

FPOA induces apoptosis in HeLa human cervical cancer cells through a caspase-mediated pathway

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Abstract. In the present study, the triterpenoid 3-acetoxy lanosta-8,24-dien-21-oic acid (FPOA) was extracted from *Fomitopsis pinicola*. The aim of the present was to elucidate the mechanism of action of FPOA in HeLa cervical cancer cells. Cell viability was examined using an MTT assay and the morphological detection of apoptosis was conducted using DAPI staining. The rate of apoptosis was examined via Annexin V-FITC/PI double staining and the expression levels of apoptosis-associated proteins were determined by western blot analysis. FPOA was observed to inhibit HeLa cell proliferation, with IC₅₀ values of 25.28, 15.30 and 11.79 µg/ml at 24, 48 and 72 h, respectively. Typical apoptotic bodies were observed in the HeLa cells following treatment with FPOA, as revealed by DAPI staining. The percentage of apoptotic cells was 3.00, 3.12, 6.18 and 32.28% following treatment with FPOA at concentrations of 0, 7.5, 15 and 30 µg/ml, respectively. Western blot analysis showed that caspase-3 and -9 were cleaved more frequently after treatment with FPOA. Furthermore, the expression of Bax was increased but Bcl-2 expression was decreased after treatment with FPOA. These results suggest that FPOA can induce HeLa cell apoptosis through a caspase-mediated pathway.

Introduction

Cervical cancer is the most common type of gynecological cancer. There are ~500,000 newly diagnosed cases, and >250,000 associated deaths worldwide each year (1). The incidence of cervical cancer has decreased in developed countries, while continuing to increase in developing countries (2). The currently available treatment routes for cancer include surgery,

radiotherapy and chemotherapy, but the efficiency of these treatments is low and they involve various adverse effects (3). Therefore, we need to develop new low-toxicity drugs for cancer treatment.

Fomitopsis pinicola (*F. pinicola*; Sw. Ex Fr.) Karst is one of the most common wood-rooting fungi in the northern hemisphere (4). It is widely distributed in Japan, Korea, China and Sweden (5). *F. pinicola* has a long history of medicinal use for the treatment of poor leg circulation in the elderly in Northeast China, and has been used for diabetes in Japan (6). *F. pinicola* is a non-toxic natural product that is becoming increasingly more attractive in academia. The extracts of *F. pinicola* have numerous pharmacological effects, including anti-inflammatory, antimicrobial, antifungal and anti-obesity properties (7-10). Wu *et al* (11), reported that the ethanol extract of *F. pinicola* could induce apoptosis in A549, HCT-116 and MDA-MB-231 cells, and inhibit SW180 cell growth *in vivo*. Chemical analysis showed that there were no ergosterol, ergosterol derivatives or lanostane triterpenes in the *F. pinicola* extract (12,13).

We isolated the triterpenoid 3-acetoxy lanosta-8, 24-dien-21-oic acid (FPOA; Fig. 1) from the fruiting body of *F. pinicola*, and determined that it was the primary active ingredient (14). In this study, we aimed to elucidate whether FPOA could inhibit the growth of HeLa cells, as well as to investigate its underlying mechanisms.

Materials and methods

Materials. The fruiting bodies were obtained in Changbai Mountain, Jilin Province, P.R.China. The 3-acetoxy lanosta-8,24-dien-21-oic acid (FPOA) was extracted from the fruiting bodies of *Fomitopsis pinicola*. The fruiting bodies were extracted with ethanol and separated by silica gel column, elution with a mixture of petroleum ether and ethyl acetate to get FPOA. The purity of FPOA was >95%, as detected via HPLC. Antibodies against caspase-3, -9, Bcl-2, PARP and Bax were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). β-actin was obtained from Wuhan Sanying Biotechnology, (Wuhan, China). BCA protein assay kit was purchased from Beijing Leagene Biotech, Co., Ltd., (Beijing, China). An Annexin V-FITC Apoptosis Detection kit was obtained from Tianjin Sungene Biotech Co., Ltd., (Tianjin, China). MTT and all other reagents were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

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Cell culture. Human cervical cancer HeLa cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The HeLa cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sijiqing; Zhejiang Tianhang Biotech Co., Ltd., Huzhou, China) and 1% penicillin-streptomycin.

