

Active fraction of clove induces apoptosis via PI3K/Akt/mTOR-mediated autophagy in human colorectal cancer HCT-116 cells

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Received March 19, 2018; Accepted June 14, 2018

DOI: 10.3892/ijo.2018.4465

Abstract. Previous studies by our group have demonstrated that extract of clove exhibits potent anticancer effects *in vitro* and *in vivo*. In the present study, the effect of an extracted and isolated active fraction of clove (AFC) on induction of cellular apoptosis in human colorectal cancer HCT-116 cells was investigated by morphological observation, flow cytometry, and western blotting analysis. The results revealed that AFC induced apoptosis of HCT-116 cells. AFC also induced autophagy, demonstrated by increased punctuate microtubule-associated protein 1A/1B-light chain 3 (LC3) staining, and LC3-II and Beclin-1 protein expression levels. Furthermore, the autophagy inhibitors 3-MA and bafilomycin A1 potentiated the pro-apoptotic activity of AFC in HCT-116 cells. AFC also inhibited the phosphorylation of the phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin signaling pathway. The present study may improve the existing understanding of the anticancer mechanisms of clove and provide a scientific rationale for AFC to be further developed as a promising novel anticancer agent for the treatment of colorectal cancer.

Introduction

Colorectal cancer (CRC) is among the most common types of cancer and the third leading cause of cancer-associated mortality worldwide. Therefore, it is an important topic among oncologists and cancer biologists (1-3). Although the mortality

rate of CRC has decreased significantly with early detection and surgery and chemotherapy treatments, recurrence and drug resistance have become more common (4). Therefore, there is an urgent requirement to develop novel anticancer drugs and/or therapeutic strategies for the treatment of colorectal cancer.

Clove, the dried bud of *Syzygium aromaticum*, is a traditional medicinal herb widely used in Asian countries. Clove has been indicated to possess various biological properties including anti-inflammatory (5,6), antiviral (7), antibacterial (8), antioxidant (8,9) and antitumor (8,10,11) activities. Our previous study demonstrated that the active fraction of clove (AFC) was effective against various types of cancer cell, including lung, breast, liver, pancreatic, ovarian and cervical cancer (12). Two components of AFC have been identified as demonstrating cytotoxicity against various types of cancer cells: Oleanonic acid (OA) and eugenol (12). It was also demonstrated that OA was able to induce apoptosis of cancer cells via the mitochondrial pathway (13). Combination of OA and fluorouracil (5-FU) treatments synergistically potentiated the cytotoxicity of 5-FU against human pancreatic cancer Pan-28 cells (13). AFC was more effective than a single isolated component of OA or eugenol against human colon cancer HT-29 xenografts *in vivo* (12). However, the mechanistic action of AFC remains unclear. Therefore, the present study had the interesting aim of elucidating the mechanism of action of AFC.

Apoptosis (programmed cell death) is a highly regulated and controlled process and serves a crucial role in the development and treatment of cancer (14-16). Apoptosis occurs through two major molecular pathways, the intrinsic (mitochondrial) and the extrinsic (death receptor-mediated) pathways (16,17). The intrinsic pathway involves the mitochondria and mainly affects the Bcl-2 and caspase families (17). The extrinsic pathway involves death signals, including TNF- α with TNF receptor 1 (TNFR1) and activates caspase-8 to cleave procaspase-3 into its active form (16). Apoptosis is an important anticancer mechanism and numerous anticancer drugs execute their anticancer activity via induction of apoptosis (18).

Autophagy is a survival-promoting pathway and serves a complicated role in cell development, growth and tumorigenesis to regulate inhibition of cancer cell proliferation or promotion of cancer cell survival (19-22). Numerous anticancer

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Key words: clove, apoptosis, autophagy, colorectal cancer, phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin pathway

agents display antitumor activity via autophagy of cancer cells (23-25). The phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin signaling pathway is a major signal transduction cascade involved in cell proliferation, survival and metabolism, and it serves an important role in the development and therapy of colorectal cancer (26,27). Upregulation of PI3K expression results in the inhibition of apoptosis in colon cancer SW480 cells (28). The PI3K/Akt/mTOR signaling pathway is also an important therapeutic target and a previous study demonstrated that the dual PI3K/mTOR inhibitor, NVP-BEZ235, was effective against colorectal cancer cells *in vitro* and *in vivo* (29). The PI3K/Akt/mTOR signaling pathway is also involved in the autophagic process, and activation of the pathway attenuates autophagy in cancer cells. A recent study also demonstrated that OA, one of the main components of AFC, is capable of inducing protective autophagy in cancer cells via the PI3K/Akt/mTOR signaling pathway (30). However, the effect of AFC on autophagy remains unclear. In the present study, AFC-induced autophagic effects were evaluated in human colorectal cancer HCT-116 cells by morphological observation, flow cytometry and western blotting. The results revealed that AFC induced apoptosis via the PI3K/Akt/mTOR-mediated autophagic pathway in colorectal cancer HCT-116 cells.

Materials and methods

Reagents and antibodies. Rapamycin and 3-methyladenine (3-MA) were purchased from Medchem Express Co., Ltd. (Shanghai, China). Baflomycin A1 (BA) was purchased from Beijing Hua MEIKO Biotechnology Co., Ltd. (Beijing, China). The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). Cell culture media, DMEM, RPMI-1640, and McCoy's5A were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell Counting kit-8 (CCK-8), Hoechst 33258, Ad-mCherry-GFP-LC3B, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), penicillin, streptomycin, monoclonal antibodies of β -actin (cat. no. AA128-1), horseradish peroxidase (HRP)-labeled goat anti-rat IgG(H+L) (#A0216) and HRP-labeled goat anti-rabbit IgG(H+L) (cat. no. A0208) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). LY294002, the caspase-3 (cat. no. ab32351), caspase-9 (cat. no. ab32539) and Poly(ADP-ribose) polymerase (PARP; cat. no. ab191217) antibodies were purchased from Abcam (Cambridge, UK). LC3B (cat. no. 3868), Beclin-1 (cat. no. 3495), PI3K (cat. no. 4292), p-PI3K at Tyr458 (cat. no. 4228), Akt (cat. no. 9272), p-Akt at Ser473 (cat. no. 9271), mTOR (cat. no. 2972) and p-mTOR at Ser2448 (cat. no. 2971) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Insulin-like growth factor-I (IGF-I) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Cell culture. The human colorectal carcinoma cell lines, HCT-116, SW620, HCT8, HT29 and LoVo were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in RPMI-1640, DMEM or McCoy's5A (Gibco; Thermo Fisher Scientific, Inc.) culture medium containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO₂. Cells

in the logarithmic phase were routinely renewed with fresh medium every 2-3 days.

