

Pristimerin demonstrates anticancer potential in colorectal cancer cells by inducing G1 phase arrest and apoptosis and suppressing various pro-survival signaling proteins

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Abstract. Pristimerin is a naturally occurring triterpenoid that has a cytotoxic effect on several cancer cell lines. However, the cytotoxic effects of pristimerin as well as its molecular mechanisms of action against colorectal cancer have never been explored. In the present study, we investigated the anticancer potential of pristimerin, and examined the different signaling pathways affected by its action in three colon cancer cell lines namely HCT-116, COLO-205 and SW-620. Pristimerin was found to possess potent cytotoxic and proliferation inhibitory effects against these cell lines. Cell cycle analysis revealed G1 phase arrest, which was strongly associated with decreased expression of cyclin D1 and cyclin-dependent kinases (cdk4 and cdk6) with concomitant induction of p21. Pristimerin also induced apoptosis in a dose-dependent manner. Cell plasma membrane alterations studied by Annexin V/PI double staining, loss of mitochondrial membrane potential ($\Delta\Psi_m$), measurements of caspase activities and the inhibitory effect of Z-VAD-FMK (a caspase inhibitor) confirmed the apoptotic effect of pristimerin. Moreover, western blot data showed that apoptotic induction was associated with activated caspase-3 and -8, PARP-1 cleavage and modulation of the expression levels of Bcl-2 family proteins. Additionally, pristimerin treatment downregulated the phosphorylated forms of EGFR and HER2 proteins, and subsequently caused a decrease in the phosphorylated forms of Erk1/2, Akt, mTOR and NF- κ B

proteins. Taken together, these results suggest that pristimerin may have potential as a new targeting therapeutic strategy for the treatment of colon cancer.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, with more than 1 million new cases diagnosed each year (1). CRC is the fourth most common cause of cancer-related mortality worldwide (1), and the third most common cause of cancer-related mortality in the US (2). CRC is a heterogeneous disease with different molecular characteristics such as abnormal genetic and epigenetic alterations in several signaling pathways which are responsible for controlling cell proliferation, apoptosis and invasion of CRC (3,4). The management of CRC has improved in the last decade, in term of screening, surgical management and adjuvant chemotherapy.

Natural compounds have become important in anticancer drug development as they are more tolerable to the human body (5). Pristimerin (Fig. 1A) is a naturally occurring quinonemethide triterpenoid compound isolated from the *Celastraceae* and *Hippocrateaceae* families. Pristimerin has long been used as an anti-inflammatory, antioxidant, antimalarial and insecticidal agent (6,7). It also has promising clinical potential as both a chemotherapeutic and chemopreventive agent for cancer. Indeed, it has been reported to exhibit a cytotoxic effect on several human cancer cell lines including breast (8), pancreatic (9), hepatic (10), cervical (11) and prostate cancer (12), and glioma tumor cells (13). However, the exact mechanism of pristimerin cytotoxicity has not been completely explored. Furthermore, pristimerin has been reported to induce cancer cell death via different mechanisms of action, including the caspase-dependent apoptotic cascade (8), potent proteasomal inhibition (14), suppression of nuclear factor- κ B (NF- κ B) activity (15), and oxidative stress induction (11).

Although pristimerin has a variety of reported antitumor activities, its effect on colon cancer cell lines and the underlying mechanism of action have never been explored. In the present study, we examined the effect of pristimerin on three

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human colon cancer cell lines namely HCT-116, SW-620 and COLO-205. Our results revealed that pristimerin strongly suppressed the growth of these cell lines by inducing G1 phase cell cycle arrest and apoptosis. Furthermore, the anticancer effects of pristimerin were associated with inhibition of EGFR and HER2 and related downstream signaling pathways.

Materials and methods

Materials. Pristimerin with a purity of >97% was purchased from Enzo Life Sciences (Lausen, Switzerland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC apoptosis detection kit was purchased from BD Pharmingen (San Diego, CA, USA). Cell cycle assay kit, caspase-3, -8 and -9 activity assay kits and mitochondrial membrane potential ($\Delta\Psi$ m) assay kit were purchased from Beyotime Institute of Biotechnology (Nanjing, China). Antibodies against cleaved caspase-3, cleaved caspase-8, Bcl-2, Bcl-xL, Bax, PARP-1, HER2, p-HER2, p-EGFR, mTOR, p-mTOR, cyclin D1, Cdk-4, cdk-6, Rb and p-Rb were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against ERK1/2, p-ERK1/2, Akt, p-Akt, NF- κ B P65 and p-NF- κ B P65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against EGFR was purchased from Abcam (Cambridge, UK); both anti-mouse IgG-horseradish peroxidase, anti-goat IgG-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase were purchased from KangChen Biotechnology (Shanghai, China).

Cell lines and cell culture. The human colon cancer cell lines HCT-116, SW-620 and COLO-205 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The HCT-116 and COLO-205 cells were cultured in RPMI-1640 medium (HyClone China Ltd., China), supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Sigma) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. SW-620 cells were cultured in Leibovitz's L-15 medium (HyClone China Ltd.), supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C under a humidified 100% atmosphere.

Cell viability assay. The inhibitory effect of pristimerin on cell viability was measured by MTT method. Briefly, 5x10³ cells/well were seeded into 96-well plates for 24 h, and then the medium was replaced with fresh medium containing various concentrations of pristimerin (0, 0.1, 0.5, 1, 5 and 10 μ M) for 12, 24, 48 and 72 h. After incubation, MTT was added to each well at a final concentration of 0.5 mg/ml. The insoluble formazan was collected and dissolved in dimethylsulfoxide (DMSO) and measured with a microplate reader (Safire²; Tecan, Switzerland) at 540 nm.

