Induction of apoptosis by 7-piperazinethylchrysin in HCT-116 human colon cancer cells

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Abstract. The antitumor activity of 7-piperazinethylchrysin (7-PEC) was investigated in HCT-116 human colon cancer cells. MTT assay revealed that the IC_{50} of 7-PEC in HCT-116 cells was 1.5 μM after 72 h of treatment, much lower than that of chrysin (>100 μM). The data showed that 7-PEC was able to inhibit the growth of HCT-116 cells in a concentration- and time-dependent manner. Topical morphological changes of apoptotic body formation after 7-PEC treatment were observed by Hoechst 33258 staining. 7-PEC reduced mitochondrial membrane potential ($\Delta\Psi m$) of cells in a concentration-dependent manner and increased the production of intracellular reactive oxygen species (ROS). After treatment with 7-PEC, a significant increase of Bax protein expression and decrease of Bcl-2 protein expression were observed at the same time. These events paralleled with activation of p53, caspase-3 and -9 and the release of cytochrome *c* (cyt-*c*), as well as poly(ADP-ribose) polymerase-1 (PARP1) cleavage and downregulation of p-Akt. However, the apoptosis induced by 7-PEC was blocked by Ac-DEVD-CHO, a caspase-3 inhibitor. These results demonstrate that 7-PEC-induced mitochondrial dysfunction in HCT-116 human colon cancer cells triggers events responsible for caspase-dependent apoptosis pathways, and the elevated ratio of Bax/Bcl-2 is likely involved in this effect.

Introduction

Apoptosis is an important continuous process of destruction of undesirable cells during development or homeostasis in multicellular organisms. This process is characterized by distinct morphological changes, including membrane bleeding, cell shrinkage, dissipation of mitochondrial membrane potential ($\Delta\Psi m$), chromatin condensation and DNA fragmentation (1,2). The extrinsic and intrinsic pathways are the two major pathways involved in the regulation of apoptosis (3): the extrinsic pathway is mediated via cell surface death receptor, leading to the activation of caspase-8; the intrinsic pathway is dependent on various cell stress stimuli, leading to altered ratio of Bcl-2 family members which affect cytochrome *c* (cyt-*c*), Smac and apoptotic protease activating factor-1 (Apaf-1) release that leads to caspase-9 and -3 activation (4). Several therapeutic agents eliminate tumor cells by inducing apoptotic cell death (5), and some natural plants have been investigated for their cytotoxicity in cancer targeting apoptosis (6).

Flavonoids are a diverse family of natural phenolic compounds commonly found in fruits and vegetables, such as flavonols, flavonones and flavans. They have demonstrated anticancer and chemopreventive properties in numerous epidemiological studies (7), and were able to inhibit the proliferation of tumor cells, such as breast, prostate and lung cancer cells, both *in vitro* and *in vivo* (8,9), although the exact mechanism is not yet fully understood. The flavonoids are generally safe with low toxicity, making them ideal candidates for cancer chemopreventive agents. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid presented in many plant extracts, including blue passion flower (*Passiflora caerulea*), honey and propolis (10). A number of studies have shown that chrysin has multiple biological activities, such as antiinflammation, antioxidation and anticancer effects (11-13). Chrysin has been reported to induce apoptosis in a panel of cancer cell lines, including HeLa cervical cancer cells, U937, HL-60 and L1210 leukemia cells (14). Chrysin was also able to inhibit tumor angiogenesis *in vivo*, which is a key step in cancer cell metastasis (15,16). We previously reported that 7-piperazinethylchrysin (7-PEC) (Fig. 1) significantly inhibited the growth of various cancer cell lines such as HCT-116 cells (17). The aim of the present study was to elucidate the mechanisms of cell growth inhibition induced by 7-PEC. Herein we report that 7-PEC can inhibit the proliferation of HCT-116 cells in a time- and dose-dependent manner, including the $\Delta\Psi m$ loss, elevating the ratio of Bax/Bcl-2, releasing cyt-*c* to cell cytoplasm, activating caspase-9, -3 and p53, followed by PARP cleavage and induction of apoptosis.

Materials and methods

Materials. 7-PEC was synthesized according to the procedure described in our previous report (17). 7-PEC (>95% purity) was dissolved in DMSO and added to the experimental media to

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give the final concentrations. Antibodies for detecting p-Akt, Akt, p53, Bcl-2, Bax, cyt-*c*, pro-caspase-9, pro-caspase-3, PARP1 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Ac-DEVD-CHO and Rhodamine 123 were purchased from the Beyotime Institute of Biotechnology (Haimen, China). Hoechst 33258 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 was purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Neonatal bovine serum (NBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. (China).

