FPOA induces the apoptosis of HepG2 cells

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Abstract. The triterpenoid 3-acetoxylanosta-8,24-dien-21-oic acid (FPOA) is isolated from the fruiting body of *Fomitopsis pinicola*. The present study reports that FPOA exerts cytotoxic activity and describes the molecular mechanism of FPOA-induced apoptosis on human HepG2 hepatoma cells. FPOA exhibited significant cytotoxic effects against HepG2, MCF-7 and HeLa cells. However, FPOA was particularly cytotoxic towards HepG2 cells, with a half maximal inhibitory concentration value of 42.10 μM, thus these cells were taken forward for further analysis. Flow cytometry results demonstrated that FPOA significantly increased the apoptotic rate of HepG2 cells in a dose-dependent manner, explaining its potent cytotoxicity. In addition, western blot analysis revealed that FPOA significantly increased the B-cell lymphoma 2 (Bcl-2)-associated X/Bcl-2 ratio, and cytochrome *c*, caspase-9 and caspase-3 release, in addition to significantly decreasing poly(ADP) ribose polymerase levels. These observations indicate that FPOA induces the apoptosis of HepG2 cells by activating members of the caspase protein family and triggering the mitochondrial apoptosis signaling pathway. Based on these results, FPOA is a potential agent for the treatment of cancer.

Introduction

Cancer is a prevalent health issue worldwide. The United States (US) National Cancer Institute reports that ~1/4 mortalities in the US are due to cancer (1). Cancer mortality has declined continuously over the past two decades; the overall risk of succumbing to cancer decreased by 20% between 1991 and 2010 (2). However, the World Health Organization reports that >14 million new cancer cases occur worldwide every year (3). In particular, cancer is the leading cause of mortality in China (3). In addition, the National Cancer Center of China reported ~4,292,000 new cancer cases and ~2,814,000 cancer-associated mortalities in China in 2015, with lung cancer being the most common cancer type and the leading cause of cancer-associated mortality (2). Stomach, esophageal and liver cancer were also commonly diagnosed, and were identified as leading causes of cancer-associated mortality (4).

Although chemotherapy-based treatments have greatly improved the survival rates of patients with cancer, current therapeutic strategies also induce significant undesirable side effects, including inflammation, ulceration and diarrhea (5). Paclitaxel, docetaxel and vinorelbine have all been widely used as anticancer agents with a good outcome (6). These drugs work through promoting tubulin polymerization and inhibiting cell mitosis; however, they induce significant side effects, including bone marrow toxicity and neutropenia (7). Therefore, it is important to develop novel drugs with low toxicities for the treatment of cancer.

Apoptosis, also called type I programmed cell death, is the tightly regulated process of cell death. Apoptosis is essential to the development and maintenance of multicellular organisms. Compelling functional studies have established the concept that apoptotic programmed cell death serves as a natural barrier to cancer development (8,9). Furthermore, abnormalities in the regulation of cell death are characteristic of neoplastic disease. Apoptotic signaling pathways are thus evident drug targets for therapeutic interventions for cancer, and the promotion of apoptosis may effectively block neoplastic progression. The B-cell lymphoma 2 (Bcl-2) family of proteins are significant regulators of apoptosis, and the rate of apoptosis can be increased by altering the ratio of Bcl-2-associated X (Bax)/Bcl-2 proteins. With increasing Bax expression, more cytochrome *c* is released by the mitochondria, which activates caspase-9 and -3, leading to apoptosis.

Triterpenoids exist widely in fungus, ferns and plants. The triterpenoid lucialdehyde c is separated from *Ganoderma lucidum*, while poricoic acid G is derived from *Poriacocos*. Triterpenoids consist of several isoprene units (10). Triterpenoids exert various biological and pharmacological activities, particularly antitumor effects (11,12). Numerous triterpenoids, including ganoderic acid B, have been used to treat various types of malignancy. Lucialdehyde c has been revealed to exert cytotoxic effects on Lewis lung carcinoma, T-47D, sarcoma 180 and meth-A tumor cell lines (13). Furthermore, 3-acetoxylanosta-8,24-dien-21-oic acid (FPOA), which was initially isolated from *Ganoderma tsugae*, has been identified to induce human hepatoma Hep3B cell death by apoptosis (14,15). With increasing research into triterpenoids, the mechanisms of their antitumor effects have been

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demonstrated to include induction of apoptosis, cell cycle inhibition and regulation of the immune response (16,17). However, the underlying molecular mechanism of the antitumor effect of FPOA remains unclear.

