A novel cycloartane triterpenoid from *Cimicifuga* induces apoptotic and autophagic cell death in human colon cancer HT-29 cells

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Abstract. The extract from *Cimicifuga,* a genus of flowering plants, has been demonstrated to have mainly therapeutic effects on menstrual and menopausal symptoms and also exhibits immunomodulatory, anti-inflammatory and antimicrobial activity. Moreover, the anticancer effects of *Cimicifuga* have been reported, but the underlying mechanism causing cancer cell death has been poorly described. The present study was designed to investigate the antitumor effects and underlying molecular mechanisms of cimigenol (KY17), a novel cycloartane triterpenoid from *Cimicifuga*. KY17-induced autophagy and apoptotic cell death in colon cancer cells (HT-29) was investigated. KY17 treatment induced growth inhibition and apoptotic cell death in a concentration-dependent manner. The induction of apoptosis was confirmed by a change in cell morphology, and an increase in the G2/M phase, as well as increased protein levels of cleaved-caspase-8 and -3; cleavage of poly(ADP-ribose) polymerase (PARP) in the HT-29 cells following KY17 treatment. In addition, autophagy was evaluated by the accumulation of acridine orange, the appearance of green fluorescent protein-light-chain 3 (LC3) punctate structures and increased levels of LC3-II protein expression. Furthermore, combination treatment with the autophagy inhibitor bafilomycin A1 enhanced the induction of apoptosis by KY17. Taken together, the present study provides new insight into the role of KY17 as a potential antitumor compound. Combination of KY17 with an autophagy inhibitor may be a valuable strategy for the chemoprevention or treatment of colon cancer.

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Introduction

Colorectal cancer (CRC) is the third most common form of cancer in both men and women worldwide (1). The incidence of CRC has increased in a number of Asian countries, including China, during the past few decades (2,3). Alarmingly, increasing numbers of reported cases of colon cancer in recent years has made this form of cancer a major health concern (4). The prognosis for CRC remains poor, as few exclusively effective agents have been developed for the treatment of CRC (5). The most effective treatment for CRC is surgery, yet, even after curative resection, the recurrence rate is high. Patients with CRC after surgery are treated with chemotherapy or radiation therapy. However, the effects of these adjuvant therapies are limited due to adverse side-effects. Therefore, the development of novel anticancer agents for patients with CRC is urgently required to increase the survival rate.

Several mechanisms of cell death are well known, as determined by morphological characteristics, including apoptosis (type I cell death), autophagy (type II cell death) and necrosis (type III cell death). Autophagy is involved in a conserved membrane trafficking pathway in all eukaryotic cells and mediates the transport of cytosolic proteins and intracellular aged organelles to lysosomes for degradation. The physiologic process of autophagy has been observed in many pathologic conditions, including infectious disease and cancer (6). Although the essential role of autophagy in cancer is not clearly identified, its role in cell death is conflicting depending on the type and stage of tumorigenesis (7). Apoptosis is distinguished from necrosis in the domain of morphological characteristics and biological function (8). Apoptosis is a self-destructive process (9), which is activated by two major pathways: the receptor-mediated extrinsic and mitochondrial-mediated intrinsic pathways (10). The extrinsic apoptotic pathway is induced by the binding of extracellular ligands (for example, FasL) to transmembrane death receptors (for example, Fas) on the cell surface leading to activation of caspase-8 (11). In contrast, the intrinsic apoptotic pathway is induced by changing the permeability of the outer mitochondrial membrane, reducing mitochondrial membrane potential, and releasing mitochondrial pro-apoptotic factors including

cytochrome *c* and Apaf-1 into the cytoplasm leading to activation of caspase-9 (12). Caspase-8 of the extrinsic pathway and caspase-9 of the intrinsic pathway activate caspase-3 and cleave poly(ADP ribose) polymerase (PARP), thus, resulting in apoptosis (13). Aberrant regulation of apoptosis can lead to inappropriate cell loss, which eventually results in various cell disorders (14).

It is generally believed that autophagy and apoptosis often occur in the same cell and that autophagy mostly precedes apoptosis (15). Furthermore, various antitumor agents that are known to trigger apoptosis also induce autophagy (16). Conversely, evident suggests that inhibition of autophagy appears to enhance the sensitivity of cancer cells toward anticancer drugs. Based on these findings, it would be useful to develop a new anticancer agent that simultaneously induces both autophagy and apoptosis.

Cycloartane triterpenoids are the major constituents of *Cimicifuga* plants. The antitumor activities of the extracts from *Cimicifuga* and their major constituent cycloartane triterpenoids have been discovered and are drawing increased attention (17,18). Cycloartane triterpenoids have been shown to inhibit the proliferation of cells via induction of apoptosis and cell cycle arrest in human cancer cell lines (19,20). Cimigenol (KY17) is a novel cycloartane triterpenoid from *Cimicifuga*, and its anticancer effect and mechanisms are still unknown.

