Anti-proliferative activity of fenretinide in human hepatoma cells *in vitro* and *in vivo*

Jianzhong Shentu[a], Bo Zhang[b], Lingling Fan[b], Qioajun He[b], Bo Yang[b] and Zhi Chen[a]

***N*-(4-hydroxyphenyl)-retinamide (fenretinide) is a synthetic derivative of all-*trans*-retinoic acid and induces apoptosis in several cancer cell lines. We determined the anti-cancer activity of fenretinide using human hepatoma cell lines, Bel-7402, HepG2 and Smmc-7721. An *in-vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that fenretinide exhibited growth inhibition in these cell lines, with IC_{50} values ranging from 13.1 to 15.5 μmol/l. In Bel-7402 cells, apoptosis with 15 μmol/l fenretinide for 0 and 48 h was 3 and 48%, respectively. *In-vivo* studies using the Bel-7402 xenografted athymic mouse model showed tumor inhibition rates ranging from 37.2 to 57.2%, with fenretinide administration once per 3 days at the rate of 25–100 mg/kg. Western blot analysis further showed down-regulation of procaspase-3, X-linked inhibitor of apoptosis protein and poly(ADP-ribose) polymerase cleavage in Bel-7402 cells treated with 15 μmol/l fenretinide for 48 h. Overexpression of p53 was observed in a time-dependent manner, along with a decrease in the Bcl-2/Bax ratio. Depolarized mitochondrial membranes were found in fenretinide-induced apoptotic cells, in a time-dependent manner. We conclude that fenretinide effectively inhibits the proliferation of Bel-7402, both *in vitro* and *in vivo*. Both procaspase-3 and p53-mediated apoptotic pathways are involved in its potent anti-cancer activity. *Anti-Cancer Drugs* 18:47–53 © 2007 Lippincott Williams & Wilkins.**

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[a]First Affiliated Hospital, College of Medicine and [b]College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang Province, PRC.

Correspondence to Z. Chen, College of Medicine, Zhejiang University. 353 Yan'an Road, Hangzhou, Zhejiang 310031, PRC.
Tel: +86 571 8721 7007;
e-mail: chenzhi@zju.edu.cn

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All authors contributed equally to this work.

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Introduction

Hepatoma is one of the main complications of liver cirrhosis and a serious health problem worldwide [1]. It is usually treated by surgical resection or liver transplantation with curative options for the patients when the disease is diagnosed at an early stage. About 70% of patients are, however, inoperable because of advanced tumor growth or liver cirrhosis and, on the other hand, hepatoma belongs to the group of cancers that are resistant to systemic chemotherapies [2]. Therefore, an alternative hepatocellular carcinoma treatment strategy focuses on the development of chemotherapy approaches.

N-(4-hydroxyphenyl)-retinamide (fenretinide) is a synthetic derivative of all-*trans*-retinoic acid that induces apoptosis in cancer cell lines, and it is used in clinical trials for adult and pediatric cancers [3–5]. It is well tolerated during long-term oral administration, as shown in chemoprevention studies [6–8] and in recent phase I trials against solid tumors [4]. Fenretinide has been demonstrated to have cytotoxicity effect against various cancer cell lines with much fewer negative effects [9]. Previous reports suggested that fenretinide induced apoptosis in several solid tumors through retinoid receptor-dependent and retinoid receptor-independent pathways *in vitro*, and in part because of the generation of free radicals or activation of the ceramide pathway [10,11]; however, the use of fenretinide is still limited to some extent.

In the present study, we evaluated both *in-vitro* and *in-vivo* anti-cancer activities and the mechanism of action of fenretinide in hepatoma models, and provided the experimental therapeutic data to expand its indications in clinical trials.

Materials and methods

Chemicals

Fenretinide was kindly provided by Dr B.J. Maurer (Childrens Hospital Los Angeles), and dissolved in ethanol to make a 10-mmol/l stock solution and stored at −20°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, Missouri, USA) and dissolved in RPMI1640 to make a 5 mg/ml solution. Antibodies to procaspase-3, poly(ADP-ribose) polymerase (PARP), p53,

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Bax, Bcl-2, β-actin, X-linked inhibitor of apoptosis protein (XIAP) and horseradish peroxidase-labeled secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Biosciences (Piscataway, New Jersey, USA). The mitochondrial fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, Oregon, USA).

