Pristimerin, a Naturally Occurring Triterpenoid, Exerts Potent Anticancer Effect in Colon Cancer Cells

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Pristimerin is a triterpene compound isolated from plant extracts that reportedly possesses antitumor, antioxidant, and anti-inflammatory activities. The current study was designed to evaluate the antitumor effects of pristimerin on human colon cancer cells. Treatment of the human colon cancer cells, HCT116 and SW480, with pristimerin led to a dose-dependent decrease in cell proliferation. Flow cytometry experiments showed that pristimerin increased cell apoptotic rate and decreased the mitochondrial membrane potential in HCT116 and SW480 cells. Western blot assay showed that pristimerin induced increased cleavage of caspase-3, -7, -8, and poly ADP ribose polymerase. Treatment with pristimerin also caused a marked decrease in the expression of Bcl-2 and Bcl-xL. Additionally, the levels of phosphorylated AKT and forkhead box O3a (FOXO3a) were decreased in pristimerin-treated colon cancer cells. Taken together, our study illustrated that pristimerin promoted apoptosis via the AKT/FOXO3a signaling pathway in colon cancer cells, elucidating that it might be considered as a potential agent for colon cancer therapy.

Key Words: Apoptosis, AKT, Colon cancer cells, Forkhead box O3a, Pristimerin

INTRODUCTION

In 2015, there were 1.7 million incident cases of colon and rectum cancer, and it caused 832,000 deaths. Colorectal cancer caused 17 million and disability-adjusted life-years in 2015 of which 96% came from years of life lost and 4% came from years lived with disability (Nagai and Kim, 2017). The odds of developing colorectal cancer before age 79 years at the global level was higher for men than for women. Globally colorectal cancer ranked third for cancer incidence and second for cancer deaths in 2015 (Nagai and Kim, 2017). Although there are many ways for management colorectal cancer such as screening, surgical management, irradiation

and adjuvant chemotherapy, it remains one of the most life-threatening malignancies, particularly when it becomes in advanced stages (Brenner et al., 2014). Thus, discovering of effective and safe compounds urgently needed to reduce colorectal cancer mortality.

During the course of chemopreventive agent identification, phytochemicals draw extensive attention for their relative safety (Lee et al., 2011). Pristimerin is a quininemethide triterpenoid compound that has been found in various species belonging to *Celastraceae* and *Hippocrateaceae* families. It displays a variety of biological activities, including antifungal, antioxidant, antibacterial, and anti-inflammatory properties (Carvalho et al., 2005; López et al., 2011; Gullo et al., 2012; Kim et al., 2013). It also has been reported that pristimerin,

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as a new proteasome inhibitor, has promising clinical potential as both a therapeutic and chemopreventive agent for cancer (Tiedemann et al., 2009). Indeed, pristimerin inhibited tumor growth of various human cancers such as breast, prostate, pancreatic, hepatic and multiple myeloma tumors (Yousef et al., 2017). In addition, a recent study reported that pristimerin inhibited cell cycle progression and induced apoptosis in colon cancer cells through blocking EGFR and HER2 activation (Yousef et al., 2016). In the present study, we further investigated anticancer potential of pristimerin on human colon cancer cells, HCT116 (as p53 normal cells) and SW480 (as p53 mutant cells) cells. In addition, the molecular mechanism of anticancer effect of pristimerin was demonstrated in both colon cancer cells.

MATERIALS AND METHODS

Reagents

Pristimerin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from Hyclone (Logan, UT, USA). Dimethylsulfoxide (DMSO) and anti- β -actin antibody were purchased from Sigma Aldrich (St. Louis, MO, USA). Protein detection BCA kit was obtained from Thermo Scientific Pierce (Rockford, IL, USA). For Western blot analysis, following antibodies were used: anti-cleaved caspase-3 rabbit polyclonal, anti-cleaved caspase-7 rabbit polyclonal, anti-cleaved caspase-8 rabbit monoclonal, anti-cleaved caspase-9 rabbit polyclonal, anti-cleaved-PARP rabbit polyclonal, anti-BAX rabbit polyclonal, anti-tBid rabbit polyclonal, anti-phospho-AKT (p-AKT) rabbit monoclonal, anti-phospho-forkhead box O3a (p-FOXO3a) rabbit polyclonal, anti-FOXO3a antibody rabbit monoclonal (all Cell Signaling Technology, Danvers, USA), anti-Bcl-2 rabbit polyclonal, anti-Bcl-xL rabbit polyclonal (all Santa Cruz Biotechnology, Santa Cruz, USA).