The purpose of the present study was to investigate whether KY17 induces apoptosis and autophagy in HT-29 cells.

Materials and methods

Plant materials and the extraction of compounds. Plant materials and the methods of compound extraction were described previously (21). In detail, *Cimicifuga dahurica* was collected from Yangla Town, Sichuang, China, in 2008. The plants were identified by Professor Ming-Hua Qiu, Kunming Institute of Botany, Chinese Academy of Science. Voucher specimens (KUN no. 200809007) have been deposited at the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China). The dried and milled roots of *Cimicifuga dahurica* (0.9 kg) were extracted by MeOH (3 x 3 l x 24 h) at room temperature to give a residue (106 g) after evaporation in a vacuum at 50˚C. The extract was subjected to silica gel column chromatography (cc) (2 kg, 10x150 cm) and eluted with CHCl$_3$-MeOH [100:0 (2 l), 50:1 (4 l), 20:1 (5 l), 10:1 (4 l), 0:100 (3 l)] to afford fractions A (21.5 g), B (13.1 g), C (14.5 g), D (16.8 g) and E (16.2 g). Fraction B (13.1 g) was divided into five sub-fractions (B.1-B.5) after performing RP-18 cc (180 g, 5x25 cm), eluting with MeOH-H$_2$O (gradient from 60:40 to 100:0, 10 l). Fraction B.3 (1.5 g) was subjected to repeated silica gel cc (40 g, 4x40 cm) eluted with CHCl$_3$-Me$_2$CO (gradient from 20:1 to 10:1, 4 l) and then to repeated semi-preparative HPLC (eluted with CH$_3$CN-H$_2$O, gradient from 60:40 to 85:15) to yield KY17 (4.0 mg). The chemical structure of KY17 that was used in the present study is shown in Fig. 1.

Chemicals. A stock solution of KY17 (10 mol/l) was prepared in dimethyl sulfoxide (DMSO) and diluted with fresh complete medium immediately before use. The DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
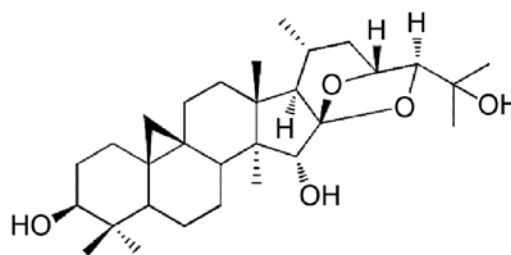

Figure 1. Chemical structure of cimigenol (KY17) a novel cycloartane triterpenoid from *Cimicifuga*.

(MTT) were obtained from Amresco LLC (Solon, OH, USA). Propidium iodide (PI), acridine orange, bafilomycin-A1 (Baf-A1), cisplatin (DDP) and doxorubicin (DOX) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Antibodies that were specific for caspase-3, poly(ADP-ribose) polymerase (PARP) and microtubule-associated protein 1 light chain 3 (LC3) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody against caspase-8 and β-actin were respectively purchased from Becton-Dickinson (BD) and Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and cell viability assay. The human colon cancer cell line (HT-29) was cultured in RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 2.0 g/l sodium bicarbonate (Sigma-Aldrich), 10% (v/v) fetal bovine serum (GE Healthcare Life Sciences) at 37˚C in a humidified 5% CO$_2$. Cell proliferation was assessed using an MTT assay. In detail, HT-29 cells were seeded onto a 96-well culture plate, cultured overnight in growth media, and then treated with or without KY17 at different concentrations for 72 h. The cells were incubated in the dark with 0.5 mg/ml MTT at 37˚C for 4 h. The formazan granules that were generated by the live cells were dissolved in DMSO, and the absorbance at 490 nm was monitored with a multiwell reader (Thermo Fisher Scientific Inc., Vantaa, Finland).

Assessment of cell cycle distribution and apoptotic cell death by flow cytometry. Cell cycle distribution was determined by propidium iodide (PI) staining. For cell cycle analysis, 5x10^5 cells were seeded in a 6-well culture plate and grown for 12 h. Following treatment with different concentrations of KY17 for 24, 48 and 72 h, the cells were trypsinized, washed with cold phosphate-buffered saline (PBS) and fixed with cold 70% ethanol at 4˚C overnight. Then, the cells were washed with PBS and incubated with 10 mg/ml RNase A, 400 mg/ml PI and 0.1% Triton-X in PBS at room temperature (RT) for 30 min. The stained cells were then analyzed by flow cytometry. Apoptotic cell death was determined using Annexin V/PI staining analyses. For the Annexin V/PI staining, the cells were treated with different conditions of KY17 for 24 and 48 h, subsequently harvested, trypsinized, washed once with cold PBS, and then suspended in 1X binding buffer (BD Biosciences, San Jose, CA, USA). The cells were stained in Annexin V-fluorescein isothiocyanate (FITC) solution (FITC Annexin V apoptosis detection kit; BD Pharmingen, Franklin Lakes, NJ, USA) and PI at RT for 15 min in the dark. The stained cells were analyzed by flow cytometry within

1 h. Flow cytometric analysis was performed on Accuri C6 (BD Biosciences).