Cell culture

Bel-7402 and Smmc-7721 cells were maintained in RPMI 1640 (2 g/l glucose) and HepG2 cells were maintained in HG-Dulbecco's modified Eagle's medium (4.5 g/l glucose); both media were supplemented with 10% fetal calf serum, 2 mmol/l glutamine and 50 U/ml penicillin. All of the cell lines were purchased from the Institute of Cell Biology (Shanghai, PRC) and grown at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity assay

Tumor cells were seeded in 96-well microtiter plates (4000 cells/well). After a 24 h incubation in the medium, cells were treated with various concentrations of fenretinide and incubated at 37°C in a 5% CO_2 atmosphere for 72 h. Afterwards, 10 μl of MTT (Sigma) stock solution was added to each well (final concentration: 0.25 mg/ml) for another 4-h incubation (37°C, 5% CO_2). After 4 h of incubation, 100 μl of dimethylsulfoxide was added to each well and the optical density was read at 570 nm. The sensitivity of cells to fenretinide was measured by IC_{50} (the drug concentration that yields 50% cell growth inhibition). Experimental conditions were tested in sextuplicate (six wells of the 96-well plate per experimental condition). All the experiments were performed in triplicate.

Anti-tumor effect of fenretinide on hepatoma cell line Bel-7402 xenografted in athymic mice

Tumors were established by injection of Bel-7402 cells (5×10^6 cells per animal, subcutaneously into the armpit of the athymic mice) to 4–5-week-old BALB/c female athymic mice (National Rodent Laboratory Animal Resource, Shanghai Branch, PRC). Treatments were initiated when tumors reached a mean group size of about 130 mm^3. Tumor volume (mm^3) was measured with calipers and calculated as $(W^2 \times L)/2$, where W is the width and L is the length. The $T/C\%$ was determined by calculating relative tumor volume (RTV) as $T/C\% = 100 \times$ (mean RTV of treated group)/(mean RTV of control group). The tumor growth inhibition rate was calculated by using the formula IR $(\%) = (1 - TW_t/TW_c) \times 100$, where TW_t and TW_c were the mean tumor weight of treatment and control groups. Athymic mice was administrated intraperitoneally with fenretinide (100.0, 50.0 and 25.0 mg/kg) dissolved in ethanol : cremophor EL : (1 : 1 : 6, volume) once every 3 days. Mice weight and tumor volume were recorded every 3 days until animals were killed. Animal care was in accordance with institutional guidelines.

Flow cytometry analysis for apoptosis

Exponentially growing Bel-7402 cells were seeded at 4×10^5 cells/flask in 75-cm^2 flasks containing 10 ml complete medium. The cells were cultured at 37°C in a 5% CO_2 atmosphere for 24 h before they were exposed to 15.0 μmol/l fenretinide for 0–48 h. Cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended in 1 ml of 0.1% sodium citrate containing 0.05 mg propidium iodide and 50 μg RNase for 30 min at room temperature in the dark. RNase was dissolved in 10 mmol/l Tris–HCl (pH 7.5) and 15 mmol/l NaCl to a concentration of 10 mg/ml, and boiled at 100°C for 15 min, then stored at −20°C. Flow cytometric analysis of apoptosis was performed on a BD FACSCalibur with a 488 nm (blue) argon laser (Becton Dickinson, San Jose, California, USA). Data acquisition was performed with CellQuest 3.1 software (Becton Dickinson) and data were analyzed with ModFit LT 3.0 software (Variety Software House, Topsham, Maine, USA). All the experiments were performed in triplicates.

