

## RESEARCH ARTICLE

# Beta-asarone Induces LoVo Colon Cancer Cell Apoptosis by Up-regulation of Caspases through a Mitochondrial Pathway *in vitro* and *in vivo*

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### Abstract

Beta-asarone is one of the main bioactive constituents in traditional Chinese medicine *Acorus calamus*. Previous studies have shown that it has antifungal and anthelmintic activities. However, little is known about its anticancer effects. This study aimed to determine inhibitory effects on LoVo colon cancer cell proliferation and to clarify the underlying mechanisms *in vitro* and *in vivo*. Dose-response and time-course anti-proliferation effects were examined by MTT assay. Our results demonstrated that LoVo cell viability showed dose- and time-dependence on  $\beta$ -asarone. We further assessed anti-proliferation effects as  $\beta$ -asarone-induced apoptosis by annexin V-fluorescein isothiocyanate/propidium iodide assay using a flow cytometer and observed characteristic nuclear fragmentation and chromatin condensation of apoptosis by microscopy. Moreover, we found the apoptosis to be induced through the mitochondrial/caspase pathway by decreasing mitochondrial membrane potential (MMP) and reducing the Bcl-2-to-Bax ratio, in addition to activating the caspase-9 and caspase-3 cascades. Additionally, the apoptosis could be inhibited by a pan-caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK). When nude mice bearing LoVo tumor xenografts were treated with  $\beta$ -asarone, tumor volumes were reduced and terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assays of excised tissue also demonstrated apoptotic changes. Taken together, these findings for the first time provide evidence that  $\beta$ -asarone can suppress the growth of colon cancer and the induced apoptosis is possibly mediated through mitochondria/caspase pathways.

**Keywords:** Beta-asarone - colon cancer cells - apoptosis - mitochondrial membrane potential - caspase

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### Introduction

Epidemiological studies have demonstrated that the incidence of colorectal cancer (CRC) rank third in men and second in women worldwide (Parkin et al., 2005; Jemal et al., 2011). Although the overall incidence and mortality have steadily declined in recent decades, CRC remains one of the leading causes of cancer related deaths.

Chemotherapy is well known as one of the effective treatments for CRC besides surgery. Conventional chemotherapy of CRC with 5-fluorouracil (5-Fu) in combination with other anti-cancer agents improves overall and disease-free survival of patients after surgery (Machover, 1997). But the effectiveness of chemotherapeutic agents is often restricted due to high frequency of severe side effects and drug resistance. Therefore, the research and development of new treatment strategies for CRC is urgently needed. Nowadays, apoptosis-inducing anticancer therapy is considered to be a promising way. More and more apoptosis-targeted drugs or agents are exploited for therapeutic benefit (Cummings

et al., 2004).

*Acorus calamus* has been widely used alone or combined with other herbs in traditional Chinese medicine over centuries. Recent studies have suggested that  $\beta$ -asarone is one of the main bioactive constituents of its essential oil. Growing evidence has demonstrated that  $\beta$ -asarone has the properties of antifungal (Lee et al., 2004) and anthelmintic activity (Lee et al., 2008; Kumar et al., 2009), modulating neurotransmission (Qiu et al., 2011), blocking cholesterol biosynthesis (Lee et al., 2010) and tranquilization or central inhibitory activity (Liao et al., 1998; Fang et al., 2008). However, little is known about its inhibitory effect on cancer cells and the precise mechanism behind it remains unclear.

In the present study, we examined the apoptosis-inducing effects of  $\beta$ -asarone on human LoVo colon cancer cells *in vitro* and *in vivo*. We further identified the possible mechanisms underlying apoptosis induction, particularly focusing on the change of mitochondrial membrane potential (MMP), the activation of caspase-9, caspase-3 and the ratios of Bcl-2/Bax and Bcl-xL/Bax. The

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results demonstrated that  $\beta$ -asarone treatment can activate caspases via depletion of MMP. Our study suggests that  $\beta$ -asarone exhibits the anticancer properties by up-regulation of caspases activity through the mitochondrial pathway in human colon cancer cells.

## Materials and Methods

### Chemicals and antibodies

Beta-asarone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and monoclonal mouse  $\beta$ -actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to caspase-3 or caspase-9 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Bcl-2 or Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase (HRP) labeled goat anti-mouse or anti-rabbit IgG antibody were from Beijing Zhongshan Biotech Company (Beijing, China). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was from BD Biosciences (San Diego, CA). carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) were from Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM), MitoProbe JC-1 (5, 5', 6, 6'-Tetrachloro-1, 1', 3, 3'-tetraethyl-imidacarbocyanine iodide) assay kit for flow cytometry, TRIzol reagent and Power SYBR Green PCR Master Mix were from Life Technologies (Grand Island, NY). Primescript RT reagent Kit with gDNA Eraser was from TaKaRa (Dalian, China). The 5-Fu was purchased from Jinyao amino acid company (Tianjing, China). All other chemicals used were of analytical grade.