Extraction, isolation and characterization of individual compounds with cytotoxic activity. Air-dried, powdered clove was purchased from Qingdao Company of Traditional Chinese Medicine (Qingdao, Shandong, China). The isolation and extraction of clove was performed as previously described (31). Briefly, 10.0 kg powdered clove was hydrated with 95% ethanol at room temperature for 72 h. Subsequent to filtration, the solution was concentrated to generate an ethanol extract and the extract was further extracted with ethyl acetate at 60°C for 2 h, and the supernatant was concentrated using a vacuum rotary evaporator (Yamato Sci., Tokyo, Japan). The ethyl acetate extract of clove (EAEC) was fractionated using silica gel column chromatography (CC; 200 and 400 mesh) and Sephadex LH-20 CC. The resulting fraction was defined as active fraction from clove (AFC). The purity was characterized by the Department of Chemistry and Molecular Engineering at Qingdao University (Qingdao) using ¹H- and ¹³C-NMR analysis, confirming that AFC contained three main compounds, including eugenol (32.32%) and oleanonic Acid (23.60). AFC underwent a comprehensive residue screen for 172 pesticides by the Pacific Agricultural Laboratory (Portland, OR, USA) and no pesticides were detected. Similarly, AFC was analyzed for heavy metal content by Avomeen Analytical Services (Ann Arbor, MI, USA) and no heavy metal was detected.

Drug preparation and treatment. AFC was diluted with cell culture medium to final concentrations of 25, 50, 100, 200 and 400 μ g/ml for the treatment of HCT-116 cells for 24, 48 or 72 h. The PI3K inhibitor, LY294002, and the autophagy-lysosomal inhibitor, BA, were dissolved in DMSO and diluted with culture medium. The final concentration of DMSO in the test solutions was <0.1%. This concentration of DMSO did not cause any adverse responses in cells. 3-MA was dissolved in heated sterile double distilled water to achieve a 100 mM stock solution, and was then diluted with culture medium for a final concentration of 2 mM. IGF-I (a PI3K activator) was reconstituted to 10 μ g/ml in sterile PBS, and diluted with culture medium for a final concentration of 50 ng/ml. HCT-116 cells were pretreated with 10 μ M LY294002, 1 nM BA, 2 mM 3-MA or 50 ng/ml IGF-I for 1 h, then further treated with 100 μ g/ml AFC for 48 h. Cells were treated with fresh medium without serum as a vehicle control.

Cell viability assay. Cell viability was determined by CCK-8 assay as previously described (12). Briefly, cells at 80-90% confluency were seeded in 96-well plates at a density of 5×10^3 cells/well. After a 24-h incubation 25, 50, 100, 200 or 400 μ g/ml AFC, or vehicle control, was added to the wells. After 24, 48 or 72 h 10% CCK-8 reagent (10 μ l/well) was added and incubated for 1 h at 37°C. The absorbance value (optical density; OD) was measured at a wavelength of 450 nm with a SpectraMAX M3 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) and the cell viability ratio was calculated using the following formula: Cell viability ratio = (OD of the experimental group/OD of the control group) $\times 100\%$. The half maximal inhibitory concentration (IC₅₀) values were determined using a nonlinear best fit method

by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). All experiments were performed 3 times in triplicate.

GFP-LC3 transfection. HCT-116 cells were seeded onto coverslips in a 24-well plate at a density of 5×10^4 cells/well and were transfected with Ad-mCherry-GFP-LC3B. Following 24 h of culture, the cells were treated with AFC (100 $\mu\text{g/ml}$) or medium (control) for 48 h then fixed with 4% polyoxymethylene and observed with an EVOS™ FL Imaging system (AMF4300; Thermo Fisher Scientific Inc.). Autophagic cells presenting ≥ 5 mRFP-GFP-LC3 dots were counted. All experiments were performed 3 times in triplicate.

Apoptotic assay of HCT-116 cells by Hoechst 33258 staining. Morphological assessment of apoptotic cells was analyzed using Hoechst 33258 staining as previously described (31). Briefly, HCT-116 cells were seeded in a 24-well plate at a density of 5×10^4 cells/well. Following culture for 24 h, the cells were treated with 25, 50 or 100 $\mu\text{g/ml}$ AFC or the same volume of vehicle control. Following incubation for another 48 h, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and the medium was removed and washed with PBS for 15 min. The cells were treated with 10 $\mu\text{g/ml}$ Hoechst 33258 staining at room temperature for 10 min, and washed with PBS for 15 min in the dark to reduce background. The morphology of the cells was visualized and photographed under a DMR fluorescence microscope at x400 magnification (Leica Microsystems GmbH, Wetzlar, Germany) using fluorescence excitation at 340 nm. The apoptotic index was calculated using the following formula: Apoptotic index = apoptotic cell number/total cell number $\times 100\%$. A minimum of 4 fields of view of each well, containing ≥ 500 cells, were required to calculate the rate of apoptosis. All experiments were performed 3 times in triplicate.

Observation of autophagosomes by transmission electron microscopy. HCT-116 cells were seeded in a 24-well plate at a density of 5×10^4 cells/well. Following 24 h of culture, the cells were treated with 25, 50 and 100 $\mu\text{g/ml}$ AFC or the equivalent volume of medium (vehicle control) for 48 h. The cells were collected by trypsinization and washed twice with PBS, then fixed in 2.5% glutaraldehyde for 90 min and post-fixed in 1% osmium tetroxide for 30 min at room temperature. Subsequent to 3 washes with PBS, the cells were progressively dehydrated in an ascending alcohol series (50, 70, 95 and 100%), and embedded in Epon resin. The ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy observation. The ultrastructure of the cells was then examined under a transmission electron microscope (JEM-1230; JEOL, Ltd., Tokyo, Japan). All experiments were performed 3 times in triplicate.

