

β -Bourbonene attenuates proliferation and induces apoptosis of prostate cancer cells

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Abstract. Sesquiterpenes have antitumor, anti-inflammation, and anti-fungal effects. β -bourbonene is a kind of sesquiterpene, but its pharmacological effect has not been studied. The present study was conducted in order to investigate the potential anticancer effects of β -bourbonene on human prostate cancer PC-3M cells. PC-3M cells were incubated with 0, 25, 50, 100 μ g/ml of β -bourbonene. Cell Counting Kit-8 (CCK-8) detection showed that compared with the control group, β -bourbonene inhibited the growth of PC-3M cells in a dose-dependent manner. G0/G1 phase arrest was observed by β -bourbonene by using flow cytometry. TUNEL staining and Annexin V/PI dual-staining method revealed that apoptosis was found in cells with β -bourbonene treatment, and the quantity of apoptotic cells was increased with the elevation in concentration. The mRNA and protein expression levels of Fas and FasL in the drug-treatment group were significantly elevated. Furthermore, the western blot assay also indicated that with an increase in the concentration of β -bourbonene, the protein expression of Bax in the drug-treatment group was significantly elevated, while a decrease was identified in the protein expression of Bcl-2. Taken together, β -bourbonene can inhibit the proliferation and simultaneously, induce apoptosis and G0/G1 arrest of prostate cancer PC-3M cells, which may be realized by upregulation of mRNA expression of Fas and FasL, increase of Bax protein expression and decrease of Bcl-2 protein expression.

Introduction

Prostate cancer is a malignant tumor with a relatively high incidence rate in the male reproductive system, and its diagnosis has been regarded as one of the medical problems to be

overcome (1). Particularly in European population, the incidence rate of solid tumor in prostate cancer is very high, and the number of patients has overtaken those with colorectal and lung cancer. Thus, prostate cancer has severe effects on male health, and is ranked first in incidence rate among solid tumors in North America and Europe (2-4). In China, the incidence rate, the cases, and death toll of prostate cancer are less than the average of the world, but there is an increasing trend in the number of patients and death toll due to the westernization of diet, prolonged average of life span and precise diagnostic technique (5). It is expected that in the near future, the incidence rate and mortality rate of prostate cancer in China will increase (6). Thus, facing the severe situation in diagnosis and treatment of prostate cancer, researchers must develop new diagnostic and therapeutic procedures and treatment methods for prostate cancer.

Sesquiterpenes are common secondary metabolites existing in nature. The larger number of derivatives with sesquiterpene as mother nucleus presents multiple bioactivities, including antitumor, anti-inflammation, and antibacterial (7). Citronella oil contains numerous sesquiterpenes, such as β -bourbonene, β -elemene, β -caryophyllene. β -elemene have proven to be useful as chemopreventive agent clinically in lung cancer, gastrointestinal carcinomas and breast cancer (8). In this present study, we observed that β -bourbonene inhibited proliferation of prostate cancer cells. This motivated us to investigate the effects of β -bourbonene on apoptosis of prostate cancer PC-3M cells to clarify the relevant mechanisms in apoptosis, results of which are expected to provide evidence for clinical treatment of prostate cancer.

Materials and methods

Materials and reagents. β -bourbonene (Sigma; Merck KGaA, Darmstadt, Germany); prostate cancer cell line PC-3M (Cell Bank of CAS, Shanghai, China); Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); Cell Counting Kit-8 (CCK-8) (Sigma; Merck KGaA); primary antibodies and horseradish peroxidase (HRP)-labeled secondary antibodies of Bax, Bcl-2 (Proteintech Group, Inc., Wuhan, China); primer synthesis (Takara Biotechnology Co., Ltd., Dalian, China), TRIzol and SYBR-GreenER™ qPCR SuperMix Universal kits (Invitrogen; Thermo Fisher Scientific, Inc.).

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Cell culture. Human androgen-independent and highly metastatic prostate cancer, PC-3M cells, were cultured in DMEM supplemented with 20% fetal bovine serum in an incubator (37°C and 5% CO₂), and cells in this experiment were divided into four groups: the blank control group (Control), the low-dose β -bourbonene group (25 μ g/ml), the mid-dose β -bourbonene group (50 μ g/ml) and the high-dose β -bourbonene group (100 μ g/ml).

