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Research Paper

Antineoplastic effect of β-elemene on prostate cancer cells and other types of solid tumour cells

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Abstract

Objectives β-Elemene, a natural compound extracted from over 50 different Chinese medicinal herbs and plants, has been effective in the treatment of hyperplastic and proliferative disorders such as prostatic hypertrophy, hysteromyoma and neoplasms. Our previous studies have demonstrated that β-elemene exhibits strong inhibitory activity in ovarian cancer cells. The aim of the present study was to assess the effect of β-elemene on prostate cancer cells as well as other types of tumour cells and to determine whether the effect of β-elemene on prostate cancer cell death was mediated through the induction of apoptosis.
Methods The MTT assay was used to evaluate the ability of β-elemene to inhibit cellular proliferation in cancer cells. Cellular apoptosis was assessed by annexin V binding, TUNEL and ELISA-based assays. Caspase activity was measured using a caspases assay kit. The protein levels of Bcl-2, caspases, cytochrome *c* and poly(ADP-ribose) polymerase (PARP) were analysed by Western blotting.
Key findings Here, we showed that β-elemene had an antiproliferative effect on androgen-insensitive prostate carcinoma DU145 and PC-3 cells. Treatment with β-elemene also inhibited the growth of brain, breast, cervical, colon and lung carcinoma cells. The effect of β-elemene on cancer cells was dose dependent, with IC50 values ranging from 47 to 95 μg/ml (230–465 μM). TUNEL assay and flow cytometric analysis using annxin V/propidium iodide staining revealed that the percentage of apoptotic prostate cancer cells was increased by β-elemene in a dose- and time-dependent manner. Moreover, β-elemene exposure resulted in a decreased Bcl-2 protein level, increased cytochrome *c* release, and activated PARP and caspase-3, -7, -9, and -10 in prostate cancer cells.
Conclusions Overall, these findings suggest that β-elemene exerts broad-spectrum antitumour activity against many types of solid carcinoma and supports a proposal of β-elemene as a new potentially therapeutic drug for castration-resistant prostate cancer and other solid tumours.
Keywords apoptosis; β-elemene; caspases; Chinese medicine; natural product; prostate cancer

Introduction

Prostate cancer constitutes a major health problem worldwide. It is the most frequently diagnosed cancer among men and the second leading cause of male cancer deaths. In 2005, for example, 185 895 men developed prostate cancer and 28 905 men died from the disease in the USA.[1] The high mortality rate of prostate cancer is the result of the progression from androgen-dependent to androgen-independent prostatic growth and metastasis to the bone and lymph nodes.[2] Both androgen ablation therapy and radical prostatectomy are considered curative for localised disease, while androgen deprivation therapy (ADT) remains a cornerstone intervention for advanced-stage disease management. Apoptotic regression of an androgen-dependent tumour is induced either chemically with luteinising hormone-releasing hormone analogues or surgically by orchiectomy, which initially reduces the intracellular concentration of dihydrotestosterone (DHT),[2] resulting in the death (by apoptosis) of androgen-sensitive prostate cancer cells. Although non-curative, androgen deprivation slows disease progression, enhances quality of life, modestly increases survival,[3] and is associated with a biochemical response of decreased prostate-specific antigen (PSA) levels.

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However, androgen signalling in prostate cancer is still prominent under androgen deprivation. Evidence has emerged that 'castration-recurrent' or 'castration-resistant' prostate cancer remains androgen receptor (AR)-dependent and is neither hormone refractory nor androgen-independent, terms once commonly used to define progression of advanced-stage disease after ADT.[4–8] Castration-recurrent or castration-resistant prostate cancer refers to patients who no longer respond to surgical or medical castration due to regrowth of prostate cancer cells that have adapted to the hormone-deprived environment of the prostate.[4–8] That ARs in castration-resistant prostate cancer function despite the paucity of circulating androgens is evidenced by the gene amplification and increased expression of the AR mRNA and protein in castration-recurrent tumour tissue relative to androgen-dependent tumours and re-expression and elevation of some androgen-regulated gene products during clinical castration resistance,[9,10] indicative of a persistently activated AR cell-signalling pathway. Furthermore, although testosterone and DHT are depleted in serum after ADT, substantial levels of androgens have been measured in locally recurrent prostate cancer tissue,[4] as well as in intratumoral metastatic castration-resistant prostate cancer tissue.[11] These androgens continue to regulate AR transcriptional activity and stimulate expression of androgen-regulated genes such as PSA, which commonly precedes clinical failure.[4,11] Nevertheless, there has been no effective therapy for the treatment of castration-recurrent and metastatic prostate cancer. Currently, chemotherapy is the major clinical treatment and medical management for castration-resistant prostate cancer,[12] and new more efficacious drugs with fewer side effects are needed to treat advanced prostate cancer patients. A better understanding of apoptosis deregulation in cancer development may suggest novel strategies for prostate cancer therapy.

