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Waltonitone induces human hepatocellular carcinoma cells apoptosis *in vitro* and *in vivo*

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ABSTRACT

Waltonitone, a new ursane-type pentacyclic triterpene isolated from *Gentiana waltonii* Burkill significantly inhibited human hepatocellular carcinoma BEL-7402 cells growth. Apoptosis induced by waltonitone was characterized by AO/EB staining and flow cytometric analysis. Apoptosis microarray assay results showed BCL-2 family genes might especially play an important role in waltonitone-induced apoptosis. RT-PCR and Western blotting analysis showed that waltonitone could induce tumor cell apoptosis via both death receptor and mitochondria pathways. Meanwhile, the inhibitory effect of waltonitone was examined *in vivo* using BEL-7402 tumor cells xenografted into athymic mice model. In summary, these studies demonstrated that waltonitone might inhibit hepatocellular carcinoma cells growth and induce apoptosis *in vitro* and *in vivo*.

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1. Introduction

Hepatocellular carcinoma (HCC) is the cancer that arises from hepatocytes, the major cell type of the liver, and usually relates to chronic liver diseases [1,2]. It is one of the most common malignancies with over 500,000 new patients diagnosed annually and be associated with a less than 5% 5-year survival rate worldwide. Current curative options for HCC mainly include surgical resection, liver transplantation, radiotherapy and chemotherapy, however, no effective systemic treatment is available [1,3,4]. Evidence suggests that the incidence of HCC is rising in several countries, including China [4,5]. Therefore, it is very important to develop new pharmacological agents of innovative and targeted therapies to overcome this deadly disease.

Recently, there has been a growing interest in developing drugs or lead compounds from traditional Chinese

medicine (TCM) and identifying the active ingredients of these herbs or studying their pharmacological mechanisms [6,7]. Indeed, there are a number of clinical practices that have evaluated these natural products for their efficacy in preventing diseases including cancer [8]. Medicinal plants from *Gentiana* genus usually have hepato- and chole-protective effects [9,10]. *Gentiana waltonii* Burkill has been commonly used for the treatment of rheumatic arthritis, liver and gall diseases in China for centuries. Our previous studies focused on the study of chemical constituents of *G. waltonii*. About 40 compounds have been obtained and identified from the plant including triterpenes, secoiridoids, sesquiterpenes and phenolic acids [11]. Triterpenes are nature products widely distributed in food, medicinal herbs and various other plants in free form or bound to glycosides [12]. Over the last decade, some pentacyclic triterpene derivatives have been reported for their notable anti-tumor, anti-bacterial, anti-viral, anti-inflammatory activities and cholesterol-lowering effect [13–16]. For instance, ursolic acid and oleanolic acid are well known for their hepatoprotective effects in both acute chemically induced liver injury and chronic

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liver fibrosis and cirrhosis. Besides, they have been shown to act at various stages of tumor development to inhibit tumor initiation and promotion, as well as to induce tumor cell differentiation and apoptosis [17–20].

Waltonitone is a new ursane-type pentacyclic triterpene isolated from *G. waltonii* (Fig. 1). In this study, we report the effect of waltonitone on cell viability, cell morphology and caspase cascade activation in human hepatocellular carcinoma BEL-7402 cells. The activities of waltonitone on BEL-7402 cells *in vivo* were also evaluated.

2. Materials and methods

2.1. Reagents and chemicals

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO BRL/Invitrogen (Gaithersburg, MD, USA). Antibodies against cleaved caspase-3, cleaved caspase-8, caspase-9 and FasL were all from Cell Signal Technology (Danvers, MA, USA). Antibody against FLIP was obtained from ProSci (San Diego, CA, USA) and antibody against β -actin was from Sigma (St. Louis, MO, USA). All other reagents unless indicated were all from Sigma (St. Louis, MO, USA).

Waltonitone isolated from *G. waltonii* in our laboratory was structurally elucidated by IR, NMR, MS, X-ray spectra and its purity determined by several conventional methods (HPLC, TLC) was above 95%. For *in vitro* experiments, waltonitone was dissolved in sterilized dimethylsulfoxide (DMSO) and diluted in RPMI1640 medium (the concentration of DMSO was less than or equal to 0.1% v/v). For *in vivo* experiments, waltonitone microemulsion was prepared using medium chain triglyceride (MCT), Cremophor EL, propylene glycol and Milli Q water, which presented as oil phase, emulsifier, co-emulsifier and aqueous phase, respectively. The mixture was diluted in injectable normal saline (NS) solution before using. The blank microemulsion without waltonitone was prepared in parallel for control group.

2.2. Cell culture

Human hepatocellular carcinoma BEL-7402 cells (Cell Bank, Type Culture Collection of Chinese Academy of

Sciences, China) were cultured in RPMI1640 medium supplemented with 10% heat-inactivated (56 °C) FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.3. MTT assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded in 96-well microplates at a density of 1×10^4 per well and were cultured for 24 h. After treatment with waltonitone for 24, 48 or 72 h, respectively, 0.5 mg/ml MTT was added and incubated with cells for 4 h in an incubator. At last the formazan was dissolved in DMSO after the medium was removed. Finally the optical density was measured using a spectrophotometer (Thermo Multiskan MK3, German) at an absorption wavelength of 570 nm.