Cells and cell culture

Human HCT116 and SW480 colon cancer cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in RPMI 1640 medium at 37°C

with 5% CO₂ in a humidified atmosphere. All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells attained 70% confluence with a normal morphology and multiplication pattern, and were used for experiments between passages 3-12. Unless otherwise mentioned, control treatment refers to the cells treated with an equal amount of DMSO; the maximum amount of DMSO was maintained at <0.1% v/v.

Cell proliferation assay

HCT116 and SW480 cells were seeded into wells of 96-well plates at a density of 1×10^4 cells/well. The rate of cell proliferation was determined at 24 h with CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instruction. Absorbance at 490 nm was measured with a microplate reader, and the average absorbance values were calculated.

Annexin V apoptosis assay

Cells (1×10^5 per well) in 24-well plates treated with indicated concentration of pristimerin were harvested, pooled in complete medium and washed repeatedly with sterile phosphate buffered saline (PBS). Cells suspended in PBS and stained with Muse® Annexin V and Dead Cell Assay Kit (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instruction. The stained cells were analyzed immediately by using Muse® Cell Analyzer (Merck Millipore).

Measurement of mitochondrial membrane potential (MMP)

MMP was determined using a JC-1 mitochondrial membrane potential detection kit (Biotium, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, cells (1×10^5 per well) were plated in 24-well plates. After 24 h incubation with indicated concentrations of pristimerin, cells were collected and mixed with 0.5 mL of JC-1 working solution at 37°C in the dark for 15 min. Cells were analyzed immediately by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Fig. 1. Effects of pristimerin on colon cancer cell proliferation. (A) The structure of pristimerin. (B) HCT116 cells and (C) SW480 cells were treated with indicated concentration of pristimerin for 24, 48 and 72 h, after which the proliferation was determined as described in Methods. The results are reported as the mean $\pm$ S.E.M. of three independent experiments in triplicate. Statistical significance is based on the difference when compared with 0 nM-treated cells, * P < 0.05, ** P < 0.01, *** P < 0.001.

Western blot analysis

Cells were lysed in Pro-prep protein extraction solution (iNtRon, Sungnam, Korea) containing $1\times$ protease and phosphatase inhibitor cocktail. The protein concentrations were determined using a BCA protein assay kit. Whole cell extracts were separated by 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. After blocking with 3% skim milk for 1 h, the membranes were incubated with primary antibody at 4°C overnight. The membranes were then washed with PBST ($1\times$ PBS, 0.1% Tween 20) and incubated for 1 h with anti-rabbit IgG or anti-mouse IgG conjugated with HRP. After washing, the protein bands were then visualized using

a WEST-One™ Western Blot Detection Spray (iNtRon) and DAVINCH-Chemi CAS-400SM (Davinch-k, Seoul, Korea). Dates were assessed using Total Lab software (Davinch-k).

Statistical analysis

Values are expressed as the mean $\pm$ S.E.M. of the results of at least three experiments. The values were then evaluated by one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post tests using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Differences with P values < 0.05 were considered statistically significant.

RESULTS

Pristimerin inhibited cell proliferation in human colon cancer cells

To evaluate anticancer effect of pristimerin in colon cancer cells, we firstly examined whether pristimerin affected the proliferation of HCT116 and SW480 cells. These cells were treated with different concentrations (0~1,000 nM) of pristimerin for 24, 48, or 72 h and cell proliferation was assessed by the MTS assay. As shown in Fig. 1B and 1C, pristimerin treatment produced a dose-dependent and time-dependent inhibition of the proliferation of colon cancer cells.