### Cell culture

LoVo, human colon cancer cell lines, were obtained from Type Culture Collection, Chinese Academy of Sciences (Shanghai, China) and propagated in DMEM supplemented with 10% bovine serum, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) at 37 °C in a water-saturated atmosphere with 5% CO<sub>2</sub>. The cells were cultured on 60mm or 100mm-diameter cell culture dishes and all studies were performed with cells at ~50% confluence. Cells treated with equal amount of vehicle (dimethyl sulfoxide, DMSO) alone were used as a negative control and treated with 5-Fu were as the positive control.

### MTT assays

Cells in the logarithmic growth phase were seeded on the 96-well plate at 5×10<sup>3</sup> cells/well. After cells adhered to the plate surface, media were replaced containing different concentrations of  $\beta$ -asarone (0  $\mu$ M as control, 200  $\mu$ M and 400  $\mu$ M) for different groups.  $\beta$ -asarone was dissolved in DMSO to make a stock solution of 500 mM, and was diluted to 200  $\mu$ M and 400  $\mu$ M with medium for treatment, which means the DMSO final concentration were equal or less than 0.08% (V/V). Each group had triplicate wells for 24 and 48 h treatment respectively. We also pre-treated the cells with the pan-caspase inhibitor, Z-VAD-FMK (100  $\mu$ mol/L) for 2 h, then induced the cells by 200  $\mu$ M  $\beta$ -asarone for 24 h. To detect the cell viability, cells were incubated with 20  $\mu$ l of 5 mg/ml MTT for 4 h at 37 °C.

Following MTT incubation, cells were lysed in 150  $\mu$ l of DMSO and the dark blue formazan precipitates were dissolved. Optical density was measured using ELx800 microplate reader (BioTek, Winooski, VT) at 490 nm, and the mean of three readings was used for calculations.

### Hoechst 33342 staining

LoVo cells were plated at a density of 3 × 10<sup>5</sup> cells per well onto cover slips in 6-well plates. After incubation for 24 h, the cells were treated with the 0.2 mM or 0.4 mM  $\beta$ -asarone for 24 h. The cells were washed with PBS and then incubated with the Hoechst labeling solution for 20 min in CO<sub>2</sub> incubator. After rinsed three times in phosphate buffer saline (PBS), the cells were analyzed via fluorescent microscope. Normal nuclei was identified as non-condensed chromatin dispersed over the entire nucleus and apoptotic nuclei was identified as condensed chromatin, contiguous with the nuclear membrane and/or fragmented nuclei.

### Electron microscopy

For electron microscopy analysis, the cells were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in PBS, postfixed in 1% osmium tetroxide in PBS, dehydrated, and embedded in epoxy resin. Ultrathin (80 nm) sections were stained with uranyl and lead acetates and examined with a JEM-1010 electron microscope (JEOL, Japan) at 80 kV.

### Apoptosis assays and measurement of MMP by flow cytometry

Cells treated with or without  $\beta$ -asarone for 24 h or 48 h were tested by annexin V-FITC apoptosis detection kits (BD Pharmingen). Briefly, cells were harvested and washed twice with cold PBS and resuspended in 1×binding buffer, and then 5  $\mu$ l annexin V-FITC and 5  $\mu$ l propidium iodide (PI) were added to about 1×10<sup>5</sup> cells. Cells were incubated at room temperature for 15 min in the dark, and then 400  $\mu$ l 1×binding buffer was added. The cells were analyzed by flow cytometry. Annexin V binds to phosphatidylserine exposed on the plasma membrane of cells undergoing apoptosis. This allows the discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (stained only with annexin V) and late apoptotic/necrotic cells (stained with both annexin V and PI). We also used Z-VAD-FMK (100  $\mu$ M) to treat the LoVo cells for 2 h, and then induced by the 0.4 mM  $\beta$ -asarone as comparison.

The lipophilic cationic probe, JC-1 was used to estimate the MMP as previously described by Ghavami et al (Ghavami et al., 2010). JC-1 is capable of selectively entering mitochondria, where it forms monomers and emits green fluorescence when MMP is relatively low. At a high MMP, JC-1 aggregates and emits red fluorescence (Ghavami et al., 2008). The ratio between red and green fluorescence provides an estimate of MMP that is independent of the mitochondrial mass. LoVo cells were plated in 60-mm culture dishes and treated with 0.2 and 0.4 mM  $\beta$ -asarone for 24 h or 48 h. The cells were incubated for another 30 min at 37 °C with the fresh culture medium containing JC-1 (2.5  $\mu$ g/ml) and 10 mM glucose, and then

washed once with DMEM. The red and green fluorescence were monitored with a flow cytometry. Decline of the ratio of red to green fluorescence indicates the loss of MMP.