Measurement of inhibition on cell proliferation through CCK-8 method. Prostate cancer PC-3M cells in logarithmic phase were trypsinized, and density of single-cell suspension was counted, and 100 μ l suspension with 0.2x10⁵ cells was inoculated per well into 96-well plate. After overnight incubation, medium was replaced with final concentration of 25, 50, and 100 μ g/ml β -bourbonene in a total volume of 100 μ l. After 72 h, complete medium was replaced without β -bourbonene, and cells were cultured for another 3 days. At every 24 h, 10 μ l CCK-8 reagent was added into the corresponding wells of the plate, and the optical density (OD) at wavelength of 450 nm was detected with a microplate reader (Thermo Fisher Scientific, MA, USA).

Measurement of apoptosis of prostate cancer PC-3M cells via TUNEL method. Prostate cancer PC-3M cells were inoculated in 6-cm dish for drug treatment, fixing in 4% paraformaldehyde for 15 min, rinsing with phosphate-buffer saline (PBS) twice (5 min/time), treating with 100 μ l Proteinase K (20 μ g/ml) for 15 min (10 to 30 min), and washing again with PBS for 3 min. TUNEL reaction mixture (500 μ l) was added into the dish for 1 h of reaction at 37°C in a dark humidifying box. After washing and blocking, 500 μ l avidin-labeled FITC was added and incubated for 30 min. Then, the cell nucleus was stained with DAPI for 10 min. The cells were observed under a laser confocal microscope and visions (Nikon, Tokyo, Japan) were selected randomly for photography.

Detection of apoptosis of PC-3M cells via Annexin V/PI dual staining method. PC-3M cells were inoculated into 6-well plate, where cells were divided into groups, i.e. the control and β -bourbonene-treated groups (25, 50, and 100 μ g/ml). After 48 h of treatment, the cells were trypsinized followed by centrifugation at 340 x g for 5 min at 4°C, and washing with PBS. Then, the cells were incubated with the mixture of 5 μ l Annexin V and 5 μ l propidium iodide (PI) for 15 min in the dark at room temperature. Cell apoptosis was detected with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell cycle analysis by flow cytometry. PC-3M cells were seeded into a 6-well plate and incubated overnight, followed by 0, 25, 50, and 100 μ g/ml of β -bourbonene. After 48 h treatment, the cells were trypsinized, harvested for single-cell suspension, and fixed with cold 70% ethanol at 4°C for 30 min. The cell pellet was incubated in a solution containing 50 μ g/ml PI, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature in the dark for 30 min. Then, the cells were analyzed by flow cytometer (BD Biosciences).

Measurement of mRNA expression of factor associated suicide (Fas) and its ligand (FasL) via RT-PCR. Cultured

Table I. Primer sequences of Fas and FasL mRNA for RT-PCR.

Genes	Primer sequences
<i>Fas</i>	F: 5'-GGCATCTGGACCCTCCTACCTCTG-3' R: 5'-CCTTGGAGTTGATGTCAGTCACTTGG-3'
<i>FasL</i>	F: 5'-GGCCTGTGTCTCCTTGTGAT-3' R: 5'-TGCCAGCTCCTTCTGAAGTA-3'
<i>GAPDH</i>	F: 5'-ACAACCTTTGGTATCGTGGAAAGG-3' R: 5'-GCCATCACGCCACAGTTTC-3'

F, forward; R, reverse.

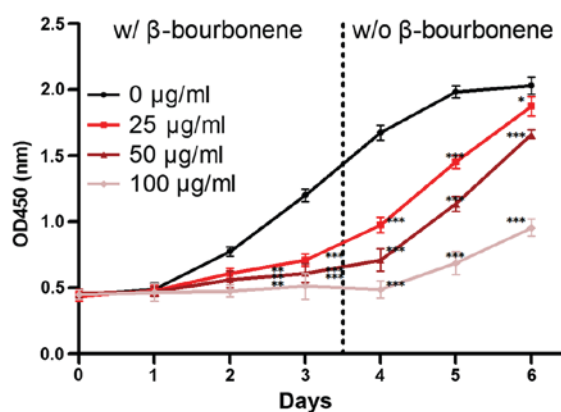


Figure 1. Effect of β -bourbonene against proliferation of PC-3M cells. PC-3M cells were incubated with serial dosage of β -bourbonene for 3 days, then were further incubated for 3 days without β -bourbonene. Cell Counting Kit-8 (CCK-8) was used to detect *P<0.05, **P<0.01 and ***P<0.001 vs. control group cell proliferation. The experiments were repeated 3 times (n=3).