DNA cell cycle analysis. Cells were treated with various pristimerin concentrations (0, 0.5, 1 and 2 μ M) in complete medium for 48 h. Both floating and adherent cells were harvested by centrifugation (3,000 x g for 5 min), washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in

ice-cold 75% ethanol at 4°C overnight. The ethanol was subsequently removed after centrifugation, and ~1x10⁶ cells were re-suspended in PBS containing 25 μ g/ml RNase. Samples were then incubated with propidium iodide (PI) (50 μ g/ml) at 37°C for 30 min and were analyzed by a flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Annexin V/PI double-staining assay. Apoptosis was measured by a flow cytometer using Annexin V/PI double staining. HCT-116, SW-620 and COLO-205 cells were cultured in a 60-mm dish at a density of 2x10⁵/dish. On the following day, the cells were treated with pristimerin (0, 0.5, 1 and 2 μ M). After 48 h, the cells were harvested, both floating and attached cells were collected, washed with ice-cold PBS twice, and re-suspended in 200 μ l binding buffer containing Annexin V and 40 ng/sample PI for 15 min at 37°C in the dark (according to the manufacturer's instructions). The number of viable, apoptotic and necrotic cells was then quantified by a flow cytometer.

Analysis of caspase-3, -8 and -9 activities. Caspase-3, -8 and -9 activities were measured using colometric assay kits as previously described (16) and following the manufacturer's guideline. Briefly, HCT-116, SW-620 and COLO-205 cells without or with (0.5, 1 and 2 μ M) pristimerin treatment, were washed with cold PBS, re-suspended in lysis buffer and left on ice for 15 min. The lysate was centrifuged at 16,000 x g at 4°C for 15 min. Activities of caspase-3, -8 and -9 were measured using substrate peptides Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, respectively. Release of p-nitroanilide (pNA) was qualified by determining the absorbance with a spectrophotometer. Data were normalized against the control untreated cells.

Pan-caspase inhibitor Z-VAD-FMK. HCT-116, SW-620 and COLO-205 cells were seeded into 96-well plates at an initial density of 5x10³ cells/well. After 24 h, the cells were pre-incubated with 50 μ M Z-VAD-FMK (Beyotime Institute of Biotechnology) for 1 h and then incubated with pristimerin (0, 0.5, 1, 2, 4 and 10 μ M) for 48 h. The inhibition rate (%) was measured by MTT in both the presence and absence of Z-VAD-FMK.

$\Delta\Psi$ m assay. The JC-1 probe was used to measure mitochondrial depolarization in HCT-116, SW-620 and COLO-205 cells. Briefly, the cells were cultured into 6-well plates; after treatment with pristimerin (0, 0.5, 1 and 2 μ M) for 48 h, they were incubated with an equal volume of a JC-1 staining solution (5 μ g/ml) at 37°C for 20 min and rinsed twice with PBS. Changes in $\Delta\Psi$ m were analyzed using a flow cytometer with the excitation and emission wavelengths of 488 and 525 nm, respectively.

Whole-cell lysates and western blot analysis. HCT-116, SW-620 and COLO-205 cells were collected following treatment and lysed by lysis buffer (KangChen Biotechnology) for 30 min on ice. Cell lysates were centrifuged at 12,000 rpm for 15 min at 4°C. Total protein concentration in the supernatants was detected by a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of lysate proteins were separated on 8-12% SDS-PAGE and transferred

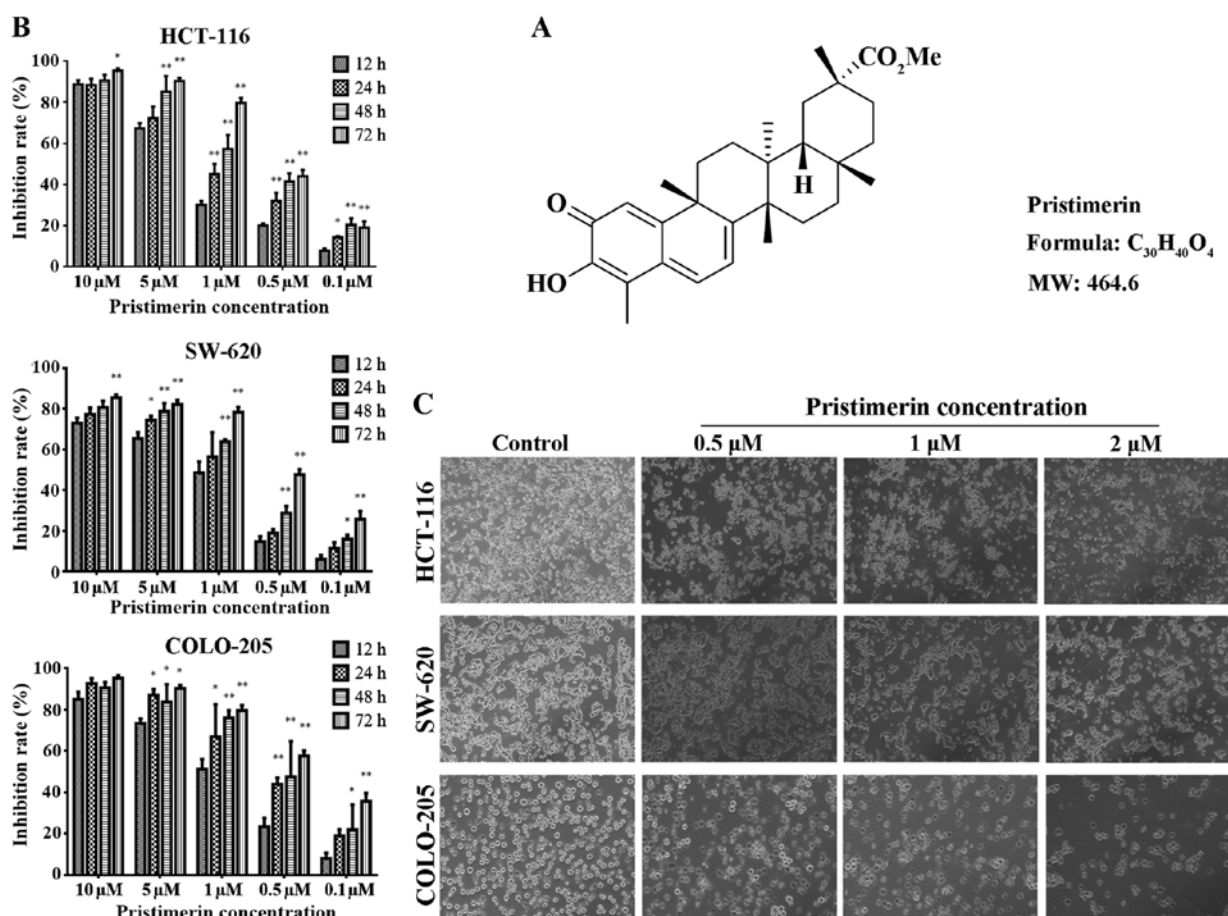


Figure 1. Pristimerin inhibits colon cancer cell growth. (A) Chemical structure of pristimerin. (B) HCT-116, SW-620 and COLO-205 cells were grown in the absence and presence of different concentrations of pristimerin for 12, 24, 48 and 72 h. Cell viability was measured by MTT assay. Inhibition rates (%) were presented as mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. 12 h of pristimerin treatment. (C) Morphological changes in cell cultures visualized by light microscopy 48 h after treatment with pristimerin. Each experiment was repeated three times.