Fomitopsis pinicola, a wood-decay fungus, has long been used in North East China as a traditional medicine to treat poor leg circulation in the elderly (18). In a previous study by our group, the triterpenoid FPOA was isolated from the fruiting body of *F. pinicola* and was identified to be its principal active component (19). A previous study has reported that FPOA induces tumor cell death by apoptosis (20). Thus, the present study assessed the antiproliferative effects of FPOA on human hepatoma HepG2 cells and investigated the underlying molecular mechanisms of these effects.

Materials and methods

FPOA extraction and isolation. *F. pinicola* was collected from Changbai mountain (Jilin, China). The fruiting bodies were extracted with petroleum ether and $CHCl_3$, and the $CHCl_3$ extract was separated using a silica gel column. Subsequent elution with petroleum ether-ethyl acetate yielded FPOA. The methods used for the extraction, isolation and purification of FPOA have been described in previous studies by our group (19,20). The isolated FPOA was characterized using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy with $CDCl_3$ solvent, as previously described (15,21), and the data were compared with the previously reported values.

Cell culture. HepG2, MCF-7, HeLa, A549 and MRC-5 cell lines were obtained from the Norman Bethune Health Science Center of Jilin University (Changchun, China). All cell lines were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 60 U/ml gentamicin, 2.0 g/l sodium bicarbonate and 2.38 g/l HEPEs. Cultures were maintained in a humidified incubator at 37˚C with 5% (v/v) CO_2.

Cytotoxicity assay. The cytotoxicity assay used was the MTT assay (MTT Cell Proliferation Assay kit; Beyotime Institute of Biotechnology, Haimen, China). Cells were plated into 96-well culture plates ($6x10^3$ cells/well) and cultured at 37˚C with 5% (v/v) CO_2. The HepG2, MCF-7, HeLa, A549 and MRC-5 cells were allowed to attach for 24 h prior to the treatment. Mitomycin C (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used as a positive control, the cells were exposed to various concentrations of FPOA dissolved in saline and Tween-80 [1 mM; 0 (negative control), 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 μg/ml], and incubation was continued at 37˚C with 5% (v/v) CO_2 for 48 h. In total, six replicates of each FPOA concentration were run and the results were averaged. Following incubation, stock MTT solution (20 μl; 5 mg/ml) was added to each well. After 4 h, dimethyl sulfoxide (150 μl) was added to dissolve the formazan crystals that had formed. The optical densities of drug-treated wells were measured using a microplate reader at 570 nm. Finally, the FPOA cytotoxicities were expressed as half maximal inhibitory concentration (IC_{50}) values.

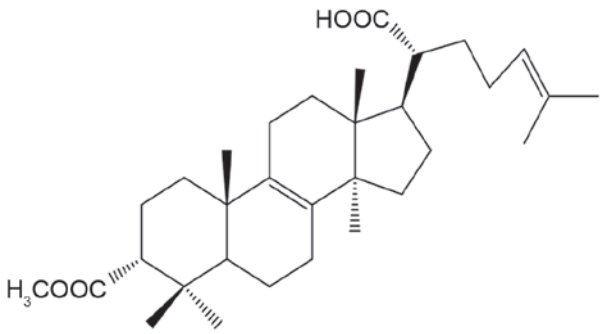

Figure 1. Chemical structure of 3-acetoxylanosta-8,24-dien-21-oic acid.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay. HepG2 cells that were cultured for 24 h in 6-well plates ($1x10^6$ cells/well) were collected, washed and stained following treatment with different concentrations of FPOA (0, 12.50, 25.00 and 50.00 μg/ml) for 24 h at 37˚C with 5% (v/v) CO_2. Each drug concentration was tested in triplicate. Cells were then washed twice with PBS and 400 μl 1X binding buffer was added followed by 5 μl annexin V-FITC conjugate as denoted by the FITC Annexin V Apoptosis Detection kit (BD Biosciences, catalogue no. 556547). The cells were then incubated in the dark for 15 min at 2-8˚C, then 5 μl PI was added and incubation was continued for 5 min. Finally, all the samples were subjected to flow cytometry analysis (FACSCalibur; BD Biosciences, San Jose, CA, USA) as previously described (11).