2.4. AO/EB staining

After incubated with or without waltonitone for 48 h, BEL-7402 cells were stained with AO/EB dye mix (10 μ g/ml acridine orange and 10 μ g/ml ethidium bromide in PBS) for 30 min followed by fixation with 4% PFA (paraformaldehyde) for 15 min in the dark. Cell apoptotic morphology was observed by a fluorescence microscope (Olympus BX51, Japan) after washing with PBS.

2.5. Apoptosis analysis by flow cytometry (FCM)

Cells treated with or without waltonitone for certain time were collected by trypsinization and washed twice with PBS, then fixed in ice-cold 70% (v/v) ethanol at 4 °C. After centrifugation, the cell pellets were resuspended in 1 ml of propidium iodide (PI) solution (50 μ g/ml PI, 50 μ g/ml RNase A, 0.03% Triton X-100 and 0.01% sodium citrate in PBS) and incubated in the dark for 30 min at room temperature. DNA histograms were obtained by fluorescence activated cell sorting analysis (FACS) (Becton Dickinson FACSCalibur, USA). Apoptosis was measured by quantifying the sub-G1 peak.

2.6. Apoptosis microarray assay

After treated with or without waltonitone for 24 h, BEL-7402 cells were harvested and total RNA was extracted using TRIZOL reagent (Invitrogen, USA). Hybridization and microarray analyses were performed by KangChen Bio-tech (Shanghai, China). Briefly, cRNA probe was synthesized and labeled from 3 μ g of total RNA by using TrueLabeling-AMP Linear RNA Amplification Kit (SuperArray Bioscience, USA). The biotin-UTD labeled cRNA was purified by ArrayGrade cRNA Cleanup Kit (SuperArray Bioscience, USA), and the cleaned cRNA was then used for hybridization with the Human Oligo Apoptosis Microarray (SuperArray Bioscience, USA) according to the manufacture's instructions. The array images were captured and data were analyzed by

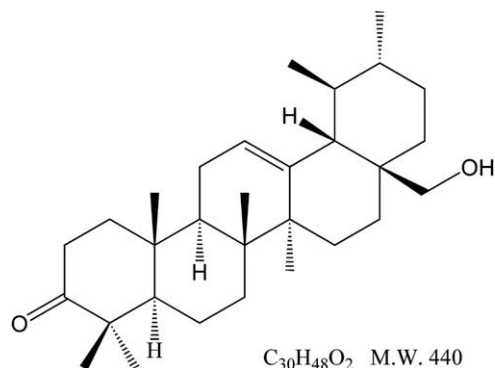


Fig. 1. Chemical structure of waltonitone (28-hydroxy-3-oxo-12-ursene).

Table 1

Gene specific primers for RT-PCR analysis.

Gene	GenBank accession no.	Primer sequences (5'-3')		Ta (°C)	Product size (bp)
Fas	NM_000043	Forward	caagggttggaattgagga	58	440
		Reverse	gacaaagcccccaggta		
FasL	NM_000639	Forward	tctcagcgttttctggctt	58	406
		Reverse	aagacagtcccccttgaggt		
Caspase-3	NM_004346	Forward	ttgtttgtgtctctgagcc	58	412
		Reverse	attctgttccaccttctgg		
Caspase-8	NM_033358	Forward	agatctggcctccctcaagt	58	217
		Reverse	ttccctttccatctcctct		
Caspase-9	NM_001229	Forward	gtttgaggacctcgaccag	58	150
		Reverse	caaagatgtctccagggtc		
Apaf-1	NM_181861	Forward	ttgtcgccttctccatgat	59	318
		Reverse	tccaactgaaaccaatgc		
BAX	NM_004324	Forward	ttgtctcagggtttcatcc	55	246
		Reverse	cagttgaagttgacctcaga		
BCL-2	NM_000633	Forward	cgacttcgaggatgtccagccag	65	389
		Reverse	acttgtggcccagataggcaccag		
18S RNA	NM_022551	Forward	gtggagcagattgtctggtt	55	201
		Reverse	acgtgagcagctcagtgta		

GEArray Expression Analysis Suite software (SuperArray Bioscience, USA).

2.7. Semi-quantitative RT-PCR analysis

Total RNA was isolated from the control and treated cells using TRIZOL reagent (Invitrogen, USA) following the manufacture's instructions. Single-strand cDNA was synthesized from 1 µg total RNA using AMV reverse transcriptase (TaKaRa, Japan). According to the specific primers described in Table 1, PCR amplification was performed with Ex Taq (TaKaRa, Japan) using the following conditions: denaturation for 50 s at 95 °C, annealing for 50 s at annealing temperature (Ta) and elongation for 50 s at 72 °C. The thermal cycling was 25 cycles for 18S RNA, 30 cycles for caspase-3, caspase-8, caspase-9, BAX, BCL-2, apaf-1, Fas and 35 cycles for FasL. The RT-PCR products were analyzed and visualized on 1.5% agarose gel containing ethidium bromide. Images were acquired by Tanon 3500 digital gel imaging system (Tanon Science & Technology, China) and quantified using ImageJ densitometry analysis software (NIH, USA).