onto nitrocellulose membranes. The non-specific binding sites were blocked with TBST buffer containing 5% free-fat milk for 2 h. Following overnight incubation at 4°C with specific primary antibodies, the membranes were washed three times with TBST buffer and incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody. After three washes with TBST buffer, the immunoblots were visualized using the enhanced chemiluminescence (ECL; Millipore Corporation) detection kit. Immunoreactive bands were scanned (GS-800) and analyzed using a ChemiDoc XRS imaging system (Quantity One, v4.4.0) (both from Bio-Rad Life Sciences, Hercules, CA, USA).

Statistical analysis. Data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). All data are expressed as mean \pm standard deviation (SD) from at least three independent experiments. The statistical comparisons between the control and treatment groups were determined using the Student's two-tailed t-test. Differences with $p < 0.05$ were considered statistically significant.

Results

Pristimerin exerts potent cytotoxicity against colon cancer cells. Pristimerin cytotoxicity in colon cancer cells was

assessed by MTT assay. As shown in Fig. 1B, pristimerin treatment caused a dose- and time-dependent inhibition of cell growth in all three tested colon cancer cell lines. Following 48 h of treatment, the IC_{50} values of pristimerin in the HCT-116, SW-620 and COLO-205 cells were 1.22 ± 0.25 , 1.04 ± 0.17 and $0.84 \pm 0.14 \mu$ M, respectively.

Next, we focused on the effect of pristimerin on colon cancer cell morphology. Untreated HCT-116, SW-620 and COLO-205 cells appeared healthy with clear skeletons, whereas the pristimerin-treated HCT-116 and SW-620 cells were detached and distorted and certain cells became rounded and floated (Fig. 1C). COLO-205 cells treated with pristimerin were distorted with an unclear cytoplasm and skeleton (Fig. 1C). These changes occurred in a dose-dependent manner. Taken together, the MTT assay and morphological data indicated that pristimerin has potent cytotoxic effects on colon cancer cells.

Pristimerin induces G1 phase arrest and modulates cell cycle-related proteins in colon cancer cells. The effect of pristimerin on the cell cycle was examined by flow cytometric analysis of cellular DNA content. HCT-116, SW-620 and COLO-205 cells were treated with pristimerin for 48 h. As shown in Fig. 2A, pristimerin caused cell cycle transition arrest in the G0/G1 phase and the proportion of cells in the G0/G1 phase