Western blotting. HepG2 cells were seeded into 10-cm-diameter culture dishes ($1x10^3$ cells/dish). After 24 h of incubation, the cells were treated with different concentrations of FPOA (0, 12.50, 25.00 and 50.00 μg/ml) for 24 h at 37˚C with 5% (v/v) CO_2. The cells were then harvested in cell lysis buffer (cat. no. 9803; Cell Signaling Technology, Inc., Danvers, MA, USA), incubated for 2 h at 4˚C and centrifuged for 15 min at 12,000 x g at room temperature. The supernatants were collected and protein concentration was determined using the Bradford assay. Equal amounts of total protein (50 μg/lane) were subjected to 20% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline-Tween-20 (TBST) for 1 h at room temperature and then incubated with the primary antibodies directed against poly(ADP-ribose) polymerase (PARP; cat. no. ENM0145; 1:3,000), Bcl-2 (cat. no. ENT0470; 1:1,000), Bax (cat. no. ENT0456; 1:1,000), caspase-9 (cat. no. ESAP14070; 1:750), caspase-3 (cat. no. ESAP10165; 1:400), cytochrome *c* (cat. no. ENT1186; 1:1,500) and GAPDH (ESAP10111; 1:1,000; all Elabscience Biotechnology Co., Ltd, Wuhan, China), dilution with 5% (w/v) non-fat dry milk in TBST, overnight at 4˚C. GAPDH served as the loading control. Next, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies in TBST for 1 h at 4˚C. The protein bands were visualized using enhanced chemiluminescence (ECL kit; cat. no. P0018; Beyotime Institute of Biotechnology, Haimen, China). Quantity one version 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for analysis.

Table I. Nuclear magnetic resonance data of 3-acetoxylanosta-8,24-dien-21-oic acid (δ_{ppm} $CDCl_3$).

Position	δ (^{13}C), ppm	δ (^{1}H), ppm
C-1	30.36	
C-2	23.38	
C-3	77.89	
C-4	36.76	
C-5	45.35	
C-6	17.95	
C-7	25.96	
C-8	133.88	
C-9	134.51	
C-10	36.89	
C-11	20.83	
C-12	28.95	
C-13	44.25	
C-14	49.56	
C-15	27.03	
C-16	30.87	
C-17	47.16	
C-18	15.98	0.759 (3, *s*)
C-19	18.84	0.973 (3, *s*)
C-20	47.59	
C-21	182.52	
C-22	32.49	
C-23	25.91	
C-24	123.57	5.093 (1, *t*)
C-25	132.24	
C-26	17.64	1.588 (3, *s*)
C-27	25.67	1.676 (3, *s*)
C-28	27.55	0.858 (3, *s*)
C-29	21.85	0.909 (3, *s*)
C-30	24.34	0.934 (3, *s*)
OCOMe (C-32)	21.32	2.06 (3, *s*)
OCOMe (C-31)	170.79	

Statistical analysis. The data are expressed as the mean ± standard deviation of six experiments. One-way analysis of the variance was performed to analyze the statistical significance of difference between groups. All statistical analyses were performed using SPSS (version 19.0, IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

FPOA extraction and isolation. FPOA (Fig. 1) is a white petroleum ether-ethyl acetate powder with a melting point of 192-194˚C and an m/z of 498. The ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) data for FPOA are shown in Table I.

Cytotoxicity of FPOA on cancer cell lines. As demonstrated in Table II, FPOA evidently inhibited the growth of HepG2, MCF-7, and HeLa cells that had IC_{50} values of 42.10, 52.25 and

Table II. FPOA and mitomycin C cytotoxicity against HepG2, MCF-7, HeLa, A549 and MRC-5 cells.