2.8. Mitochondrial and cytosolic fractionation

To determine the release of pro-apoptotic factors from mitochondria to cytosol by Western blotting, the isolation of mitochondria and cytosol was performed using the Cell Mitochondria Isolation Kit (Beyotime Institute of Biotechnology, China). Briefly, cells were incubated in 100 µL ice-cold mitochondrial lyses buffer on ice for 10 min. Cell suspension was then taken into a glass homogenizer and homogenized for 30 strokes using a tight pestle on ice. The homogenate was subjected to centrifuging at 600g for 10 min at 4 °C to remove nuclei and unbroken cells. Then the supernatant was collected and centrifuged again at 12,000g for 30 min at 4 °C to obtain the cytosol (supernatant) and mitochondria (deposition) fraction. Samples of cytosol and mitochondria were dissolved in lyses buffer and proteins were subjected to Western blotting, respectively, described as below.

2.9. Western blotting analysis

Cells treated with or without waltonitone for certain time were washed with cold PBS and lysed with RIPA buffer containing 0.2% (w/v) SDS, 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate supplemented with 1 mM PMSF

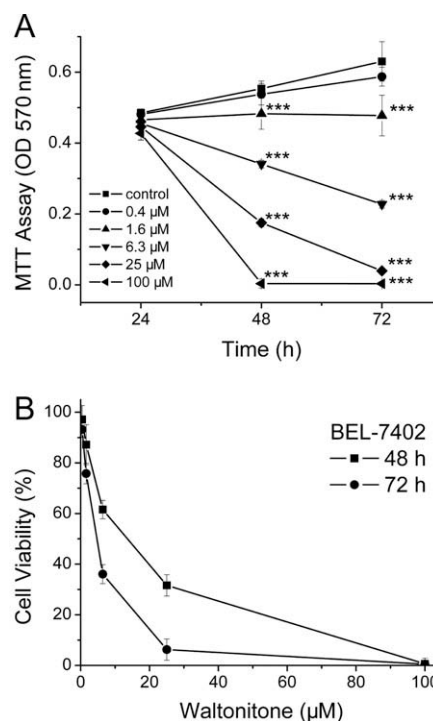


Fig. 2. Waltonitone inhibits BEL-7402 cells growth. Cells were plated at a density of 1×10^4 per well into 96-well plates and treated with different concentrations of waltonitone for 24, 48 or 72 h. Cell viability was measured by MTT assay. (A) Time course of the viability of BEL-7402 cells treated with different concentrations of waltonitone for 24, 48 or 72 h, respectively. (B) Dose course of the viability of BEL-7402 cells treated with waltonitone for 48 or 72 h, results were expressed as the ratio compared to untreated cells. Data were means \pm SD for triplicate experiments ($***p < 0.001$, *t*-test).

and 1% protease inhibitor cocktail (Sigma, USA) for 30 min on ice. Total protein content was measured by Protein Assay Reagent (Bio-Rad, USA). Equivalent amounts of protein of each sample were subjected to 4–15% SDS-PAGE and transferred to PVDF membranes (Bio-Rad, USA). The membranes were blocked with 5% nonfat milk in TBST buffer [20 mM Tris (pH 8.0), 150 mM NaCl and 0.1% Tween-20] and incubated overnight at 4 °C with the primary antibodies then probed using horseradish peroxidase conjugated secondary antibody (Jackson, USA). Visualization of bands by enhanced chemiluminescence kit (PIERCE, USA) was performed according to manufacturer's instructions.

2.10. Tumor growth in vivo

Male athymic mice (BALB/c nu/nu) (Shanghai Experimental Animal Center of Chinese Academy of Sciences, China) were housed in barrier facilities with food and water *ad libitum* under specified-pathogens free (SPF) conditions. Cell suspensions with density of 1×10^7 cells/ml in serum-free medium were subcutaneously (s.c.) injected into the right flank of the 4-week-old mice. After tumor transplantation for 1 week, waltonitine or blank micro-emulsion was intravenously (i.v.) injected once every two days for 15 consecutive days. The length (a) and width (b) of tumor were regularly measured for 3–4 times every

week by a caliper and the tumor volumes were then calculated according to the formula $[(a \times b^2)/2]$. All mice were sacrificed after administration for 15 days and the tumors were removed and weighted. Proteins were extracted from tumor tissue samples by homogenizing with RIPA buffer on ice, and then subjected to Western blot analysis as described herein.

2.11. Statistical analysis

All data represent at least three independent experiments and are expressed as means \pm standard deviation (SD). Student's *t*-test was used to determine the significances of differences in multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Waltonitine inhibits BEL-7402 cells growth

The potential effect of waltonitine was investigated on the viability of human hepatocellular carcinoma BEL-7402 cells using MTT assay. Cells were treated with different concentrations of waltonitine for 24, 48 or 72 h. Their viabilities were determined by formazan dye uptake and expressed as percent compared to untreated cells. As showed in Fig. 2A and B, waltonitine showed a dose- and time-dependent inhibitory effect on BEL-7402 cells growth. It caused 50% growth inhibition (IC_{50}) at $13.9 \pm 5.2 \mu\text{M}$ for 48 h and $4.2 \pm 0.3 \mu\text{M}$ for 72 h, respectively.