cells were seeded on a 6-well plate at density of 1x10⁴/well, and after 24 h, the supernatant was discarded. Following treatment with 25, 50 and 100 μ g/ml of β -bourbonene for 48 h, the cells in each group were collected for extraction of the total RNA using RNAPure Tissue Cell kit according to manufacturer's instructions (CW BIO, Beijing, China). After detection, the total RNA that was qualified using a spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) for this experiment, was used as a template for synthesis of complementary DNA (cDNA) through reverse transcription in following reaction conditions: incubation at 42°C for 15 min and 95°C for 3 min, and cooling on ice using the First-Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed on cDNA by using SYBR-GreenER™ qPCR SuperMix Universal kit (Invitrogen; Thermo Fisher Scientific, Inc.) using the following conditions: at 50°C for 2 min; at 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min; followed by 72°C for 10 min. Data were analyzed through the 2^{- $\Delta\Delta$ C_q} method and normalization to GAPDH as an endogenous reference (9). Regular amplification was performed for primer sequences in Table I.

Detection of protein expression levels of Bax and Bcl-2 via western blot assay. The cultured cells were inoculated into a

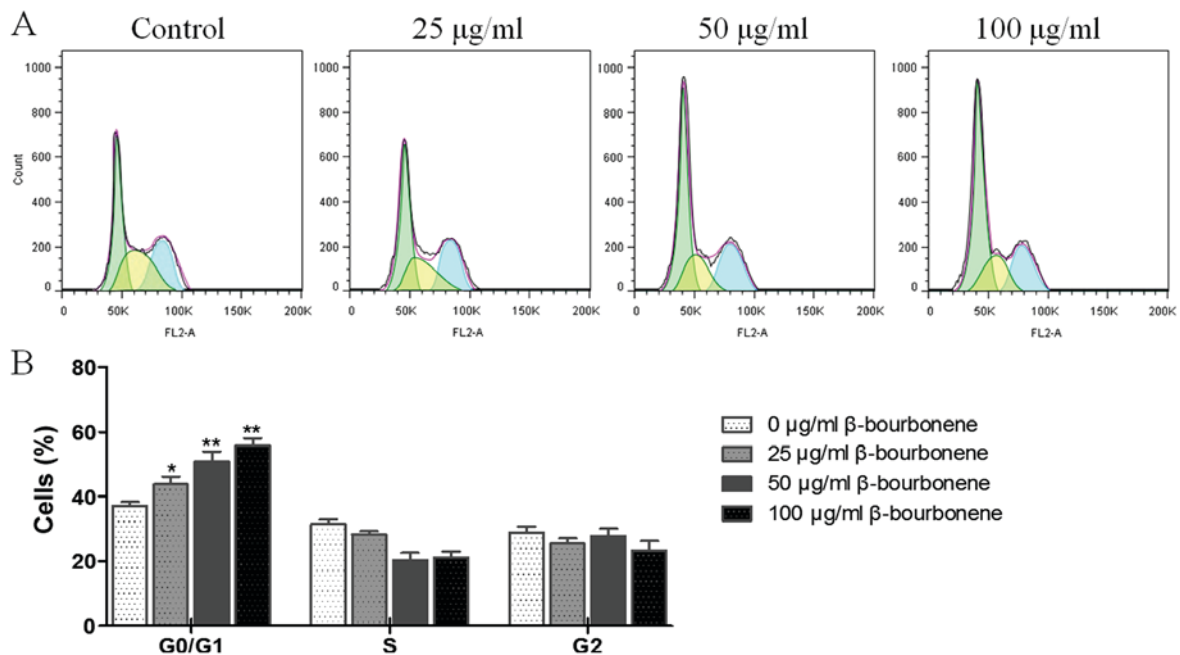


Figure 2. Effects of β -bourbonene on cell cycle. PC-3M cells were treated by various concentration of β -bourbonene for 48 h. Then cells were stained with propidium iodide (PI) and cell cycle detected by flow cytometry. (A) Representative images of PC-3M cell cycle. (B) Percentage of G0/G1, S, and G2 phase. Values are mean \pm SD (n=3). *P<0.05 and **P<0.01 vs. control group.