Western blot analysis. The total cells were lysed in lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol and 2% SDS]. Equal amounts of the protein extracts were denatured by boiling at 100˚C for 5 min in sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The total proteins were separated by SDS-PAGE electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking for 1 h at RT using PBST containing 10% dried fat-free milk with gentle shaking, the membranes were incubated with primary antibodies (1:1,000) overnight at 4˚C with gentle shaking, incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), and then visualized with the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Autophagy detection with acridine vesicular orange. As a marker of autophagy, the volume of the cellular acidic compartment was visualized by acridine orange staining. Cells were seeded into 6-well plates and treated as described above for the cell viability assay for 24 and 48 h. The cells were then stained with acridine orange (3 μM) for 15 min, trypsinized, and then washed with PBS. The stained cell were photographed and images were captured using fluorescent micrographs. The intensity of acridine orange fluorescence (red) reflects the degree of cellular acidity, which positively correlates with autophagy levels. The numbers of red dots in 200 cells were counted for each slide.

GFP-LC3 assay. Autophagy was confirmed by the formation of punctate LC3-positive structures, which are essential for the dynamic process of autophagosome formation. When autophagy is induced, LC3 aggregates in the autophagosome membrane, thus, an increase in puntuate GFP-LC3 is a specific indicator of autophagy. GFP-LC3 plasmid is specifically used to detect autophagy in cultured cells through direct fluorescence microscopy. HT-29 cells were seeded in 6-well plates with coverslips at a density of 1×10^5 cells/well overnight. Cells were treated with KY17, and then transfected with 8 μg GFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions. After 24 and 48 h, the cells were fixed with 4% paraformaldehyde at 4˚C for 30 min, and the GFP-LC3 dots were examined using fluorescence microscopes (Nikon Eclipse 80i). Two hundred cells were counted for each slide and the numbers of GFP-LC3 dots in the cells were calculated.

Statistical analyses. The results are expressed as mean ± standard deviation (SD). Statistical differences were evaluated using the two-tailed Student's t-test and analysis of variance (ANOVA) followed by q-test, and were considered significant at p<0.05, p<0.01 or p<0.001.

Results

Ky17 exerts potent antiproliferative activity. To investigate whether KY17 inhibits the growth and proliferation of HT-29
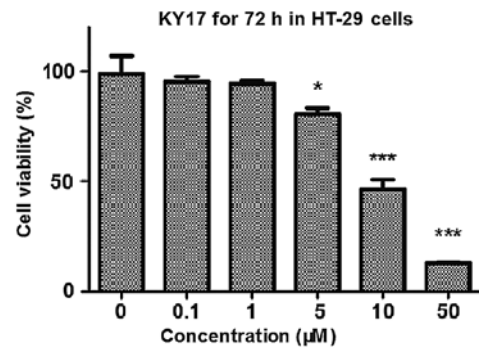

Figure 2. Effects of KY17 on the viability of HT-29 cells. The cells were treated with the indicated concentrations of KY17 for 72 h. Proliferation was determined with an MTT assay. The results are expressed as mean ± standard deviation (SD; n=3) and as a percentage of the control. The significance was determined by Student's t-tests and ANOVA; *p<0.05 and ***p<0.001 compared with the control cells.

cells *in vitro*, we treated the cells for 72 h with increasing concentrations of KY17. The KY17-mediated growth inhibition was measured by MTT assay. As shown in Fig. 2, KY17 significantly suppressed the cell growth of HT-29 cells in a concentration-dependent manner, and the IC$_{50}$ value of KY17 was 8.84±1.1 μM. The result suggests that KY17 suppressed the growth of HT-29 cells in a concentration-dependent manner.