MTT assay. Cell viability was evaluated using an MTT assay, as previously described (15). Briefly, HeLa cells were seeded into 96-well plates with 4,500 cells/well and treated with different concentrations of FPOA (12.5, 25, 50, 100 and 200 $\mu\text{g/ml}$). After incubation for 20, 44 and 68 h, 37°C, 10 μl MTT (5 mg/ml) was added to each well and then incubated for another 4 h, 37°C. Subsequently, dimethyl sulfoxide (100 μl) was added to each well and the plates were agitated for 10 min. The absorbance was read at a wavelength of 570 nm using a microplate reader. The inhibitory concentration 50% (IC_{50}) was defined as the concentration of experimental compound required to inhibited 50% cell proliferation, as compared with the untreated cells (16). The IC_{50} was calculated with GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

DAPI staining. DAPI staining was performed as previously described (17). Briefly, HeLa cells were seeded into 6-well plates with 140,000 cells/well and treated with FPOA for 24 h. The cells were fixed with 4% polyoxymethylene at room temperature for 10 min. The coverslips were equilibrated in PBS, following which 300 μl DAPI (300 nM) staining solution was added to the coverslips and incubated for 5 min at room temperature. The coverslips were rinsed two times in PBS and viewed using a fluorescence microscope (Nikon TE-2000 U; Nikon Corporation, Tokyo, Japan). The normal cell nuclei were faint staining (cells were alive). The apoptotic cell nuclei were brightness (18).

Apoptosis assay. Annexin V-FITC/PI staining was performed, followed by flow cytometry as previously described (15). Briefly, HeLa cells were seeded into 6-well plates with 140,000 cells/well and treated with FPOA for 24 h, after which the cells were collected and washed with cold PBS. Then, the cells were resuspended in 300 μl binding buffer. The cells were incubated with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) in the dark for 15 min at room temperature. Finally, the cells were analyzed via flow cytometry (BD FASCCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The total percentage of apoptotic cells was defined as the sum of both early apoptosis (annexin V-FITC positive, PI negative) and late apoptosis (annexin V-FITC PI positive), top and bottom right quadrants in a flow cytometric dot plots, respectively (19). For each sample, 10,000 events were collected and analyzed by CellQuest™ Pro (v6.0) software (BD Biosciences).

ROS detection. ROS generation was assessed as previously described (20). Briefly, HeLa cells were seeded into 6-well plates with 140,000 cells/well and treated with FPOA for 24 h. Following this, HeLa cells were incubated with non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 20 min at 37°C, and then cells were washed two times with

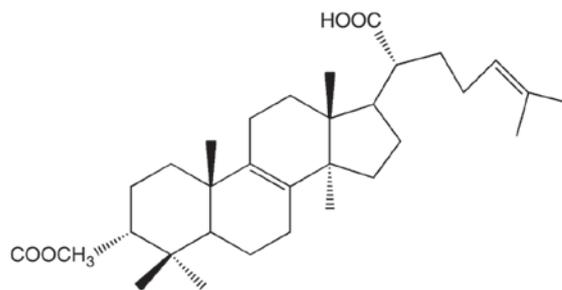


Figure 1. Structure of 3-acetoxyylanosta-8,24-dien-21-oic acid.