6-well plate at a density of 1×10^4 /well, and after 24 h, the supernatant was discarded. Following the treatment with 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ of β -bourbonene for 48 h, the cells in each group were collected and lysed in a cell lysis buffer (Sigma-Aldrich; Merck KGaA) containing 1 mM PMSF. Total protein concentration was measured by Coomassie brilliant blue assay using BSA as the quantitative standard (P0006; Beyotime Institute of Biotechnology, Shanghai, China). From the protein samples, 50 μg protein was electrophoresed on 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) with marker ladder (P0060S, Beyotime Institute of Biotechnology). Proteins were then transferred electrically on a polyvinylidene fluoride (PVDF) membrane followed by blocking with blocking buffer (Candor Bioscience GmbH, Wangen, Allgäu, Germany) for 1 h. Proteins were then incubated with primary antibodies at 4°C overnight. The dilution multiple of primary antibodies are 1:200, 1:500, 1:1,000, 1:500 and 1:1,500 for Fas, FasL, Bax, Bcl-2, and GAPDH, respectively and then, the membrane was washed with Tween 20/Tris-buffer saline (TTBS). Then, the secondary antibody (1:2,000) was added on the membrane for incubation at room temperature for 1 h followed by washing with TTBS, color development with ECL western blot substrate developing solution (32109, Pierce; Thermo Fisher Scientific, Inc.) and photographing. The protein bands were determined as relative quantity by an image analysis system (Odyssey 3.0 software) and normalized to control GAPDH. The following antibodies were used: Fas (1:200; cat. no. BM4868) and GAPDH (1:500; cat. no. BM1623) were purchased from Wuhan Boster Biological Technology (Wuhan, China), FasL (1:500; cat. no. bs-0216R), Bax (1:1,000; cat. no. bsm-33283M), and Bcl-2 (1:500; cat. no. bsm-33047M) were purchased from BIOSS (Beijing, China), goat anti-rabbit IgG (1:400; cat. no. 926-32211) and goat anti-mouse IgG (1:400; cat. no. 926-32210) were purchased from Licor (Lincoln, NE, USA).

Statistical processing. Data processed with Statistical Product and Service Solutions 19.0 (SPSS 19.0). One-way ANOVA was used to identify group difference and followed by Dunnett's post test for group difference. Values are expressed as mean \pm standard deviation (SD) with replicate number of 3. P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory effect of β -bourbonene on cell proliferation. As shown in Fig. 1, compared with the control group, PC-3M cell proliferation was significantly inhibited in groups treated with 25, 50 and 100 $\mu\text{g/ml}$ β -bourbonene. The proliferation rate notably decreased in a dose-dependent manner. After β -bourbonene was removed, the proliferation profile of PC-3M cells was observed for the following 3 days. Compared to the proliferative activity (according to the curve slope) since day 0 to day 3 with β -bourbonene treatment, the proliferative activity at day 4 to day 6 dramatically increased without treatment of β -bourbonene. The significantly different proliferation profile with or without β -bourbonene treatment indicated that the decreased proliferation before day 3 was induced by β -bourbonene.

Effect of β -bourbonene on cell cycle arrest. As shown in Fig. 2, compared with the control group, the number of cells in G0/G1 phase of PC-3M cells changed significantly in groups treated with 25, 50 and 100 $\mu\text{g/ml}$ β -bourbonene (P<0.05). The increased trend was dose-dependent. Cell amount in the S and G2 phase did not present any trend. This result indicated that the progression of cell cycle was arrested at G0/G1 phase.