#### Western blotting analysis

To isolate total proteins, both untreated and  $\beta$ -asarone-treated cells (0.2 mM and 0.4 mM for 24 h) cultured in 100 mm-diameter culture dishes for 24 h were rinsed twice with ice-cold PBS, harvested by using cell scraper. After centrifugation, the cell pellets were resuspended in 250  $\mu$ l of RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate) containing protease inhibitor cocktail (P8340, Sigma-Aldrich), lysed for 30 min on ice, and spun by centrifugation at 12000 g for 15 min at 4°C. Protein concentrations were determined by the Bradford method. Samples containing equal proteins (25  $\mu$ g) were loaded and analyzed by Western blot assay. Briefly, proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Membrane were incubated with blocking buffer (5% non-fat dry milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20) for at least 30 min at room temperature. Membranes were then incubated with appropriate primary antibodies in the above solution on an orbit shaker at room temperature for at least 1 hour or at 4 °C overnight. Then, membranes were washed in Tris-buffered saline Tween (TBST, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20) for three times and incubated with HRP-linked secondary antibodies (anti-rabbit or anti-mouse IgG). After the membranes were washed three times with TBST, the signals were detected by enhanced chemiluminescence kit (Millipore, Billerica, MA). Beta-actin was used as a loading control.

#### Caspase-3 activity assays

For caspase-3 activity detection, the Apo-ONE homogeneous Caspase-3/7 assay kit (Promega, Madison, WI) was used according to the manufacturer's instructions. The reaction buffer containing substrate (Ac-DEVD-R110) were added to the equal amounts of cells with or without  $\beta$ -asarone treatment for 24 h and then incubated at 37 °C for 4 h in the dark. Absorbance of the released Rhodamine 110 was measured at 499 nm using a fluorescence ELISA reader (Molecular Devices, Sunnyvale, CA).

#### Total RNA isolation and quantitative RT-PCR

Total RNA was isolated using the TRIzol reagent, as described by the manufacturer. Briefly, after treated with  $\beta$ -asarone for 24 h, the cells of treated and control were resuspended in 1 ml of TRIzol. The suspension was then extracted with 0.2 ml of chloroform. After centrifugation the aqueous phase was mixed with 0.5 ml of isopropyl alcohol. The resulting pellet was washed with 0.7 ml of 75% ethanol and finally resuspended in 50  $\mu$ l of RNase-free water. All total RNA samples were kept at -80°C until use.

Expressions of target genes were analyzed by ABI

**Table 1. Sequences of Primers Used in the Real-time PCR Amplifications**

Gene	Primer sequences (5'-3')	Length of PCR Product (bp)
Bax	F: TTTGCTTCAGGGTTTCATCC R: GCCACTCGGAAAAAGACCTC	213
Bcl-2	F: TCGCCCTGTGGATGACTGAG R: CAGAGTCTTCAGAGACAGCCAGGA	143
Bcl-xl	F: ATGAACTCTTCCGGGATGG R: TGGATCCAAGGCTCTAGGTG	166
Survivin	F: TTCTCAAGGACCACCGCATC R: GCCAAGTCTGGCTCGTTCTC	127
ATF4	F: CCACTAGGTACCGCCAGAAG R: GCCTTGCGGACCTCTTCTAT	157
XBP1	F: TGCTGAGTCCGCAGCAGGTG R: GCTGGCAGGCTCTGGGGAAG	169
CHOP	F: ATGGCAGCTGAGTCATTGCCTTTC R: AGAAGCAGGGTCAAGAGTGGTGAA	177
GAPDH	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC	66

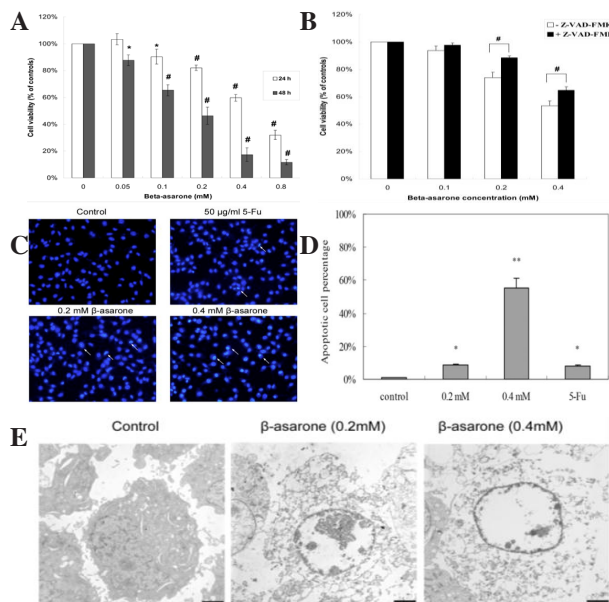
7900HT fast real time PCR System and Power SYBR Green PCR Master Mix. The primers for human Bax, Bcl-2, Bcl-xL, surviving, CHOP, XBP1, ATF4 and GAPDH were designed using Primer Express were as shown in Table 1. The cDNA was synthesized from 500 ng RNA using the reverse transcription reagent Kit. The optimized concentrations for real-time PCR were 0.2  $\mu$ M for both primers in a 10  $\mu$ l reaction volume. Human GAPDH expression was used as internal controls. Each sample was tested in triplicate. Cycle threshold (Ct) values were obtained graphically for the target genes and GAPDH. The difference in Ct values between GAPDH and target genes were represented as  $\Delta$ Ct values.  $\Delta\Delta$ Ct values were obtained by subtracting  $\Delta$ Ct values of control samples from those of treated samples. The relative fold change in gene expression was calculated as  $2^{-\Delta\Delta Ct}$ .