Cell line and culture conditions. HCT-116 human colon cancer cells were kindly provided by Shanghai Jiao Tong University. The cells were routinely cultured in RPMI-1640 medium, supplemented with 10% NBS. The culture was maintained at 37˚C with a gas mixture of 5% CO_2/95% air. All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell viability assay. The cells were seeded in 96-well microtiter plates ($3x10^4$/ml). After 12 h of incubation in the appropriate medium, cells were treated with various concentrations (1, 10, 25 and 50 μM) of 7-PEC for another 72 h (24 or 48 h). Subsequently, 10 μl of MTT stock solution was added to each well for an additional 4 h of incubation. Then, 100 μl of DMSO was added to each well and the absorbance at 570 nm was determined with a microplate reader. Using the MTT method, cell numbers were obtained as absorbance values. The results were expressed as viability compared with that of control cells. Each treatment and time-point had three independent wells. The representative data shown in this study are the results of three independent experiments.

Cell morphological assessment. Cell morphological changes were assessed by Hoechst 33258 staining. Briefly, following exposure to 7- PEC for 48 h, the cells were washed twice with PBS and fixed with 4% formaldehyde at 4˚C for 10 min. The samples were then washed with PBS and stained with Hoechst 33258 solution (0.5 μg/ml) for 10 min at room temperature. Finally, the cells were observed under the fluorescence microscope (Nikon Eclipse Ti-s, Nikon Corp., Tokyo, Japan).

Detection of mitochondrial membrane potential. $\Delta\Psi m$ was measured using Rhodamine 123. Briefly, cells under different concentrations of 7-PEC treatment were incubated with Rhodamine 123 (5 μg/ml) at 37˚C for 30 min, and washed with PBS. The cell pellet was collected by centrifugation (1,500 x g, 3 min), and resuspended in 1 ml of PBS. Fluorescence intensities of Rhodamine 123 in cells were analyzed by flow cytometric analysis.

Cell cycle analysis. For cell cycle analysis, HCT-116 cells ($1x10^5$ cells/ml, 3 ml) were cultured in 6-well plates, with or without 7-PEC (1.25, 2.5 and 5 μM) for 48 h. Cells were collected and resuspended in 500 μl of PBS containing 0.025 mg of propidium iodide (PI) and 50 μg of RNase for 30 min at room temperature in the dark. Flow cytometry was performed on Quanta SC (Beckman Coulter, Fullerton, CA, USA).

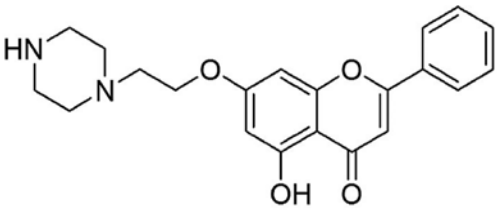

Figure 1. Chemical structure of 7-PEC.

Annexin V-FITC/PI assay of apoptotic cells. Briefly, HCT-116 cells ($1x10^5$ cells/ml) exposed to 7-PEC for 48 h were determined by flow cytometry (Quanta SC, Beckman Coulter) using a detection kit. Following 7-PEC treatment, cells were collected and washed twice in cold PBS and resuspended in 200 μl of binding buffer ($1x10^5$ cells/ml). The samples were incubated with 5 μl of Annexin V-FITC and 5 μl PI in the dark for 15 min at room temperature. Finally, samples were analyzed by flow cytometry and evaluated based on the percentage of cells for Annexin V-positive.

Western blot analysis. HCT-116 cells were treated with 7-PEC (1.25, 2.5 and 5 μM) for 48 h. Proteins were extracted with cell lysis buffer for western and IP (Beyotime Institute of Biotechnology). Equal amounts (40 μg/lane) of protein were separated on 10 or 15% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA) and blocked at room temperature for 1 h in 3% (w/v) non-fat milk in TBST. The blots were incubated overnight at 4˚C with the primary antibodies diluted in TBST buffer. The membranes were incubated with anti-Akt, p-Akt, Bcl-2, Bax, p53, cyt-*c*, pro-casapse-3, pro-caspase-9, PARP1 and β-actin primary antibodies (1:1000). After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:5000), and visualized with the ECL detection kit (Thermo, USA), according to the manufacturer's instructions.