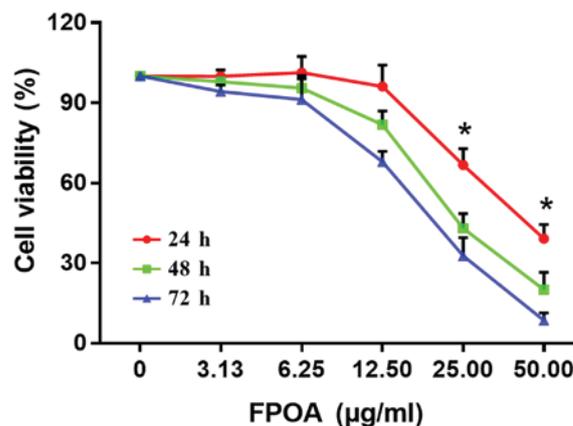


Figure 2. Cytotoxicity of FPOA on HeLa cells. HeLa cells were seeded in 96-well plates and incubated with different concentrations of FPOA for 24, 48 and 72 h. Cell viability was determined using an MTT assay. Values are presented as the mean + SD of three experiments. * $P < 0.05$ vs. control group. FPOA, 3-acetoxyylanosta-8,24-dien-21-oic acid. The inhibitory concentration 50% (IC_{50}) in μM is defined as the concentration of experimental compound required to inhibited 50% of the conversion of MTS to formazan, as compared with the absorbance produced by untreated cells after 16 h of incubation.

medium without serum. ROS was examined by fluorescence microscopy or flow cytometry.

Western blot analysis. HeLa cells were seeded into 6-well plates with 140,000 cells/well and treated with FPOA for 24 h, following which the cells were harvested and lysed on ice with RIPA buffer (150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.4) for 30 min. After centrifugation at 13,000 \times g for 15 min, the protein concentration was determined using a BCA protein assay kit. Then, 20 μg cell lysate proteins were loaded onto 12% SDS-polyacrylamide gels, resolved via electrophoresis and then electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) non-fat milk for 1 h. After blocking, the membrane was incubated overnight at 4°C with primary antibodies: caspase-3 (cat. no. 9662, 1:1,000 dilution), caspase-9 (cat. no. 9508, 1:1,000 dilution), Bcl-2 (cat. no. 2876, 1:1,000 dilution), PARP (cat. no. 9542, 1:1,000 dilution) and Bax (cat. no. 2772, 1:1,000 dilution) supplied by Wuhan Sanying Biotechnology. β -actin (20536-1-AP, 1:1,000 dilution; Wuhan Sanying Biotechnology), was also used. Membranes were then washed twice with PBST and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. IH-0011, 1:5,000 dilution) and anti-mouse