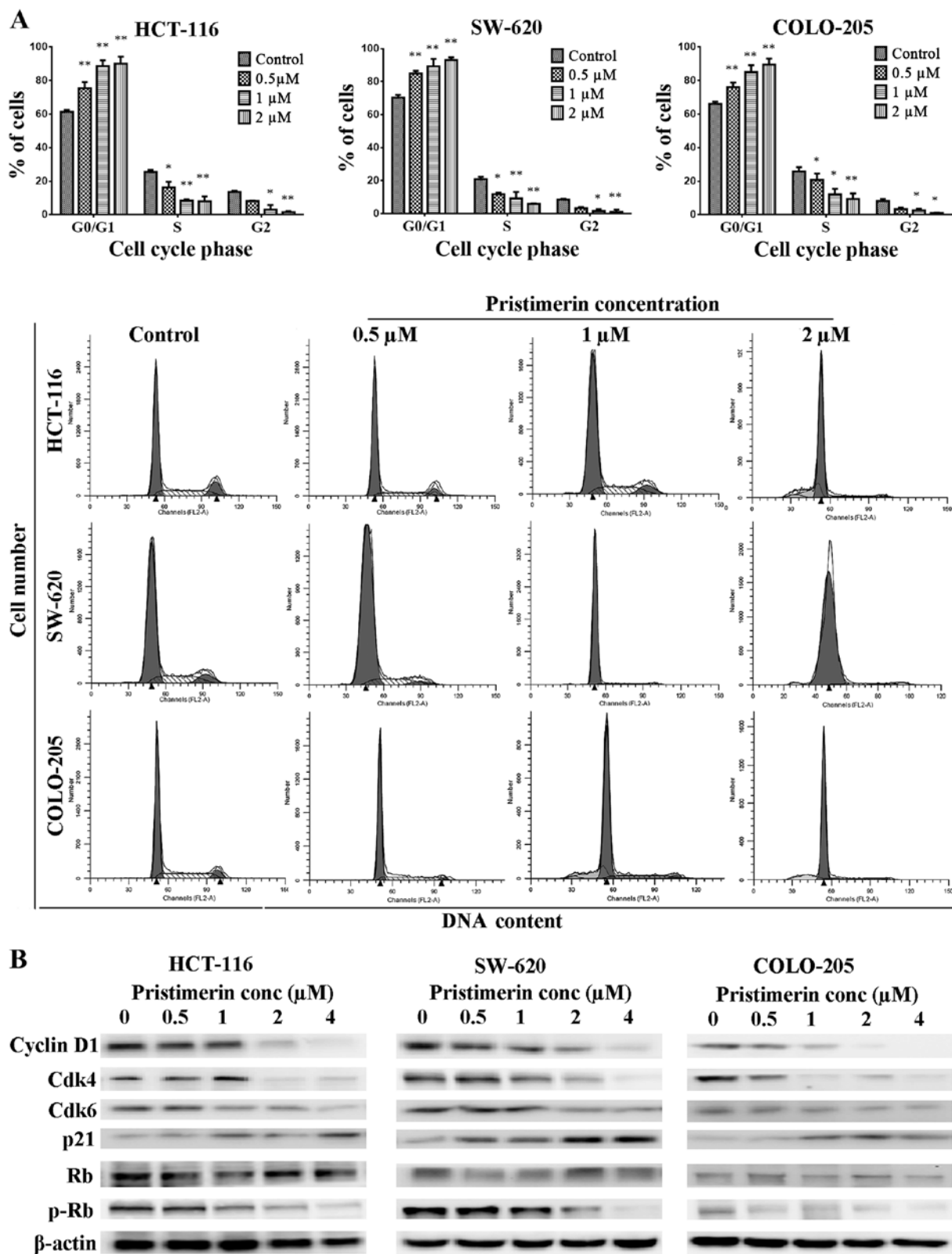


Figure 2. Pristimerin-induced cell cycle arrest. (A) The effects of pristimerin on cell cycle distribution are shown. HCT-116, SW-620 and COLO-205 cells were treated either with pristimerin (0.5, 1 and 2 μ M) or DMSO (control) for 48 h, and the cells were collected, stained and analyzed for the percentage of cells in different phases of the cell cycle. * p <0.05, ** p <0.01 vs. the control. (B) The effects of pristimerin on cell cycle-associated protein expression in the HCT-116, SW-620 and COLO-205 cells which were treated either with pristimerin (0.5, 1, 2 and 4 μ M) or DMSO (control) for 48 h. Total cell lysates were analyzed by western blot analysis (three independent experiments) for G1 cell cycle regulatory proteins (cyclin D1, cdk4, cdk6, p21, Rb and p-Rb). β -actin was used as the control.

was markedly increased in a concentration-dependent manner. G1 phase cell cycle distribution was 61.2, 74.5, 86.9 and 90% (HCT-116); 70.5, 81.3, 87.9 and 91.2% (SW-620); 65.8, 74.5, 86.1 and 90.4% (COLO-205) when the cells were treated with

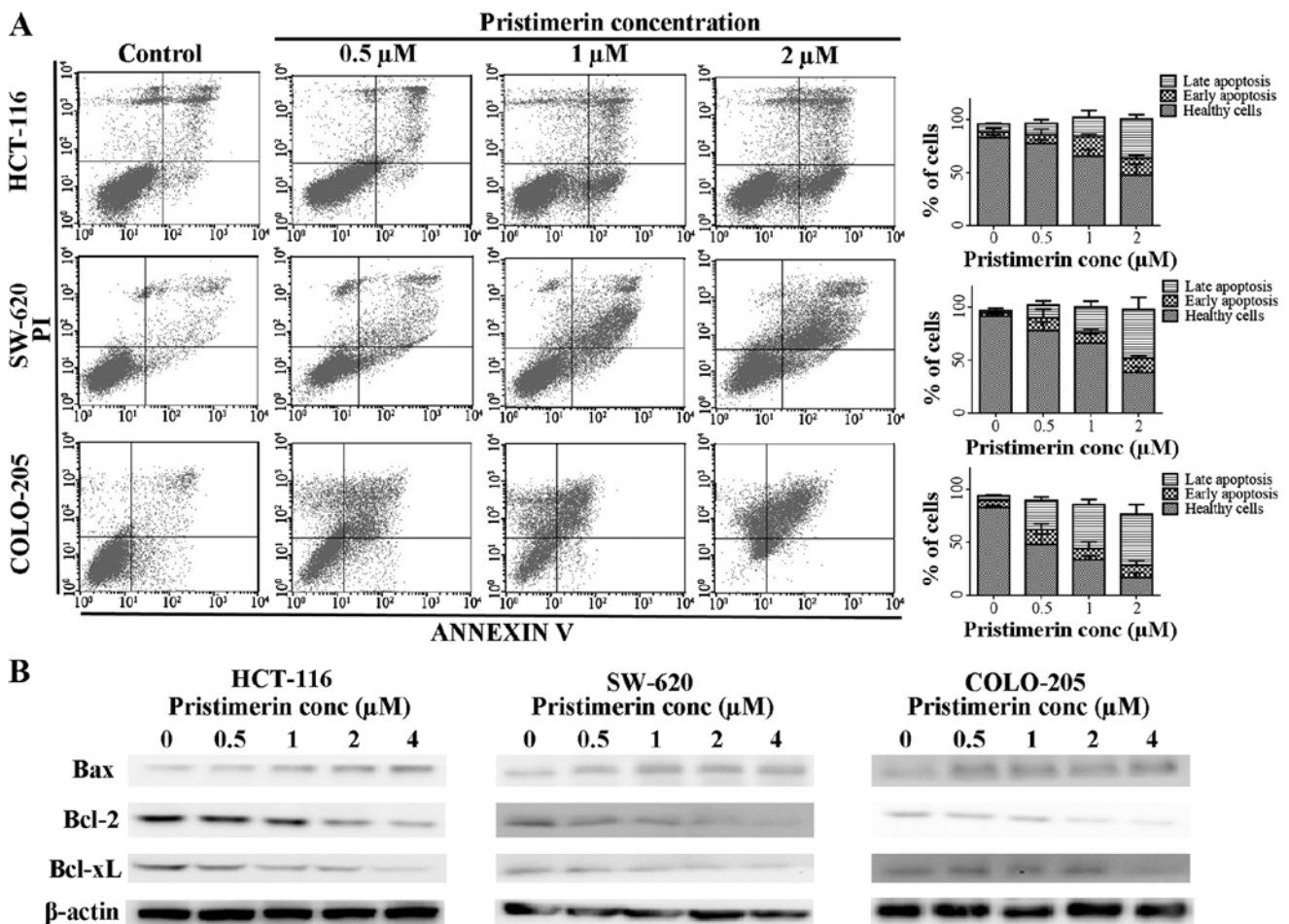


Figure 3. Pristimerin-induced apoptosis in colon cancer cells. (A) Induction of apoptosis as assessed by Annexin V/PI method. HCT-116, SW-620 and COLO-205 cells were treated either with pristimerin (0.5, 1 or 2 μM) or DMSO (control) for 48 h and analyzed using flow cytometry. (B) Effect of pristimerin on Bcl-xL, Bcl-2 and Bax levels. HCT-116, SW-620 and COLO-205 cells were treated either with pristimerin (0.5, 1, 2 and 4 μM) or DMSO (control) for 48 h. Total cell lysates were analyzed by western blot analysis (three independent experiments). β-actin was used as the control.

pristimerin at 0, 0.5, 1 and 2 μM, respectively. The increments in the G0/G1 phases were accompanied by concomitant reduction in the proportion of cells in the S and G2/M phases of the cell cycle in the CRC cell lines, with the presence of a small proportion of sub-G1 cells at higher concentrations, which indicated apoptosis. Taken together, these findings suggest that pristimerin-induced G1 arrest in colon cancer cells may be responsible for its growth inhibitory effects.