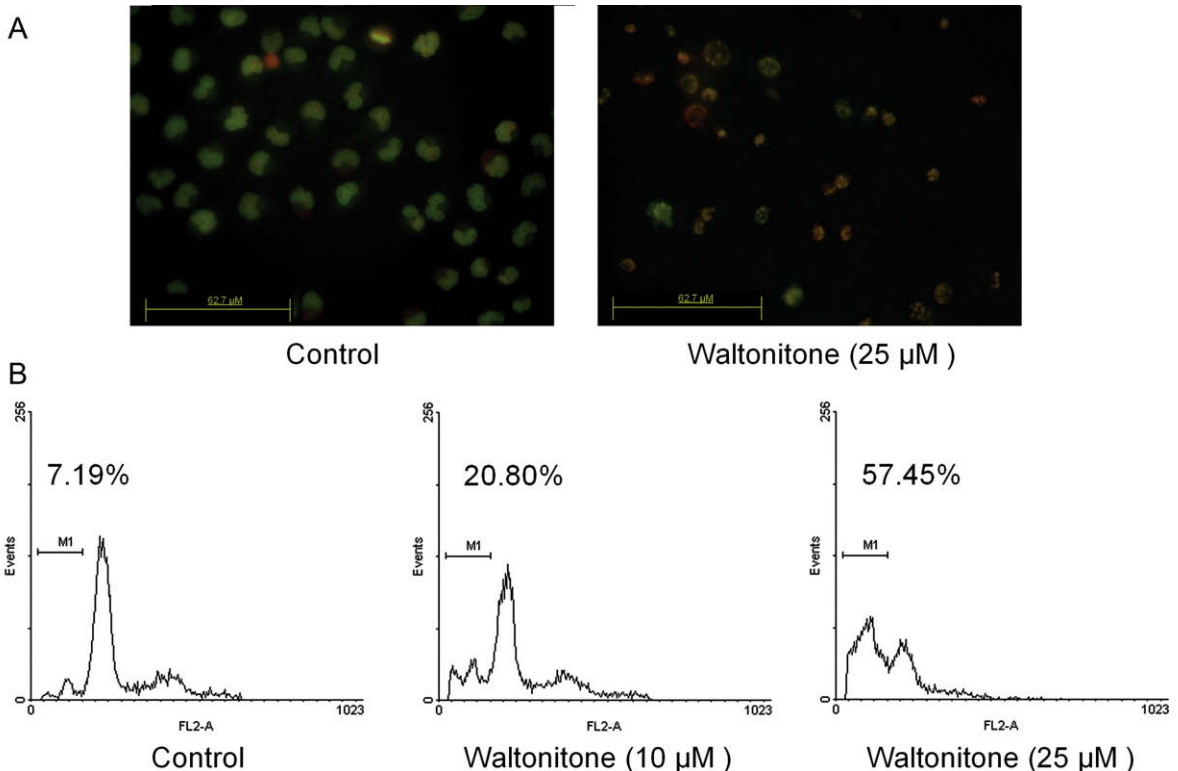


Fig. 3. Waltonitine induces BEL-7402 cells apoptosis. (A) Morphology changes in BEL-7402 cells treated with waltonitone (25 μM). After exposure to waltonitone (25 μM) for 48 h, cells were stained with AO/EB and examined using fluorescence microscopy. Apoptotic cells with fragmented nuclei (right) were observed in the waltonitone-treated cells (magnification 400 \times). (B) Flow cytometric analysis of propidium iodide stained DNA of BEL-7402 cells following waltonitone (10 μM , 25 μM) treatment for 48 h. Increased sub-G1 fraction (M1 fraction) in the waltonitone-treated cells provided an estimate of apoptotic cells. Data represented one of three times independent experiments.

3.2. Waltonitone induces BEL-7402 cells apoptosis

To determine whether the cell death was due to apoptosis induced by waltonitone, the morphological analysis with AO/EB staining and the fluorescence microscopy were employed. After 48 h exposure to waltonitone (25 μ M), BEL-7402 cells showed typical apoptotic characters, including cell shrinkage, orange nuclear margination, chromatin condensation and fragmentation, while the green live cells with normal morphology were observed in the untreated group (Fig. 3A). The percentage of apoptotic cells was determined by cell flow cytometric analysis following PI staining. Treatment with waltonitone (10 μ M, 25 μ M) for 48 h resulted in an increment of sub-G1 population (M1 fraction of Fig. 3B) in BEL-7402 cells, which were 20.80%, 57.45%, respectively, compared to 7.19% of untreated cells. These results revealed that the growth inhibition of waltonitone in BEL-7402 cells might be due to inducing cell apoptosis.

3.3. Waltonitone alters apoptosis-related genes expression

A human Oligo Apoptosis Microarray was used to assess mRNA level changes of 112 apoptosis-related genes in BEL-7402 cells by waltonitone treatment for 24 h. This array includes tumor necrosis factor (TNF) ligands and their receptors, members of the BCL-2 family, caspase family, death domain and death effector domain family, cell death-inducing DNA fragmentation factor (DAFF)-like effector (CIDE) domain family

and inhibitors of apoptosis (IAP), TNF receptor-associated factors (TRAF), caspase recruitment domain (CARD) family as well as genes involved in the ATM/p53 and anti-apoptotic pathways. Results showed that many apoptotic regulator genes expression altered in waltonitone-treated cells compared to the control (Fig. 4A). In details, 29 genes were found to be up-regulated more than twofolds, and 11 genes to be down-regulated. Of these, 15 genes belong to the BCL-2 family which indicated that BCL-2 family played an important role in the apoptosis induced by waltonitone. Up-regulation of apoptotic protease activating factor 1 (apaf-1), caspase-3, 9 and were also observed (Fig. 4B).