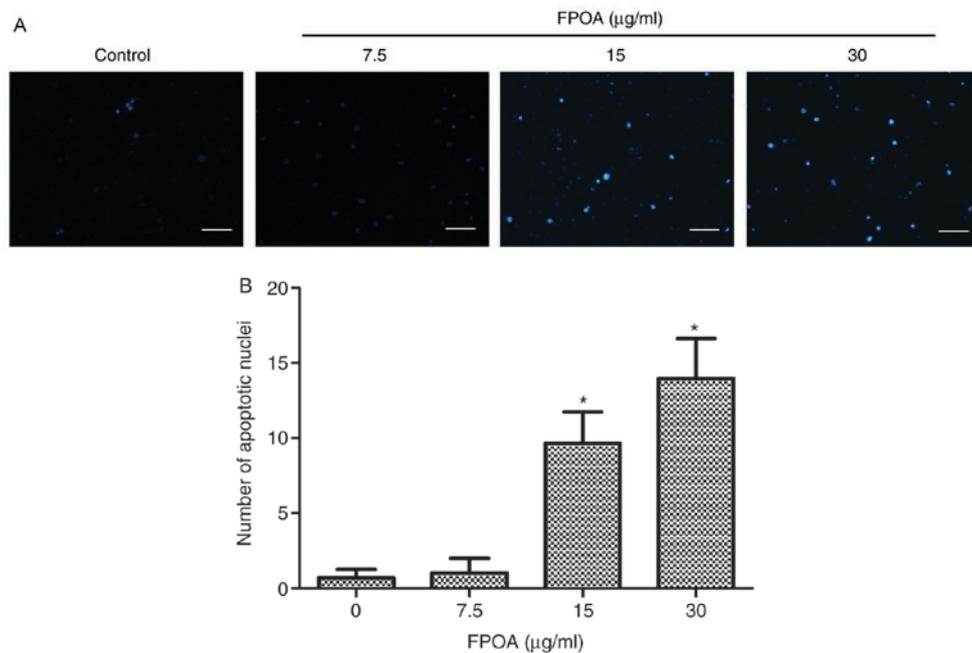


Figure 3. Apoptosis of HeLa cells induced by FPOA. HeLa cells were incubated with various concentrations of FPOA for 24 h and then stained with DAPI. There were few apoptotic cells in control group. However, apoptotic cells appeared after 24 h of treatment with FPOA (15 and 30 µg/ml). (A) Fluorescence microscopy images of DAPI stained cells (magnification, x100). The normal cell nuclei were faint staining (cells were alive). The apoptotic cell nuclei were brightness. Scale bar: 100 µm. (B) The number of cells with brightness was quantified from 3 wells and 5 vision fields from each well was presented. The graphs represent the mean ± SD. *P<0.05 vs. control group. Each experiment was repeated three times. FPOA, 3-acetoxylanosta-8,24-dien-21-oic acid.

antibodies (cat. no. IH-0031, 1:5,000 dilution) supplied by Dingguo Changsheng Biotechnology, (Beijing, China) at room temperature for 1 h. Then the membranes were then washed twice with PBST at room temperature. Western blot bands were detected using Odyssey v1.2 software.

Statistical analysis. Results are expressed as the mean ± SD. Statistical comparisons were evaluated by SPSS v21.0 (IBM SPSS, Armonk, NY, USA) using the Student's t-test or one-way analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

FPOA inhibits cell proliferation. We investigated the growth inhibitory effect of FPOA on HeLa cells using an MTT assay. As shown in Fig. 2, FPOA could inhibit the proliferation of HeLa cells in a dose- and time-dependent manner. IC₅₀ values of 25.28, 15.30 and 11.79 µg/ml were calculated at 24, 48 and 72 h, respectively.

FPOA induces the apoptosis of HeLa cells. In order to elucidate whether FPOA induces apoptosis, DAPI staining was performed. As shown in Fig. 3, numerous apoptotic bodies containing unclear fragments were observed following FPOA treatment, but comparatively few were present in the control group. These results indicated that FPOA could induce the apoptosis of HeLa cells.

Furthermore, in order to quantify the rate of apoptosis, Annexin V-FITC/PI staining was performed. As shown in Fig. 4, the percentage of apoptotic cells was 3.00, 3.12, 6.18 and

32.28% following treatment with 0, 7.5, 15 and 30 µg/ml FPOA, respectively.

FPOA induces ROS in HeLa cells. In order to test whether mitochondria play a role in FPOA-induced apoptosis, we performed ROS generation assays. As shown in Fig. 5, FPOA could increase ROS production in a dose-dependent manner.

Induction of apoptosis via the caspase cysteine protease family It is known that the cysteine protease family is an integral part of the apoptotic signaling cascade. As shown in Fig. 6, the levels of cleaved caspase-9 and caspase-3 were increased following treatment with FPOA. PARP is a substrate of caspase-3 (21). After treatment with FPOA, PARP was hydrolyzed to an 89-kDa fragment, corresponding to the activation of caspase-3.

FPOA regulates the expression of Bcl-2 family proteins. Bcl-2 family members are critical regulators of the apoptotic cascade (15). We further investigated the effect of FPOA on the Bcl-2 protein family. As shown in Fig. 7, the expression of Bcl-2 was decreased while the expression of Bax was increased following treatment with FPOA. These results suggested that FPOA induced apoptosis by regulating the ratio of Bcl-2 to Bax.