Western blotting

Bel-7402 cells were incubated with 15 μmol/l fenretinide for 12, 24 and 48 h, the proteins were extracted in immunoprecipitation buffer [150 mmol/l NaCl, 50 mmol/l Tris, 2 mmol/l ethylene glycol-bis (*b*-aminoethyl ether), 2 mmol/l ethylenediaminetetraacetic acid, 25 mmol/l NaF, 25 mmol/l β-glycerophosphate, 0.2% Triton X-100, 0.3% Nonidet P-40, 0.1 mmol/l phenylmethylsulfonyl fluoride]. The extracted total protein was loaded 40 μg per lane on 10–12% Tris-glycine gels and then transferred to a polyvinylidine diflouride membrane (Millipore, Billerica, Massachusetts, USA). The membrane was blocked in 5% nonfat milk dissolved in PBS with 0.1% Tween 20 (T-PBS), and, subsequently, probed with the primary antibody and horseradish peroxidase-labeled secondary antibody. The bands were visualized using enhanced chemiluminescence Western blotting detection reagents. All the experiments were performed in triplicates and proteins were normalized against actin before analyzing.

JC-1 stain for mitochondrial membrane potential

The harvested cells (2×10^6) were resuspended in 0.5 ml of complete medium containing 10.0 μg/ml of JC-1 for 10 min at 37°C. JC-1 was a cationic dye that exhibited potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 ± 10 nm) to red (610 ± 10 nm). Samples (1×10^4 cells/sample) were analyzed by a FACSCalibur using an argon laser (488 nm). Mitochondria depolarization was

specifically indicated by a decrease in the red to green fluorescence intensity ratio.

Results

Cytotoxicity assay

Using the MTT method, we determined the cytotoxic activity of fenretinide in three human hepatoma cell lines (Bel-7402, HepG2 and Smmc-7721) and the dose–response curves are shown in Fig. 1. All three cells exhibited dose-dependent sensitivity to fenretinide (0–20.0 μmol/l) at 72 h with IC_{50} values ranging from 13.1 to 15.5 μmol/l.

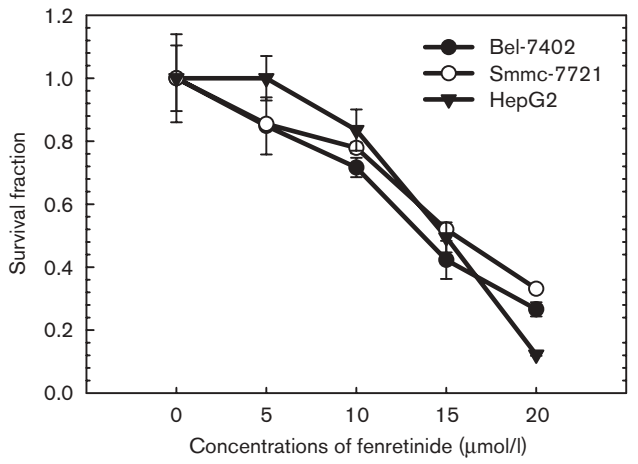
Effect of fenretinide on tumor growth in Bel-7402 xenografted athymic mice

The in-vitro anti-proliferation activity of fenretinide was evaluated using human tumor models xenografted in athymic mice as described in Materials and methods. Fenretinide inhibited tumor growth in a dose-dependent manner (Table 1). Compared with the control group, the tumor growth was significantly inhibited ($P < 0.05$–0.001) from day 12 to day 21 in the groups treated with fenretinide (25.0–100.0 mg/kg) with tumor inhibition rate at 38.9, 45.8 and 58.3% in fenretinide 25.0, 50.0 and 100.0 mg/kg treatment groups, respectively (Fig. 2). Furthermore, there was no significant change on athymic mice body weight during the experiment, which could be considered as the anti-hepatoma activity of fenretinide taking precedence over the toxicity on athymic mice.

Apoptosis assay for the anti-hepatoma activity of fenretinide against Bel-7402

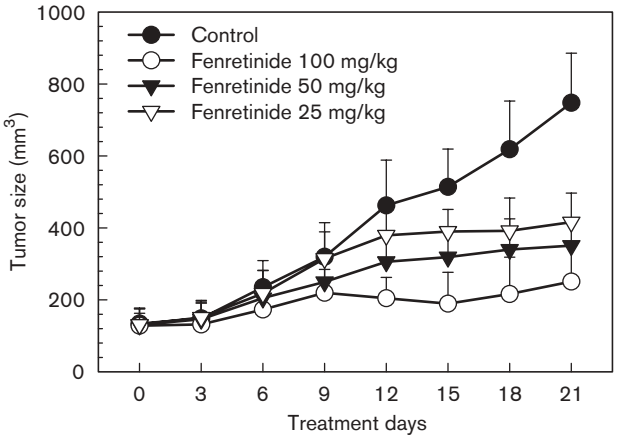
We intended to identify whether the cause of tumor inhibition *in vivo* the cause of tumor inhibition *in vivo* was due to the fenretinide-induced apoptosis. We collected cells that were treated with 15 μmol/l of fenretinide for 12, 24 and 48 h. The apoptotic cells were detected by flow cytometry. The percentages of apoptotic Bel-7402 cells induced by 15.0 μmol/l fenretinide for 0, 12, 24 and 48 h were 3, 7, 24 and 40%, respectively (Fig. 3). This indicated that the increase in fenretinide-mediated apoptotic cells was in a time-dependent manner.