Apoptosis is controlled by many factors that interact in a complex web of regulation, as would be expected for a process that is critical to the maintenance of tissue homeostasis.[13] Nonetheless, the molecular basis for apoptosis implies that cell death, like any other metabolic or developmental programme, can be disrupted by alterations or defects in the apoptotic pathway, which contribute to a number of human diseases, including the initiation and progression of prostate cancer.[13,14] In mammalian cells, two major apoptosis pathways have been proposed.[15] One is an extrinsic pathway, which involves signal transduction through cell-surface death receptors, and the other is an intrinsic pathway, which is triggered by various stressors such as ultraviolet radiation or chemical agents. Although chemotherapeutic agents may induce apoptosis through the death-receptor pathway, the mitochondrial pathway is indicated as the primary pathway in anticancer drug-mediated cell death.[16] In response to stressors such as chemical agents or chemotherapeutic drugs, the mitochondria are damaged and the mitochondrial proteins, including cytochrome *c*, are released into the cytosol. Cytosolic cytochrome *c* then binds apoptotic activator 1 (Apaf-1) and, in the presence of ATP, coordinates a series of conformational changes to form the apoptosome. The apoptosome binds and activates caspase-9, which in turn recruits and activates caspase-3, -6, and -7 to initiate and execute a series of proteolytic events.[16,17]

Studies over the past decade have demonstrated that alterations of apoptotic regulatory genes play pivotal roles in prostate cancer progression. Although prostate cancer cells contain intact cell-death programmes, the cells fail to initiate or execute these programmes in response to conventional modes of treatment, thus shifting the balance in favour of cell proliferation.[14,18] A novel therapeutic strategy for advanced prostate cancer therefore is to trigger tumour-selective apoptotic cell death without producing clinically significant effects on the host.

Elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane) has been extracted from numerous plants[19] and has been identified in more than 50 different medicinal herbs and plants.[20] These plants grow in tropical areas around the world and include *Rhizoma zeodaria*, *Radix inulae*, *Radix ginseng, Curcuma wenyujin*, *Cymbopogon citrates*, *Cymbopogon winterianus Jowitt*, *Zhangzhou Aglaia odorata* flowers and leaves, *Fuzhou Aglaia odorata* flower, *Chunging Aglaia odorata* flower and leaves, *Yibin geranium* leaves, *Kunmin geranium* leaves, *Litchi chenensis cinnamonmi folium*, dry *Lauris nobilis*, *Citrus limona* leaves, *Vitis vinifera* grape leaves, *Clausena lansium* leaves, *Fortunella margarita* leaves, *Fortunella odoranta*, *C. Wenyunjin Chen* and *Magnolia sieboldi* among others.[21] In China, β-elemene, the major active component of elemene, has been used effectively in the treatment of hyperplastic and proliferative disorders, including prostatic hypertrophy, hysteromyoma and neoplasms, and one of its preparation formulations (85% β-elemene) has been approved by the State Food and Drug Administration of China for the treatment of primary and secondary brain tumours and other carcinomas.[22] The major advantages of β-elemene as an anticancer drug are that it has antitumour activity toward a broad spectrum of cancers, including leukaemia, it is associated with a low level of toxicity, and it is well tolerated by cancer patients[22–28] Our previous studies have demonstrated that β-elemene exhibits strong inhibitory activity in ovarian cancer cells and non-small cell lung cancer cells.[24–27] However, the effect of β-elemene on human prostate tumours is unknown, and the mechanism of β-elemene as a chemotherapeutic agent remains to be further determined.