To understand the mechanism behind pristimerin-induced G1 phase arrest, we investigated the effect of pristimerin on levels of proteins that regulate the progression of cells in the G1 phase including p21, cyclin D1, cdk4, cdk6, retinoblastoma (Rb) and p-Rb. As shown in Fig. 2B, the protein level of cyclin D1 was significantly decreased by pristimerin in a concentration-dependent manner in all three colon cancer cell lines. Similarly, a dose-dependent reduction in the expression levels of cdk4 and cdk6 were observed (Fig. 2B). In addition, pristimerin decreased the p-Rb level in a dose-dependent manner without change in total Rb protein levels. Furthermore, exposure to pristimerin generated a dose-dependent induction of p21 expression in all three colon cancer cell lines (Fig. 2B). We assumed that the pristimerin modulation of cell cycle regulatory proteins may have contributed to the pristimerin-mediated G1 phase arrest in the colon cancer cells.

Pristimerin-induced apoptosis in the colon cancer cells. Pristimerin-induced apoptosis was also studied in colon cancer cells. Cells were treated with varying pristimerin concentrations for 48 h, stained with Annexin V/PI, and subjected to analysis using a flow cytometer. As shown in Fig. 3A, pristimerin treatment of colon cancer cells provoked significant dose-dependent enhancement in both early and late stages of apoptosis. The apoptotic indices were 11.5, 21.4, 39.9 and 55.8% (HCT-116); and 5.7, 23.4, 36.1 and 64.5% (SW-620); and 11, 35.2, 62.7 and 72.1% (COLO-205) at 0, 0.5, 1 and 2 μM concentrations of pristimerin, respectively (Fig. 3A).

To explore how pristimerin induced apoptosis, we studied the action of pristimerin on Bcl-2 family proteins, which play an important role in the regulation of apoptosis, including pro-apoptotic proteins (Bax) and anti-apoptotic proteins (Bcl-2 and Bcl-xL). Western blot analysis showed that pristimerin treatments resulted in a dose-dependent reduction in the levels of anti-apoptotic proteins Bcl-2 and Bcl-xL (Fig. 3B). In contrast, the level of pro-apoptotic protein Bax was significantly increased upon treatment with pristimerin in a dose-dependent manner (Fig. 3B). Thus, pristimerin treatment modulated the Bcl-2 family protein levels in a manner that led to increased ratios of Bax/Bcl-2 and Bax/Bcl-xL, which may have contributed to the observed apoptotic effect of pristimerin.