3.4. Activation of caspase-3 induced by waltonitone in BEL-7402 cells

To identify whether the caspases were involved in the mechanism of apoptosis herein, the mRNA expression and activation of caspase-3 were firstly detected in the waltonitone-treated cells by RT-PCR and Western blot analysis. Results showed the mRNA expression of caspase-3 significantly increased after 12 and 24 h treatment (Fig. 5A). This was confirmed by the appearance of 17 kD and 19 kD fragments of active enzymes following exposure to waltonitone for 48 h (Fig. 6A), suggesting waltonitone could effectively activate caspase-3 in BEL-7402 cells.

3.5. Waltonitone induces the apoptosis through the mitochondrial and Fas-FasL signaling pathways

To further explore the mechanism underlying the activation of caspase-3 by waltonitone, we study both the intrinsic mitochondria and the extrinsic death receptor signaling pathway involved in cell apoptosis.

Firstly, waltonitone (25 μ M) treatment caused an increment of BAX mRNA expression at 24 h (Fig. 5B). A temporal increase of caspase-9 mRNA expression accompanied with similar up-regulation tendency of apaf-1 was also observed (Fig. 5C). Moreover, cleavage of caspase-9 (35 kD and 37 kD) in waltonitone-treated cells was also detected with a concomitant decrease of full length of pro-caspase-9 (47 kD) (Figure 6B), suggesting a mitochondria pathway involved. Besides, waltonitone promoted the release of cytochrome *c* from mitochondria to cytosol (Fig. 6C) which confirmed our prediction.

In addition, the death receptor Fas and Fas ligand in BEL-7402 cells treated with waltonitone were examined. Results showed that the mRNA expression of Fas receptor and Fas ligand increased in a time-dependent way up to 12 or 24 h (Fig. 5D). Increasing expression of Fas ligand protein was also observed. The appearance of cleaved caspase-8 active fragments (43, 41 and 18 kD) and a reciprocal decrease of caspase-8 inhibitor FLIP (FLICE/caspase-8-like inhibitory protein) support the implication of Fas death receptor signaling pathway in waltonitone-induced apoptosis (Fig. 7).

Overall, these results demonstrated that waltonitone might induce tumor cell apoptosis via intrinsic mitochondrial and extrinsic death receptor signaling pathways.

3.6. Waltonitone inhibits BEL-7402 tumor growth in athymic mice in vivo

To test whether waltonitone could inhibit tumor growth, BEL-7402 cells xenografted into athymic mice model was used. Treatment of mice bearing tumors with waltonitone (20 mg/kg or 50 mg/kg once every two days intravenously) resulted in a significant tumor growth delay (Fig. 8A). However, this was not the case when the mice were administered with 10 mg/kg of waltonitone (data not shown). Moreover, treatment with waltonitone caused significant inhibition of tumor weight (Fig. 8B) without mice body weight significant changes compared to the control group (Fig. 8C). Furthermore, the increase of cleaved caspase-3 (17 kD), cleaved caspase-9 (35 kD) and concomitant decrease of pro-caspase-9 (47 kD) were also detected by Western blotting, however, without changes of FasL expression and cleaved caspase-8 (Fig. 8F) in the tumor tissues (Fig. 8D).

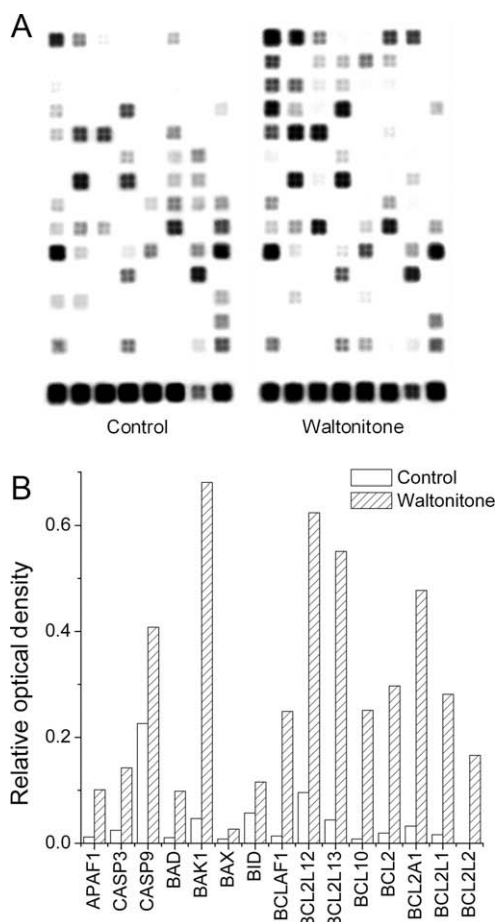


Fig. 4. Waltonitone alters several apoptosis-related genes expression. After treated without or with waltonitone (100 μ M) for 24 h, BEL-7402 cells were harvested and total RNA was extracted for microarray assay with Human Oligo Apoptosis Microarray. (A) Gene profiling of BEL-7402 cells treated without or with waltonitone. (B) Graphic representation of genes expression differences in BEL-7402 cells treated without or with waltonitone.