#### Animals and tumor implantation

All mice were handled according to the Guide for the Care and Use of Laboratory Animals. Mouse studies were carried out following the procedures approved by the Institutional Animal Care and Use Committee at Nanjing University of Chinese Medicine. LoVo cancer xenografts were established by the subcutaneous implantation of LoVo cells ( $5 \times 10^6$  cells) at the right flank area of 6- or 7-week-old nude mice (nu/nu strain, Shanghai laboratory animal center, Chinese academy of sciences). For analysis of efficacy, when the average tumor volume reached approximately 200 mm<sup>3</sup> (about 2 weeks after injection), mice were treated with  $\beta$ -asarone p.o. (50 mg/kg body weight, n=6) daily for 10 days, or the placebo (0.9% saline) p.o. daily. When the treatment began, the mean tumor volumes were calculated for each group by caliper measurements using the following formula: tumor volume = (length  $\times$  width<sup>2</sup>)/2. The animals were sacrificed three weeks (24 days) after injection of LoVo cells and the tumor weight were measured.

#### Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay

Histological analysis of DNA fragmentation was used to identify dying cells in paraffin sections of the



**Figure 1. Anti-proliferation of  $\beta$ -asarone on LoVo Cells and Cellular Structure Change.** (A) MTT assays showed that LoVo cells viability demonstrated a dose- and time-dependence on  $\beta$ -asarone treatment at the indicated concentration (0 – 0.8 mM) for 24 or 48 h. \*  $p < 0.05$ , #  $p < 0.01$ . The data represent the means  $\pm$  SE from four independent experiments. (B) MTT assays showed that LoVo cells viability demonstrated pre-treatment of Z-VAD-FMK (100  $\mu$ M) can attenuate the apoptosis induced by  $\beta$ -asarone. (C) Cells morphology was observed under fluorescence microscope by staining with Hoechst 33342. Arrows indicated apoptotic cells with fragmented nuclei and nuclear shrinkage. Magnification  $\times 200$ . (D) The quantifications of apoptotic cells in under fluorescence microscope. (E) Transmission electron microscopic observation (8000 $\times$ ). Compared with normal microstructure, the treated cells undergone nuclear shrinking, cytoplasmic vacuolization, and expansion of endoplasmic reticulum and accumulation of condensed chromatin, which indicates apoptosis. Scale bar = 2  $\mu$ m

xenografts. The in situ Apoptosis Detection Kit (Boster Company, Wuhan, China) was used to detect apoptotic cells according to the manufacturer's protocols. Briefly, biotinylated nucleotides were added to DNA breaks with free 3'-hydroxyl residues by terminal deoxynucleotidyl transferase. Labeled DNA was then identified with the avidin peroxidase, with diaminobenzidine used as a substrate. Hematoxylin was used as a counterstain. The apoptotic cells show brown articles in the nuclei.

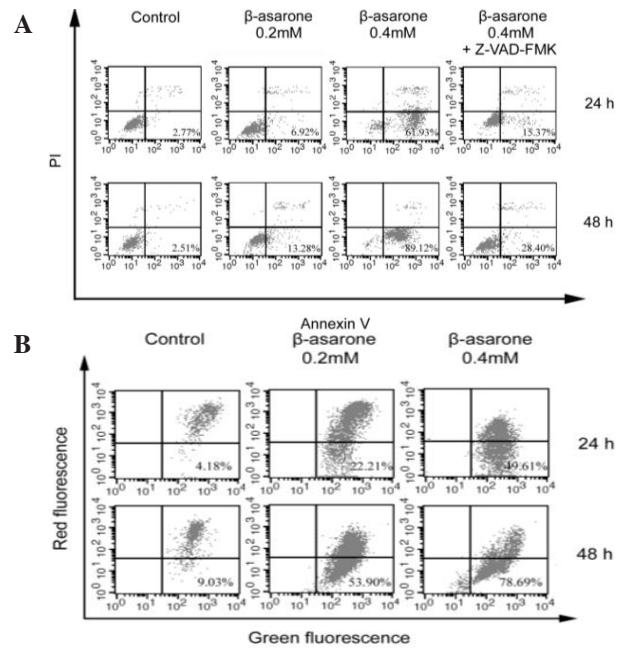
#### Statistical Analysis

Each experiment was replicated for at least three times. Results were presented as the mean  $\pm$  standard deviation (SD), and statistical comparisons were made using the student's t test or one-way ANOVA test. Significance was defined as the  $P < 0.05$  or 0.01.