The goal of the present study was to investigate the antitumour effect of β-elemene against prostate cancer cells and cells of other solid tumours and to delineate the molecular mechanism of β-elemene-induced cell killing in androgen-insensitive human prostate cancer DU145 and PC-3 cells. We found that β-elemene effectively kills prostate cancer cells through the induction of apoptosis and that β-elemene treatment inhibits the growth and proliferation of several types of tumour cells, including brain, breast, cervical, colon and lung cancer cells. Furthermore, the induction of apoptosis by β-elemene is mediated by the caspase cascade of the mitochondrial pathway, as β-elemene enhances the activities of caspase-3, -7, -9 and -10, increases the release of cytochrome *c*, and induces the cleavage of caspases and poly(ADP-ribose) polymerase (PARP). Thus, inducing apoptosis through the mitochondrial pathway may contribute, at least in part, to β-elemene-mediated anticancer activity.

Materials and Methods

Chemicals and immunoreagents

The (–)-β-elemene (98% purity) was obtained from Dalian Yuanda Pharmaceuticals, Ltd (Dalian, China). Propidium iodide (PI) and glycine were from Sigma-Aldrich (St Louis, MO, USA). The primary antibodies against Bcl-2, cytochrome *c*, caspase-9, caspase-3, PARP and β-actin, the secondary HRP-conjugated goat anti-rabbit-IgG and HRP-conjugated goat anti-mouse-IgG antibodies, nitrocellulose membranes, Blotto, and the chemiluminescence luminal reagent were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The CellTiter 96 Aqueous One Solution cell proliferation assay kit was from Promega Corp. (Madison, WI, USA), the Vybrant Apoptosis Assay Kit 2 was from Molecular Probes, Inc. (Eugene, OR, USA), and the Cell Death Detection ELISA^{PLUS} kit and TUNEL labelling kit were from Roche Diagnostics Corp. (Indianapolis, IN, USA). The CasPASE-9, -3, -7 and -10 assay kits were from Geno Technology (St Louis, MO, USA).

Cell lines and cell culture conditions

The two androgen-insensitive human prostate cancer cell lines, DU145 and PC-3, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Three brain tumour cell lines, two lung carcinoma cell lines, two colon carcinoma cell lines, three cervical carcinoma cell lines and two breast cancer cell lines (all listed in Table 1) were also purchased from ATCC. The cell culture medium RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin-glutamine (100×) and 0.25% trypsin-EDTA solution were from Invitrogen Corp. (Carlsbad, CA, USA). All of the carcinoma cell lines used in this study were maintained in RPMI-1640 supplemented with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin, and were grown at 37°C in a humidified atmosphere of 5% CO_2.

Table 1 The effect of β-elemene on in-vitro cytotoxicity in human cancer cells

Cancer cell type	β-Elemene IC50 (μg/ml)[a]	β-Elemene IC50 (μM)[a]
A-172 brain glioblastoma	65 ± 5.8	318 ± 29
U-87MG brain glioblastoma	88 ± 9.2	431 ± 46
CCF-STTG1 brain astrocytoma	82 ± 7.5	401 ± 38
CCL-222 colon carcinoma	47 ± 5.3	230 ± 27
CCL-225 colon carcinoma	67 ± 6.2	328 ± 31
NCI-H596 lung adenosquamous carcinoma	95 ± 8.4	465 ± 42
NCI-H69 small cell lung carcinoma	52 ± 4.6	255 ± 23
CCL-2 HeLa cervical adenocarcinoma	63 ± 5.8	308 ± 29
ME-180 cervical carcinoma	68 ± 6.2	333 ± 31
HTB-33 cervical carcinoma	68 ± 7.1	333 ± 36
MCF-7 breast adenocarcinoma	93 ± 7.8	455 ± 39
T47D breast carcinoma	63 ± 5.4	308 ± 27

Cytotoxicity to β-elemene was assessed by the MTT assay, as described in the Materials and Methods section.