Measurement of ROS production. The elevations of intracellular ROS induced by 7-PEC in HCT-116 cells were detected by DCFH-DA (2',7'-dichlorofluorescein diacetate) using flow cytometry. This compound is a cell-permeant indicator for ROS that is non-fluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Briefly, cells were seeded at $1x10^5$ cells/well in 6-well plates, and treated with or without 7-PEC (5, 10 and 20 μM). At the indicated times, cells were harvested and washed with PBS, then resuspended in PBS containing DCFH-DA (10 μM) and incubated for 20 min at 37˚C. After the inhibition, cells were washed twice by PBS and then analyzed by flow cytometry.

Statistical analysis. Results are expressed as the mean ± SD for three independent experiments. Statistical differences were evaluated using Student's t-test or one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate statistically significant differences.

Table I. The cytotoxicity of compound 7-PEC against the DU-145, SGC-7901, HCT-116, HeLa and HEK-293 cell lines.

	Cytotoxicity (IC_{50}, μM)[a]				
Compound	DU-145	SGC-7901	HCT-116	HeLa	HEK-293
7-PEC	3.08	2.78	1.50	2.46	41.90
5-FU	2.95	2.19	1.93	9.70	>100

[a]Data are the mean of three independent experiments.

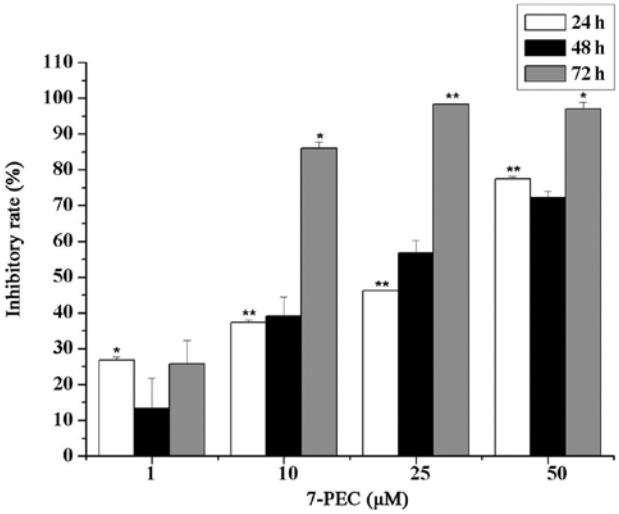

Figure 2. Effects of 7-PEC on cell viability. MTT assay was used to detect HCT-116 cells viability after treatment of different concentrations of 7-PEC for 24 h, 48 h and 72 h, respectively. The data shown are the mean from three parallel experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared to control.

Results

Effects of 7-PEC on cell viability. The cytotoxic effects of 7-PEC on five different cell lines were examined by MTT assay. The results showed that the cytotoxicity of 7-PEC on HCT-116 cells is most potent; it is comparable with that of 5-FU as a positive control (Table I). HCT-116 cells in exponential growth were treated with graded concentrations of 7-PEC (1, 10, 25 and 50 μM) for 24, 48 and 72 h. Under the experimental conditions, 7-PEC treatment exhibited strong inhibition on the survival of HCT-116 cells in a time- and dose-dependent manner as shown in Fig. 2. The IC_{50} values were calculated as 16.25, 5.49 and 1.5 μM in cells treated for 24, 48 and 72 h, respectively.

Effects of 7-PEC on the morphology of HCT-116 cells. In order to elucidate whether the loss of HCT-116 cell viability induced by 7-PEC was associated with apoptosis, the occurrence of apoptosis was identified with Hoechst 33258 staining. HCT-116 cells were incubated with various concentrations of 7-PEC (2.5 and 5 μM) for 48 h and stained by Hoechst 33258 for observation of the morphology. As is clearly shown in Fig. 3E, significant nuclear condensation and morphological changes for HCT-116 cells were observed, whereas in the control group, the cells without 7-PEC treatment demonstrated normal nuclear morphology. These data confirmed that 7-PEC could induce apoptosis in HCT-116 cells.