4. Discussion

Hepatocellular carcinoma (HCC) is a common hepatic highly malignant tumor with poor prognosis, which is characterized by rapid cell proliferation and strong anti-apoptosis genes expression signatures [21,22]. Recently,

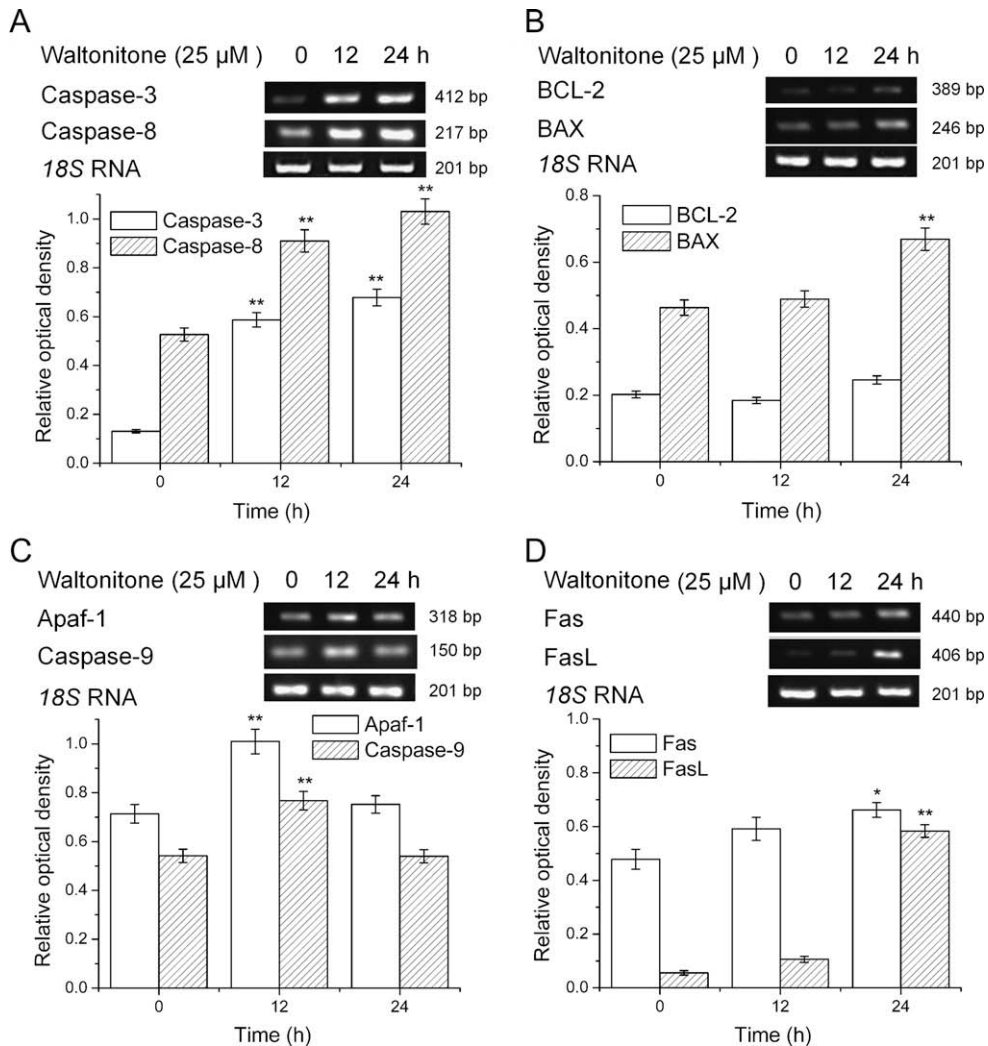


Fig. 5. Waltonitone up-regulates the mRNA expression of caspase-3, caspase-8, BAX, caspase-9, apaf-1, Fas and FasL in BEL-7402 cells. Cells were treated with waltonitone (25 μM) for indicated time, total RNA was immediately extracted for RT-PCR analysis using specific gene primers. (A) Caspase-3 and caspase-8. (B) BAX and BCL-2. (C) Apaf-1 and caspase-9. (D) Fas and FasL. 18S RNA was used as an internal standard to normalize loadings. Quantification of each band was performed and results were expressed as the ratio of genes/18S RNA. Data were means \pm SD for triplicate experiments (** $p < 0.01$, t -test).

people believe that apoptosis, especially the caspase mediated cell death, plays an important role in the etiology, pathogenesis and therapy of a variety of human malignancies, such as human HCC [23–25]. Apoptosis is one of the main types of programmed cell death (PCD). When occurs, the cell initiates a series of biochemical events leading to a variety of morphological changes, including budding, cell shrinkage, nuclear fragmentation, chromatin condensation and formation of membrane-bound apoptotic bodies. It is distinct from the necrosis of entire cell swell, lysis and then intracellular materials spilling out into the surrounding environment [26,27]. Many anti-cancer therapy drugs induce apoptosis to achieve therapeutic efficacy [25,28].

In this study, our results indeed demonstrate that waltonitone, a new ursane-type pentacyclic triterpene isolated from *G. waltonii* can significantly inhibit human hepatocellular carcinoma BEL-7402 cells growth. Although BEL-7402

cells growth was not inhibited after waltonitone addition for 24 h, but they became so over time (48 h or 72 h treatment) as waltonitone induced tumor cell apoptosis which confirmed by AO/EB cell staining and FCM analysis (Fig. 3).