Effect of β -bourbonene on apoptosis of PC-3M cells. Annexin V/PI dual staining method was used to detect the

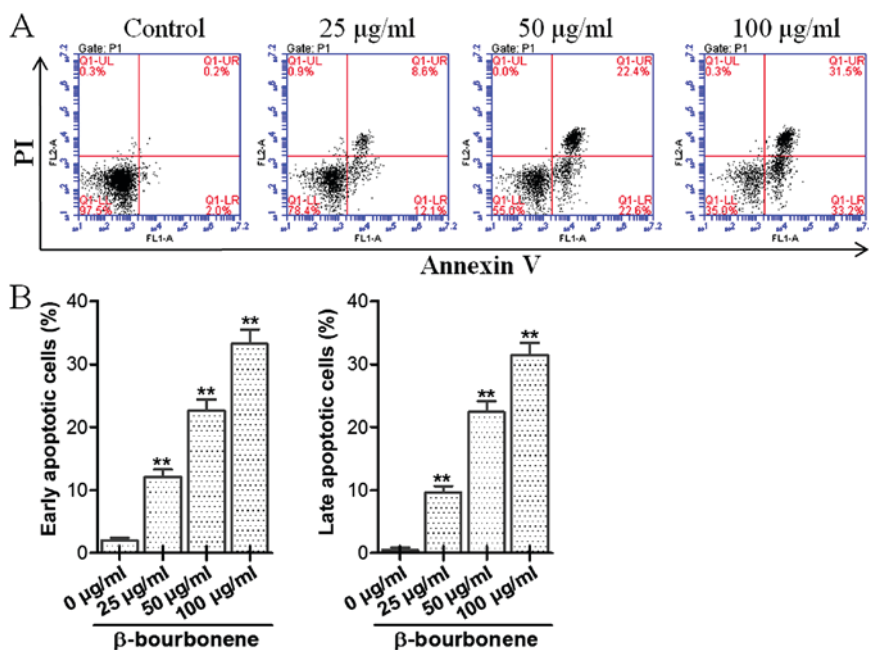


Figure 3. Effect of β -bourbonene on apoptosis of PC-3M cells. PC-3M cells or cells treated with β -bourbonene for 48 h at 0 (control), 25, 50 and 100 $\mu\text{g/ml}$, and then harvested, stained with Annexin V-FITC and propidium iodide (PI). (A) PC-3M cytogram from flow cytometry. (B) Percentage of Annexin V⁺/PI⁺ and Annexin V⁺/PI⁻ positive cells. Values are mean \pm SD (n=3). **P<0.01 vs. the control group.