Pristimerin induces apoptosis of human colon cancer cells

To determine if apoptosis mediated the inhibition of HCT-116 and SW480 proliferation, the loss of phosphatidylserine asymmetry was quantified by cytometry of 7-AAD/Annexin V double-stained cells. HCT116 and SW480 cells treated with 500 and 1,000 nM pristimerin for 24 h were significantly increased Annexin V-positive cells indicating that pristimerin induced apoptosis (Figs. 2A-2C).

Apoptosis was further examined by measuring cleaved caspases and PARP in HCT116 and SW480 cells treated with pristimerin for 24 h because activation of caspases and the subsequent cleavage of PARP are classic molecular markers of apoptosis (Elmore, 2007). Consistent with Annexin V staining results, the levels of cleaved caspase-3, -7, -8, and -PARP were increased in cells treated with pristimerin (Fig.

Fig. 2. Pristimerin induces apoptosis in human colon cancer cells. (A) HCT116 and SW480 cells were treated with indicated concentration of pristimerin for 24 h and apoptosis was evaluated using Annexin V Assay Kit. The lower right quadrant (Annexin V⁺/7-AAD⁻) represents early apoptosis, and the upper right quadrant (Annexin V⁺/PI⁺) represents late apoptotic cells. Percentages of early and late apoptotic cells in (B) HCT116 and (C) SW480 cells are shown. (D) HCT116 and (E) SW480 cells were treated with indicated concentration of pristimerin for 24 h. Equal amounts of cell lysates were subjected to Western blot analysis for indicated proteins. β -actin was served as an equal loading control. Relative protein expression is presented in the bar graphs below. The results shown are the mean $\pm$ S.E.M. of four independent experiments. Statistical significance is based on the difference when compared with 0 nM-treated cells, * P < 0.05, ** P < 0.01, *** P < 0.001.

2D and 2E). In contrast, no detectable changes in amount of cleaved caspase-9 following pristimerin treatment were observed.

Treatment of pristimerin reduces the mitochondrial membrane potential (MMP)

Cell apoptosis appears to be controlled by Bcl-2 family proteins (Elmore, 2007; Kale et al., 2018). Bcl-2 protein is a cell death inhibitor, whereas BAX has a critical role in facilitating apoptosis. Thus, we measured the expression of Bcl-2 family proteins in HCT116 and SW480 cells by Western blot analysis after treatment with pristimerin. The expression of Bcl-2 and Bcl-xL was decreased, while the levels of BAX were unchanged (Fig. 3A and 3B). The expression of tBid was also elevated by pristimerin treatment (Fig. 3A and 3B). These results suggest that the apoptotic effect of pristimerin may be mediated by Bcl-2, Bcl-xL and tBid.

Disruption of the function of Bcl-2 protein leads to permeabilization of MMP. We therefore investigated the effects of pristimerin on MMP using JC-1 fluorescent dye and flow cytometry. JC-1, a cationic dye, produces red fluorescent J-aggregates in mitochondria with high MMP and green fluorescence with low MMP. An increased proportion of HCT116 and SW480 cells experiencing mitochondrial depolarization and undergoing cell death. Exposure of colon cancer cells to 500 and 1,000 nM pristimerin for 24 h led to a significant reduction in the MMP (Fig. 3C and 3D).

Role of AKT and FOXO3a signaling pathway in pristimerin-treated colon cancer cells

As reported, AKT is constitutively activated in majority of colon cancer (Manning and Toker, 2017). Therefore, we hypothesized that the anticancer effect of pristimerin in colon cancer cells was due to inhibition of AKT pathway. To test our hypothesis, constitutive protein and phosphorylation levels of AKT were examined in both colon cancer cells by Western blot analysis. As shown in Fig. 4, phosphorylation of AKT was dramatically suppressed by 100, 500 and 1,000 nM pristimerin treatment. However, there was no variation in the expression of AKT. Next, we investigated FOXO3a, which is an important downstream molecule of AKT pathway and a tumor suppressor in the human cancers (Link et al., 2017; Manning and Toker, 2017). The results showed that the phosphorylated levels, but not protein levels of FOXO3a