Diverse groups of molecules are involved in the apoptotic pathway. One set of crucial mediators implicated in apoptosis belong to the cysteine aspartic acid protease (caspase) family. A member of this family, caspase-3 has been identified as the key executioner of apoptosis [29]. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated fragments [30]. We examined whether the possible apoptotic effect of waltonitone on BEL-7402 cells might link to an activation of caspase-3. Results showed that waltonitone induced an increase in caspase-3 mRNA expression and active cleaved caspase-3 fragments, suggesting the involvement of caspase in the apoptosis caused by waltonitone.

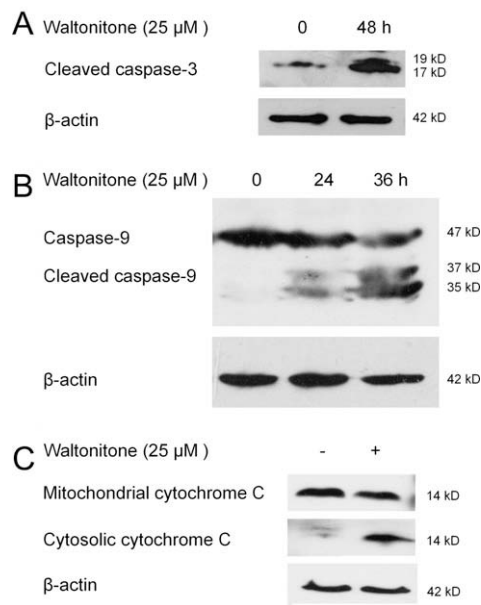


Fig. 6. Waltonitine activates caspase-3, caspase-9 and promotes cytochrome *c* release in BEL-7402 cells. Cells were treated with waltonitine (25 μM) for indicated time, caspase-3, caspase-9, cytochrome *c* and β-actin were determined by Western blotting. (A) Effect of waltonitine on activation of caspase-3 in BEL-7402 cells. Antibody for caspase-3 recognized the cleaved form (17, 19 kD). (B) Effect of waltonitine on activation of caspase-9 in BEL-7402 cells. Antibody for caspase-9 recognized the full length pro-enzymes (47 kD) and cleaved form (35, 37 kD). (C) Effect of waltonitine on cytochrome *c* distribution in mitochondria and cytosol. Mitochondria and cytosol were fractionated with a Cell Mitochondria Isolation Kit. Data represented one of three times independent experiments.

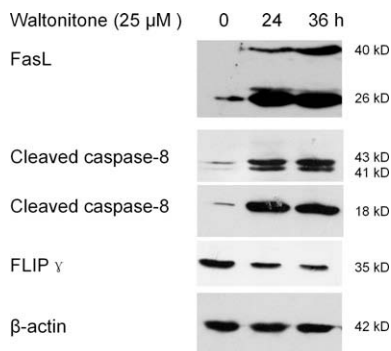


Fig. 7. Waltonitine increases FasL protein expression, decreases FLIP expression and activates caspase-8 in BEL-7402 cells. Cells were treated with waltonitine (25 μM) for indicated time, FasL, FLIP, caspase-8 and β-actin were determined by Western blotting. Antibody for caspase-8 recognized the cleaved form (18, 41, 43 kD). Data represented one of three times independent experiments.

Sequential activation of caspases plays a central role in the execution of cell apoptosis. It is reported that two major signaling pathways, the death receptor and the mitochondria pathway converge at the level of caspase-3 activation [31,32]. In terms of mitochondrial pathway, caspase activation is closely linked to mitochondrial outer

membrane permeabilization (MOMP) which is regulated by members of BCL-2 family. Pro- and anti-apoptotic BCL-2 family members compete to regulate an array of molecules such as cytochrome *c* exit. If the pro-apoptotic camp wins, cytochrome *c* leak out and be associated with apoptotic protease activating factor 1 (apaf-1) and then pro-caspase-9 to form the apoptosome in turn to activate the caspase-3 and cause apoptotic cell death [33–36]. The death-receptor pathway triggered death ligands (such as FasL) binding to their receptors (Fas) transmits a signal to the cytoplasm resulting in activation of caspase-8 and initiation of a caspase cascade. Caspase-8 activation can be blocked by FLICE/caspase-8-like inhibitory protein (FLIP) [36–38]. Actually, two pathways may have a cross-talk mediated by caspase-8, which can promote activities of pro-apoptotic BCL-2 members [27]. Data obtained in the present study allow us to propose a model for the regulation of apoptosis by waltonitine. The apoptosis microarray analysis showed the balance in cells treated with waltonitine was broke and a series of apoptotic-related genes were alerted especially BCL-2 family members. Many pro-apoptotic genes of BCL-2 family members such as BAD, BAX, BID, BAK, BCL2L12, BCL2L13 and BCLAF1 with apaf-1 and caspase-9 were up-regulated (Figs. 4 and 5) under waltonitine treatment. Furthermore, waltonitone promoted the release of cytochrome *c* from mitochondria to cytosol and activates caspase-9 (Fig. 6). Waltonitine could also increase the Fas/FasL expression, inhibit FLIP protein expression and activate caspase-8 (Figs. 5 and 7). These results indicated that waltonitine engages in multiple and distinct apoptosis pathways, including Fas/FasL and the mitochondrial signaling pathways. However, many anti-apoptotic BCL-2 family members such as BCL-2, BCL-10, BCL2A1 and BCL2L1 were also up-regulated. Although we speculated that it might be due to the negative feedback regulation or drug resistance, and the pro-apoptotic and anti-apoptotic members were competing until the pro-apoptotic camp won finally leading to apoptosis [33]. That was not the only feedback phenomenon since apoptosis is a quite complicated event. Apoptosis is also regulated by extracellular signals except for BCL-2 family as intracellular regulator. Recent studies showed that BCL-2 family is also concerned with tumorigenesis and cellular responses to anti-cancer therapy, and some BCL-2 inhibitors are designed for cancer therapies [39]. Whether BCL-2 in apoptosis induced by waltonitine is implicated in cellular feedback to anti-cancer therapy and drug resistance needs further studies.