Ky17 induces G2/M phase arrest and apoptosis in HT-29 cells. In order to study the mechanisms underlying the effects of KY17 on the suppression of proliferation of HT-29 cells, the effects of KY17 on cell cycle distribution and apoptosis were examined by flow cytometry. We found that KY17 induced G2/M phase arrest and increased the percentage of cells in the sub-G1 phase in a concentration- and time-dependent manner (Fig. 3A and B). Consistently, Annexin V assays also indicated that KY17 increased cellular apoptosis in the HT-29 cells. As shown in Fig. 3C and D, there was a prominent increase in the percentage of late apoptotic cells (11.6%) following 10 μM KY17 treatment for 48 h compared with the untreated controls (0.2%). During the apoptosis process, several caspases are active, which is induced by various apoptotic stimuli (22). To examine the mechanism underlying the KY17-mediated cell growth inhibition and death, caspase involvement was evaluated using western blot analysis. The exposure of HT-29 cells to KY17 (1, 5 and 10 μM) at 24 and 48 h markedly increased the protein levels of cleaved-caspase-8 and -3 in a concentration- and time-dependent manner (Fig. 4A and B). In addition, after treatment of KY17 the full length form of the PARP protein (a selective substrate for caspase-3) was degraded to the cleaved form (Fig. 4C). These results further demonstrated that KY17 promoted cell death in the HT-29 cells through apoptosis.

Ky17 induces cell autophagy in HT-29 cells. In addition to apoptosis, many modes of cell death have been identified such as autophagy and necroptosis (23). In the present study, we determined whether KY17 induces autophagy in HT-29 cells. Since the formation of cytosolic acidic vesicular organelles (AVOs) is one of the typical features of autophagy, fluorescence microscopy was performed after staining the
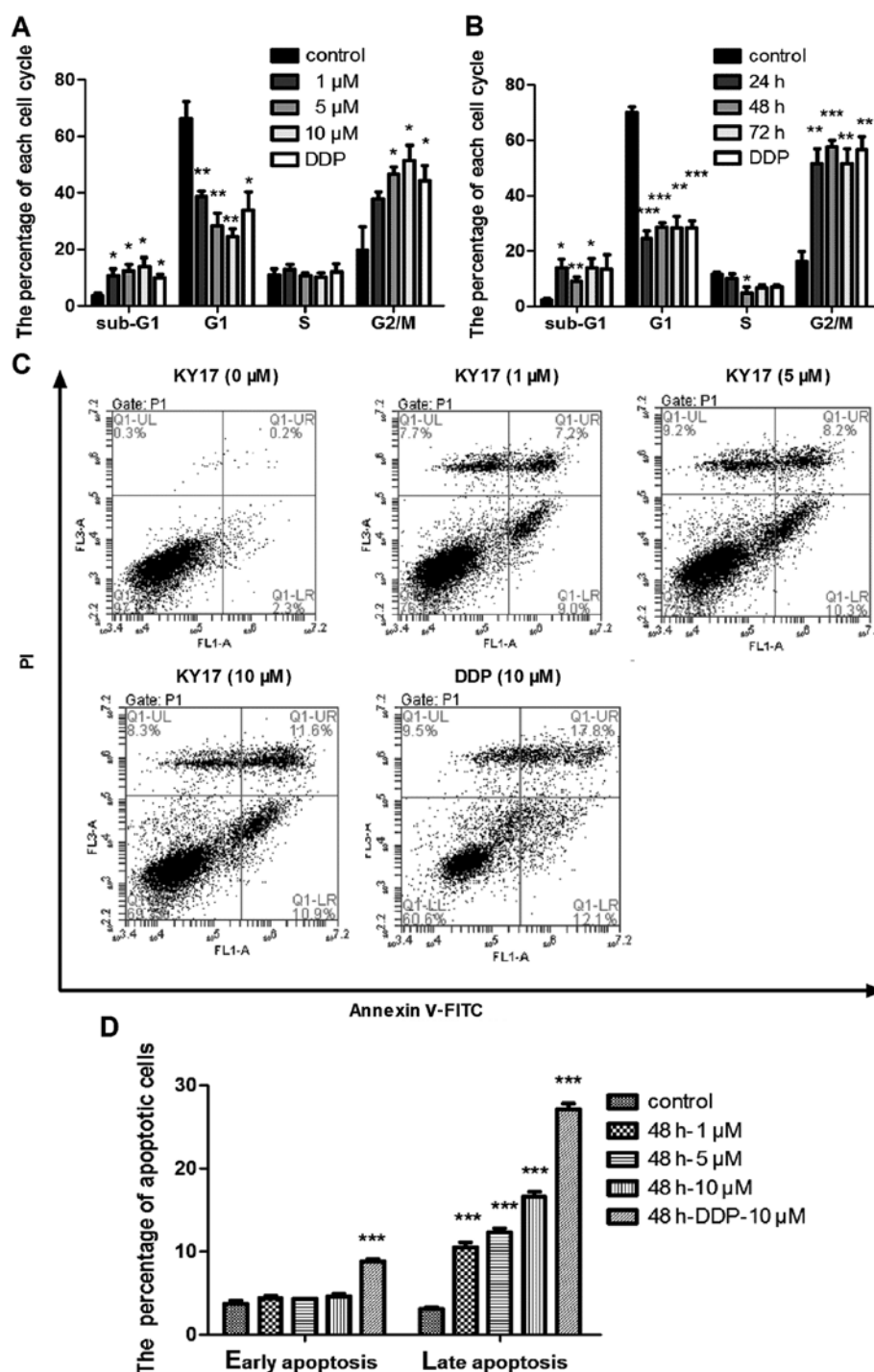
Figure 3. Induction of cell cycle arrest and apoptosis in HT-29 cells by KY17. (A) Effect of KY17 on cell cycle progression of HT-29 cells at 24 h. (B) Effect of KY17 on cell cycle progression of HT-29 cells. (C) Annexin V-fluorescein isothiocyanate (FITC) binding and propidium iodide (PI) uptake in non-permeabilized cells were analyzed by flow cytometry. (D) The presence of cells with different cell cycle content following treatment with different concentrations of KY17 for 48 h was evaluated using flow cytometry. DDP (cisplatin) was used as a positive control. The significance was determined by Student's t-tests and ANOVA; $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared with the control cells.