Apoptotic assay of HCT-116 cells with Annexin V-FITC/PI and flow cytometry. AFC-induced apoptosis in HCT-116 cells was also evaluated by flow cytometry with Annexin V-FITC apoptosis detection kit (cat. no. 556547BD Pharmingen; BD Biosciences), according to the manufacturer's protocol. Briefly, HCT-116 cells at 80-90% confluency were seeded in 6-well plates at a density of 4×10^5 cells/well and cultured for 24 h. Then, the cells were treated with 100 $\mu\text{g/ml}$ AFC or vehicle

control for 48 h. After treatment, the cells were harvested by cryogenic centrifugation at 4°C , $1,500 \times g$ for 5 min and washed twice with 4°C PBS. The cells were resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. A total of 100 μl of the solution (1×10^5 cells) was transferred to a 5-ml culture tube, and 5 μl Annexin V-FITC and 5 μl PI were successively added to the cells and incubated at room temperature in the dark for 15 min. The quantity of stained cells was analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). All experiments were performed 3 times in triplicate.

Western blotting analysis. HCT-116 cells were seeded at a density of 4×10^5 cells/well in 6-well plates, and the culture medium (2 ml) was replaced after 24 h in culture, with 25, 50 or 100 $\mu\text{l/ml}$ AFC, or vehicle control for 24 h prior to harvesting. The cells were lysed on ice with radioimmunoprecipitation assay buffer [0.5% NP-40, 50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 0.1 mM Na_3VO_4 , 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 $\mu\text{g/ml}$ leupeptin, pH 7.5] for 30 min in the presence of PhosSTOP (Roche Molecular Systems, Inc., Basel, Switzerland) with PMSF and then centrifuged at $9600 \times g$ for 20 min at 4°C . The supernatant was collected and stored in aliquots at -80°C until analysis. Protein concentrations were determined using BCA protein assay kit (Beyotime Institution of Biotechnology, Shanghai, China) and equalized prior to loading. Equal amounts of protein (40 μg) were separated by 15% SDS-PAGE and blotted onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat dry milk or bovine serum albumin in 1X Tris-buffered saline with Tween (TBST; 20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20) for 1 h at room temperature, the membranes were incubated with primary antibodies at a dilution of 1:1,000 (caspase-9, PARP, LC3B, Beclin-1, PI3K, p-PI3K at Tyr458, Akt, p-Akt at Ser473, mTOR, p-mTOR at Ser2448) and 1:5,000 (β -actin and caspase-3) at 4°C overnight. Then, the membranes were incubated secondary antibodies at a dilution of 1:2,000 at room temperature for 1 h. β -actin was used as a loading control. Following 3 washes in TBST, the membranes were developed by incubation with ECL Western detection reagents (Thermo Fisher Scientific Inc.). The specific protein bands were visualized using a chemiluminescence reagent (EMD Millipore) and imaged using a VersaDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA). All experiments were performed three times in triplicate. The relative protein expression was quantified by Image J software (National Institutes of Health, Bethesda, MD, USA). For each sample, the grayscale value of each band was normalized to that of corresponding β -actin and the ratio of LC3-II/I was calculated using the normalized value of LC3-II/LC3-I.

Statistical analysis. All experiments were repeated ≥ 3 times. Data were analyzed using SPSS 19.0 statistical software (IBM Corporation, Armonk, NY, USA) and expressed as the mean \pm standard deviation. One-way analysis of variance and Tukey's test were used to compare the means among groups. $P < 0.05$ was considered to indicate a statistically significant difference, and highly significant differences were indicated by $P < 0.01$ and $P < 0.001$.

Results

AFC inhibits the proliferation of colorectal cancer cells. We initially investigated the effects of AFC on various types of human colorectal cancer cells using a CCK-8 assay. The results demonstrated that AFC was able to significantly inhibit proliferation of HCT116, HT-29, SW620, HCT8 and LoVo cells (Fig. 1; Table I). Compared with other cells, HCT116 cells were the most sensitive to AFC. Therefore, HCT116 cells were selected for use in subsequent experiments to investigate the potential mechanisms of the effect AFC in human colorectal cancer cells.

AFC inhibits cell viability and induces apoptosis of HCT-116 cells. The effects of AFC on cell viability of human colorectal cancer HCT-116 cells was investigated by CCK-8 assay. The results demonstrated that AFC inhibited the viability of HCT-116 cells in a dose- and time-dependent manner (Fig. 2A and B). Next, the effect of AFC on the induction of apoptosis of HCT-116 cells was investigated by Hoechst 33258 staining, which is commonly used to detect cell apoptosis by observation of chromatin condensation under a fluorescence microscope (31). As demonstrated in Fig. 2C, following treatment with AFC (25, 50 and 100 $\mu\text{g/ml}$) for 48 h, the cells exhibited typical apoptotic morphological features, including chromatin condensation, nuclear shrinkage and the formation of apoptotic bodies. Annexin V is a sensitive method for detection of early apoptosis of cancer cells by using fluorescein (FITC) as fluorescent probe (32). Therefore, the rate of apoptosis of HCT-116 cells was investigated by flow cytometric analysis using Annexin V-FITC/PI double staining. The results demonstrated that the percentage of apoptotic cells increased when HCT-116 cells were treated with AFC compared with control (Fig. 2D and E). To study the underlying mechanism of AFC-induced apoptosis, the cleavage of PARP, caspase-3, and caspase-9 was analyzed by western blotting. As demonstrated in Fig. 2F and G, AFC cleaved PARP, pro-caspase-3 and pro-caspase-9 into their active forms. These data indicate that AFC inhibits cell viability through inducing apoptosis of HCT-116 cells.

AFC induces autophagy in HCT-116 colorectal cancer cells. To study AFC-induced autophagy, the autophagic flow of LC3-puncta was firstly determined. HCT-116 cells were transfected with Ad-mCherry-GFP-LC3B and GFP-LC3 puncta were observed under a fluorescence microscope. As indicated by Fig. 3A, fluorescence of GFP-LC3 puncta was frequently observed in HCT-116 cells treated with AFC, whereas the cells treated with vehicle control exhibited a relatively homogeneous LC3 expression pattern. In addition, a large number of autophagic bodies and autophagy-lysosomes were observed in HCT-116 cells treated with AFC (Fig. 3B) using transmission electron microscopy.