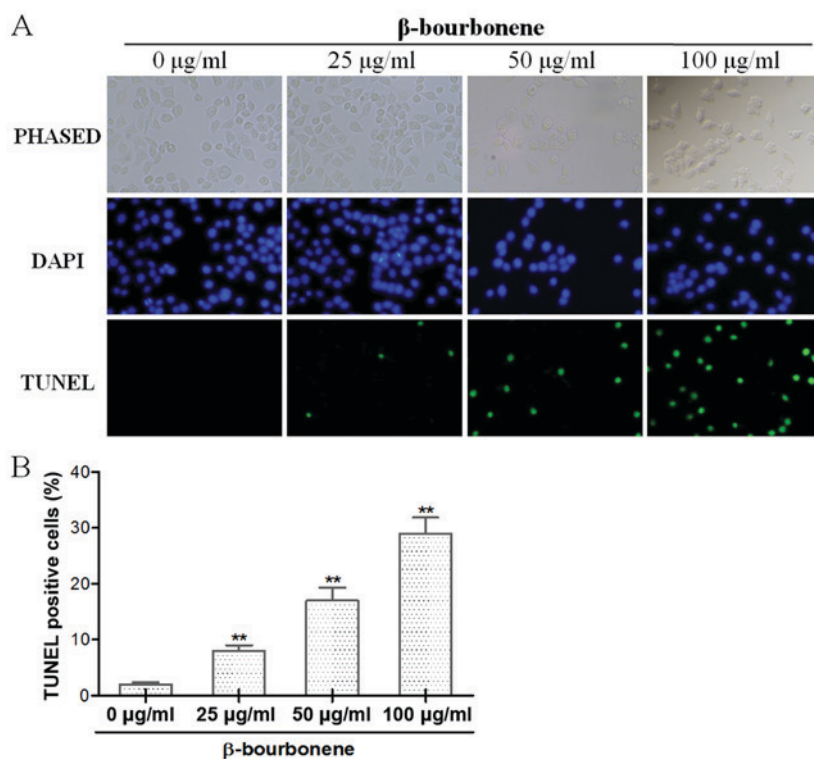


Figure 4. Increased TUNEL staining in PC-3M cells treated by β -bourbonene. PC-3M cells, treated by various concentrations of β -bourbonene, were grown in a 6-cm dish and fixed for TUNEL and DAPI staining as described in Materials and methods. (A) Representative images (x200 magnification) of bright field, DAPI and TUNEL staining 48 h after β -bourbonene treatment. Blue fluorescence represents DAPI-positive cells and green fluorescence represents TUNEL positive cells. (B) Percentage of TUNEL-positive cells in each sample. Values are mean \pm SD (n=3). **P<0.01 vs. the control group.

effect of β -bourbonene on apoptosis of PC-3M cells, and the results are shown in Fig. 3. The four quadrants of the dual parameter fluorescent dot plots represent different states of the cells. The viable cell population was in the lower left quadrant (Annexin V⁻/PI⁻). The early apoptotic cells were in the lower

right quadrant (Annexin V⁺/PI⁻) and the ones in late apoptosis were in the upper right quadrant (Annexin V⁺/PI⁺). As shown in Fig. 3, compared with the control group, significant increases in both early and late apoptosis were seen in groups treated with β -bourbonene in different concentrations, and

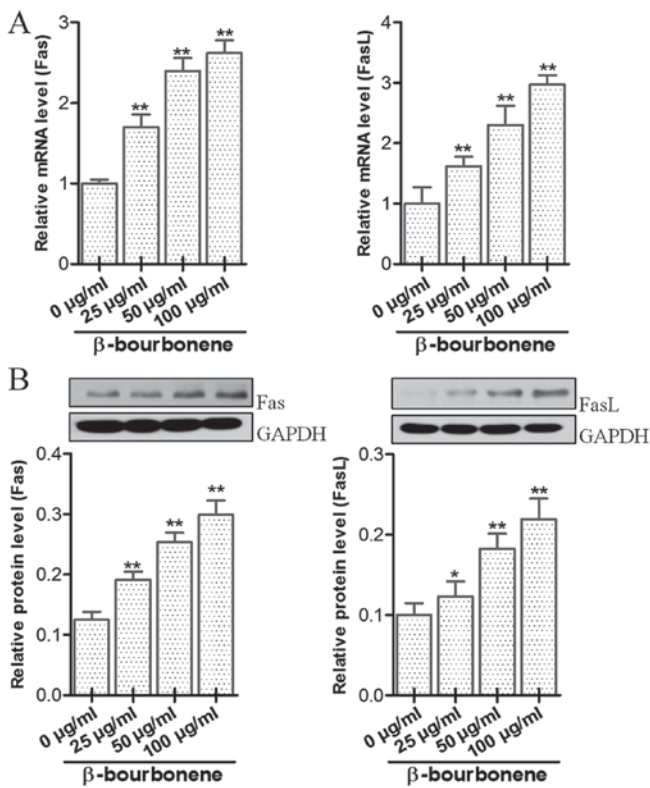


Figure 5. Effects of β -bourbonene on mRNA and protein expression levels of Fas and FasL in cells. After 48 h treatment by β -bourbonene, RNA and total proteins were extracted from PC-3M cells for analysis of RT-qPCR and western blot analysis, respectively. (A) RT-qPCR result for Fas and FasL mRNA levels. (B) Western blot results for Fas and FasL protein levels and densitometric quantification with GAPDH as loading control. Values are mean \pm SD (n=3). *P<0.05 and **P<0.01 vs. control group.

the difference had statistical significance (P<0.01), suggesting that β -bourbonene can remarkably induce both early and late apoptosis in prostate cancer PC-3M cells.