Discussion

In a previous study, the fungal extracts exhibited anti-cancer effects in multiple tumor types (11). FPOA, isolated from the fruiting body of *F. pinicola*, was determined to be the main active ingredient (14). Here, we studied the anticancer activity of FPOA, and found that FPOA could inhibit the proliferation

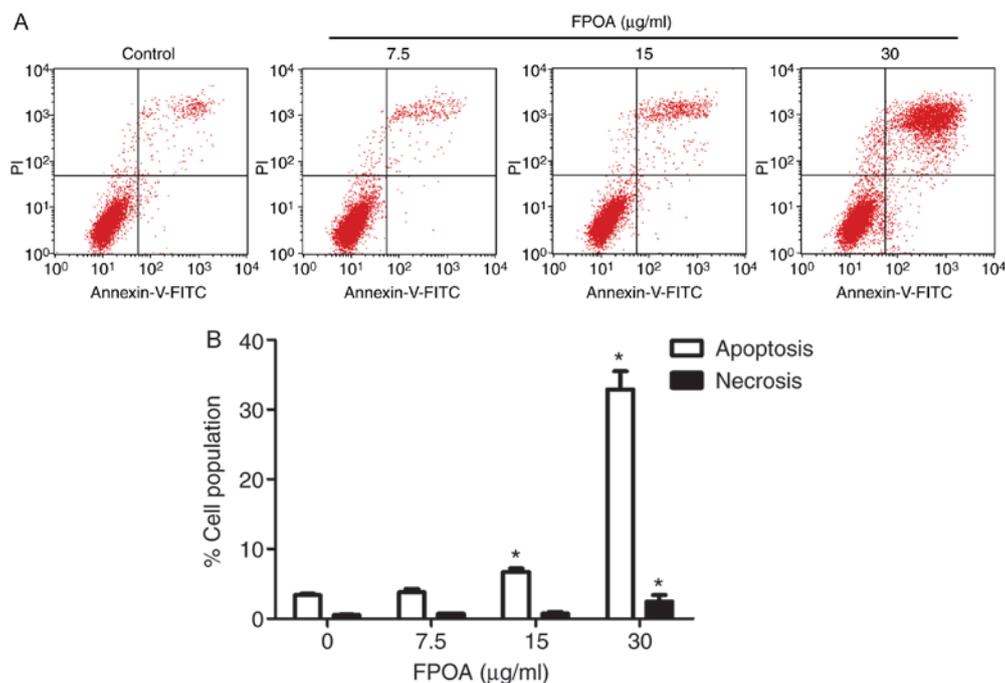


Figure 4. Annexin V-FITC/PI staining of HeLa cells incubated with FPOA. (A) HeLa cells were incubated with various concentrations of FPOA (7.5, 15 and 30 $\mu\text{g/ml}$) for 24 h. Cells were then subjected to Annexin V-FITC/PI staining and analyzed via flow cytometry. (B) The total percentage of apoptotic cells were stained with annexin V-FITC defined as the sum of both early apoptosis and late apoptosis (white bars). Cells that were stained only with PI, due to the loss of plasma membrane integrity, were considered necrotic cells (black bars). The data shown in the bar charts represent the mean \pm SD of three independent experiments. * $P < 0.05$ vs. control group. FPOA, 3-acetoxylanosta-8,24-dien-21-oic acid; FITC, fluorescein isothiocyanate; PI, propidium iodide.