It has been well established that the microtubule-associated protein 1A/1B-light chain 3 (LC3) is a central protein in the autophagy pathway and closely associated with autophagosome appearance. Thus, it serves as a reliable marker to monitor autophagy (33). Beclin-1 was the first gene identified to induce autophagy (34). Therefore, the effect of AFC on the expression of LC3 and Beclin-1 was next investigated. As demonstrated by Fig. 3C-E, the expression levels of LC3-II and Beclin-1 in HCT-116 cells were significantly increased

Table I. IC₅₀ of active fraction of clove in terms of proliferation of various cell lines.

Cell line	Tumor type	IC ₅₀ $\mu\text{g/ml}$ (mean \pm standard deviation)
HCT116	Human colorectal cancer cell	113.5 \pm 7.83
SW620	Human colorectal cancer	174.9 \pm 9.52
HCT8	Human colorectal cancer	211.7 \pm 7.29
HT29	Human colorectal cancer	232.3 \pm 15.42
LoVo	Human colorectal cancer	280.9 \pm 12.61

IC₅₀, half maximal inhibitory concentration.

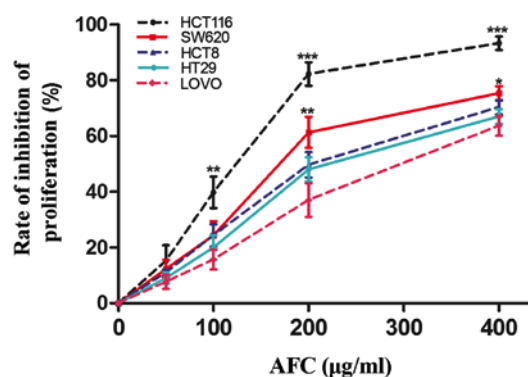


Figure 1. The human colorectal cancer cell lines, HCT-116, SW620, HCT8, HT29 and LoVo, were incubated with 0-400 $\mu\text{g/ml}$ AFC for 48 h. Inhibition of cell viability was measured by cell counting kit-8 assay. All data are representative of 3 independent experiments (mean \pm standard deviation). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. LoVo group. AFC, active fraction of clove.

by AFC in a dose- and time-dependent manner. These data indicated that AFC treatment not only results in apoptosis, but also induces autophagy in HCT-116 cells.

Inhibition of autophagy enhances AFC-induced apoptosis in HCT-116 cells. It has been documented that autophagy could facilitate cell survival in adverse microenvironments and that inhibition of autophagy leads to increased cytotoxicity and induction of apoptosis (35). The effect of inhibition of autophagy on apoptosis induced by AFC in HCT-116 cells was investigated using 2 inhibitors, 3-MA and BA. 3-MA suppresses class III phosphatidylinositol 3-kinase (PI3K), essential for the initiation of the early stages of autophagy (36), while BA, an inhibitor of the vacuolar-type ATPase, inhibits the fusion of autophagosomes with lysosomes, preventing autophagic degradation (37). As demonstrated in Fig. 4A, cell viability was significantly decreased in HCT-116 cells treated with the combination of AFC (100 $\mu\text{g/ml}$) and 3-MA (2 mM) or BA (1 nM). The resulting cell viability was 40.33 \pm 2.52 and 54.34 \pm 4.04%, respectively, compared with 73.25 \pm 5.13% for cells treated with AFC alone. This result indicated that the inhibitory effect of AFC on HCT-116 cell proliferation was enhanced by autophagic inhibitors of 3-MA and BA.

Recent studies suggest that increased LC3 expression could reflect either increased autophagosome formation, due