## Results

### *Beta-asarone exerted the anti-proliferative effects on LoVo cells in vitro*

We first examined whether  $\beta$ -asarone has the inhibitory effect on colon cancer cells proliferation. MTT assay results showed that the growth of LoVo colon cells was

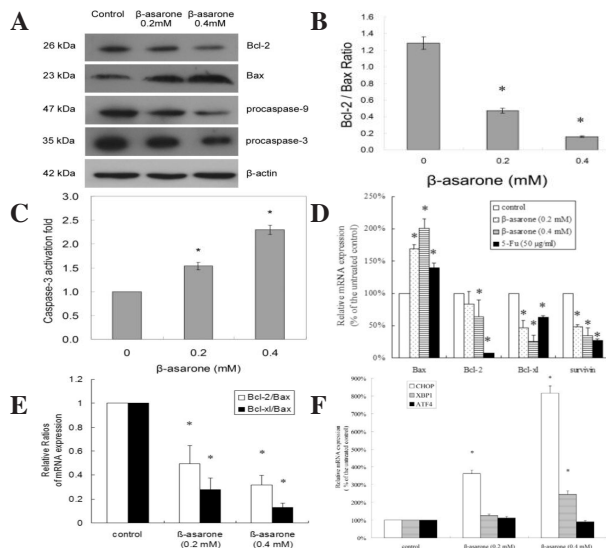


**Figure 2. Flow Cytometric Analysis of Apoptosis by Annexin-V/propidium Iodide (PI) Staining and MMP by JC-1 Staining.** (A) LoVo cells were treated with 0.2 mM and 0.4 mM  $\beta$ -asarone for 24 h or 48 h. The dual parameter dot plots combining annexin V-FITC and PI fluorescence showed the viable cell population in the lower left quadrant (annexin V-PI-), the early stage apoptotic cells in the lower right quadrant (annexin V+PI-), the late stage apoptotic or dead cells in the upper right quadrant (annexin V+PI+). (B) The MMP after treatment were measured using JC-1 staining by flow cytometry. The reduced MMP indicated by a decrease in red fluorescence. The green fluorescence represents the monomeric form of JC-1. The data are representative of three independent experiments

suppressed by  $\beta$ -asarone in both dose- and time-dependent manner (Figure 1A). When we treated LoVo cells with 0.2 mM  $\beta$ -asarone, the cell viability rate declined to  $82.0\% \pm 2.0\%$  at 24 h and further down to  $46.0\% \pm 6.4\%$  at 48 h. The inhibitory rate of cell growth was obviously higher at 48 h than that at 24 h in each dose of  $\beta$ -asarone. After 0.8 mM  $\beta$ -asarone treatment for 48 h, the growth of  $88.0\% \pm 2.1\%$  cells was suppressed. Meanwhile, before treated with 0.2 mM  $\beta$ -asarone, if the cells were pre-treated with Z-VAD-FMK, the corresponding cell viability rates were increased (Figure 1B).

### *Beta-asarone changed the ultra structure of LoVo cells*

To check the ultra structure change of LoVo cells after treatment, we used both fluorescence microscope and transmission electron microscope to observe the change (Figure 1C, D). The Hoechst staining showed that after  $\beta$ -asarone treatment, some nuclei become condense and fragmented, which indicated apoptosis. The transmission electron microscope showed that LoVo cells lost normal structure after treatment and exhibited expansion of the endoplasmic reticulum, vacuolization in the cytoplasm, shrinkage and fragmentation of the nucleus and densification of the chromatin, all of which are the signs of early-stage apoptosis. In contrast, the LoVo cells used as negative control had regular nucleus and evenly distributed chromatin structures. From the figures, we can easily conclude that the normal nucleic structures were



**Figure 3. Western Blot and Real-time PCR Analysis of the Apoptosis-associated Molecules.** (A) Representative image of the western blot analysis assaying for Bcl-2 (26 kDa), Bax (23 kDa), procaspase-9 (47 kDa) and procaspase-3 (35 kDa). Expressions of Bcl-2, procaspase-9, procaspase-3 were significantly reduced when the LoVo cells were treated with 0.2 mM or 0.4 mM  $\beta$ -asarone for 24 h, while the reverse was true for the expression of Bax. (n=3) (B) Ratios of Bcl-2 to Bax expression based on Western blot results. \* $p < 0.01$  vs. the control group. (C) Caspase-3 activity, determined by a colorimetric assay kit using the specific substrate Ac-DEVD-R110. Data show mean  $\pm$  SD (n = 6 in each group). \* $p < 0.01$  vs. the control group. (D) Treatment with  $\beta$ -asarone changes Bax, Bcl-2, Bcl-xL, and survivin mRNA expression. LoVo cells were treated for 24 h with  $\beta$ -asarone (0.2 mM or 0.4 mM) or DMSO as vehicle. The mRNA expressions were determined by quantitative RT-PCR and calculated as % of DMSO-only control. GAPDH was as internal control and 50  $\mu$ g/ml 5-Fu as a positive control. \* $p < 0.05$ . (E) The Bcl-2/Bax and Bcl-xL/Bax ratios of mRNA expression in LoVo cells treated with 0.2 mM or 0.4 mM  $\beta$ -asarone. \* $p < 0.05$ . The data represent the means  $\pm$  SE from three independent experiments. (F) The change of CHOP, XBP1, ATF4 mRNA expression induced by  $\beta$ -asarone. \* $p < 0.05$  vs. the control group

changed by  $\beta$ -asarone, indicating that it might have the apoptosis-inducing activity.