		Inhibition ratio (%) of different concentrations (μg/ml)								
Compound	Cell line	0	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00	IC_{50}(μM)
FPOA	MRC-5	0.00±1.88	3.99±0.78	6.56±1.41^{a}	5.62±1.76	2.14±0.65	4.61±0.97	14.13±1.48^{b}	31.00±2.96^{b}	53.03±3.45^{b}	365.09
	HepG2	0.00±3.43	-4.47±1.90	-16.94±2.83^{b}	21.73±4.36^{b}	24.01±0.68^{b}	58.84±3.02^{b}	85.47±0.84^{b}	81.89±0.47^{b}	83.62±0.73^{b}	42.10
	MCF-7	0.00±2.48	7.03±4.42	9.97±2.84^{a}	15.78±4.57^{a}	27.40±3.01^{b}	47.69±2.32^{b}	87.79±0.42^{b}	88.36±1.03^{b}	87.70±0.48^{b}	52.25
	HeLa	0.00±3.03	-0.13±2.02	17.83±2.71^{b}	19.33±3.09^{b}	27.20±1.94^{b}	47.96±0.51^{b}	72.33±2.09^{b}	89.36±0.74^{b}	90.17±0.62^{b}	53.19
	A549	0.00±2.17	9.95±3.21^{b}	11.61±1.92^{b}	14.67±1.76^{b}	14.39±2.10^{b}	11.13±1.09^{b}	34.41±2.64^{b}	37.19±2.66^{b}	64.15±1.34^{b}	279.14
Mitomycin C	MRC-5	0.00±1.35	3.32±0.77	2.70±0.43	4.84±1.23^{a}	5.89±1.42^{a}	6.29±1.59^{a}	18.09±1.78^{b}	40.74±5.49^{b}	52.68±4.74^{b}	397.13
	HepG2	0.00±3.35	7.15±1.50	18.63±4.05^{b}	8.26±2.59	34.25±3.41^{b}	48.95±3.75^{b}	63.66±3.57^{b}	77.99±3.02^{b}	79.23±4.01^{b}	78.58
	MCF-7	0.00±3.15	9.94±3.35	19.62±3.34^{b}	20.19±5.08^{a}	38.86±0.89^{b}	53.33±2.29^{b}	66.72±3.91^{b}	84.24±3.75^{b}	82.25±2.39^{b}	63.76
	HeLa	0.00±4.03	6.51±2.04	15.34±3.08^{a}	26.96±4.75^{b}	39.86±3.99^{b}	50.13±3.96^{b}	62.72±4.35^{b}	76.82±2.71^{b}	79.19±4.17^{b}	74.13
	A549	0.00±3.03	7.92±1.16^{a}	6.79±1.42	19.99±2.68^{b}	22.53±2.17^{b}	43.09±2.69^{b}	61.31±3.79^{b}	73.56±2.70^{b}	69.53±4.47^{b}	94.75

Compounds with an IC_{50} >100 μM were considered inactive. The inhibition ratio and the percentage of growth inhibition compared with the control group (0 μg/ml) were used to express the cell viability. Cells were exposed to various concentrations (0, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 μg/ml) of FPOA or mitomycin C and incubated at 37˚C with 5% CO_2 for 48 h. ^{a}P<0.05, ^{b}P<0.01 vs. the control group. FPOA, 3-acetoxylanosta-8,24-dien-21-oic acid; IC_{50}, half maximal inhibitory concentration.