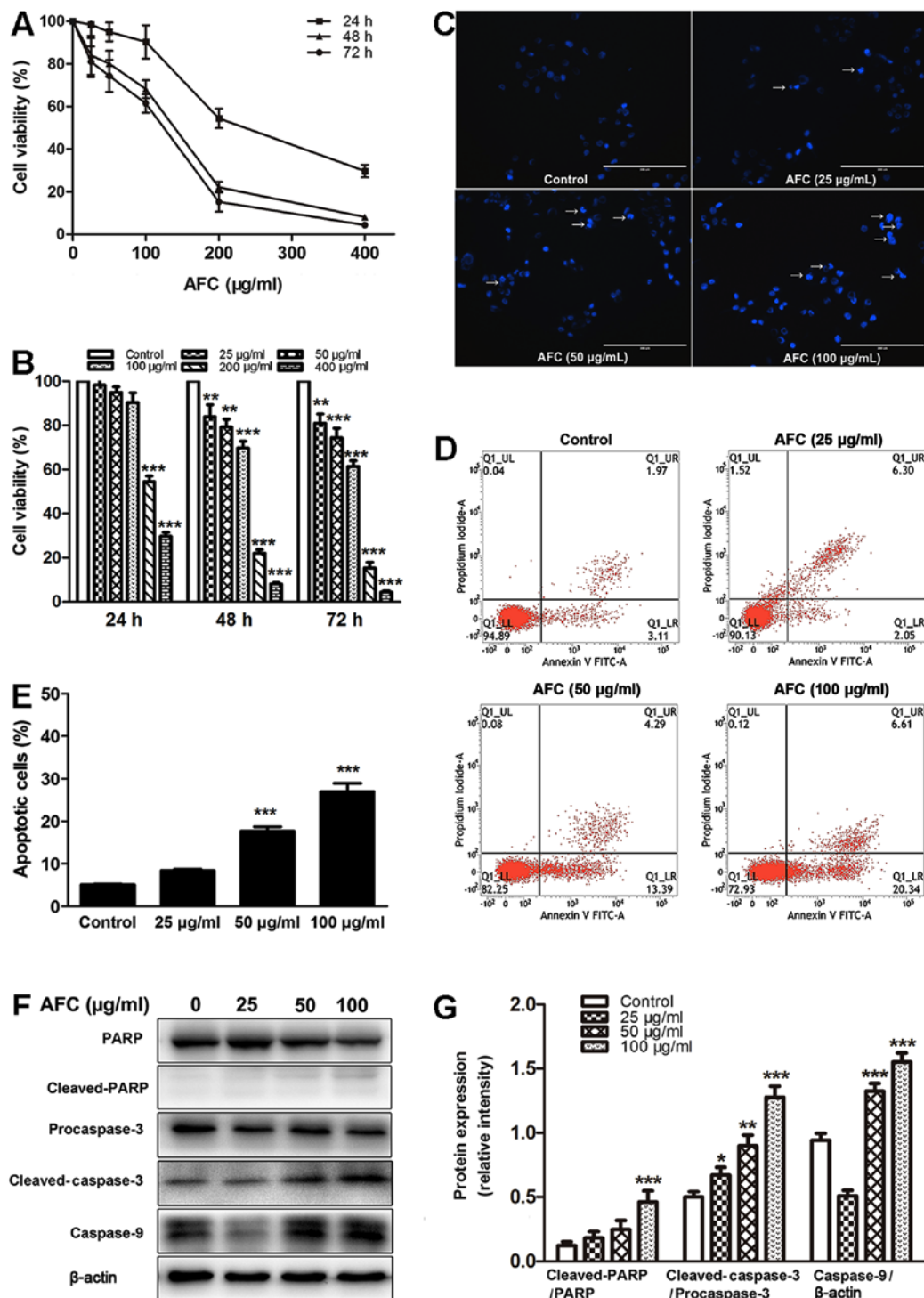


Figure 2. Effects of AFC on cell viability and apoptosis of HCT-116 cells. (A) HCT-116 cells were treated with AFC and cell viability was analyzed by cell counting kit-8 assay. (B) The percentage of cell viability quantified by a histogram for 3 independent experiments (mean \pm standard deviation). (C) Hoechst 33258 staining was used to detect apoptosis, indicated by arrows (x200, magnification). (D) HCT-116 cells were treated with 25, 50 or 100 μ g/ml AFC for 48 h, and apoptosis was determined by flow cytometry. (E) The percentage of apoptotic cells quantified by a histogram for 3 independent experiments (mean \pm standard deviation). (F) Cleavage of PARP, caspase-3 and caspase-9 was detected by western blotting, using actin as a loading control. (G) Quantification of western blotting results. All data are expressed as mean \pm standard deviation of 3 independent experiments in triplicate. * P <0.05, ** P <0.01 and *** P <0.001 vs. vehicle control. AFC, active fraction of clove; PARP, Poly (ADP-ribose) polymerase.

to increased autophagic activity, or reduced autophagosome turnover (33). Therefore, the effects of AFC on LC3 expression in the presence of 3-MA or BA were also studied. As demonstrated in Fig. 4B, 2 mM 3-MA decreased the expression of LC3, but 1 nM BA increased the level of LC3 expression. The opposite effects of 3-MA and BA on LC3 are associated with

blocking autophagy at different stages: 3-MA inhibits autophagosome formation, whereas BA prevents degradation of LC3 in autophagolysosomes and in turn increases the LC3 expression level (38). Additionally, 3-MA significantly decreased the LC3 expression induced by AFC, whereas BA increased the LC3 expression induced by AFC treatment (Fig. 4B). These data