Effects of 7-PEC on cell cycle and apoptosis. To investigate the effects of 7-PEC on apoptosis and the cell cycle of HCT-116 cells, sub-diploid DNA-content and phosphatidylserine (PS) externalization were measured by FACS after PI and Annexin V-FITC/PI staining. For the cell cycle study, HCT-116 cells were treated with 7-PEC (1.25, 2.5 and 5 μM) for 48 h, and the DNA content of 10, 000 events was analyzed by flow cytometry. Fig. 3A and B show a dose-dependent increase of apoptosis induction which is indicated by percentage of sub-diploid DNA content. Apoptotic cells reached ~8.53 and 22.27% when the cells were exposed to 2.5 and 5 μM of 7-PEC, respectively. For the apoptosis study, HCT-116 cells were incubated with different concentrations of 7-PEC (4, 6, 8 and 10 μM) for 48 h, and then the cells were subjected to Annexin V-FITC/PI staining and analyzed by flow cytometry. Significant apoptosis for HCT-116 cells is observed in Fig. 3C and D. Upon treatment with 2 and 10 μM of 7-PEC, the percentage of apoptotic cells increased from 3.09 to 50.03%. These results suggest that the Annexin-V-FITC assay is more sensitive than sub-diploid DNA-content measurement for the evaluation of apoptosis.

Effects of 7-PEC on caspase-3 activity. Caspase, a family of cysteine proteases, is known to form integral parts of the apoptotic pathway (18). Caspase-3 activation is considered the central and final apoptotic marker enzyme for both mitochondrial intrinsic and death-domain receptor-dependent extrinsic pathways. Poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair, is a substrate for caspase-3 (19). Therefore, we investigated the protein levels and activity of caspase-3. As is shown in Fig. 4E, the pro-caspase-9 and -3 protein levels were significantly decreased and cleavage of PARP1 was detected in 7-PEC-treated HCT-116 cells.

To confirm whether 7-PEC specifically triggers caspase-3 expression, caspase-3 protein expression was investigated in HCT-116 cells by treating with 5 μM of 7-PEC for 48 h in the presence or absence of caspase-3 inhibitor, Ac-DEVD-CHO. As shown in Fig. 4C, activation of caspase-3 induced by 7-PEC is blocked in the presence of Ac-DEVD-CHO. MTT results demonstrated that the cell growth inhibition activity of 7-PEC was also weakened by Ac-DEVD-CHO (Fig. 4F). These data suggest that 7-PEC-induced apoptosis might engage caspase-3 dependent signaling cascades. Taken together, our results