cells with acridine orange for the quantification of the AVOs. The number of AVOs in the KY17-treated cells was clearly increased (Fig. 5A and C).

The conversion of microtubule association protein LC3 is a special autophagic marker. Therefore, we examined the LC3 distribution in the KY17-treated HT-29 cells with fluorescence microscopy. As shown in Fig. 5B and D,

compared with the untreated control cells, green fluorescent protein (GFP)-tagged-LC3 (GFP-LC3) formed cytoplasmic puncta in the cells that were treated with KY17 at different times and concentrations. In order to further confirm that autophagy is induced by KY17, a western blot analysis of LC3 was detected. The results showed that LC3 underwent conversion from LC3-I (the soluble form) to LC3-II (the lipidized
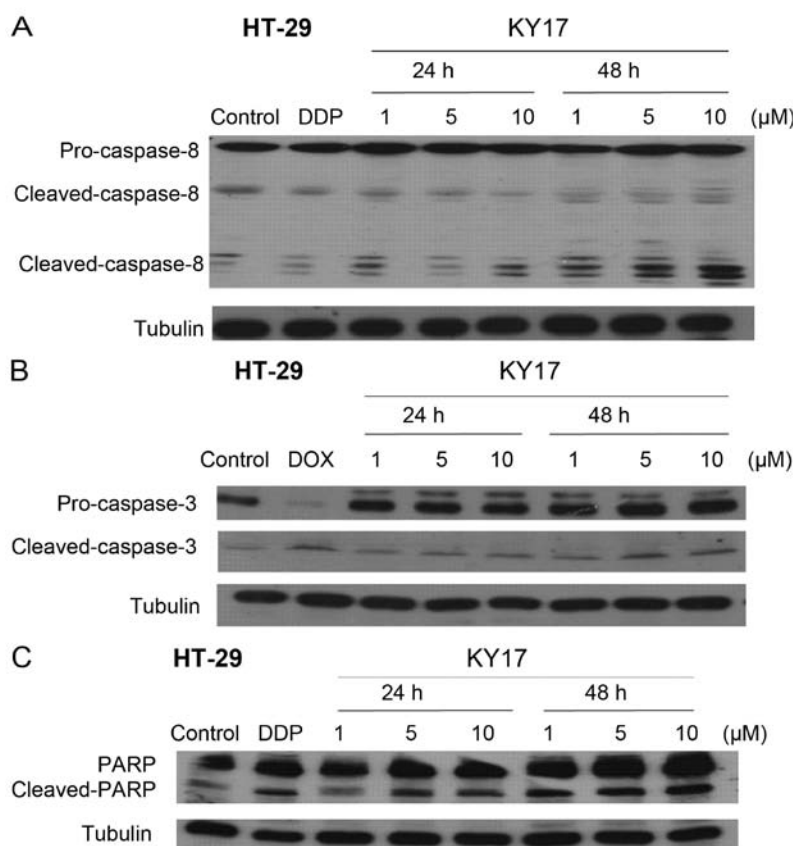
Figure 4. Effects of KY17 on apoptosis-related protein expression in HT-29 cells. (A) Membranes were probed with pro-caspase-8 and cleaved-caspase-8. DDP was used as a positive control. (B) Membranes were probed with pro-caspase-3 and cleaved-caspase-3. DOX (doxorubicin) was used as a positive control. (C) Membranes were probed with poly(ADP ribose) polymerase (PARP). DDP (cisplatin) was used as a positive control.

form) in the KY17-treated cells, thus confirming induction of autophagy (Fig. 5E).