#### *Beta-asarone induced LoVo cell apoptosis*

To further confirm the apoptosis-inducing activity of  $\beta$ -asarone, we examined LoVo cells by annexin V-FITC/PI staining. Induction of apoptosis was rigorously substantiated by examining annexin V-FITC stained cells by flow cytometry. After analyzing the results, we could find the apoptotic cells accounted for 6.92% and 61.93% of the cells in early apoptosis (lower right quadrant) with  $\beta$ -asarone treatment for 24 h at 0.2 mM and 0.4 mM, respectively (Figure 2A). When we prolonged the treatment time to 48 h, the corresponding ones (lower right quadrant) were account for 13.28% and 89.12% respectively. Meanwhile, when we pretreated the cells with pan-caspase inhibitor, Z-VAD-FMK (100  $\mu$ M) for 2 h, the 0.4 mM  $\beta$ -asarone induced apoptosis had been partially suppressed. Taken together, our results showed that  $\beta$ -asarone can effectively initiate apoptosis.

#### *Beta-asarone induced loss of MMP in LoVo cells*

Since the change of MMP is commonly involved in the series of events leading to the activation of caspases as effectors, we performed an assay to examine MMP by means of the potential sensitive JC-1, a cationic dye that exhibits potential dependent accumulation in mitochondria. As shown in Figure 2B, after LoVo cells were treated with 0.2 mM and 0.4 mM  $\beta$ -asarone for 24 h, flow cytometric data suggested the red fluorescence intensity were significantly decreased and revealed that disruption of MMP, which were 22.21% and 49.61% respectively, compared with only 4.18% in the control group. From these data, we could conclude that  $\beta$ -asarone can down-regulate MMP in a dose-dependent manner, indicating the important role of mitochondria in  $\beta$ -asarone induced apoptosis.

#### *Beta-asarone altered the levels of Bcl-2 family proteins and induced caspase-3 and caspase-9 activation in LoVo cells*

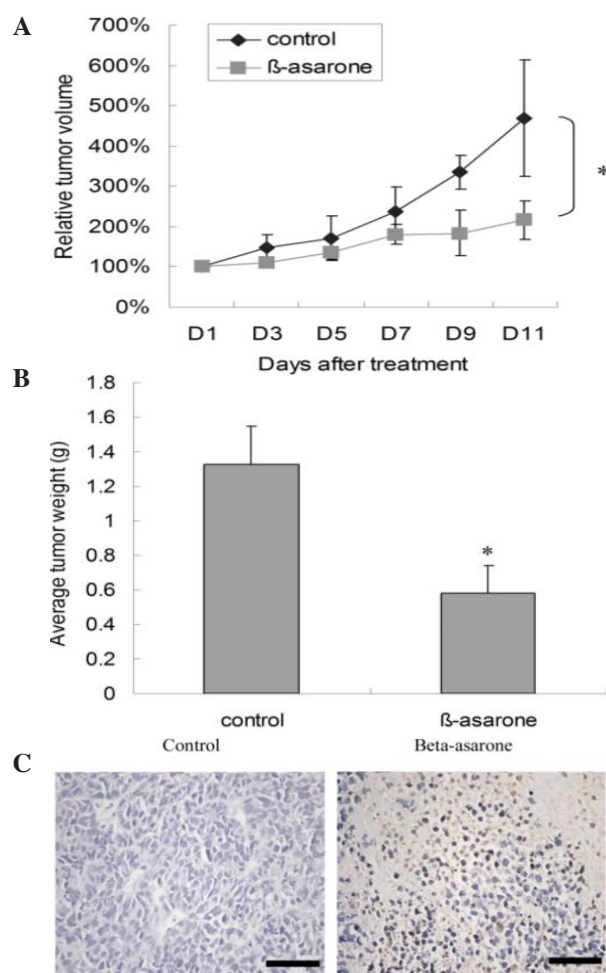
In order to characterize molecular change of  $\beta$ -asarone-treated LoVo cells, we also performed Western blot to analyze the apoptotic proteins of interest. As shown in Figure 3A, the results demonstrated that Bax protein levels increased gradually in a dose-dependent manner within total cell lysates while Bcl-2, pro-caspase-3 and pro-caspase-9 decreased, indicating that increasingly higher  $\beta$ -asarone concentrations enhanced caspase-3 and caspase-9 in their active forms. A significant decrease of Bcl-2/Bax ratio was observed when cells were treated with  $\beta$ -asarone as compared to the control group (Figure 3B). Caspase-3 activity assay using a colorimetric method further confirmed its activation in the presence of  $\beta$ -asarone. Treatment of LoVo cells resulted in a dose-dependent increase of caspase-3 activity (Figure 3C). Therefore,  $\beta$ -asarone mediated a cascaded series of molecular events that led to an attenuated level of Bcl-2, augmented level of the pro-apoptotic protein Bax, and activation of the executor apoptosis enzyme caspase-3.