Fig. 1



Fenretinide inhibited the proliferation of three human hepatoma cell lines. Three human hepatoma cells (Bel-7402, Smmc-7721 and HepG2) were treated with fenretinide (5.0–20.0 μmol/l) for 72 h and cytotoxicity was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Fenretinide exhibited cytotoxicity in a dose-dependent manner. Points represent the mean fractional survival; error bars represent standard deviation.

Fig. 2



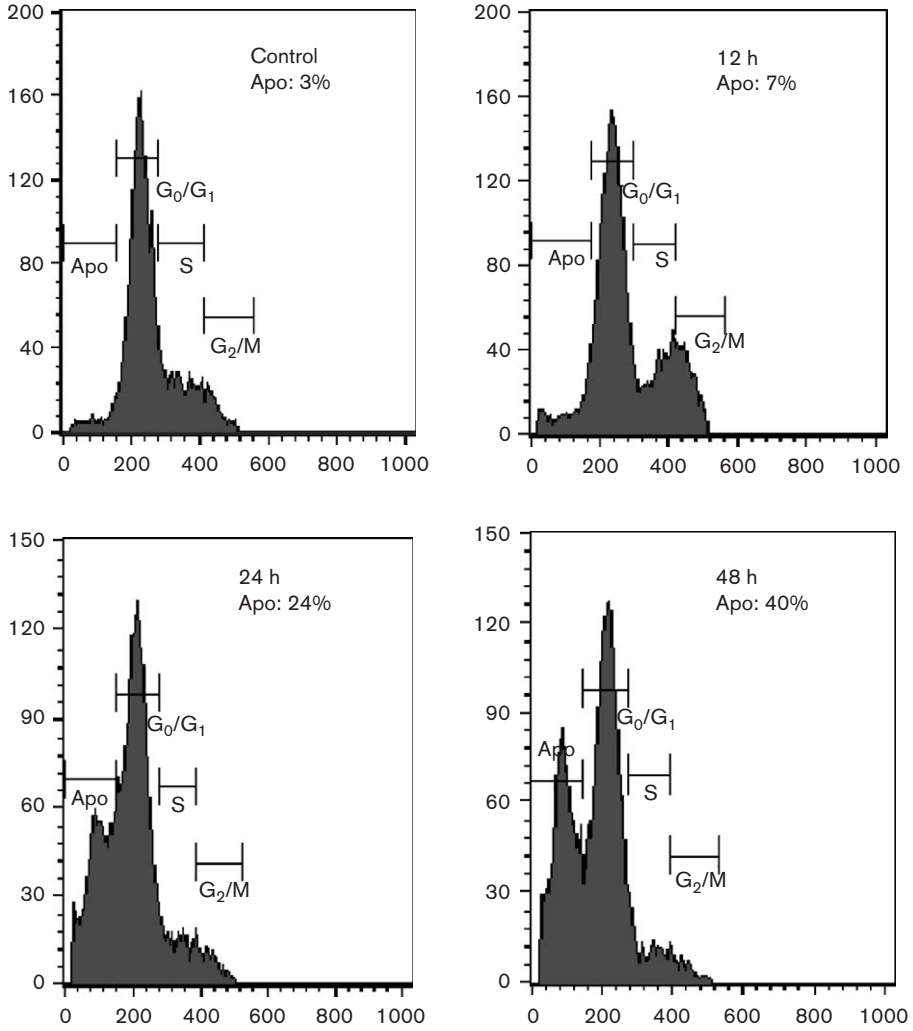
Fenretinide significantly inhibited Bel-7402 cell growth in athymic mice. Treatment was initiated when average tumors reached a mean group size of about 130 mm^3. Fenretinide was dissolved in ethanol, cremophor and saline (1 : 1 : 6) and administrated intraperitoneally once per 3 days with 25, 50 or 100 mg/kg fenretinide, respectively. Mice weight and tumor volume were recorded every 3 days until animals were killed at day 21.