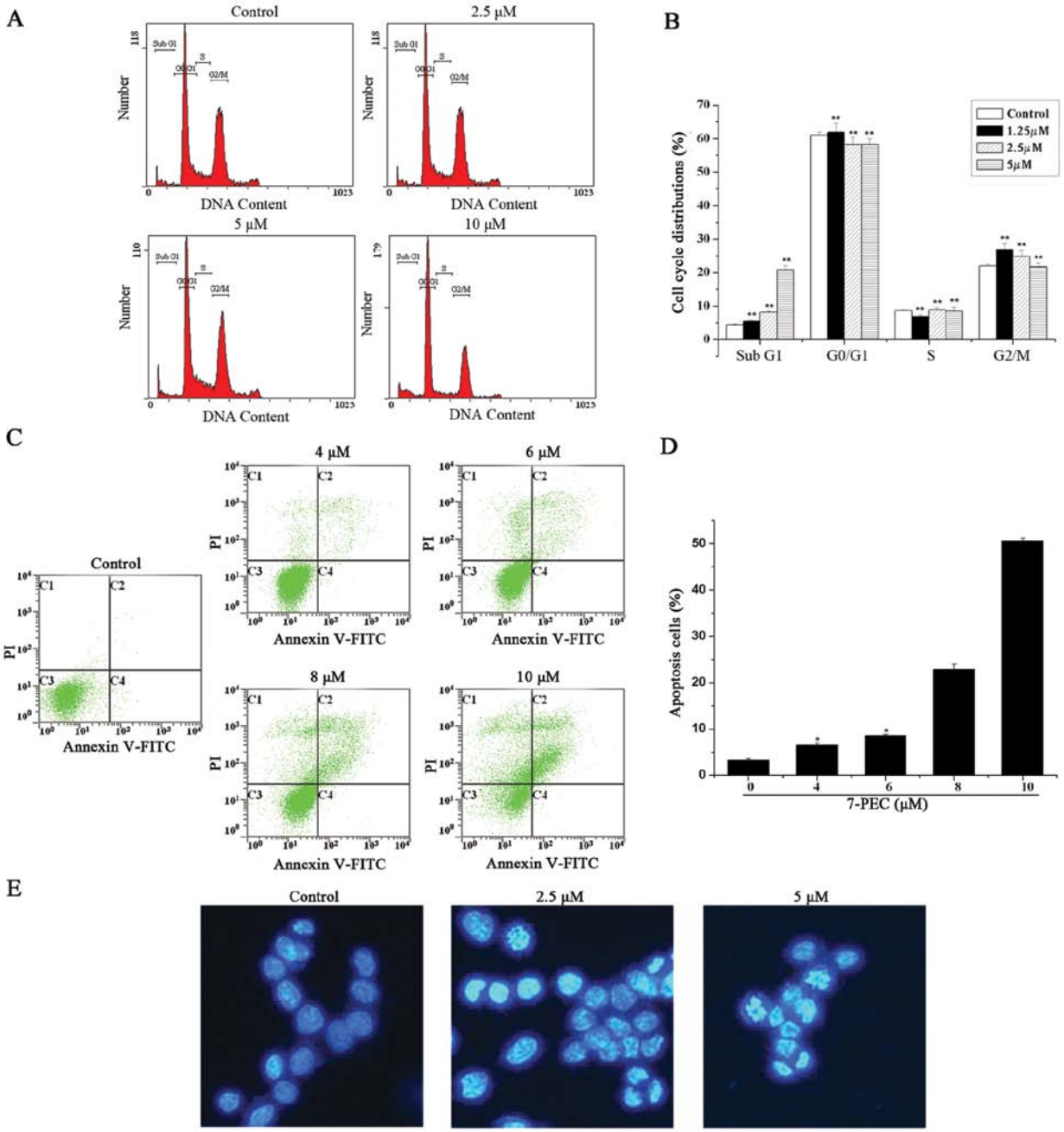

Figure 3. Effects of 7-PEC on cell cycle and apoptosis of HCT-116 cells. (A) Effects of 7-PEC on the cell cycle of HCT-116 cells. Cells were treated with 7-PEC (1.25, 2.5 and 5 μM) for 48 h, and the DNA content of 10,000 events was analyzed by flow cytometry. (B) The proportions (%) in each phase of the HCT-116 cells. *p<0.05, **p<0.01 compared to control. (C) Effect of 7-PEC on cell apoptosis of HCT-116 cells. Induction of apoptosis was measured by Annexin-V/PI double-staining assay after treatment with 7-PEC (4, 6, 8 and 10 μM) for 48 h by flow cytometry. (D) The apoptotic proportion of HCT-116 cells treated with 7-PEC. *p<0.05, **p<0.01 compared to control. (E) Effect of 7-PEC on the morphological changes of HCT-116 cells. HCT-116 cells were incubated with various concentrations of 7-PEC for 48 h and stained by Hoechst 33258 to observe the morphology.

indicate that 7-PEC-induced apoptosis is possibly via the caspase-dependent apoptotic pathway in HCT-116 cells.

Effects of 7-PEC on p53/mitochondria-related apoptotic markers. The expression of Akt, p-Akt, p53, pro-caspase-3, pro-caspase-9, PARP1 and cyt-*c* was measured in HCT-116 cells treated with 7-PEC (1.25, 2.5 and 5 μM). As is shown in Fig. 4, 7-PEC treatment resulted in the decrease of antiapoptotic protein Bcl-2 and increase of the Bax (Fig. 4A), with an increase in the Bax/Bcl-2 ratio (Fig. 4B). In addition, upregulation of p53, cyt-*c*, pro-caspase-3, pro-caspase-9 and subsequent cleavage of PARP1 were detected in 7-PEC-treated HCT-116 cells. The exposure to 7-PEC had no effects on steady-state levels of total Akt protein, whereas p-Akt levels were decreased significantly in a dose-dependent manner (Fig. 4E). These findings suggest the activation of the mitochondria-based intrinsic apoptosis in HCT-116 cells after 7-PEC treatment.