Fig. 3. The dissipation of mitochondrial membrane potential (MMP) of colon cancer cells upon treatment with pristimerin. Western blot analysis showing BAX, Bcl-2, Bcl-xL and tBid protein levels in (A) HCT116 and (B) SW480 cells with the same treatments as in Fig. 2. Equal amounts of cell lysates were subjected to Western blot analysis for indicated proteins. β-actin was served as an equal loading control. Relative protein expression is presented in the bar graphs below. HCT116 (C) and SW480 cells (D) treated with indicated concentrations of pristimerin for 24 h were stained with JC-1 and the average ratio of red/green fluorescence intensity was analyzed by flow cytometry. The data shown are representative of three independent experiments. Relative protein expression is presented in the bar graphs below. The results shown are the mean $\pm$ S.E.M. of four independent experiments. Statistical significance is based on the difference when compared with 0 nM-treated cells, ** P < 0.01, *** P < 0.001.

were decreased in pristimerin-treated cells (Fig. 4). The results indicated that pristimerin specifically targeted the activation of AKT/FOXO3a pathway.

DISCUSSION

Colon cancer is the third most common malignancy both in terms of estimated new cases and estimated deaths in 2016 (Siegel et al., 2016). Despite the development of several therapies for its treatment, there is still no effective cure for patients with advanced stages of the disease and the prognosis for advanced tumors remains reserved. Therefore, more

Fig. 4. Effect of pristimerin on the activation of AKT and FOXO3a signaling pathway. (A) HCT and (B) SW480 cells were treated with indicated concentration of pristimerin for 24 h. Equal amounts of cell lysates were subjected to Western blot analyses for p-AKT, AKT, p-FOXO3a and FOXO3a. Bar diagram showed the quantitation of respective Western blots. Each bar represents means $\pm$ S.E.M. of at least three independent experiments. Statistical significance is based on the difference when compared with 0 nM-treated cells, * P < 0.05, ** P < 0.01, *** P < 0.001.

studies are still needed to develop non-toxic new antitumor approaches with minimal side effects. Phytochemicals constitute a prolific source of therapeutic compounds that exhibit antitumor activities.

In present study, we found that pristimerin suppressed the proliferation of colon cancer cells in a dose-dependent manner in both HCT116 and SW480 cells (Fig. 1). By flow cytometry using Annexin V binding assay, we demonstrated that pristimerin triggers apoptosis in HCT116 and SW480 cells (Figs. 2A-2C). This effect implies increased expression level of cleaved caspase-3, -7, -8 and -PARP, a hallmark of apoptosis (Fig. 2D and 2E). The extrinsic pathway of apoptosis is initiated by the binding of ligands to death receptors. The transmembrane death signal induces formation of the death-inducing signaling complex through adaptor proteins, subsequently leading to caspase-8 activation (Elmore, 2007). Presently, caspase-8 also played a role in apoptosis of colon cancer cells treated with pristimerin.

The p53 protein becomes stabilized and activated in response to a number of stimuli, including exposure of cells to DNA-damaging agents and oncogene activation. The activation of p53 allows it to carry out its function as a tumor suppressor through cell cycle arrest, apoptosis, DNA repair, differentiation, and antiangiogenesis (el-Deiry, 1998; Wang