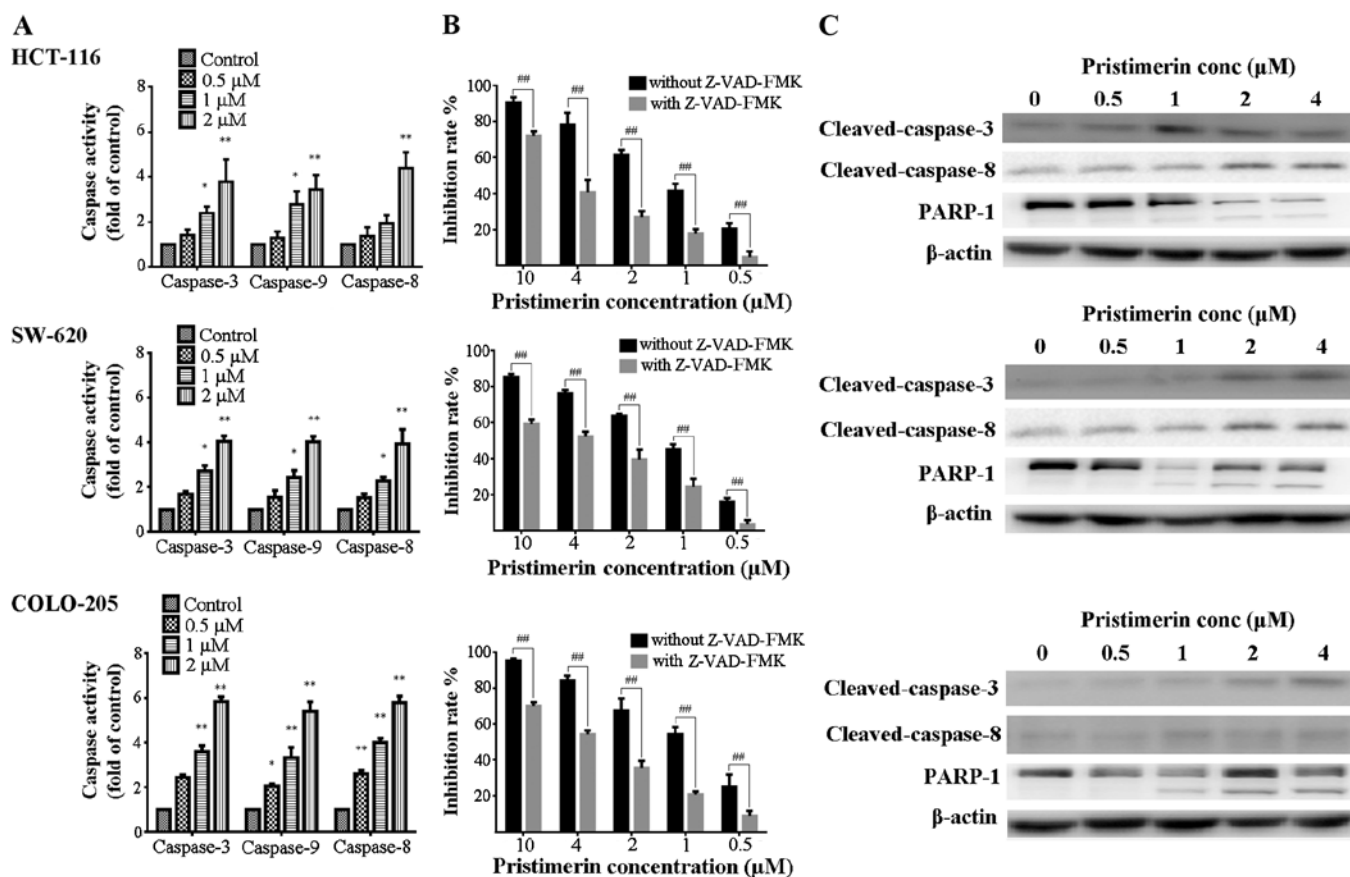


Figure 4. Pristimerin-induced apoptosis via caspase activation in the colon cancer cells. (A) Effect of pristimerin on apoptosis induction as assessed by caspase-3, -8 and -9 activity assays. (B) Effect of pristimerin in both the presence and absence of pan-caspase inhibitor Z-VAD-FMK. Inhibition rate (%) was determined by MTT assay. Each data are presented as mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, vs. control. ## $p < 0.01$ compared to pristimerin treatment in the absence and presence of Z-VAD-FMK. (C) Effect of pristimerin on cleaved-3 and -8 caspase, and PARP-1 protein expression, as assessed by western blotting. β -actin was used as the control. Western blot analyses represent three independent experiments.

To further understand the effects of pristimerin on cell apoptosis, we studied its effects on caspase-3, -8 and -9 activities and PARP-1 activation. Pristimerin treatment caused a dose-dependent enhancement in caspase-3, -8 and -9 activities in all the tested cell lines compared with these levels in the control after 48 h of treatment (Fig. 4A). Pretreatment of cells for 1 h with 50 μ l of Z-VAD-FMK, a pan-caspase inhibitor caused a significant reduction in pristimerin cytotoxicity in the colon cancer cells (Fig. 4B). Subsequently, we studied the expression of cleaved caspase-3 and -8, and PARP-1 in all three tested cell lines by western blotting. Pristimerin treatment also increased the expression of cleaved caspase-3 and -8, with increased cleavage of PARP-1 in a concentration-dependent manner (Fig. 4C). Taken together, pristimerin induced caspase-dependent apoptotic cell death in the colon cancer cells.

Pristimerin-induced loss of $\Delta\psi_m$. Since the intrinsic apoptotic pathway is predominantly triggered by changes in $\Delta\psi_m$, we further investigated the theory that pristimerin treatment-induced mitochondrial apoptosis was due to the changes in $\Delta\psi_m$. HCT-116, SW-620 and COLO-205 cells treated with pristimerin (0.5, 1 and 2 μ M) for 48 h were loaded with $\Delta\psi_m$ JC-1 probe, and the fluorescent shift in cells was measured by a flow cytometer. There was a significant change in mito-

chondrial potential after pristimerin treatment as shown in Fig. 5; pristimerin induced mitochondrial depolarization in a dose-dependent manner.

Pristimerin inhibits EGFR, HER2 activation and related signaling pathways in colon cancer cells. To understand the molecular basis of pristimerin-mediated anticancer activity, we examined the effect of pristimerin on EGFR and HER2 receptors, which are overexpressed in colon cancer (17). In the present study, we demonstrated that pristimerin exhibited anticancer activity against HCT-116 (EGFR strong), COLO-205 (EGFR weak) and SW-620 (EGFR negative), while all three cell lines showed detectable HER2 levels. Our results showed that pristimerin inhibited EGFR phosphorylation in a dose-dependent manner in the HCT-116 and COLO-205 cells, and inhibited HER2 phosphorylation in the three tested cell lines in a dose-dependent manner, with insignificant change in total EGFR and HER-2 protein expression (Fig. 6). Next, we evaluated whether the pristimerin-dependent reduction in EGFR and HER2 activities was associated with reduced downstream kinase activity including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR and NF- κ B signaling pathways (18). We found that pristimerin significantly suppressed phosphorylation of ERK1/2, Akt, mTOR and NF- κ B in a dose-dependent manner (Fig. 6), with