Table 1 **Effects of fenretinide on athymic mice body weight, tumor volume and tumor weight at before and after dosing**

Groups	No. of animals		Body weight (g)		Tumor size (mm^3)		*T*/*C* (%)	Tumor weight (g)	Inhibition rate (%)
	Start	End	Start	End	Start	End			
Control	8	8	25 ± 2	27 ± 3	134 ± 89	748 ± 276	–	0.72 ± 0.28	/
Fenretinide (mg/kg)									
25	8	8	26 ± 2	27 ± 2	131 ± 31	416 ± 81	56.9	0.44 ± 0.15[a]	38.9
50	8	8	26 ± 1	26 ± 2	131 ± 45	351 ± 74	48.0	0.39 ± 0.11[a]	45.8
100	8	8	24 ± 2	24 ± 3	128 ± 59	251 ± 99	35.1	0.30 ± 0.16[b]	58.3

Athymic mice with Bel-7402 transplant tumor were treated with (intraperitoneal) fenretinide 25, 50 or 100 mg/kg every 3 days for 21 days.
[a]$P < 0.05$, [b]$P < 0.01$ vs. control. $T/C(\%) = T_{RTV}/C_{RTV} \times 100$; RTV, relative tumor volume.

Fig. 3



The fenretinide-induced apoptotic percentage was in a time-dependent manner. Cells were treated with fenretinide (15.0 μmol/l) for 12–48 h. After treatment, apoptosis was assessed by propidium iodide staining of lysed cell nuclei, as described in Materials and methods. Apo: apoptotic percentage. Two additional experiments yielded equivalent results.

Determination of the expression level of apoptosis-related proteins

To explore the mechanisms of fenretinide-induced apoptosis, we examined the expression level changes of apoptosis-related proteins. After incubating the Bel-7402 cells with 15.0 μmol/l fenretinide for 12, 24 and 48 h, we collected the cells and extracted proteins. The expression of p53 protein increased visibly in a time-dependent manner, accompanied with the decrease in the proportion of Bcl-2/Bax. The expression of procaspase-3 and XIAP decreased at 48 h, and cleaved fragment of PARP appeared after exposure to fenretinide for 48 h (Fig. 4).

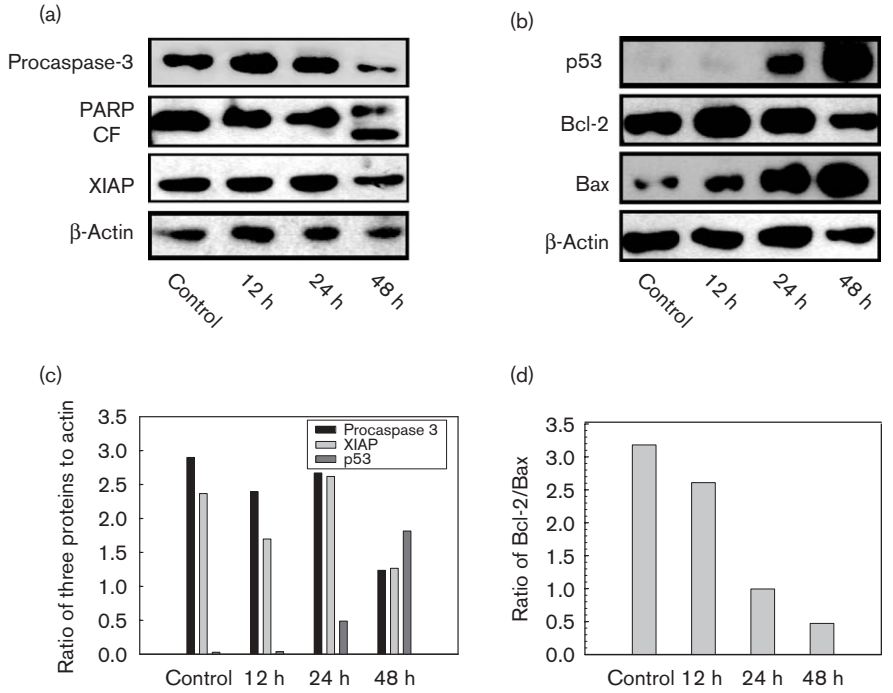
Changes of mitochondria membrane potential induced by fenretinide