Suppression of autophagy increases KY17-induced apoptotic cell death in HT-29 cells. Accumulating research has shown that the suppression of autophagy may enhance the chemosensitization in human cancer cells (24). Thus, we analyzed the role of autophagy in KY17-induced cell death by examining the effects of pharmacological autophagy inhibitors. We first treated cells with Baf-A1 which is a vacuolar-type H^+-ATP inhibitor to prevent the maturation of autophagic vacuoles for 1 h, and then incubated them with KY17 for another 48 h. Next, we used Annexin V/PI staining to evaluate cell death. As shown in Fig. 6A and B, compared with KY17 treatment the combination of the two agents effectively increased the percentage of late apoptotic cells. In addition, western blot analysis showed that KY17 significantly increased the cleaved form of the PARP protein combined with Baf-A1 compared with KY17 treatment alone (Fig. 6C). These data indicate that the inhibition of autophagy with autophagy inhibitors, such as Baf-A1, enhanced the KY17-induced cell death in the HT-29 cells.

Discussion

Colorectal cancer (CRC) is the third most lethal malignancy worldwide, and surgery is the most common therapy for

patients with CRC, and advanced CRC also needs treatment with chemotherapy or radiation therapy. However, the effects of these adjuvant therapies are limited due to adverse side-effects. In the present study, we investigated the anticancer mechanisms of KY17, which is a novel cycloartane triterpenoid from plants from the genus *Cimicifuga*. Our results indicated that KY17 induced both apoptosis and autophagy in HT-29 cells. Furthermore, we found that inhibition of autophagy resulted in higher levels of apoptotic cell death in response to KY17 treatment. Collectively, our findings suggest that inhibition of autophagy during cancer treatment with KY17 may augment the therapeutic effects.

Regulation of apoptosis and cell cycle is an important process to preserve cell homeostasis between cell death and cell proliferation (25), which means that the induction of apoptosis and suppression of cell cycle progression is an advantageous strategy for cancer therapy. Autophagy is also a crucial component of the cellular stress adaptation response that maintains mammalian homeostasis (26). In the present study, we found that KY17 effectively inhibited the proliferation of HT-29 cells through induction of apoptosis and cell cycle arrest in a concentration- and time-dependent manner. Moreover, KY17 also induced cell autophagy in the HT-29 cells. These results suggest that *Cimicifuga* may have beneficial effects for the reduction of colon cancer growth.

In addition, we found that KY17 mediated anticancer activity by modulating the expression of genes that are involved
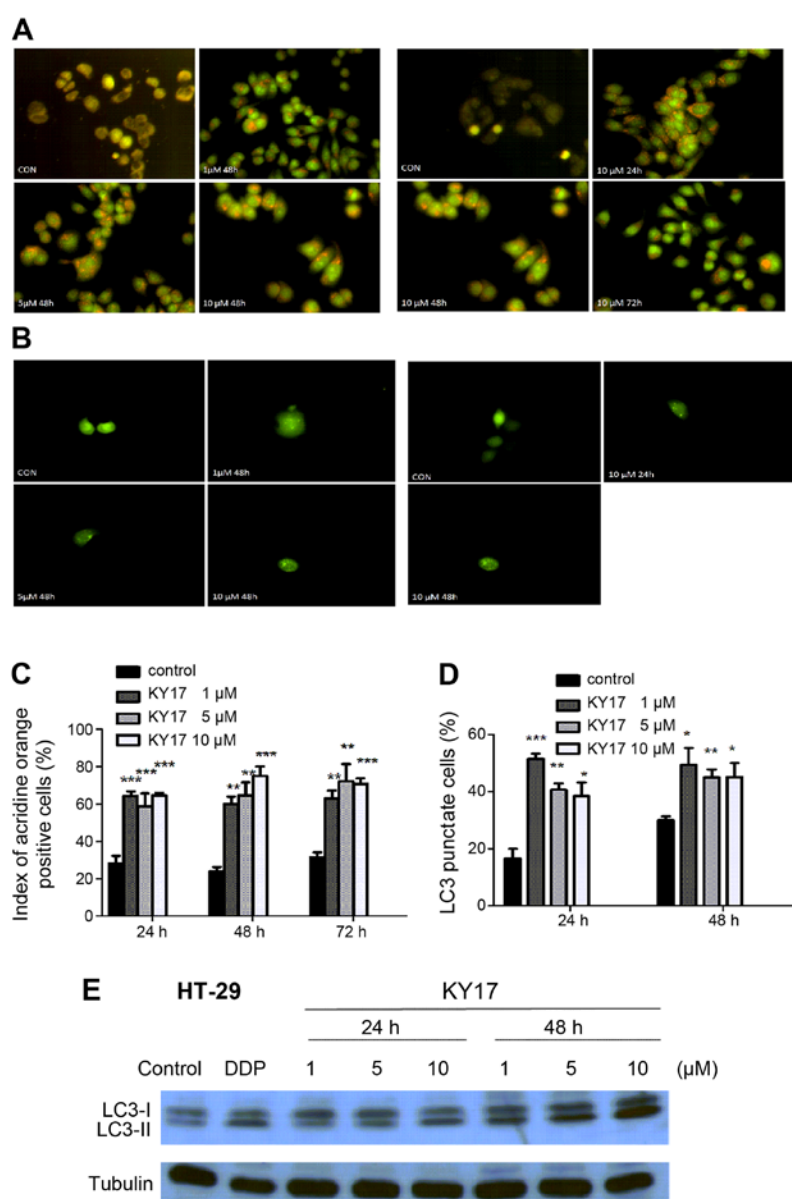
Figure 5. Induction of autophagy in HT-29 cells by KY17. (A and C) HT-29 cells were treated with KY at different concentrations for 48 h or different times, stained with acridine orange, and then analyzed by fluorescence microscopy to quantify acidic vesicular organelle (AVO) formation. (B and D) HT-29 cells were transfected with the light-chain 3 (LC3)-green fluorescent protein (GFP) plasmid for 24 h, and the medium was then replaced with KY17 at different concentrations for 48 h or for different times at 10 μM. GFP-LC3 puncta formation was observed with fluorescence microscopy. The results are expressed as mean $\pm$ standard deviation (SD; n=3) and as a percentage of the control. The significance was determined by Student's t-tests and ANOVA; *p<0.05, **p<0.01 and ***p<0.001 compared with the control cells. (E) The levels of LC3 proteins were analyzed by western blot analysis.