The strong tumor inhibition properties as well as the caspase mediated apoptotic actions of waltonitine prompted us to evaluate its efficacy and safety to inhibit the tumor growth *in vivo*. In our preliminary experiments, waltonitine suppressed tumor growth at a dose of 50 mg/kg to an extent without any significant changes of body weight (Fig. 8C). Side effects, such as weight loss, hair loss, lethargy or dysphoria, macroscopical visceral pathogenic changes were not observed (data not shown). By the end of experiments, the final tumor weight was decreased by 50% compared to the control (Fig. 8A and B). These experiments firmly established the *in vivo* anticancer activity of waltonitine against the BEL-7402 cells

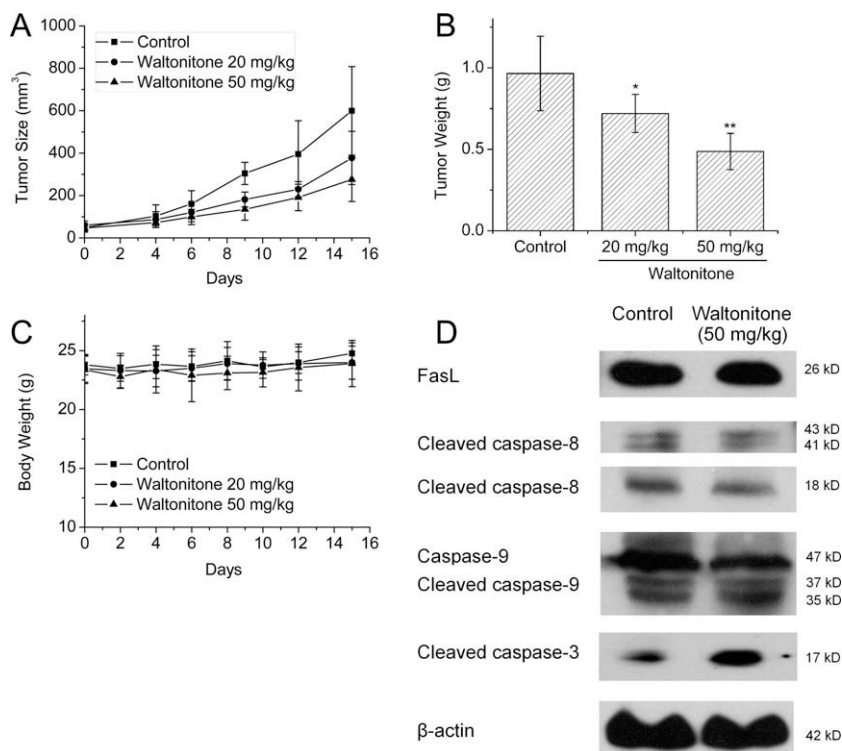


Fig. 8. Waltonitone inhibits BEL-7402 tumor growth in athymic mice *in vivo*. All mice were implanted by subcutaneously injection with BEL-7402 cells (1×10^7 cells/ml) in serum-free medium on the right flank. Seven days later, mice received intravenous injection of solvent vehicle (control) and waltonitone (20 mg/kg, 50 mg/kg) once every two days. (A) Tumor growth kinetics during administration of waltonitone for 15 days. (B) The final tumor weight at necropsy 15 days after administration. (C) Effect of waltonitone on the body weight of mice during 15 days. (D) Western blot analysis of apoptotic related proteins in the tumors from control and waltonitone (50 mg/kg) groups. Data were presented as means \pm SD, $n = 6$, * $p < 0.05$ and ** $p < 0.01$ compared with control.

xenografted hepatocellular carcinoma model with a reasonable safety margin. To validate the mechanism involved in the inhibition of tumor by waltonitone *in vivo*, we also examined the caspase cascade activation in tumors. Results showed that the mitochondria pathway maybe accounted for the apoptotic effect of waltonitone *in vivo*.

Taken together, these results suggest that the waltonitone may inhibit tumor cells growth by inducing apoptosis via death receptor and mitochondria pathways on human hepatocellular carcinoma *in vitro* and *in vivo*.

Conflicts of interest

We have no personal or financial conflict of interest on the submission of the manuscript entitled "Waltonitone induces human hepatocellular carcinoma cells apoptosis *in vitro* and *in vivo*" by Zhang Zhang, Shuo Wang, Hong Qiu, Chaohui Duan, Kan Ding and Zhengtao Wang to Cancer Letters. We also agree with the authorship as listed on the manuscript.

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