was not consistent with its inhibitory effects on colorectal cancer cells, suggesting that other mechanisms may be involved in the anticancer activity of  $\alpha$ -hederin.

Previous studies had demonstrated that autophagy regulated by  $\alpha$ -hederin was differently in different cells:  $\alpha$ -hederin induced autophagy in neuronal PC12 cell (45) but inhibited autophagy in non-small cell lung cancer cells (44). In the present study, autophagosomes were observed in two colorectal cancer cell lines (HCT116 and HCT8) treated with  $\alpha$ -hederin by fluorescence microscopy using LC3 lentivirus and electron microscopy. In view of the high toxicity of  $\alpha$ -hederin, low-dose (2 mg/kg body weight)  $\alpha$ -hederin was used for intraperitoneal injection. The results also demonstrated that  $\alpha$ -hederin could induce autophagy in colorectal cancer cells *in vivo*.

The mTOR pathway is a typical signaling pathway that regulates cell autophagy (40). LC3, a subunit of the

microtubule-associated proteins involved in the formation of autophagic vacuoles, is regarded as an autophagy marker (41). It was demonstrated that  $\alpha$ -hederin was able to decrease the expression of p-mTOR and increase the ratio of LC3II/I in a concentration-dependent manner. Which suggested that  $\alpha$ -hederin could activate autophagy mediated by the mTOR signaling pathway.

AMPK signaling is one major regulatory signal of mTOR signaling pathway and autophagy (46). In addition,  $\alpha$ -hederin is able to induce AMPK/mTOR dependent autophagy and promote the degradation of neurodegenerative mutant disease protein (45). The expression of AMPK/mTOR signaling pathway related proteins and autophagy-related proteins were also detected. The results demonstrated that  $\alpha$ -hederin could induce AMPK-mTOR dependent autophagy in HCT116 cells. In addition, this autophagy could be blocked by the AMPK siRNA. It suggested that the AMPK/mTOR signaling pathway served a major role in  $\alpha$ -hederin-induced autophagy.