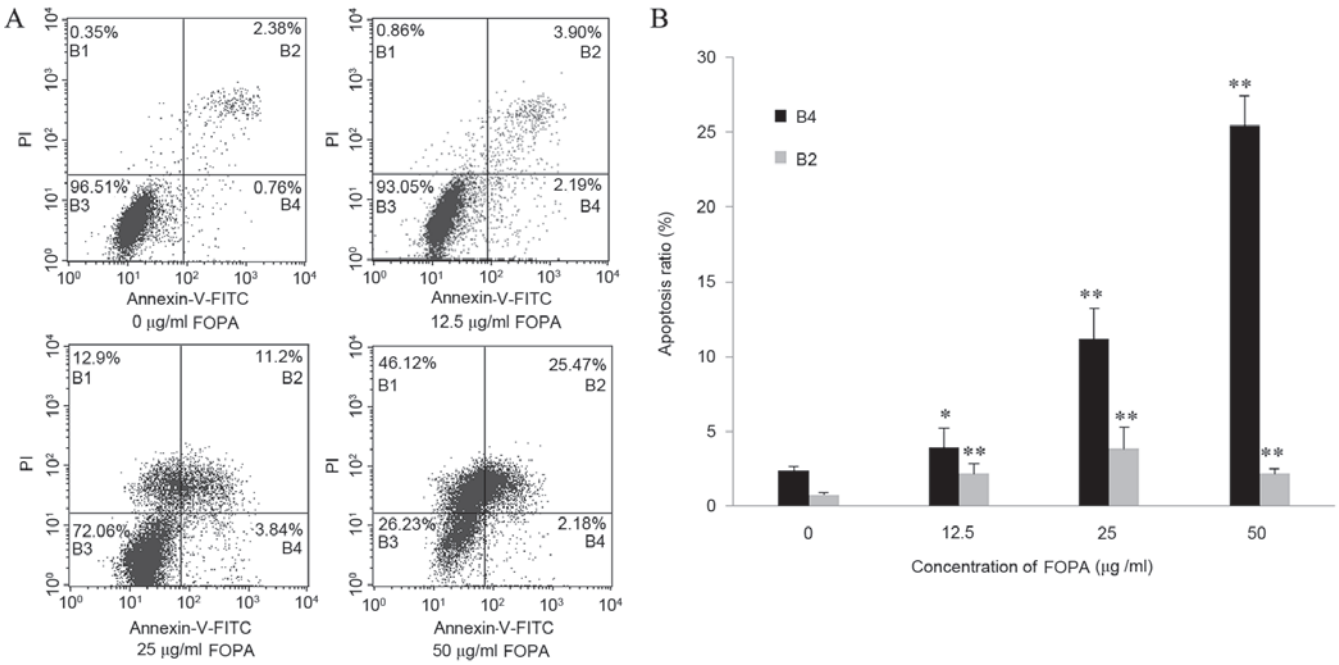

Figure 2. FPOA induces the apoptosis of HepG2 cells. (A) HepG2 cells were exposed to different concentrations of FPOA (0, 12.5, 25 and 50 μg/ml) for 24 h. Cells collected were subjected to annexin V-FITC/PI staining and analyzed by flow cytometry. (B) Quantification of the flow cytometry results demonstrated the fractions of cells in the early and late stages of apoptosis. *P<0.05, **P<0.01 vs. the control group. FPOA, 3-acetoxylanosta-8,24-dien-21-oic acid; FITC, fluorescein isothiocyanate; PI, propidium iodide.

53.19 μM, respectively. However, it had no effect on MRC-5 and A549 cells that had IC_{50} values of 365.09 and 279.14 μM, respectively. Furthermore, when mitomycin C was used as a positive control the IC_{50} values on the MCF-7 and A549 tumor cells were 63.76 and 94.75 μM, respectively, confirming that FPOA had no effect on these cell lines. Furthermore, the mitomycin C results confirmed that FPOA was particularly toxic to the HepG2 cells. Therefore, the HepG2 cell line was selected to further evaluate the antitumor actions of FPOA.

FPOA induces HepG2 cell apoptosis. As demonstrated in Fig. 2A, HepG2 cells were distributed into four quadrants by flow cytometry after Annexin/PI staining: Viable ($Annexin^{-}/PI^{-}$); early apoptotic ($Annexin^{+}/PI^{-}$); late apoptotic ($Annexin^{+}/PI^{+}$) and necrotic ($Annexin^{-}/PI^{+}$). The rate of apoptosis in the control group (early and late apoptotic) was 3.14±0.47%. The apoptosis rate of the HepG2 cells significantly increased following FPOA treatment at all doses (P<0.05 vs. the control group; Fig. 2B). This effect was observed in a dose-dependent manner, with the apoptotic rate of the 50 μg/ml FPOA group reaching 27.65±0.79% (Fig. 2B). These results indicate that FPOA induces significant apoptosis in HepG2 cells in a dose-dependent manner.