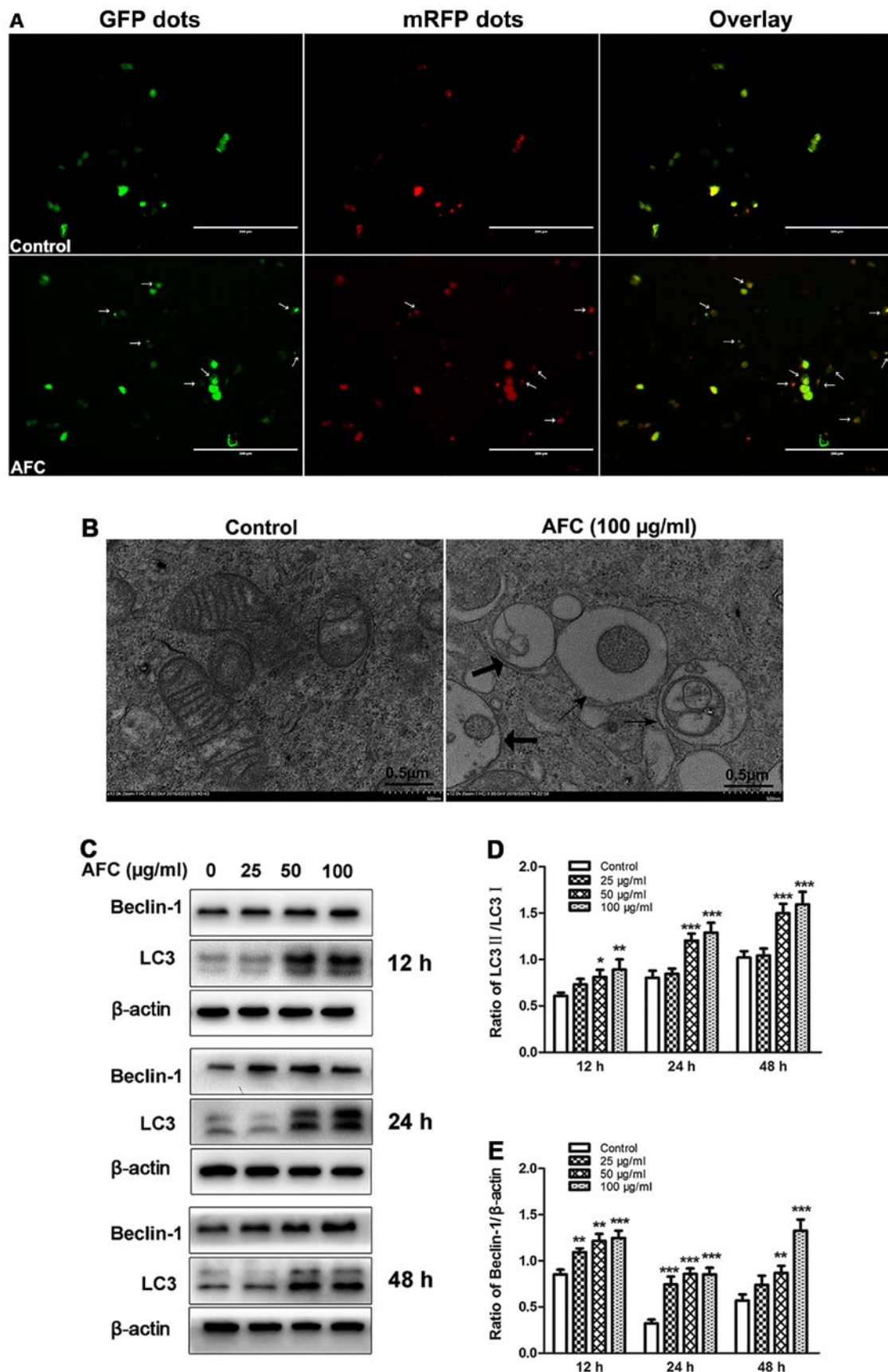


Figure 3. AFC induces autophagy in HCT-116 cells. (A) Stable expression of GFP-LC3 was observed under a fluorescence microscope in HCT-116 cells following treatment with 100 µg/ml AFC for 48 h. Arrows indicate autophagic cells. (B) Autophagy body (thin arrows) and autophagy-lysosome (thick arrows) were observed in HCT-116 cells under a transmission electron microscope (x400, magnification), following treatment with 100 µg/ml AFC for 48 h. (C) Expression of LC3 and Beclin-1 in HCT-116 cells. (D) Quantification of the LC3-II/I protein expression ratio, using actin as a loading control. (E) Quantification of Beclin-1 protein expression, using actin as a loading control. All data are expressed as the mean \pm standard deviation of 3 independent experiments in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle control. AFC, active fraction of clove; GFP, green fluorescent protein; LC3, microtubule-associated proteins 1A/1B light chain 3; mRFP, monomeric red fluorescent protein.

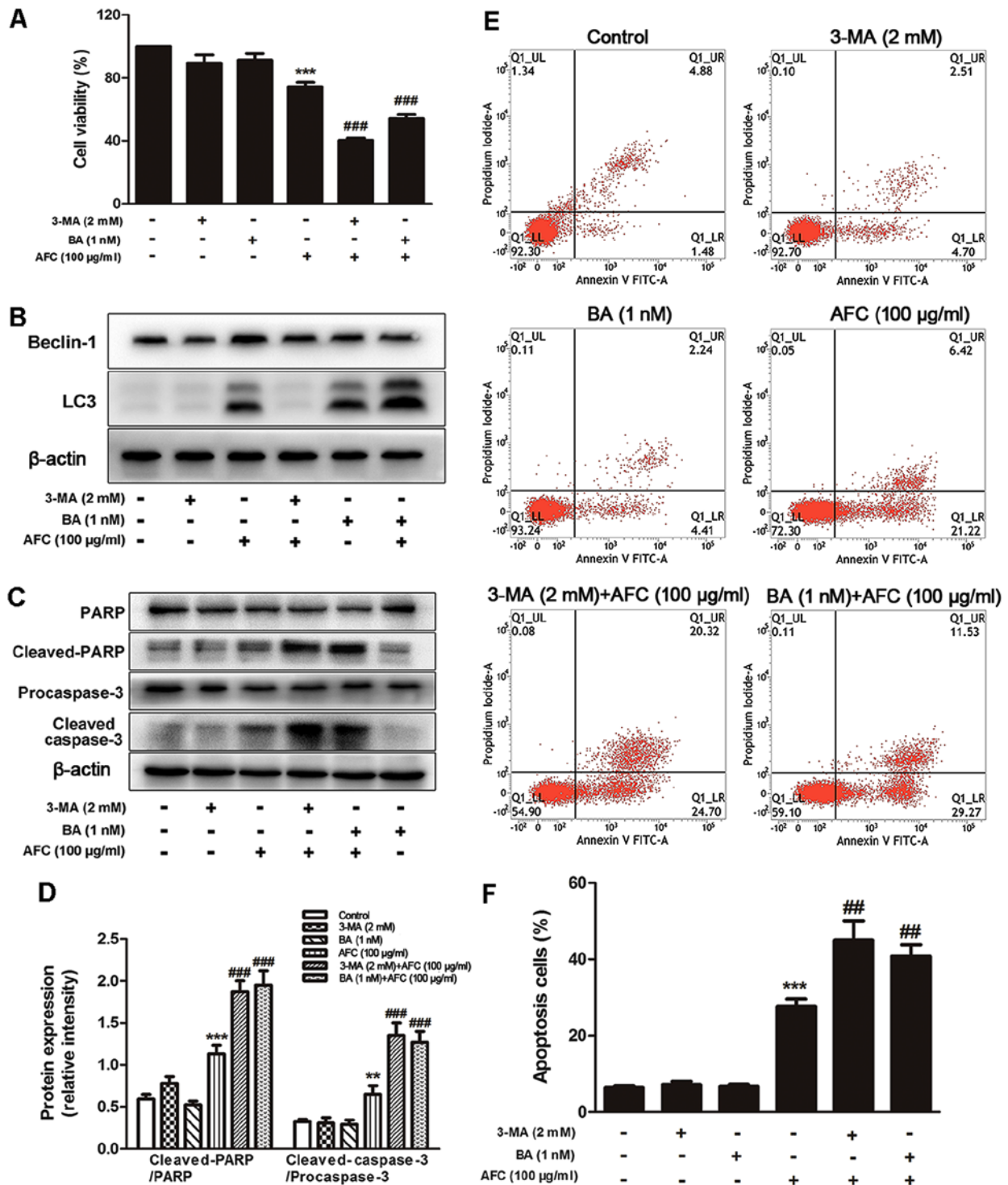


Figure 4. Inhibition of autophagy enhances apoptosis induced by AFC in HCT-116 cells. (A) The effect of 3-MA and BA on cell viability induced by AFC in HCT-116 cells. Cells were treated with 100 μg/ml AFC for 48 h with or without pretreatment with 2 mM 3-MA or 1 nM BA for 1 h. (B) The protein expression levels of LC3 and Beclin-1 proteins in HCT-116 cells. Cells were treated with 100 μg/ml AFC for 48 h with or without pretreatment of 2 mM 3-MA or 1 nM BA for 1 h. (C) The protein expression levels of cleaved caspase-3 and PARP. Cells were treated with 100 μg/ml AFC for 48 h with or without pretreatment of 2 mM 3-MA or 1 nM BA for 1 h. (D) Quantification of western blotting results. (E) The apoptotic ratios of HCT-116 cells analyzed by Annexin V-FITC/PI double staining and flow cytometry. Cells were treated with 100 μg/ml AFC for 48 h with or without pretreatment with 2 mM 3-MA or 1 nM BA for 1 h. (F) The percentage of apoptotic cells represented by a histogram. Results are expressed as the mean ± standard deviation, and the experiment was performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle control. ##P<0.01 and ###P<0.001 vs. AFC alone. AFC, active fraction of clove; 3-MA, 3-methyladenine; BA, Baflomycin A1; LC3, microtubule-associated proteins 1A/1B light chain 3; PARP, Poly (ADP-ribose) polymerase; PI, propidium iodide.

suggested that 3-MA and BA inhibited autophagy induced by AFC at different stages.