Effects of 7-PEC on mitochondrial membrane potential. Early apoptosis is always accompanied by the disruption of the mitochondrial membrane, resulting in a rapid collapse in the electrochemical gradient (20). In this study, we explored

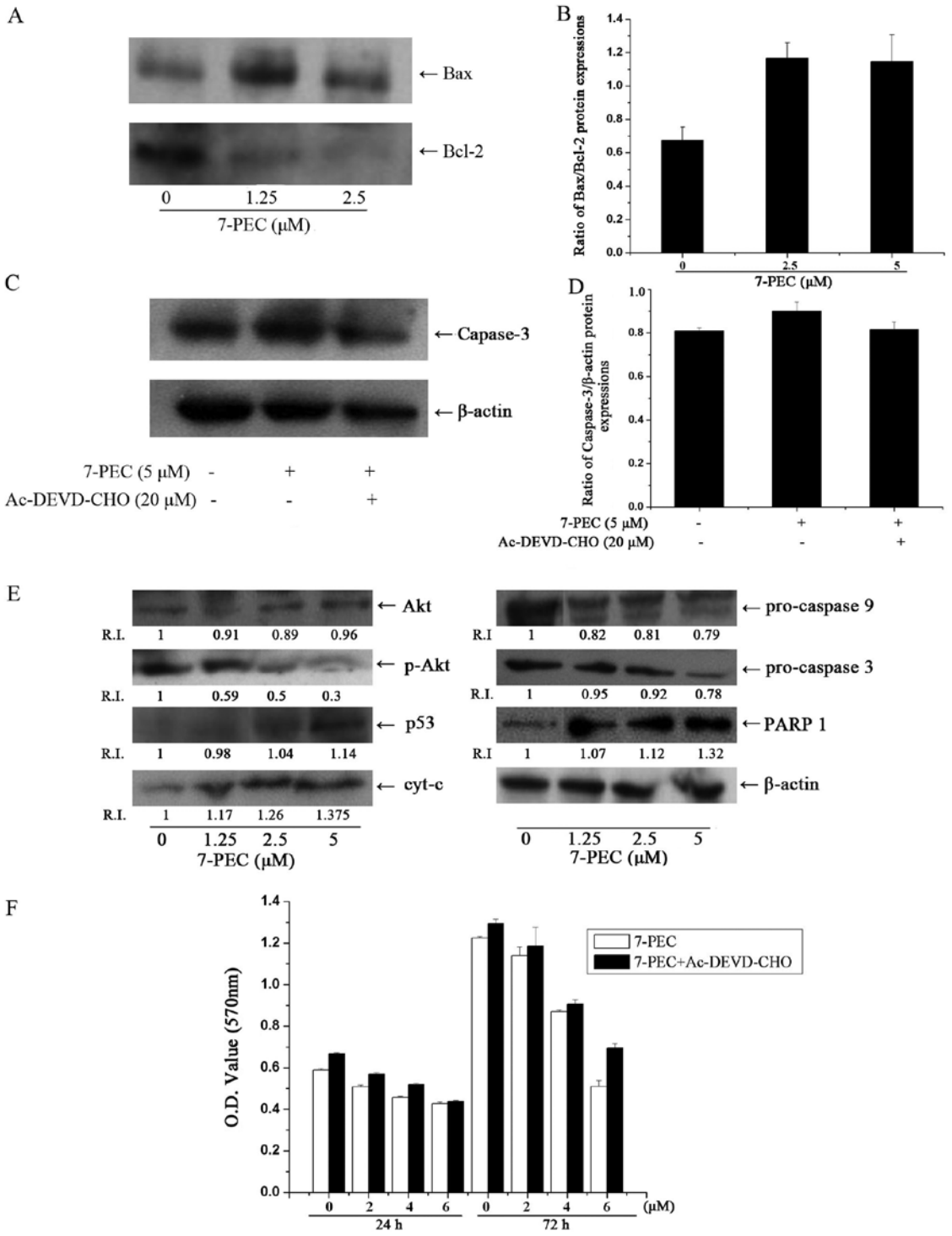

Figure 4. Effects of 7-PEC on apoptosis-related proteins of HCT-116 cells. (A) Bcl-2 and Bax protein expressions. HCT-116 cells were treated with 7-PEC (1.25 and 2.5 μM) for 48 h. Western blot analysis was performed. (B) Ratio of Bax/Bcl-2 protein expressions using densitometric analysis. (C) Effect of 7-PEC on the activity of pro-caspase-3 in HCT-116 cells. HCT-116 cells were incubated with 5 μM 7-PEC in the presence or absence of 10 μM Ac-DEVD-CHO for 48 h. (D) Ratio of caspase-3/β-actin protein expressions. (E) Expression of apoptosis-related proteins in HCT-116 cells treated with 7-PEC. The levels of proteins including Akt, P-Akt, p53, cyt-*c*, pro-caspase-9, pro-caspase-3, PARP1 and β-actin in HCT-116 cells were assessed by western blot assay. RI values indicate relative intensity (of upper band) using expression of proteins in control untreated cells as 1. (F) Effect of capase-3 inhibitor on 7-PEC induced cell proliferation inhibition. MTT assay was carried out while HCT-116 cells in exponential growth were treated with graded concentrations (2, 4 and 6 μM) of 7-PEC in the presence or absence of 10 μM Ac-DEVD-CHO for 24 or 72 h.