Effect of FPOA on apoptosis-associated protein expression. As demonstrated in Fig. 3, the protein expression of Bcl-2 and PARP were downregulated in HepG2 cells exposed to FPOA for 24 h, while the protein expression levels of Bax, cytochrome *c*, caspase-9, caspase-cleaved PARP and caspase-3 increased. These results demonstrated that expression of the antiapoptotic protein Bcl-2 was inhibited by FPOA in a dose-dependent manner, whereas the levels of proapoptotic Bax were increased. Furthermore, expression of caspases-3, a key apoptotic protein, was activated, indicating that apoptosis occurred.

Discussion

Apoptosis is a fundamental process that takes place throughout an organism's life. A regulated amount of apoptosis allows for the development and maintenance of normal organs (22). The majority of anticancer drugs in clinical use induce tumor cell apoptosis, via activating several apoptotic signaling pathways, including those of the mitochondria and endoplasmic reticulum.

FPOA has previously been revealed to cause Hep 3B cell death by apoptosis (14). However, the apoptotic pathway induced by FPOA remains unclear. The structure of the triterpenoid ganoderic acid from fungi is similar to FPOA, the primary difference being the carboxyl position. Previous studies have revealed that ganoderic acid induces apoptosis in numerous tumor cells, including Bel-7402, HepG2 and HeLa cells (23,24). Additionally, various molecular mechanisms for this effect have been reported, including apoptosis-associated proteins, death receptors, oxidative stress and immunomodulation (23,24).

Bcl-2 family members serve an important role in regulating apoptosis, particularly at the mitochondrial level (25). Bcl-2 family proteins can be divided into proapoptotic, including Bax, Bcl-2 homologous antagonist/killer and Bcl-2-interacting killer, and antiapoptotic, including Bcl-2, B-cell lymphoma-extra large and Bcl-2-like protein 2. Bax and Bcl-2 are important proteins in the process of apoptosis. The ratio of proapoptotic Bax to antiapoptotic Bcl-2 is a critical determinant of the extent of cellular susceptibility to apoptosis (26). The present study revealed that FPOA downregulated