[a]Each value represents the mean ± SD from three independent determinations.

Cell growth inhibition assay

Cytotoxicity to β-elemene was assessed using the MTT assay, as described previously.[24–27] In brief, 5×10^3 tumour cells (DU145, PC-3, or others) were evenly distributed in each well of a 96-well plate and incubated overnight. The cells were then treated with various concentrations of β-elemene (0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μg/ml) for 24, 48 or 72 h. Following β-elemene exposure, 20 μl of CellTiter 96 Aqueous One Solution reagent was added to each well of a 96-well assay plate containing the cells in 100 μl of culture medium, and the plates were incubated at 37°C in 5% CO_2 for 1–4 h, after which the optical density at 590 nm was determined using a 96-well plate reader. The optical density was used to calculate proliferation rates after exposure to the different concentrations of β-elemene relative to that of control cells with no β-elemene exposure (100%).

Flow cytometric analysis for apoptosis

Prostate carcinoma (DU145 or PC-3) cells were treated with different concentrations of β-elemene (0, 40, 60, and 80 μl/ml) for 24 or 48 h. After treatment, the cells were harvested by trypsinisation, washed twice with phosphate buffered saline (PBS), and stained with annexin V and PI using a Vybrant Apoptosis Assay Kit 2 following the manufacturer's step-by-step instructions. The stained cells were measured by fluorescence-activated cell sorting analysis at the Flow Cytometry Core of the West Virginia University Health Sciences Center.

Cell death detection by ELISA assay

Prostate cancer (DU145 or PC-3) cells (1×10^4/well) were evenly distributed in 96-well plates and incubated overnight, followed by treatment with various concentrations of β-elemene (0, 40, 60 and 80 μg/ml) for 24 or 48 h. The cells were harvested and then assayed for apoptosis using a Cell Death Detection ELISA kit, following the step-by-step protocol provided by the manufacturer. This assay is based on the quantitative sandwich-enzyme-immunoassay principle and uses mouse monoclonal antibodies directed against DNA and histones. Using this assay, histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cell lysates can be quantitatively determined photometrically at 405 nm *in vitro*.

TUNEL assay for apoptosis

Prostate carcinoma (DU145 or PC-3) cells were treated with β-elemene at 0, 40, 60 and 80 μg/ml and harvested after 24 or 48 h. The cells were washed twice with PBS, placed in Bouin's fixative for at least 24 h, dehydrated in alcohol and paraffin-embedded using standard protocols. Sections of 5-μm thickness were cut and mounted on glass slides for staining with haematoxylin and eosin (H&E) and terminal deoxynucleotidyltransferase-mediated deoxy-UTP-fluorescein nick end labelling (TUNEL). For the TUNEL assay, the sections were rehydrated, postfixed in 4% paraformaldehyde-PBS (pH 7.2), rinsed in PBS and incubated for 1 h at room temperature with a mix containing fluorescein-deoxy-UTP and terminal deoxynucleotidyltransferase following the manufacturer's instructions.

Caspase activity assay

Caspase activities were assayed using a CasPASE apoptosis assay kit according to the manufacturer's instructions. In brief, 2×10^7 prostate cancer (DU145 or PC-3) cells were treated with different concentrations of β-elemene (0, 40, 60 and 80 μg/ml) for 24 or 48 h. Then the cells were collected by trypsinisation and washed once with PBS. The cell pellets were resuspended in 350 μl of lysis buffer and lysed using five freeze–thaw cycles. The lysates were centrifuged at 12 000 rpm for 30 min at 4°C, and the supernatants were used for measuring the activities of caspase-3, -7, -9 and -10. Caspase-3, -7 and -10 activities were detected using DEVD peptide conjugated to 7-amino-4-trifluoromethyl coumarin (AFC), which is a fluorogenic substrate specific for these caspases. Caspase-9 activity was detected using the caspase-9-specific fluorogenic substrate LEHD-AFC. The assay results were determined using a microplate reader at 405 nm.