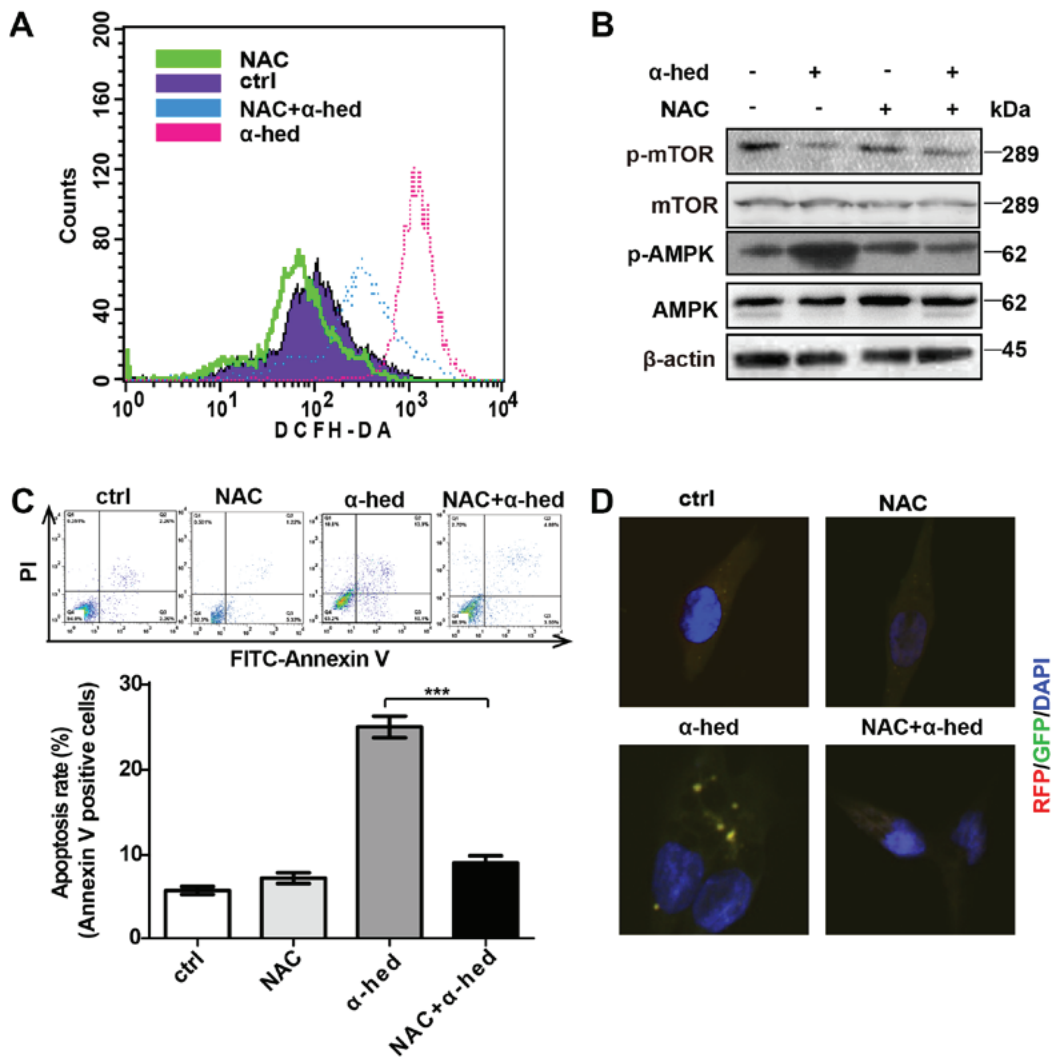


Figure 6.  $\alpha$ -hederin-induced AMPK pathway activation depended on ROS. (A) After being treated with  $10 \mu\text{M}$   $\alpha$ -hederin or ROS inhibitor NAC ( $10 \mu\text{M}$ ) for 24 h, HCT116 cells were dyed with oxidation-activated fluorescent dye DCFH-DA and analyzed using flow cytometry to detect intracellular ROS. (B) After being treated with  $10 \mu\text{M}$   $\alpha$ -hederin or ROS inhibitor NAC ( $10 \mu\text{M}$ ) for 24 h, western blotting was used to test HCT116 cell lysates for p-mTOR and p-AMPK expression levels. Following treatment with  $\alpha$ -hederin, NAC and NAC plus  $\alpha$ -hederin for 24 h, (C) The apoptosis rate of HCT116 cells was tested by flow cytometry. (D) Light chain 3 fluorescence of HCT116 cells was photographed via confocal microscopy (magnification,  $\times 400$ ). \*\*\* $P < 0.001$ . AMPK, AMP-activated protein kinase; ROS, reactive oxygen species; mTOR, mechanistic target of rapamycin; p, phosphorylated;  $\alpha$ -hed,  $\alpha$ -hederin; ctrl, control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

ROS production has been regarded as the ideal anticancer mechanism for many drugs (37,47) given that it activates both apoptosis and autophagy to kill cancer cells (48). In the present study, ROS levels of HCT116 cells treated with  $\alpha$ -hederin were determined using DCFH-DA. The results demonstrated that although  $\alpha$ -hederin increased ROS levels, NAC reversed this effect. The results of western blotting demonstrated that NAC blocked the  $\alpha$ -hederin-induced activation of the AMPK/mTOR signaling pathway. This suggested that the  $\alpha$ -hederin-induced AMPK/mTOR signaling pathway activation depended on ROS production. In addition, NAC could reverse the apoptosis and autophagy induced by  $\alpha$ -hederin. These results suggested that increasing ROS may serve a key role for the anticancer effects of  $\alpha$ -hederin.

Autophagy is one of the major mechanisms involved in cancer cell apoptosis and drug resistance during the treatment of various cancers (49). This is primarily because drugs stimulate the cell's protective autophagy and inhibit cell apoptosis (50).

However, a number of studies have demonstrated that certain drugs cause not only excessive autophagy in specific tumor cells, but also autophagic cell death (51,52), making autophagy their main anticancer mechanism. Therefore, considering that different drugs act on different cells, their autophagic and apoptotic outcomes may be different. This may have been caused by differing activation of the signaling pathway (53). In the present study, it was demonstrated that autophagy inhibitor 3-MA significantly reduced the anticancer effects of  $\alpha$ -hederin. Furthermore, 3-MA had no significant effects on  $\alpha$ -hederin-induced apoptosis in HCT116 cells. These results suggest that autophagy and apoptosis are equally important mechanisms through which  $\alpha$ -hederin exerts its anticancer effects.

In conclusion, the present findings suggest that  $\alpha$ -hederin may stimulate ROS production in colorectal cancer cells and activate both autophagy and apoptosis, both of which increase the anticancer effects of  $\alpha$ -hederin. Autophagy is