On the basis of the results of Western blotting, especially the changes of expression level of p53 and Bcl-2/Bax, we attempted to identify whether mitochondria were involved in fenretinide-induced apoptosis. The experiment described above was performed after cells were treated with fenretinide for 8–24 h and it showed an increased ratio of cells (1.5-fold for 8 h, 2.2-fold for 16 h and 8.0-fold 24 h) with depolarized mitochondrial membranes (Fig. 5), which indicated that mitochondria were involved in the fenretinide-induced apoptosis.

Discussion

Fenretinide is a synthetic derivative of all-*trans*-retinoic acid that induces apoptosis in various cancer cell lines, including human neuroblastoma cells, cervical, breast, human ovarian carcinoma cells, head and neck, lung, and human malignant hematopoietic cells [3]. In our experiments, we first found that fenretinide exerted potent

Fig. 4



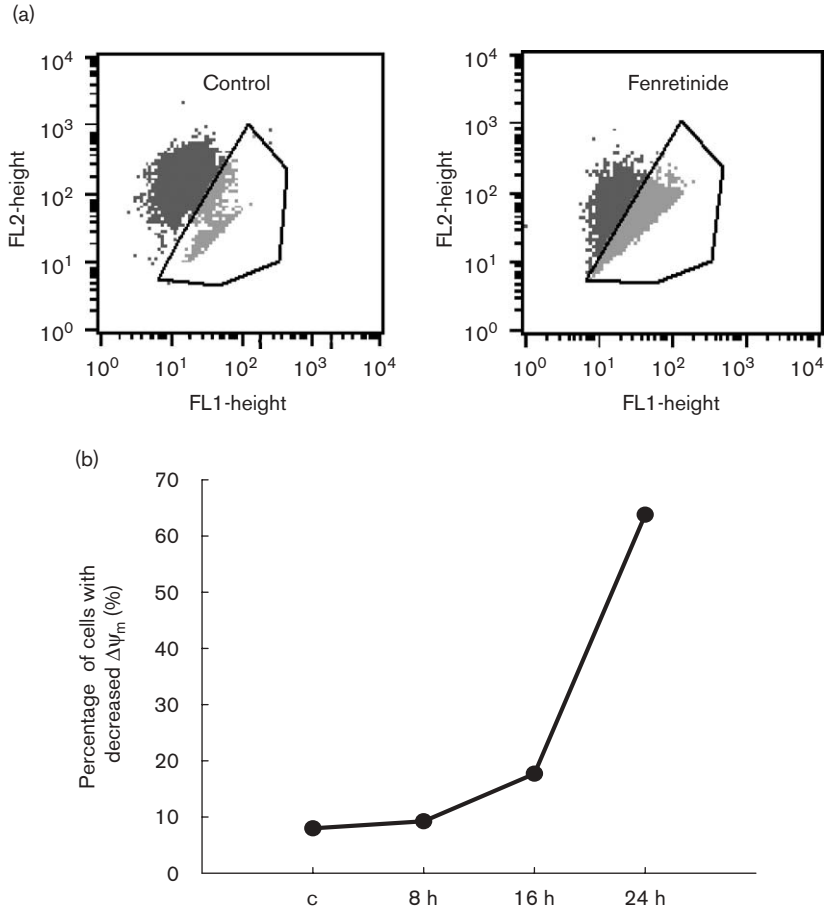
Fenretinide changed the expression level of some apoptotic proteins. Cells were treated with fenretinide (15.0 μmol/l) for 12–48 h. Equal amounts (40 μg/lane) of cellular protein were fractionated on sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidine diflouride membranes, followed by immunoblotting with anti-procaspase-3 protein, X-linked inhibitor of apoptosis protein (XIAP) and poly(ADP-ribose) polymerase (PARP) antibodies (a), with anti-Bcl-2 protein, Bax protein and p53 antibodies (b). Immunoblotting of β-actin was shown to demonstrate loading equivalency. Normalization was performed to demonstrate the exact changes of each protein (c). The columns represent the ratio of Bcl-2/Bax (d). Two additional experiments yielded equivalent results.