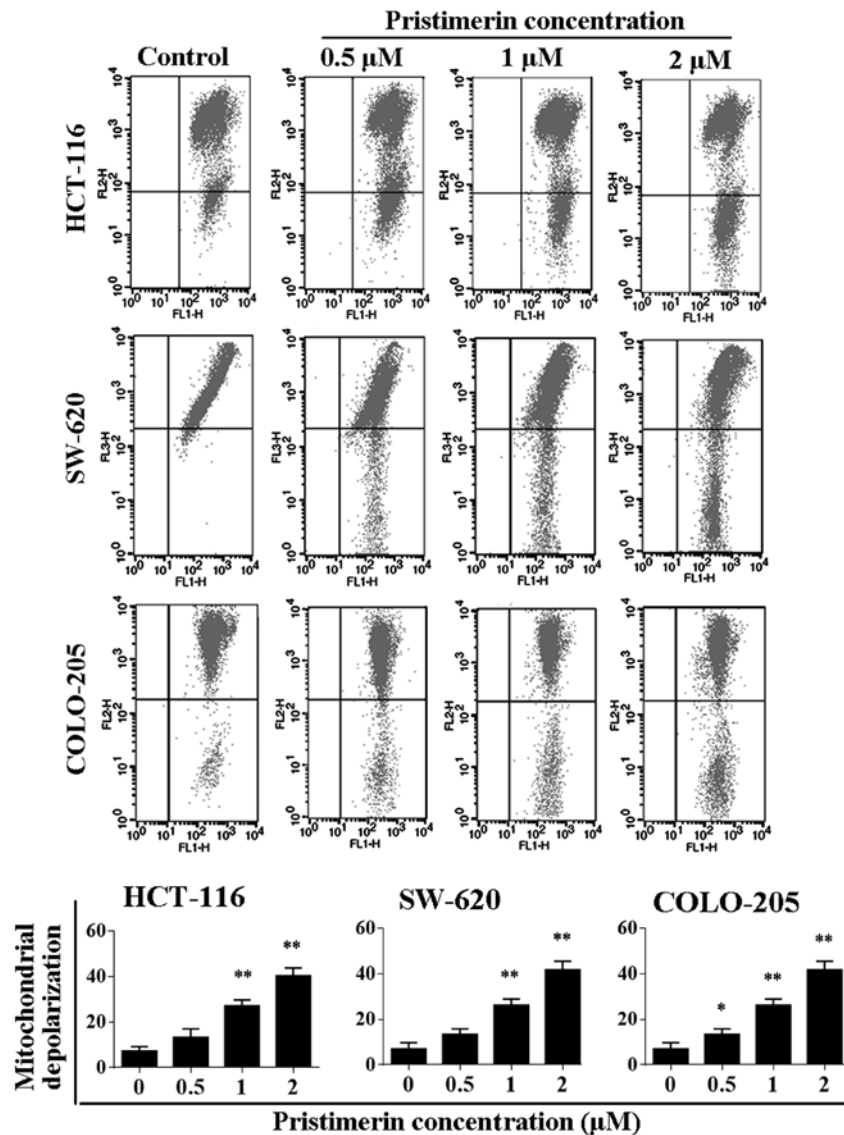


Figure 5. Pristimerin-induced mitochondrial depolarization in colon cancer cells. HCT-116, SW-620 and COLO-205 cells were treated with pristimerin (0.5, 1 and 2 μ M) or DMSO (control) for 48 h. Cells were collected and stained with mitochondrial potential sensor JC-1 and analyzed for mitochondrial membrane potential loss. Data are represented as the mean \pm SD from three independent experiments. * p <0.05, ** p <0.01 vs. the control.

no effect on total protein expression. The results suggest that pristimerin exerts its anticancer effects through both inhibition of EGFR and HER-2 activation on colon cancer cell surfaces and suppression of downstream ERK1/2, Akt, mTOR and NF- κ B signaling pathways.

Discussion

Plants are important sources of biologically active natural products which have become more important in anticancer drug development as they are more tolerable to the human body (5). Pristimerin is a natural triterpenoid that has been shown to possess a variety of biological activities. It has been reported to exhibit anticancer activity against different human cancer cells (19). In the present study, we examined the pristimerin cytotoxicity against three colon cancer cell lines for the first time. Our experimental results showed that pristimerin displayed dose- and time-dependent potent cytotoxicity in colon cancer cells. The cytotoxic effects were

further confirmed by studied morphological changes induced by pristimerin on human colon cancer cells.

Persistence of cell cycle progression in cancer cells is regarded as one of the hallmarks of cancer (20). Therefore, it is considered as a target for the treatment of human malignancies (21). Pristimerin was found to induce cell cycle arrest in a variety of human cancer cell lines, including pancreatic cancer (9) and myeloma tumors (15). Our results revealed that pristimerin treatment of colon cancer cells induced a dose-dependent arrest of cells in the G1 phase. Cell cycle progression is highly controlled by many processes involved in activation of cyclin-dependent kinases (CDKs), which are paired with the cyclins (e.g. cyclins A, B, E, D1-3) (22). Activation of CDKs by cyclins leads to phosphorylation of Rb and leads to diminished binding of Rb to the E2F transcription factor, which enhances transcription of various genes necessary for cell cycle progression (23). The activities of CDKs are antagonized by CDK inhibitors, such as p27 and p21, which directly interact with CDKs (24). In order to investigate the

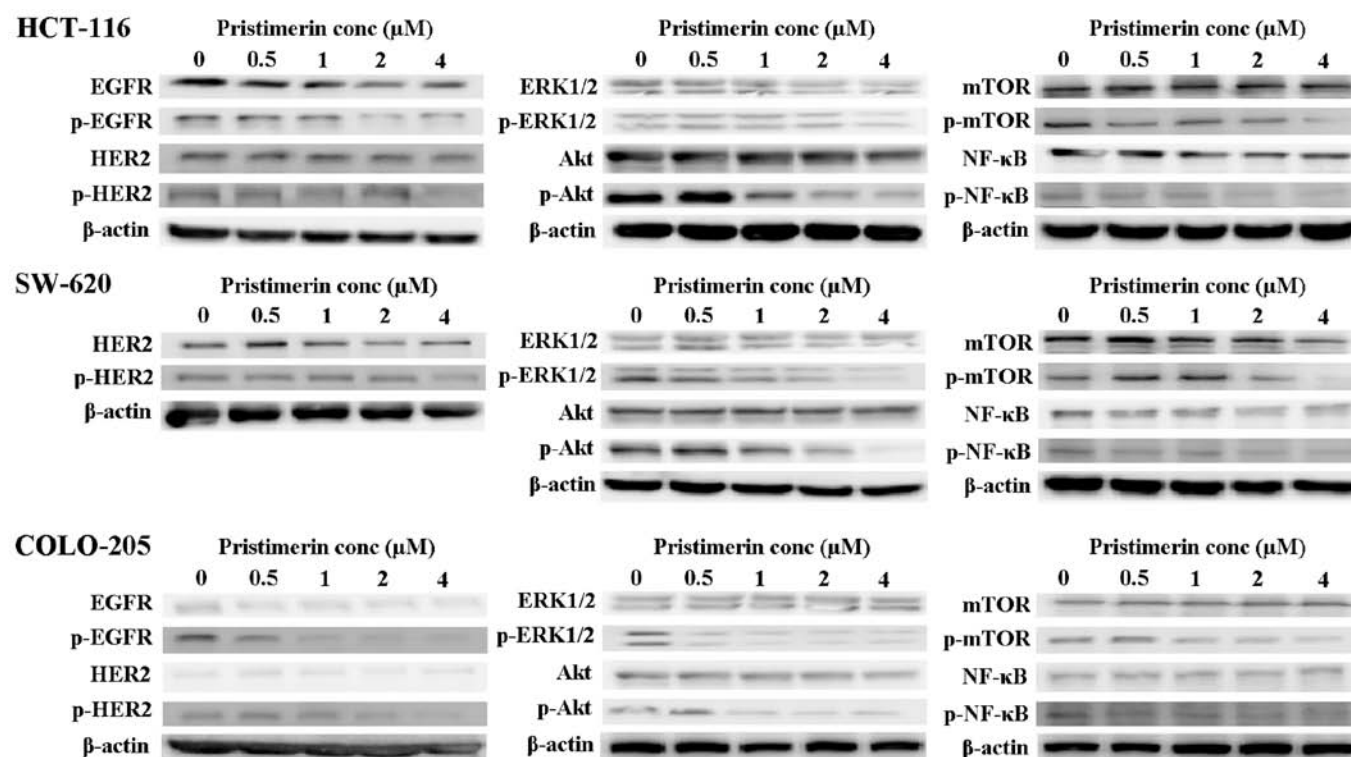


Figure 6. Pristimerin inhibited EGFR, HER2 and related downstream signaling proteins in colon cancer cells. HCT-116, SW-620 and COLO-205 cells were treated either with pristimerin (0.5, 1 and 2 μ M) or DMSO (control) for 48 h. Total cell lysates were analyzed by western blot analysis (three independent experiments) for EGFR, p-EGFR, HER2 and p-HER2. In addition, downstream targets (ERK1/2, p-ERK1/2, Akt, p-Akt, mTOR, p-mTOR, NF- κ B and p-NF- κ B) also were detected. β -actin was used as control.