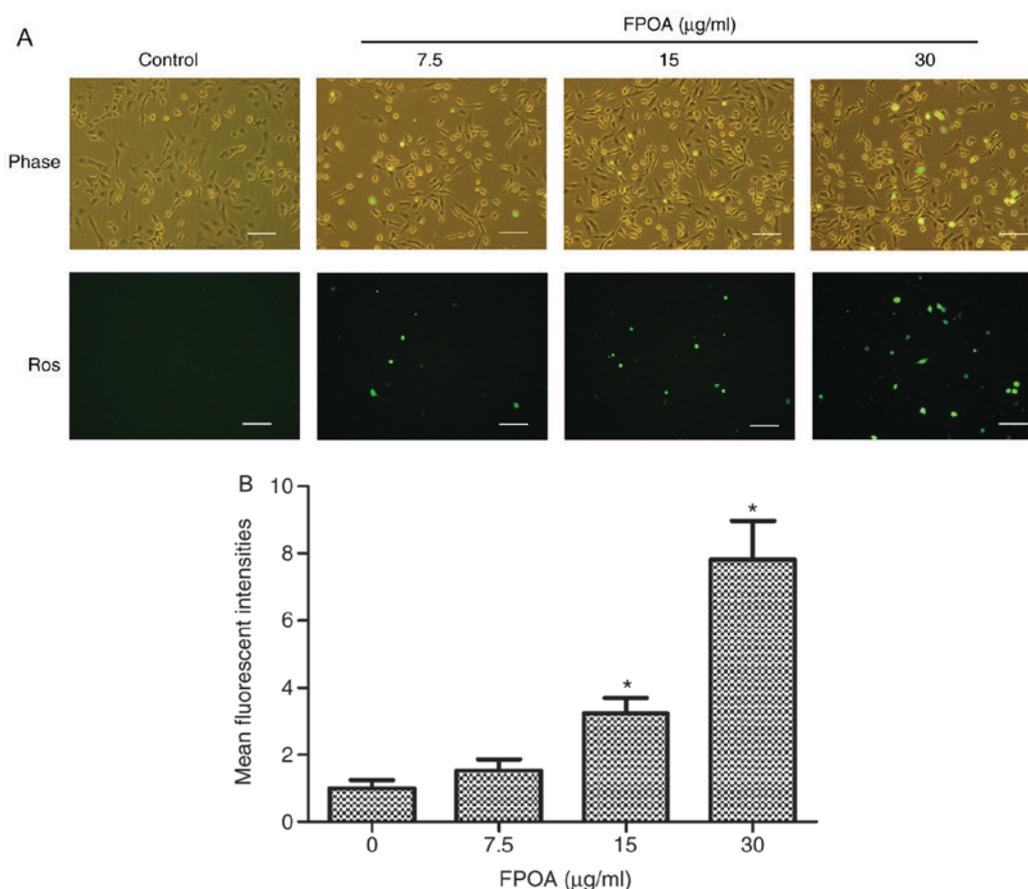


Figure 5. Measurement of ROS. The generation of ROS was assessed using non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining. (A) Fluorescence intensity was observed using a fluorescence microscope. DCF fluorescence intensity is an indication of the amount of ROS present in the cells. Up: Phase morphology; Down: DCFH-DA staining. Scale Bar: 50 μm . (B) Then the cells were collected and fluorescence was measured by flow cytometry. * $P < 0.05$ vs. control. Each experiment was repeated three times and similar results were obtained. ROS, reactive oxygen species.

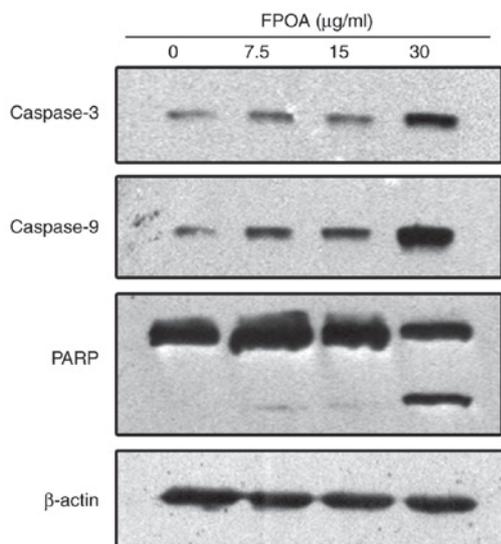


Figure 6. HeLa cells were incubated with various concentrations of FPOA for 24 h. HeLa cells were incubated with different concentrations of FPOA for 24 h, lysed, and resolved via 12% SDS-PAGE, following which western blotting was carried out to detect the activation and expression of caspase-9, -3 and poly (ADP-ribose) polymerase. The results are representative of three separate experiments, and β -actin was used as the internal control. FPOA, 3-acetoxyloganosta-8,24-dien-21-oic acid.