in apoptosis. Death receptor-mediated apoptosis (extrinsic apoptotic pathway) is activated by the interaction of pro-apoptotic and pro-inflammatory cytokines, such as tumor-necrosis factor-α (TNF-α) and their receptors (27). Caspases are major components of the apoptotic system, which is associated with proteolytic processes (23). The interplay between ligands and receptors induces the formation of intracellular death-induced signaling complexes (DISCs), which activate caspase-8 and release DISC into the cytoplasm (28). Caspase-8 can directly activate caspase-3. In particular, caspase-3 plays a pivotal role in apoptosis, which may be controlled through death receptors or mitochondria. Our data showed that KY17 markedly increased the protein levels of cleaved-caspase-8 and -3 in HT-29 cells and the full length form of the PARP protein

was degraded to the cleaved form suggesting that KY17 has a promoting effect on apoptosis in HT-29 cells.

As with many other anticancer therapies, we observed an increase in the number of AVOs and LC3 puncta expression in the KY17-treated cells. This indicated that KY17 induced autophagy, and autophagy appears to act as a cytoprotective mechanism (29). Numerous data have also shown that the suppression of autophagy enhances the chemosensitization in human cancer cells (24). Thus, we investigated the role of autophagy in KY17-induced cell death by examining the effects of pharmacological autophagy inhibitors. Our data showed that the lysosomal H^{+}-ATPase inhibitor Baf-A1, which interrupted autophagolysosome formation, successfully enhanced KY17-mediated apoptosis. These results indicated
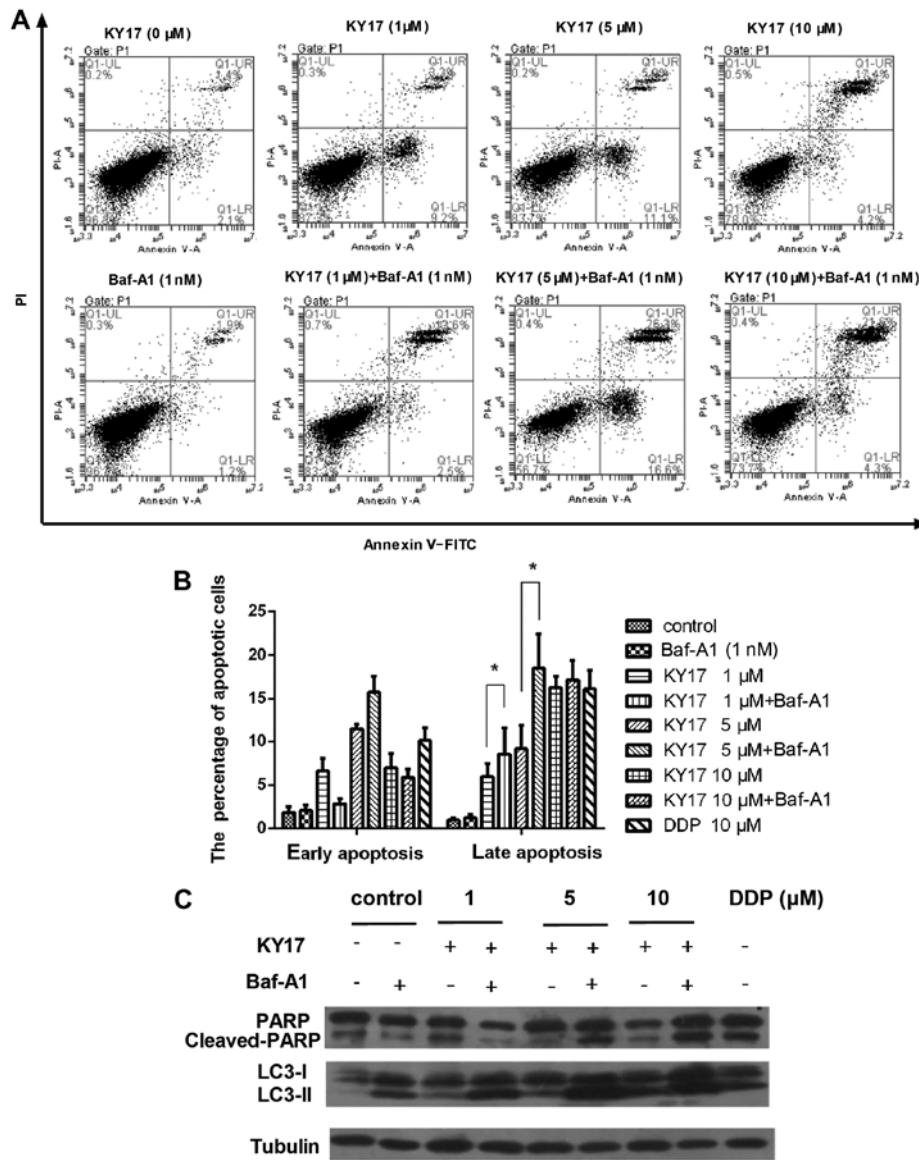