the effects of 7-PEC on the loss of $\Delta\Psi m$ using a cationic dye Rhodamine 123, which can diffuse into the mitochondria matrix and reflect the change of $\Delta\Psi m$ (21). Thus, HCT-116 cells were incubated with different concentrations of 7-PEC (4, 6, 8 and 10 μM) for 24 h, and then incubated with Rhodamine 123 dye for another 30 min. Fluorescence emission was measured by flow cytometry. As shown in Fig. 5, the $\Delta\Psi m$ was significantly decreased by 7-PEC in a dose-dependent manner.

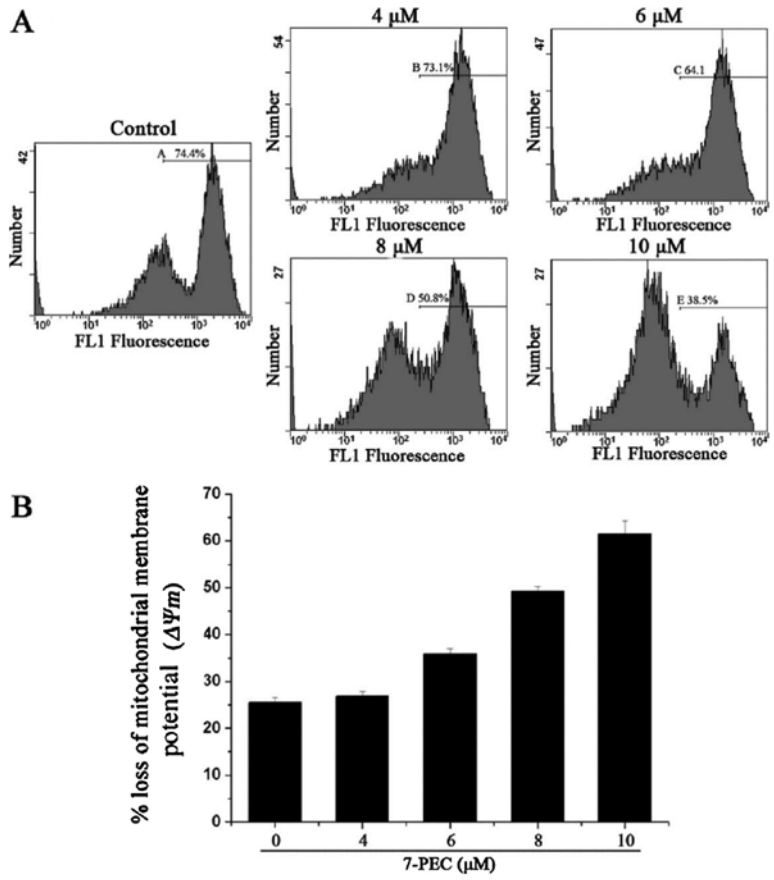

Figure 5. Effect of 7-PEC on the mitochondrial membrane potential (*ΔΨm)* of HCT-116 cells. (A) Cells treated with 7-PEC for 24 h were incubated with Rhodamine 123 and measured by flow cytometry. The percentages of cells in the right section of the fluorocytogram indicate the number of *ΔΨm* collapsed cells. (B) Percentage loss of *ΔΨm* in the control and 7-PEC-treated cells.

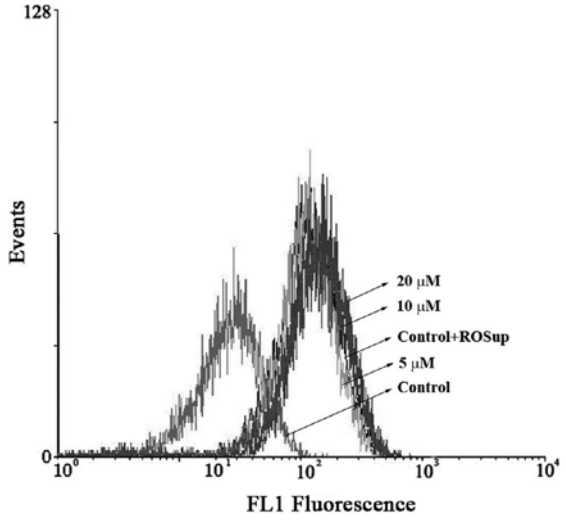

Figure 6. Effect of 7-PEC on intracellular ROS of HCT-116 cells. Cells treated with 7-PEC for 24 h were incubated with DCFH-DA and measured by flow cytometry.