Detection of the effect of β -bourbonene on cell apoptosis via TUNEL staining. As shown in Fig. 4A, the cells with green-stained nuclei were regarded as apoptotic cells, and all cell nuclei were stained with DAPI to show the total number of PC-3M cells. The results showed that in comparison with the control group, the number of apoptotic cells was gradually increased against an elevation in drug concentration in groups treated with β -bourbonene in concentrations of 25, 50 and 100 μ g/ml for 48 h (Fig. 4A and B). Beyond that, PC-3M cells showed shrinkage and detachment as drug concentration increased, indicating the cytotoxic effect of β -bourbonene.

Effects of β -bourbonene on mRNA and protein expression levels of Fas and FasL in cells. Results of mRNA and protein levels of Fas and FasL are shown in Fig. 5. In groups that were treated with β -bourbonene in concentrations of 25, 50 and 100 μ g/ml for 48 h, mRNA expression levels of Fas and FasL were significantly higher than those in the control group (P<0.01). Protein levels of Fas and FasL were also notably enhanced by various concentrations of β -bourbonene compared to the control group (P<0.05).

Effect of β -bourbonene on protein expression levels of Bax and Bcl-2. As shown in Fig. 6, after treatment with β -bourbonene

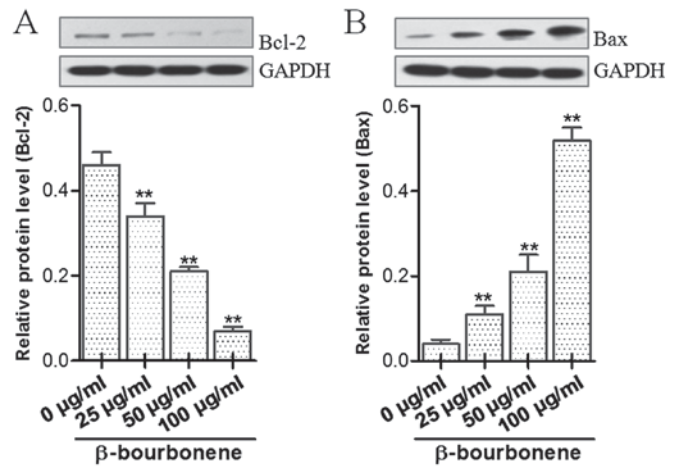


Figure 6. Detection of the effects of β -bourbonene on Bcl-2 and Bax protein expression levels in PC-3M cells via western blot analysis. (A) Representative protein bands and densitometric quantification of Bcl-2; (B) Representative protein bands and densitometric quantification of Bax. Values are mean \pm SD (n=3). **P<0.01 vs. control group.

in concentrations of 25, 50 and 100 μ g/ml for 48 h, the protein expression levels of Bax and Bcl-2 in each group were significantly increased and decreased, respectively, with an increase in concentration of β -bourbonene, showing a significant dose-dependent manner.

Discussion

Currently, endocrine therapy remains the major method for clinical treatment of prostate cancer, including surgical resection and anti-androgen therapy, and its objective is to reduce the level of androgen or the completion to the receptor of androgen (10-12), thereby activating the biological effect of androgen receptor. Despite that it is conducive to the treatment of androgen-dependent prostate cancer, endocrine therapy shows poor efficacy on the hormone-independent patients in advanced prostate cancer (13,14). Moreover, our preliminary data (not shown) indicated that β -bourbonene was not an antagonist of androgen receptor. Thus, it is prioritized to develop a new effective compound to treat hormone-independent prostate cancer. For compound of β -bourbonene, it may be more meaningful to first explore its effect and mechanism on androgen-independent cellular model. In the present study, PC-3M, a highly metastatic cell line was used as the objective. Both PC-3M and PC-3 cell lines are androgen-independent (15), whereas PC-3M shows higher metastasis than PC-3. Therefore, PC-3M is more suitable for the study of advanced prostate cancer.