#### *Beta-asarone altered Bcl-2 family and endoplasmic reticulum (ER) stress associated molecules mRNA expression levels*

Apoptosis can be initiated and balanced by the pro- and anti-apoptotic factors of the BCL-2 family. We determined the relative mRNA expression levels of Bax, Bcl-2, Bcl-xL, survivin, GAPDH and further calculated the Bcl-2/Bax and Bcl-xL/Bax ratios in LoVo cells after treated with  $\beta$ -asarone for 24 h. Both ratios shifted in favor of the pro-apoptotic Bax (Figure 3D, E). Meanwhile, we detected the mRNA expression levels of the ER stress associated molecules, including CHOP, XBP1, and ATF4. The results showed that CHOP and XBP1 mRNA expression were up-regulated while the change of ATF4 was not obviously (Figure 3F).

#### *Beta-asarone inhibits the growth of LoVo tumor xenografts in nude mice*

After the growth-suppressing activity of  $\beta$ -asarone was verified in vitro, we next investigated its effect in



**Figure 4. The Inhibitory Effect of  $\beta$ -asarone on the LoVo Cancer Xenografts.** (A) The comparison of tumor relative volumes between groups since treatment. The tumor volumes were measured every other day. Results are expressed as the means  $\pm$  SE (n=6). (B) The tumor weight comparison between the two groups when the mice were sacrificed. \*  $p < 0.05$ . (C) TUNEL assay. Apoptotic cells were seen in the tumors treated with  $\beta$ -asarone (original magnification  $\times 200$ ). Bar=100  $\mu$ m

vivo on the growth of LoVo tumor xenografts. As shown in Figure 4A, the size of LoVo tumor xenografts treated with the placebo increased proportionally during the treatment. Treatment with  $\beta$ -asarone at 50 mg/d/kg body weight concentration suppressed the growth of the tumors significantly. In comparison with the placebo controls, the tumor suppression by  $\beta$ -asarone reached statistical significance ( $P < 0.01$ ). Meanwhile, the suppressive effect of  $\beta$ -asarone on the tumor growth is obvious when we compared the excised tumor weights between the groups (Figure 4B).

#### TUNEL assay indicates the apoptosis in tumor xenografts

To determine whether the tumors in the  $\beta$ -asarone-treated animals were growth-inhibited through apoptosis, we subjected the isolated tumors to the TUNEL assay. The degree of apoptosis in the tumors in the treatment group was significantly higher than that seen in the control group. TUNEL assay confirmed the fragmentation of DNA in the growth-inhibited tumors for these mice (Figure 4C).

## Discussion

In the present study, for the first time we demonstrated that: (1)  $\beta$ -asarone decreases the viability of LoVo colon cancer cells in a time- and dose-dependent manner; (2) Apoptosis and necrosis in  $\beta$ -asarone-treated LoVo cells were revealed with flow cytometry by annexin V-FITC and PI labeling and with microscope observations; (3) Mechanistic evidence of apoptosis was drawn from the results that  $\beta$ -asarone depleted MMP, activated caspase-9 and caspase-3, and decreased the ratio of the survival protein Bcl-2 or Bcl-xL to the pro-apoptotic protein Bax; (4) The  $\beta$ -asarone inhibits the tumor growth and induces apoptosis in mice tumor xenografts model. Therefore, we provided experimental evidence that the anti-cancer effect of  $\beta$ -asarone results from cell apoptosis.

It has been already reported that  $\beta$ -asarone can serve as the anti-epileptic (Fu et al., 2008) because the pharmacokinetics studies confirmed that it can easily pass the blood brain barrier (Wu and Fang, 2004). It is also used as the antifungal agent (Lee et al., 2004), anthelmintic (Kim et al., 2008) or nematocide (Sugimoto et al., 1995). To our knowledge, the action of  $\beta$ -asarone in colon cancer cell lines has never been studied. For the first time we reported the growth inhibitory effect of  $\beta$ -asarone on LoVo cells by inducing the cells apoptosis, which might extend its potential usage.

Liu et al. (2010) and Zou et al. (2011) have reported that beta-asarone can attenuate neuronal apoptosis by amyloid in rat hippocampus. Their results had shown that beta-asarone has the ability to attenuate the apoptosis, which is different from ours. However, in those studies, the researchers used the rat hippocampus of Alzheimer's disease model rather than human LoVo colon cancer cells. At the same time, we do not know the interaction of beta-asarone with amyloid beta peptide used in the modeling.

In our study we also performed the assays to observe whether apoptosis accounts for the anti-proliferation effect. After flow cytometry analysis, we confirmed the apoptosis can be induced by  $\beta$ -asarone. Although the effective inhibitory concentration of  $\beta$ -asarone is not exciting, the extension of its effects to anticancer will cause researcher's attention.