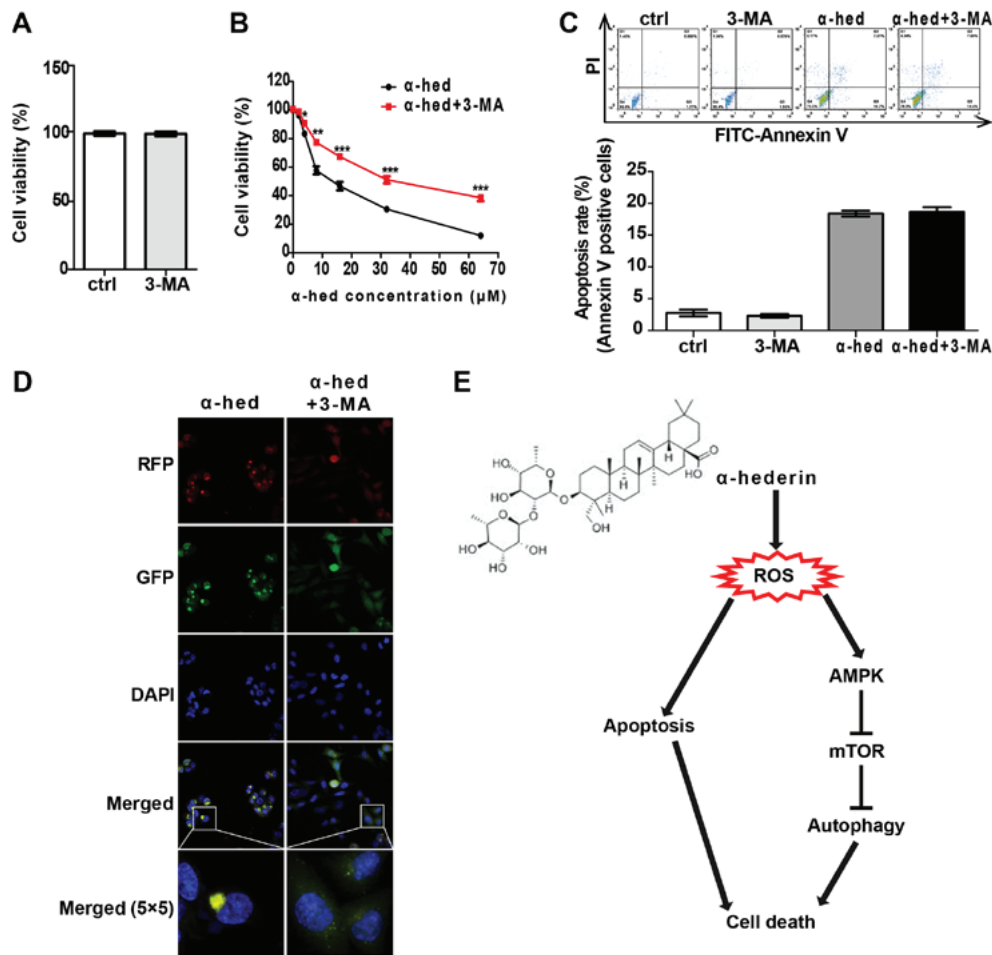


Figure 7. Cell death induced by  $\alpha$ -hederin depended on apoptosis and autophagy. (A) HCT116 was treated with 2 mM 3-MA for 24 h. CCK8 was used to evaluate the effects of 2 mM 3-MA alone on the cell viability. (B) HCT116 was incubated with 10  $\mu$ M  $\alpha$ -hederin or 10  $\mu$ M  $\alpha$ -hederin plus 2 mM 3-MA for 24 h. Thereafter, CCK8 assay was used to detect cell viability. (C) HCT116 was incubated with 10  $\mu$ M  $\alpha$ -hederin, 2 mM 3-MA or 10  $\mu$ M  $\alpha$ -hederin plus 2 mM 3-MA for 24 h. Apoptosis assay by flow cytometry was used to determine the effect of 3-MA on  $\alpha$ -hederin induced apoptosis. (D) Light chain 3 lentivirus-infected HCT116 cells were treated with 10  $\mu$ M  $\alpha$ -hederin or 10  $\mu$ M  $\alpha$ -hederin plus 2 mM 3-MA for 24 h. Autophagosomes were then photographed using laser confocal microscopy (RFP, GFP, DAPI and Merge magnification, x400; Merge 5x5 magnification, x10,000). (E) Model of the mechanism for  $\alpha$ -hederin induced colorectal cancer cell death. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs.  $\alpha$ -hed. CCK8, cell counting kit 8; ctrl, control;  $\alpha$ -hed,  $\alpha$ -hederin; PI, propidium iodide; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species.

produced by the ROS-activated AMPK/mTOR signaling pathway (Fig. 7E). With its strong anticancer effect and multiple anticancer mechanisms,  $\alpha$ -hederin may potentially become a more reliable anticancer drug. However, due to its strong toxicity (54-57), protein absorption (58) and hemolytic effect (59), further molecular modifications and nanotarget drug carriers should be applied during the anticancer study of  $\alpha$ -hederin.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

### Authors' contributions

JS, YF, YW, QJ, GC, LS, YW, YH, JZ and QL performed the experiments and data analysis, and wrote the manuscript. JS made substantial contributions to the design of the present study and acquired experimental materials. JZ and QL served a key role in guiding the subject, providing funding and writing the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The animal protocol was approved by the Ethics Committee of Shuguang Hospital affiliated with Shanghai University of Traditional Chinese Medicine.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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