mechanism underlying pristimerin-induced G1 phase cell cycle arrest, cell cycle regulatory proteins, CDKs and CDK inhibitors were analyzed using western blot analysis. Our results showed that pristimerin upregulated p21 expression, along with decreased cyclin D1, Cdk-4 and Cdk-6 expression. In addition, pristimerin decreased phosphorylated Rb expression, which led to G1 phase arrest. These findings suggest that pristimerin-induced G1 phase cell cycle arrest may be mediated through the cyclin-CDK checkpoint.

Apoptotic resistance is another important hallmark of cancer cells (20). Thus, activation of apoptotic pathways is a key mechanism by which anticancer drugs kill tumor cells (25). Apoptosis is a highly regulated and organized cell death process. Two major pathways of apoptosis, depending on different apoptotic stimuli, are the mitochondrial pathway (intrinsic) and the death receptor pathway (extrinsic) (26). In the present study, we observed that pristimerin induced apoptotic cell death in a dose-dependent manner, which was confirmed by Annexin V/PI double staining, confirming membrane alterations related to apoptosis in colon cancer cells. Moreover, our caspase activity assay showed that pristimerin was able to induce caspase-dependent apoptosis in human colon cancer cells in a dose-dependent manner. This caspase-dependent apoptosis was further explored by the presence of pan-caspase inhibitor (Z-VAD-FMK), which reduced the cytotoxic activity of pristimerin. We found that pristimerin induced apoptosis via the cell death receptor pathway (extrinsic) by activating caspase-8 and -3. These results were in line with a previous study on human breast cancer cells (27). Furthermore, pristimerin showed activation of caspase-9 and -3, which are

modulators of the mitochondrial pathway (intrinsic) (28). The activation of caspases are crucial steps in apoptotic cell death, which consequently induce PARP-1 cleavage, another marker of cell apoptosis (29).

Mitochondria have been shown to play a central role in the apoptotic process, through both intrinsic and extrinsic pathways. Mitochondrial membrane permeability is one of the most important features of the mitochondria, and permeability change, mainly through permeability transition pores, leads to pro-apoptotic factor release, loss of mitochondrial membrane potential ($\Delta\Psi$ m) and cell death (30). Our results showed that pristimerin induced conformational changes of mitochondria, followed by redistribution of pro-apoptotic factors and $\Delta\Psi$ m loss in a dose-dependent manner. Bcl-2, Bcl-xL and Bax have been implicated as major regulators of the role of mitochondria in apoptosis (31). Pro-apoptotic Bcl-2 members bind to the mitochondrial outer membrane and block cytochrome *c* efflux. In apoptosis, Bax translocates from the cytosol to the mitochondria where it increases the efflux of cytochrome *c* through the mitochondrial outer membrane (32). Thus, changes in anti-apoptotic and pro-apoptotic Bcl-2 family proteins are critical for the induction of apoptosis (33). Pristimerin treatment of colon cancer cells increased Bax protein expression and decreased both Bcl-2 and Bcl-xL expression thereby increasing the Bax/Bcl-2 and Bax/Bcl-xL ratios leading to apoptosis. Collectively, our results revealed that pristimerin induced both intrinsic and extrinsic apoptotic pathways in the colon cancer cell lines.

Overexpression of growth factors and their receptors (EGFR and HER2) play a vital role in the tumorigenesis of

colorectal carcinoma, which leads to constitutive activation of tyrosine kinase activity of EGFR and related downstream signaling pathways (17,34). Therefore, agents that target EGFR and/or HER2 receptor show anticancer activity against CRC (35). Our results showed that the tested colon cancer cell lines had different expression patterns of growth factor receptors, as previously reported (36). Pristimerin inhibited EGFR and HER2 activation in a dose-dependent manner. The same results were previously reported in glioma tumors (13) and breast cancer cells (27). Upregulation and activation of EGFR and HER-2 were found to lead to the activation of multiple intracellular signaling cascades that are responsible for tumorigenesis of colon cancer including MAPK, PI3K/Akt/m-TOR and NF- κ B signaling pathways (18,37). The downstream MAPK/ERK signaling pathway plays an important role in cell proliferation and apoptosis regulation (38). Our results showed that pristimerin blocked ERK1/2 phosphorylation. The same results were obtained in hepatic (10) and breast cancer cells (39). PI3K/AKT is another downstream signaling pathway of EGFR/HER2 that controls cell proliferation, survival, apoptosis and malignant transformation (40). Our results illustrated that pristimerin reduced Akt activation as a consequence of EGFR/HER2 inhibition. Anti-apoptotic/pro-survival NF- κ B and mTOR signaling pathways are downstream targets of activated PI3K/AKT, which controls cell growth, survival, division and motility (41,42). Pristimerin also blocked both phosphorylation and activation of these pathways. Other researchers found the same results, in which pristimerin inhibited pro-survival Akt/NF- κ B/m-TOR signaling proteins in pancreatic cancer cells (43). Pristimerin inhibited the expression of p-ERK, p-Akt, p-NF- κ B and p-mTOR in colon cancer cells, which indicated that inhibition of those pro-survival signaling proteins was essential for pristimerin-induced apoptosis.

In conclusion, our findings revealed that pristimerin induced cell cycle arrest and apoptosis in human colon cancer cells HCT-116, SW-620 and COLO-205 through cyclin-CDK, mitochondrial dysfunction and caspase-dependent mechanisms. The mechanisms may involve inhibition of phosphorylated EGFR and HER2 expression, and subsequently caused inhibition of related downstream kinases. These findings suggest that pristimerin may be a novel candidate for colon cancer therapy.

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