et al., 2015). Because of these abilities, the tumor suppressor p53 is an attractive target in cancer therapy (Bo et al., 2014). However, most of the tumor malignancies are associated with mutation of p53 gene and the state of p53 is pivotal for the response of tumor cells to anticancer therapies (Muller and Vousden, 2013). Many studies indicated that colon cancer cells with mutant p53 were more chemo-resistant than those with wild type p53 due to their poor ability to activate apoptosis and initiation of apoptosis is the most important functions of p53 (Iacopetta, 2003; Chen, 2016). There is no correlation between cell sensitivity to pristimerin and p53 status, because the anti-proliferative effect of pristimerin for SW480 cells, which carry mutant p53 are similar to those for HCT116 cells, which have wild-type p53 (Figs. 1B and 1C). Interestingly, pristimerin was able to affect the viability of SW480 colon adenocarcinoma cells that express a p53 mutated form (R273H), a very well-known hot spot mutation in tumors. Besides, the percentage of apoptotic cells in pristimerin-treated SW480 cells was much higher than HCT-116 cells (Figs. 2A-2C). This result suggests that pristimerin effects on the viability of colon cancer cells did not rely on p53. Also, pristimerin might be a potential agent to induce apoptosis against p53 mutant cancer cells.

The intrinsic apoptotic pathway involve non receptor mediated stimuli that produce mitochondrial mediated signals, resulting in an opening of mitochondrial permeability transition pore, loss of the MMP and release of pro-apoptotic proteins such as cytochrome c. The Bcl-2 family of proteins, which includes anti-apoptotic proteins such as Bcl-2 and Bcl-xL and pro-apoptotic proteins such as BAX and Bid regulates the release of cytochrome c from mitochondria via control of the mitochondrial membrane's permeability. Release of cytochrome c contributes to the activation of caspase-9 followed by sequential activation of caspase-3 and cleavage of PARP (Decker and Muller, 2002; Elmore, 2007). Our results showed that pristimerin induced apoptosis of colon cancer cells was associated with the down regulation of the Bcl-2 level and the up regulations of the tBid levels (Fig. 3A and 3B), as well as the loss of MMP (Fig. 3C and 3D), indicating that pristimerin induced mitochondrial dysfunction through regulation of the Bcl-2 family, leads to apoptosis.

Pristimerin did not change the levels of cleaved caspase-9 in both colon cancer cells (Fig. 2D and Fig. 2E). Until recently, the sole apical initiator in apoptosis pathways was assumed to be caspase-9, which is activated in a complex termed the apoptosome by the scaffold protein Apaf-1 and its cofactor cytochrome c (Shakeri et al., 2017). Caspase-9 is of particular interest because it initiates cell death in response to non-cytokine-mediated cellular stress, such as UV or chemical treatment (Sakai et al., 2004). However, the apoptosome is not universally essential for Bcl-2-regulated apoptosis, because certain cells lacking Apaf-1 or caspase-9 readily undergo apoptosis in response to diverse insults (Marsden et al., 2002; Tomicic et al., 2005). Here we found that pristimerin-mediated apoptosis in HCT116 and SW480 cells was independent of caspase-9. As processing of this caspase did not occur, it is possible that the other initiator caspase may be involved in pristimerin-induced apoptosis.

FOXO3a, a member of FOXO transcription factors family, is a promising target to evaluate the tumorigenesis in different cancer types and it is required to induce the expression of various genes necessary for cell death (Fernández de Mattos et al., 2008). The major upstream regulator of FOXO3a is AKT signaling axis (Manning and Toker, 2017). AKT-dependent phosphorylation of FOXO3a leads to its cytoplasmic retention by 14-3-3 proteins and loss of target gene activation (Manning and Toker, 2017). AKT-mediated phosphorylation of FOXO3a promotes cell survival through inhibiting proapoptotic proteins such as TRAIL, Bim and promoting anti-apoptosis protein such as Bcl-xL (Fernández de Mattos et al., 2008). Our results reveal that the phosphorylation of FOXO3a, but not protein expression, was decreased with pristimerin treatment in colon cancer cells (Fig. 4A and 4B). Previous study also showed decreased levels of phospho-FOXO3a in pristimerin-treated pancreatic cancer cells (Deeb et al., 2014). Furthermore, FOXO3a responsive Bcl-xL were decreased by pristimerin. These results suggest that pristimerin induces apoptosis by inhibiting the AKT/FOXO3a pathway. This evidence showed that pristimerin was efficacious for the growth control of colon cancer cells, demonstrated the potential utility of pristimerin in clinical settings.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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