Figure 6. Role of autophagy inhibitor (Baf-A1) on KY17-induced apoptosis in HT-29 cells. (A and B) HT-29 cells were pretreated with 1 nM of bafilomycin-A1 (Baf-A1) for 1 h, and were then treated with the indicated concentrations of KY17 for 48 h. Annexin V-FITC binding and PI uptake in non-permeabilized cells were analyzed by flow cytometry. The results are expressed as mean ± standard deviation (SD; n=3) and as a percentage of the control. The significance was determined by Student's t-tests and ANOVA; *p<0.05 compared with the control cells. (C) Total proteins were prepared and immunoblotted for PARP and LC3. Tubulin was used as a loading control.
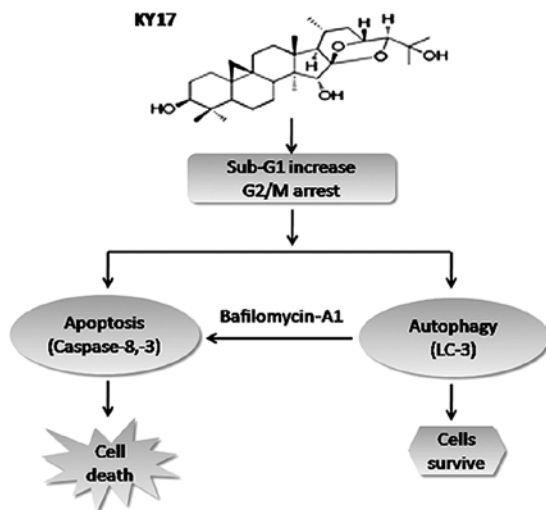
that the disruption of autophagolysosome formation effectively sensitized cells to KY17 apoptosis. However the molecular mechanisms of autophagy in the KY17 anticancer effects warrant further investigation. Moreover, clinically relevant animal models also need to be explored.

Altogether, the present study suggested that the novel cycloartane triterpenoid KY17 from *Cimicifuga* possesses anticancer activity by induction of apoptosis and autophagy in HT-29 cells (Fig. 7). The results from the present study provide evidence of KY17-induced autophagy, and autophagy inhibition by Baf-A1 significantly increased the apoptotic cell death induced by KY17 in HT-29 cells. Our findings suggest

Figure 7. Proposed model depicting the mechanism of action of KY17 in HT-29 cells.

that KY17 in combination with Baf-A1 may be a useful candidate for the chemoprevention or treatment of colon cancer. However, the potential of this fascinating molecule in the treatment of other types of cancer remains unknown. Thus, evaluation of the application of this compound in other cancer cell lines will be carried out in future research.

Acknowledgements

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