anti-proliferative activity on three human hepatoma cells lines, Bel-7402, HepG2 and Smmc-7721. We further selected one hepatoma cell line Bel-7402 and evaluated the effects of fenretinide on Bel-7402 xenografted athymic mice. The results of in-vivo experiments indicated the potent anti-hepatoma activity of fenretinide in inhibiting tumor growth in a dose-dependent manner, without any significant influences on mice body weight. On the basis of the previous analysis, it was most likely that the anti-hepatoma effect on Bel-7402 xenografted athymic mice was due to fenretinide-mediated apoptosis and the results of the apoptosis assay were consistent with our hypothesis.

Furthermore, we identified the mechanisms involved in the process of fenretinide-induced apoptosis and our work revealed the obvious changes of expression levels of several proteins that played key roles in apoptosis. Bax is a member of the Bcl-2 family that triggers apoptosis and counters the protective effect of Bcl-2 [12]. It has been demonstrated that Bax is a downstream effector of p53-induced apoptosis and is transcriptionally regulated by p53 [13,14]. In our study, the expression of p53 and Bax proteins were enhanced greatly by fenretinide in a time-dependent manner, while the amount of Bcl-2 remained at the same level. As Bax has the ability to heterodimerize with Bcl-2 protein, the ratio of Bcl-2 to Bax appears as one of the important markers for the occurrence of apoptosis [15]. The data in our study showed that the Bcl-2/Bax ratio decreased in a time-dependent manner. On the other aspect, mitochondria, which might play a pivotal role in the interaction among p53, Bcl-2 and Bax, were also an important indication of apoptosis; therefore, the mitochondria membrane potential was investigated and depolarized mitochondrial membranes were observed during fenretinide-induced apoptosis. We further determined the expression of p53 and the expression increased obviously especially at 24 and 48 h, and we concluded that p53, the Bcl-2 family and mitochondria were involved in fenretinide-mediated apoptosis.

Activation of the caspase cascade is another crucial gateway that is involved in the execution of apoptosis in a variety of cellular systems, among which procaspase-3 is an ultimate executioner of the caspase family that is essential for the nuclear changes associated with apoptosis, including chromatin condensation [16]. PARP is a highly conserved nuclear enzyme that binds tightly to

Fig. 5



Fenretinide induced the decrease of mitochondrial membrane potential in cells treated with fenretinide. Cells were treated with 15 μmol/l fenretinide for 8, 16 and 24 h, and stained with the mitochondria-selective JC-1 dye. The percentage of cells with fluorescence emission shifting from red to green (a) presented a time-dependent manner (b), which indicated the corresponding tendency of depolarized mitochondrial membranes.

DNA, and plays a role in DNA repair, recombination, proliferation and genomic stability [17–19]. It is also known that when the caspase cascade is activated, procaspase-3 disassembles PARP into cleaved fragments. Therefore, the appearance of the cleaved PARP is solid proof to demonstrate the activation of the caspase family. Our observation showed that the decreased expression of procaspase-3 and cleaved PARP appeared simultaneously at 48 h after the addition of fenretinide, which indicated that the activation of the caspase family is related to fenretinide-induced apoptosis. The inhibitors of apoptosis (IAP) family, including cIAP-1, cIAP-2, XIAP, neuronal apoptosis inhibitory protein and survivin, were initially identified in baculovirus and highly conserved across species. These proteins acted directly on caspases, distal to mitochondrial perturbation [20]. We found that the expression of XIAP slightly decreased at 48 h, which further demonstrated the postulate that the caspase family contributes to the process of fenretinide-induced apoptosis.

In conclusion, we shown that fenretinide could induce apoptosis in human hepatoma cell lines both *in vivo* and *in vitro*, and its mechanisms of actions might be associated with p53 and caspase apoptotic pathways.

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