Bcl-2, as a gene with biological functions, such as inhibitory effect on cell apoptosis (16), is pivotal in mechanism of cell apoptosis. *Bcl-2* can protect cells from death in diverse forms, thereby improving the survival of cells and increasing the quantity of cells. Thus, in some tumor cells, the upregulation of *Bcl-2* can help cells escape from death or prolong the life span (17), indicating that *Bcl-2* is closely related to tumors. On the contrary, *Bax* can promote cell apoptosis (18). Even though *Bax* and *Bcl-2* belong to the same family, *Bax* can not only antagonize the inhibitory effect of *Bcl-2* on cell

apoptosis, but also directly act on cells to facilitate apoptosis. The homodimer of *Bcl-2* can inhibit cell apoptosis, while the dimers constituted by highly expressed Bax proteins and *Bcl-2* protein, or Bax protein itself, will facilitate cell apoptosis (19). Kim *et al* (20) also found that the downregulation of *Bcl-2*, as the target gene, can induce apoptosis of cells in multi-onset myeloma. Fas, also known as factor associated suicide, is a kind of cell death factor that can induce cell apoptosis, and when it combines with FasL, the intracellular apoptosis-associated signal pathway can be activated (21,22).

CCK-8 experiment revealed that compared with the control group, β -bourbonene in different concentrations could inhibit the proliferation of PC-3M cells, and with an increase in concentration, the inhibition rate on proliferation was significantly elevated. Result of G0/G1 arrest further verified the anti-proliferation effect of β -bourbonene to PC-3M cells. Since various proteins influence G0/G1 arrest, it is speculated according to this result, that β -bourbonene can be regulated by multiple molecules, such as the p21 (23). The results of TUNEL staining showed that apoptosis emerged gradually in cells in drug-treatment groups, and the increase in concentration of drug led to an elevation in quantity of apoptotic cells. According to the results of Annexin V/PI dual staining experiment, we found that β -bourbonene could induce apoptosis in PC-3M cells in a concentration-dependent manner. Therefore, proliferation inhibition by β -bourbonene might be the combined effect of cell cycle arrest and pro-apoptosis. In addition, compared with the control group, the mRNA and protein expression levels of Fas and FasL in the drug-treatment group were significantly elevated, which further confirmed that β -bourbonene can initiate the apoptosis pathway to induce apoptosis in PC-3M cells through upregulation of Fas and FasL.

With the results of western blot assay, we found that in comparison with the control group, the protein expression level of Bax in the drug-treatment group was significantly elevated with an increase in concentration of β -bourbonene, but the *Bcl-2* expression was significantly decreased with the increase in concentration of β -bourbonene. Zhang *et al* (24) reported that some antitumor drugs can downregulate the expression of *Bcl-2* to induce apoptosis in tumor cells. Messaris *et al* (25) also found that the abnormal downregulation of *Bcl-2* and upregulation of Bax in gastrointestinal epithelial tumors may be the early variations in gastrointestinal tumors. The above results, consistent with the results in this study, further proved that β -bourbonene-induced apoptosis in PC-3M cells may be achieved through upregulation of Bax protein and downregulation of *Bcl-2* protein.

Moreover, adaptive upregulation of some anti-apoptosis genes has been verified by substantial literature. For example, the adaptive upregulation of *Bcl-2* may exert a cell protective effect against apoptosis due to androgen removal. In this study, the inhibition of *Bcl-2* by β -bourbonene indicated that β -bourbonene might produce inhibitive effect to androgen-independent prostate cancer.

In conclusion, the results of this study confirmed that β -bourbonene can inhibit the proliferation of PC-3M cells in prostate cancer, and induce cell apoptosis, which may be realized through upregulating Fas, FasL, Bax, and downregulating *Bcl-2*. However, the development of prostate cancer, especially castration-resistant prostate cancer, is closely related to

androgen receptor. It is a limitation of this study to explore the efficacy of β -bourbonene using a single cell line, and it is necessary to detect the profile of β -bourbonene against androgen receptor on androgen-receptor-overexpressing cells or androgen-dependent cells. Therefore, attention should be paid more on a comprehensive understanding for β -bourbonene efficacy *in vitro* or *in vivo*.

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Availability of data and materials

The original data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions

The concept and study design was performed by JZJ. The conduct of experimental parts, supervision, resources, materials, data collection and processing were performed by ZW, JJY and FL. The analysis and interpretation of the data were performed by JZJ and ZW. The literature search and writing the manuscript were performed by ZW and JZJ. The critical review was performed by JZJ. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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