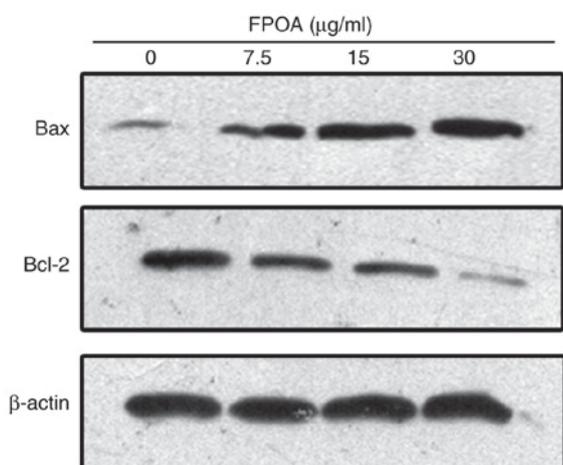


Figure 7. Alterations in the levels of Bcl-2 family proteins in HeLa cells incubated with FPOA. HeLa cells were incubated with different concentrations of FPOA (7.5, 15 and 30 μ g/ml) for 24 h, lysed, and then whole cell lysates were subjected to western blot analysis to examine the expression of Bcl-2 and Bax. β -actin was used as the internal control. Each experiment was repeated 3 times and similar results were obtained. The results are representative of three separate experiments, and β -actin was used as the internal control. FPOA, 3-acetoxyloganosta-8,24-dien-21-oic acid.

of HeLa cells in a dose-dependent manner. Morphological examination revealed that HeLa cells exhibited cellular alterations after treatment with FPOA. Chromatin condensation, a change typically observed during apoptosis, was revealed by DAPI staining. In addition, the Annexin V-FITC/PI double-staining assay confirmed the apoptosis induced by FPOA (Fig. 4). The results verified FPOA exerted anticancer activity through inducing apoptosis.

Apoptosis is an important mechanism for regulating the development of organisms, the renewal of cells and the

stability of the intracellular environment (22,23). At present, there are two pathways involved in the apoptosis cascade, including the mitochondrial-mediated intrinsic pathway, and the endoplasmic reticulum pathway; these pathways are inter-linked and interact to promote apoptosis (24). The Bax/Bcl-2 protein family is involved in the mitochondrial-mediated intrinsic pathway. Specifically, Bcl-2 and Bax can regulate the permeability of mitochondrial membranes. When the Bax/Bcl-2 ratio increases, the permeability of the mitochondrial membrane increases, which induces apoptosis. We detected the expression of certain Bcl-2 family members via western blotting; the data suggested that FPOA treatment leads to elevated Bax levels, accompanied by a decrease in Bcl-2 levels (Fig. 7).

Activation of the caspase protease family has an important role in the apoptosis regulated by the Bcl-2 family. Caspases are produced as inactive zymogens and undergo proteolytic activation during apoptosis. Caspase-9 is a regulator in the caspase cascade reaction and is involved in the mitochondrial-mediated intrinsic pathway; and caspase-3 is a primary effector that triggers the initiation of apoptosis (25). Our results indicated that FPOA treatment induced the proteolytic activation of caspase-9 and -3 in a dose-dependent manner (Fig. 6). PARP has a protective effect on DNA; as a substrate for caspase-3 involved in apoptosis, PARP is cleaved by caspase-3, leading to DNA damage and apoptosis. We found that FPOA also resulted in the cleavage of 116 kDa PARP into an 89-kDa fragment in HeLa cells (Fig. 6). Taken together, our data suggested that FPOA induced the mitochondrial-mediated apoptosis of HeLa cells by activating the caspase cascade.

In conclusion, this study demonstrated that FPOA could inhibit the proliferation of HeLa cells by inducing apoptosis. Furthermore, our results demonstrated that FPOA induces apoptosis in HeLa cells via the mitochondrial-mediated intrinsic pathway. The present results suggested that FPOA could be a potential anticancer agent for human cervical cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TB and HB conceived and designed the study. XL performed the experiments. XL and HB wrote the paper. TB, HB and XL reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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