Effects of 7-PEC on cellular reactive oxygen species production. Increased production of reactive oxygen species (ROS) triggers cytotoxicity and cell death by increasing oxidative stress. The intracellular production of ROS in HCT-116 cells was measured while treating with 7-PEC (5, 10 and 20 μM) and using DCFH-DA staining. The 7-PEC treatment of HCT-116 cells induced a dose-dependent increase of ROS production. Fig. 6 shows an example of FACS analysis of DCFH-DA-stained HCT-116 cells after 7-PEC treatment. The experiments were triplicated and similar results were obtained.

Discussion

MTT assay revealed that 7-PEC significantly exerts growth inhibitory effects on various cell lines, particularly on HCT-116 human colon cancer cells with IC_{50} at 1.5 μM after treating with 7-PEC for 72 h. We speculated that apoptosis may be the main mechanism for 7-PEC-induced growth inhibitory effects on HCT-116 cells. Previous studies have shown that a number of anticancer drugs induce apoptosis through the activation of the caspase pathways and the mitochondrial membrane dysfunction. Accumulating evidence indicates that mitochondria play a pivotal role in the apoptotic process in mammalian cells (22-24). Disruption of mitochondrial *ΔΨm* is considered to be an indicator of mitochondria damage and is generally defined as an early stage of apoptosis, preceding efflux of small molecules from the mitochondria (including cytochrome *c*, apoptosis-inducing factor) and followed by caspase-9/-3 cascade activation (25-28). In the present study, we found the marked decrease of pro-caspases (pro-caspase-3 and -9) by 7-PEC after the breakdown of *ΔΨm*, suggesting

that the mitochondria-mediated pathway is involved in 7-PEC-triggered apoptosis. Sequential disruption of $\Delta\Psi m$, increased Bax/Bcl-2 ratio and activation of caspases-9 and -3 was involved in 7-PEC-induced apoptosis. We showed that 7-PEC treatment activated caspase-3 in a dose-dependent manner and resulted in the cleavage of PARP1, a well-known caspase-3 substrate. A more significant accumulation of the p53 protein in HCT-116 cells was also observed after 7-PEC treatment, and this result indicates that the 7-PEC-induced apoptosis could be p53-dependent.

Akt, a serine/threonine protein kinase, is activated by phosphorylation and protects cells from apoptosis (29), and this protection is the result of the fact that p-Akt increases expression of the FLICE inhibitory protein (FLIP), which inhibits caspase-8 activity (30). We found that 7-PEC induced downregulation/dephosphorylation of p-Akt.

The overexpression and integration of Bax in the mitochondrial membrane were responsible for the commitment of the cells to apoptosis (31). Bcl-2 is localized in the mitochondria, endoplasmic reticulum, and nuclear membranes, where most of the oxygen-free radicals are generated and where the free radicals exert their apoptotic effects. Bcl-2 possibly acts to prevent apoptosis by scavenging oxygen derived free radicals inside the cells (32,33). The increase of the Bax/Bcl-2 ratio could induce cell apoptosis (34,35). Treatment of HCT-116 cells with 7-PEC decreased the Bcl-2 and increased the Bax protein levels. We speculate that ROS might modulate the cellular distribution and content of Bcl-2. Generation of ROS may contribute to mitochondrial damage and lead to cell death by acting as apoptotic signaling molecules (36-39). In the present study, we found that in addition to its effect on $\Delta\Psi m$, 7-PEC caused an increase in ROS production in HCT-116 cells. The 7-PEC-mediated disruption of $\Delta\Psi m$ and apoptosis in HCT-116 cells are apparently dependent on ROS generation.

In conclusion, the present study demonstrates that the significant growth inhibitory effects of 7-PEC on HCT-116 human colon cancer cells is associated with induction of apoptosis, involving sequential events, such as ROS production, reducing the mitochondrial membrane potential ($\Delta\Psi m$), and increasing the Bax/Bcl-2 protein ratio.

Acknowledgements

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