Apoptosis is a process of programmed cell death, which plays a crucial role in maintaining cellular homeostasis between cell division and cell death (Wong, 2011). Apoptosis results in cellular self-destruction that involves specific morphological and biochemical changes in the nuclei and cytoplasm. Therefore, researchers nowadays had performed anti-cancer studies on natural compounds as well as herbal extracts based on the biochemical properties of apoptosis (Li et al., 2009; Meiyanto et al., 2012). Our results showed that after treatment with 0.2 mM and 0.4 mM  $\beta$ -asarone the nuclei of cells showed shrinkage and fragmentation with the vacuolization in the cytoplasm, which indicated the cellular apoptosis.

The caspases, known as cysteine proteases, play crucial roles in cell apoptosis. Caspases can be classified into two groups according to their function and structure: the initiator caspases (caspase-2, 8, 9, 10) and the

executioner caspases (caspase-3, 6, 7) (Thornberry and Lazebnik, 1998; Kuribayashi et al., 2006). After we demonstrated the apoptosis induced by  $\beta$ -asarone, we continued to examine the expression levels of caspase-3 and caspase-9, which are the key enzymes required in the caspase cascade activation and execution (Degterev and Yuan, 2008).

The caspase-dependent apoptotic pathways can be generally divided into the extrinsic and the intrinsic. The former includes signaling through the death receptors while the latter involves a mitochondria pathway (Denault and Boatright, 2004). To clarify whether the intrinsic pathways is involved in  $\beta$ -asarone-induced apoptosis, we performed western blotting experiments. Caspase-9 is in the intrinsic pathway and sequential activation of caspase-9 and caspase-3 results in cell apoptosis (Cho and Choi, 2002). We found procaspase-9 and procaspase-3 were all cleaved in LoVo cells following  $\beta$ -asarone treatment (Figure 3A), which suggests that  $\beta$ -asarone contributes to LoVo apoptosis by activating the intrinsic caspase-9/caspase-3 controlled pathway. Our results indicated that the apoptosis might be through the intrinsic pathway. In addition, more research need to perform to confirm whether the extrinsic pathway play a role in the apoptosis.

Mitochondria played a major role in apoptosis triggered by many stimuli. Mitochondrial originated apoptotic signals include the increase of outer mitochondrial membrane permeability, loss of MMP, failure of  $\text{Ca}^{2+}$  flux homeostasis, generation of ROS, and release of caspase activators (Suzuki et al., 2001). The early loss of MMP is a hallmark of apoptosis (Liu et al., 2004). Our data showed that after treatment, the JC-1 red fluorescence intensity was significantly decreased in a dose-dependent manner, which means the loss of MMP and the initiation of the apoptosis process.

In order to further understand  $\beta$ -asarone-induced apoptosis in LoVo cells, we also examined some apoptosis-associated proteins by Western blotting and the corresponding mRNA by real-time PCR. Besides caspases, we focused on Bcl-2 family because they serve as vital regulators of the mitochondrial pathway involved in apoptosis (Reed, 1998). The Bcl-2 protein, known as an anti-apoptotic protein, binds to the outer membrane of the mitochondria and prevent the release of cytochrome c. Bax protein is identified to be pro-apoptotic effectors and responsible for permeabilizing the membrane due to damaging cellular stress (Ewings et al., 2007). As shown in Figure 3, the protein level of Bax was increased in a dose-dependent manner, while the Bcl-2 protein was decreased. Our results suggest that the decreased ratio of Bcl-2 to Bax might be a key indicator in  $\beta$ -asarone inducing apoptosis of LoVo cells.

The ER has the function of mediating and controlling the folding of proteins. ER stress is the result of a number of insults, including exposure to chemicals that reduce of disulfide bonds, nutrient deprivation, oxidative-reduction imbalance, changes in  $\text{Ca}^{2+}$  level, inhibition of glycosylation, and overexpression of mutant proteins (Schroder and Kaufman, 2005; Bernales et al., 2006). ER stress is one of the reasons to induce apoptosis. One of the

markers of ER stress is CCAAT/enhancer binding protein (C/EBP), which is also known as CHOP. Its increasing can down-regulate Bcl-2 (McCullough et al., 2001). Another marker of ER stress is inositol requiring 1 (IRE1), which is also suggested to be a contributor to apoptosis during certain ER stress arrangements. Its phosphorylation can splice and activate XBP1 (Ron and Walter, 2007). Activating transcription factor 4 (ATF4) is suggested to be required for full activation of ATF6 in response to ER stress by enhancing ATF6 transcription. In our results, we found the increase of CHOP and spliced XBP1 mRNA expression, which indicates that  $\beta$ -asarone can induce ER stress in LoVo cells.

In this study, we investigated the anti-proliferative effect of  $\beta$ -asarone on LoVo colon cancer cells *in vitro* and *in vivo* and its apoptosis-inducing mechanism. The mechanistic implication of our data is that  $\beta$ -asarone initiates the mitochondrial dysfunction and ER stress, which is a determinant that promotes apoptosis of the cancer cells through activation of the caspase-9 and caspase-3 cascades. These findings provide a basis for further studies on  $\beta$ -asarone as chemicals against colon cancer.

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