Furthermore, the effect of AFC on the expression of apoptotic genes in the presence of 3-MA or BA was determined.

The expression of cleaved-PARP and cleaved-caspase-3 were increased significantly with combined treatment of AFC and 3-MA or BA compared with either AFC, 3-MA or BA alone in HCT-116 cells (P<0.05; Fig. 4C and D). Similar effects were

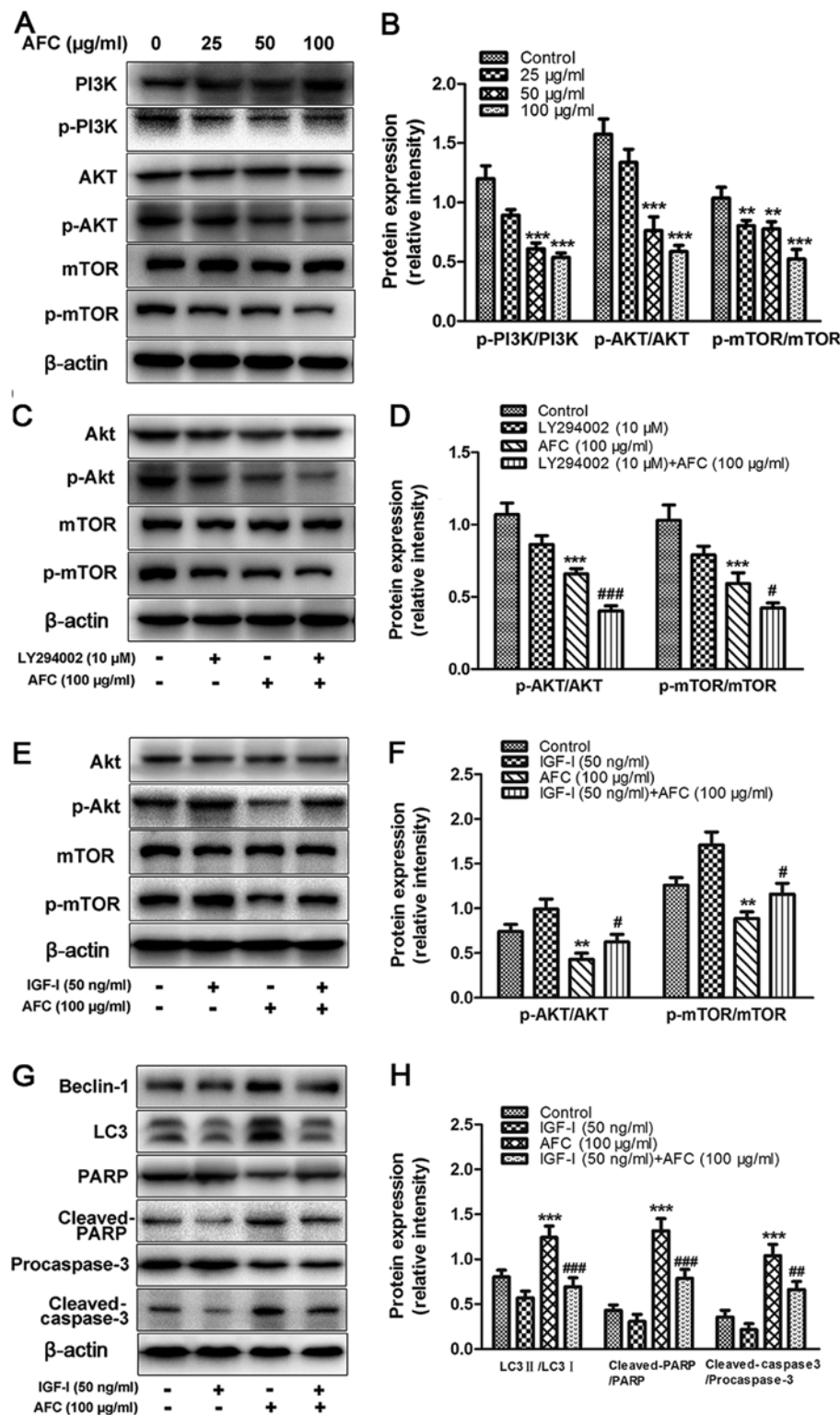


Figure 5. AFC inhibits activation of the PI3K/Akt/mTOR signaling pathway. (A) The protein expression levels of phospho-PI3K, phospho-Akt and phospho-mTOR in HCT-116 cells following treatment with 25, 50 or 100 μ g/ml AFC for 48 h. (B) The intensities of bands were quantified by densitometric analysis. (C) The protein expression levels of phospho-Akt and phospho-mTOR in HCT-116 cells following treatment with 100 μ g/ml AFC with or without 10 μ M LY294002 for 48 h. (D) Quantification of the western blotting results. (E) The expression levels of phospho-Akt and phospho-mTOR in HCT-116 cells following treatment with 100 μ g/ml AFC with or without 50 ng/ml IGF-I for 48 h. (F) Quantification of the western blotting results. (G) The expression levels of LC3, Beclin-1, caspase-3 and PARP protein in HCT-116 cells following treatment with 100 μ g/ml AFC for 48 h with or without IGF-I pretreatment (50 ng/ml) for 1 h. (H) Quantification of the western blotting results, using actin as a loading control. All data are expressed as the mean \pm standard deviation from 3 independent experiments in triplicate. ** P <0.01 and *** P <0.001 vs. vehicle control; # P <0.05, ## P <0.01 and ### P <0.001 vs. AFC alone. AFC, active fraction of clove; PI3K, phosphoinositide 3-kinase; mTOR, mechanistic target of rapamycin; IGF-I, Insulin-like growth factor-I; p-, phosphorylated.

evident in the Annexin V-FITC/PI double staining assay, in which combined treatment of AFC and 3-MA or BA increased

apoptosis compared with AFC, 3-MA or BA alone in HCT-116 cells (Fig. 4E and F). These data suggest that autophagy

induced by AFC exerted a suppressive effect on the apoptotic pathways of HCT-116 cells.