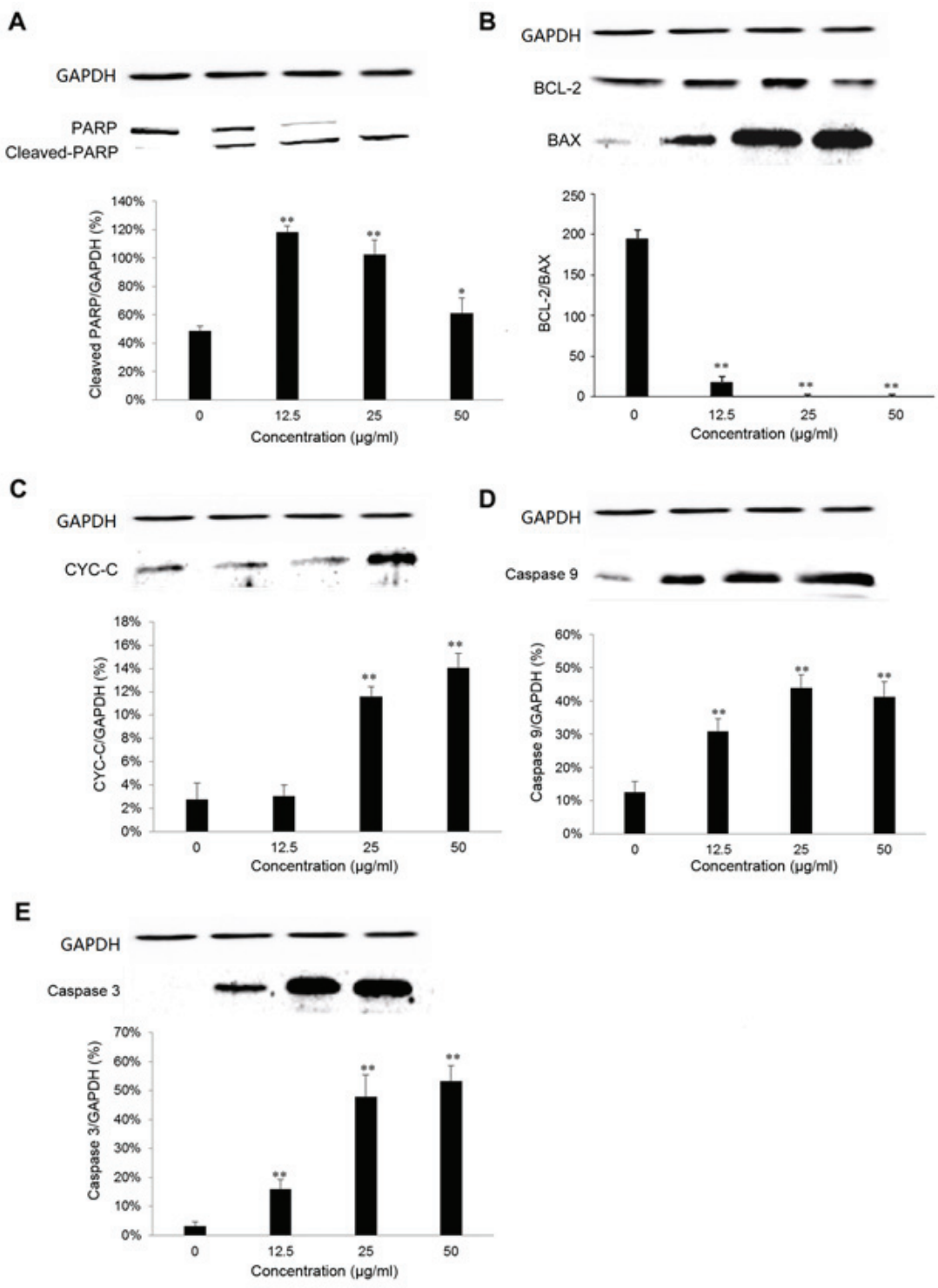

Figure 3. Western blotting analysis of protein extracts obtained from HepG2 cells treated with FPOA (0, 12.5, 25 and 50 μg/ml) for 24 h. Western blot and quantification of (A) PARP and cleaved PARP proteins, (B) Bax/Bcl-2, (C) cyt *c*, (D) caspase-9 and (E) caspase-3. *P<0.05, **P<0.01 vs. the control group. FPOA, 3-acetoxylanosta-8,24-dien-21-oic acid; PARP, poly (ADP) ribose polymerase; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; cyt *c*, cytochrome *c*.

the expression of the Bcl-2 protein and upregulated the expression of Bax protein in HepG2 cells, resulting in a significant increase in the Bax/Bcl-2 ratio and inducing apoptosis. An increased Bax/Bcl-2 ratio increases mitochondrial membrane permeability, allowing cytochrome *c* to combine with other factors and activate caspase family proteins, triggering apoptosis (27). The present study also identified an increase in cytochrome *c* release from the mitochondria as the Bax/Bcl-2 ratio increased.

The caspase family of proteins serve an important role in the process of apoptosis. Caspase-9 is an upstream protease in the apoptotic signal transduction pathway and is a key enzyme of apoptosis, while caspase-3 functions further downstream. Cleaved PARP is the protein substrate of caspases-3. The

present study used western blot analysis to detect cleaved PARP, caspase-9 and caspase-3 levels. Cleaved PARP, caspase-9 and caspase-3 levels were determined to be increased significantly in FPOA-treated cells. In conclusion, the results of the present study demonstrated that the FPOA-induced apoptosis of HepG2 cells was associated with activated caspase family proteins. Furthermore, the mitochondrial apoptotic pathway was determined to be the underlying molecular mechanism for this effect. These results indicate that FPOA is a potential candidate for the development of anticancer drugs.

Acknowledgements

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