Western blot analysis

Prostate carcinoma (DU145 or PC-3) cells were exposed to β-elemene at 0, 40, 60 and 80 μg/ml for 24 or 48 h. Then the cells were harvested and lysed with 200 μl of RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1.0% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM aprotinin, 1 μg/ml PMSF, leupeptin and pepstatin. The protein concentration was quantified by BCA assay, and equal amounts of protein from control and drug-treated cells were boiled for 5 min in Laemmli buffer, separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with primary antibodies against Bcl-2, cytochrome *c*, caspase-9, caspase-3 and PARP, followed by secondary HRP-conjugated antibodies. In all cases, the blots were stripped with a buffer containing 62.5 mM Tris (pH 6.7), 2% SDS, and 90 mM 2-mercaptoethanol, and reprobed with an anti-actin antibody as a loading control. The signals were detected by enhanced chemiluminescence.

Detection of released cytochrome *c*

Cytosolic and mitochondrial extracts were prepared with a mitochondria isolation kit (Sigma-Aldrich, St Louis, MO, USA), essentially according to the manufacturer's instructions. Briefly, prostate carcinoma (DU145 or PC-3) cells were treated with β-elemene at 0, 40, 60 and 80 μg/ml for 24 or 48 h, then collected by centrifugation. The cell pellets were washed twice with cold PBS, resuspended in ice-cold Buffer A (50 mM HEPES, pH 7.5, 1 M mannitol, 350 mM sucrose, 5 mM EGTA, 10 μg/ml leupeptin and 10 μg/ml aprotinin), and homogenised with a glass homogeniser. Nuclei and intact cells were cleared by centrifugation at 600*g* for 10 min at 4°C. The supernatant was centrifuged at 14 000*g* for 20 min at 4°C to separate the mitochondrial fraction. The resulting supernatant was used as the soluble cytosolic fraction, and the mitochondrial pellet was washed once and suspended in Buffer A. Samples of the mitochondrial and cytosolic fractions containing equal amounts of protein were subjected to Western blot analysis, as described above, with an anti-cytochrome *c* antibody.

Statistical analysis of data

All quantitative values are presented as the mean ± SD. Data were statistically analysed using a two-way analysis of variance (ANOVA) for comparison among different groups. Student's *t*-test was used to analyse the statistical significance of the differences between the untreated control and β-elemene-treated groups in different assays. A value of $P < 0.05$ was considered statistically significant.

Results

β-Elemene inhibited the growth of prostate cancer cells and other types of carcinoma cells

To investigate the antitumour effect of β-elemene against prostate cancer cells, we first assessed the ability of β-elemene to inhibit prostate cancer cell proliferation *in vitro* as determined with the MTT assay. We characterised the dose–response relationship and time course of β-elemene inhibition of cell proliferation in the androgen-insensitive prostate cancer cell lines DU145 and PC-3. Our results showed that β-elemene at a concentration range of 20–160 μg/ml caused dose-dependent inhibition of DU145 cell proliferation for three different treatment times (Figure 1; data at 48 and 72 h not shown). The half-maximal inhibitory concentrations (IC50) of β-elemene in DU145 cells were 75, 70 and 66 μg/ml at 24, 48 and 72 h, respectively; the IC50 values of β-elemene in PC-3 cells were 105, 102 and 96 μg/ml at 24, 48 and 72 h, respectively (Figure 1; data at 48 and 72 h not shown). The results indicate that β-elemene inhibits growth in both of the prostate cancer cell lines, with a greater effect in DU145 cells than in PC-3 cells.

We have previously shown that β-elemene has antitumour efficacy in ovarian cancer cells and non-small cell lung cancer