AFC inhibits the activation of the PI3K/Akt/mTOR signaling pathway in HCT-116 cells. It has been well documented that the PI3K/Akt/mTOR signaling pathway serves a key role in regulating both apoptosis and autophagy through divergent pathways (39). Therefore, the effect of AFC on the PI3K/Akt/mTOR signaling pathway in HCT-116 cells was investigated by western blotting. Treatment with AFC inhibited PI3K phosphorylation, decreased the levels of phospho-Akt, and downregulated phospho-mTOR in a dose-dependent manner (Fig. 5A). The ratios of p-Akt/Akt and p-mTOR/mTOR were significantly decreased following AFC treatment (Fig. 5B). LY294002 is a well-characterized inhibitor of PI3K (40), and the effect of combined AFC (100 μ g/ml) and LY294002 (10 μ M) on the expression of p-Akt/Akt and p-mTOR/mTOR on HCT-116 cells was investigated by western blotting. The results revealed that the combination of AFC and LY294002 treatment was more effective in decreasing of the ratios of p-Akt/Akt ($P<0.001$) and p-mTOR/mTOR ($P<0.05$) compared with AFC or LY294002 alone (Fig. 5C and D). Insulin-like growth factor-I (IGF-I), a PI3K activator, is capable of upregulating PI3K expression, as well as its downstream targets, Akt and mTOR (41). IGF-I treatment (50 ng/ml) significantly increased the phosphorylation of Akt and mTOR (Fig. 5E and F). When cells were pretreated with IGF-I (50 ng/ml), the effect of AFC was significantly attenuated. Furthermore, significant differences in the expression of the LC3-II/LC3-I, cleaved-PARP/PARP and cleaved-caspase-3/procaspase-3 ratios were observed between AFC treatment alone and the combination of AFC and IGF-I (Fig. 5G and H). These data indicate that the effect of AFC on induction of apoptosis and autophagy is associated with inhibition of the PI3K/Akt/mTOR signaling pathway.

Discussion

In the present study, the antitumor effect of AFC was investigated in human colorectal cancer cells. It was demonstrated that AFC inhibited cell proliferation and apoptosis and induced autophagy in a concentration- and time-dependent manner (Figs. 2-4). The effect of AFC on induction of apoptosis and autophagy was demonstrated to occur via inhibition of the PI3K/Akt/mTOR signaling pathway (Fig. 5). Furthermore, autophagy induced by AFC was demonstrated to suppress apoptotic pathways, and inhibition of autophagy by autophagic inhibitors, 3-MA and BA, enhanced the effects of AFC on cytotoxicity and apoptosis of HCT-116 cells (Fig. 4).

In our previous study, two active components of AFC, OA and Eugenol, were identified (12). OA has been demonstrated to exhibit anticancer efficacy against various types of human cancer cells (12,42,43). Our previous study indicated that treatment of human pancreatic pan-28 cancer cells with OA induced apoptosis via a mitochondrially-mediated apoptotic pathway (13). It was also demonstrated that OA is able to induce protective autophagy in multiple types of cancer cells (30). In the present study, it was indicated that AFC-induced autophagy decreased its effect on induction of apoptosis in human HCT-116 cancer cells. Eugenol has also been demonstrated

to possess moderate antitumor activity (44,45), and to trigger apoptosis of breast cancer cells through E2F1/survivin down-regulation (46). The combination of myricetin and methyl Eugenol enhanced the anticancer activity of cisplatin against HeLa cervical cancer cells (47). However, whether a synergistic effect of anticancer activity exists of OA and Eugenol remains unclear and requires further investigation. AFC may contain other anticancer components. Studies are on-going in our laboratory to address the complicated mechanism of the anticancer activity of clove.

Clove is traditionally believed in Chinese medicine to aid gastrointestinal function and to alleviate pain, and historically used to treat nausea, gastric spasm and sore throat (48). In a previous study it was demonstrated that oral administration of aqueous clove (100 μ l/mouse/day for 21 weeks) decreased the incidence of tumor development by >50% in a mouse model of benzo[*a*]pyrene (BP)-induced lung carcinogenesis, and that the chemo-preventive effect of clove may be due to inhibition of anti-apoptotic gene expression, including that of Bcl-2, VEGFA and CD44 (10). Our previous study also revealed that clove extracts were capable of inducing apoptosis via mitochondrial pathways in a number of cancer cell lines (12). Considering its low toxicity and its effectiveness, AFC has potential for development as a novel anticancer agent.

In the present study, AFC treatment was demonstrated to enhance autophagy in HCT-116 cells in a dose- and time-dependent manner. Autophagy has been indicated to be induced by anticancer drugs during the induction of apoptosis. Therefore, inducing autophagy-associated cell death of cancer cells may be useful in cancer treatment (49-52). An extract from the tuber of *Amorphaphallus* was reported to suppress the growth of proliferation of SGC-7901 and AGS cancer cells by induction of apoptosis and autophagy (53). Fenugreek extract also displayed anticancer effects through induction of autophagy and autophagy-associated death in human T lymphoma jurkat cells (54). The present study suggests that AFC extracts may be used in combination with classical chemotherapeutic agents to achieve an optimized outcome in the treatment of cancer.

In conclusion, the data from the present study indicate that AFC was able to induce typical apoptosis and autophagy in human colorectal cancer HCT-116 cells. Furthermore, the autophagy inhibitors, 3-MA and BA, potentiated the pro-apoptotic activity of AFC in HCT-116 cells. AFC also inhibited the phosphorylation of members of the PI3K/Akt/mTOR signaling pathway. These data may provide scientific rationale for improving the existing understanding of the anticancer mechanism of clove and to further develop AFC as a promising novel anticancer agent used alone or in combination with other chemotherapeutic agents for the treatment of colorectal cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the National innovative drug development projects of China (grant no.014ZX-09102043-001), National Natural Science Foundation

of China (grant nos. 81302906, 81273550 and 41306157), and the Distinguished Professor Research Startup Funding (SC and XKL) from Southwest Medical University. This study was also supported in part by the grants from Sichuan Sci. and Tech Dept., China (grant nos. 2017SZ0201 and 17GJHZ0074).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ML, SC and XL designed the experiments and analysed the data. GZ, DZ, WA and HL performed the experiments. ML, GZ, SC and XL wrote the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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