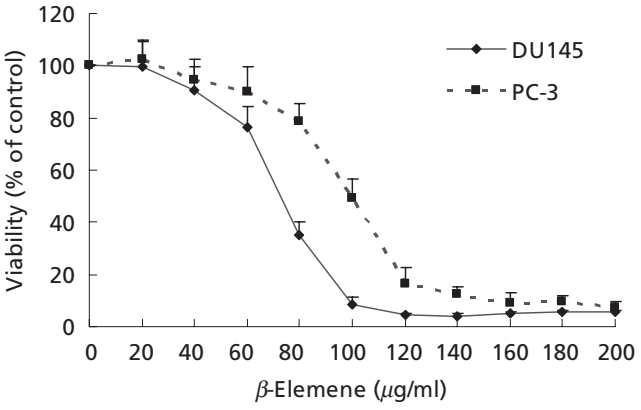


Figure 1 β-Elemene dose-dependently inhibits the proliferation of prostate cancer cells. DU145 or PC-3 prostate cancer cells (5×10^3/well) were evenly distributed in 96-well plates and exposed to the indicated concentrations of β-elemene for 24, 48 or 72 h (data at 48 and 72 h not shown). The ability of β-elemene to inhibit cell proliferation was determined by the MTT assay, as described in the Materials and Methods section. Values are expressed relative to those for cells with no β-elemene exposure (control value, 100%). The results are expressed as the mean ± SE of eight determinations. A representative of two separate experiments is shown.

cells.[24–27] To further explore the spectrum of cancer cells inhibited by β-elemene, we tested the in-vitro cytotoxicity of β-elemene in five other types of human cancer cells, using the MTT assay. As shown in Table 1, β-elemene exhibited antitumour activity in all cancer cell lines examined, which included two breast carcinoma lines, three brain tumour lines, three cervical carcinoma lines, two colon carcinoma lines and two lung carcinoma lines. The IC50 values of β-elemene ranged from 47–67 μg/ml (230–328 μM) in brain, colon and cervical carcinoma cells, to 93–95 μg/ml (455–465 μM) in breast and lung carcinoma cells (Table 1). These data suggest that β-elemene exerts antitumour activity toward a broad spectrum of solid carcinomas.

β-Elemene-induced apoptosis in prostate cancer cells

A major goal of chemotherapy is to commit cancer cells to apoptosis following exposure to anticancer agents.[16,29] To investigate whether β-elemene effectively inhibits prostate cancer cell growth (as was shown in Figure 1) by inducing apoptosis, we treated DU145 cells with β-elemene at 0, 40, 60 and 80 μg/ml for 24 or 48 h and quantified the apoptotic cells by flow cytometric analysis. Figure 2 shows that β-elemene at a concentration of 40 μg/ml induces cellular apoptosis at 24 h and that its effect on cell death continues to increase with increases in the dose and treatment time (data at 48 h not shown). After 24 h, β-elemene at 40 μg/ml had induced apoptosis in 7% of the DU145 cell population, and the percentage of apoptotic cells reached 41% after 48 h with β-elemene at 80 μg/ml (data not given). Similar results were obtained in PC-3 cells (data not shown). These observations suggest that β-elemene inhibits the proliferation of prostate cancer cells by inducing apoptosis.

To confirm the results obtained by flow cytometric analysis, we assessed β-elemene-induced apoptosis in DU145 and PC-3 cells using two other methods: ELISA-based assay and TUNEL assay. DNA fragmentation is an early and characteristic event of apoptosis.[30] The ELISA assay, based on the quantitative sandwich-enzyme-immunoassay principle, uses mouse monoclonal antibodies directed against DNA and histones to provide a quantitative in-vitro determination of histone-associated DNA fragments.[31] Figure 3 shows that after DU145 cells were exposed to β-elemene for 24 or 48 h, apoptosis was significantly increased at all three β-elemene concentrations (40, 60 and 80 μg/ml). Similar results were observed in PC-3 cells (data not shown).

The induction of apoptosis by β-elemene was further measured using the TUNEL technique, a classic method for detecting apoptotic cells in culture.[32] With the TUNEL assay, the induction of apoptosis was detected in DU145 cells starting at 40 μl/ml of β-elemene. In contrast, few apoptotic cells were clearly seen in the negative control group (data not shown). A quantitative analysis of TUNEL staining revealed that β-elemene, at concentrations of 40, 60 and 80 μg/ml, induced a significant increase of apoptosis in DU145 cells (Figure 4). Taken together, the results from all three apoptosis assay methods demonstrate that β-elemene induces apoptosis in prostate carcinoma cells, in a dose- and time-dependent manner.

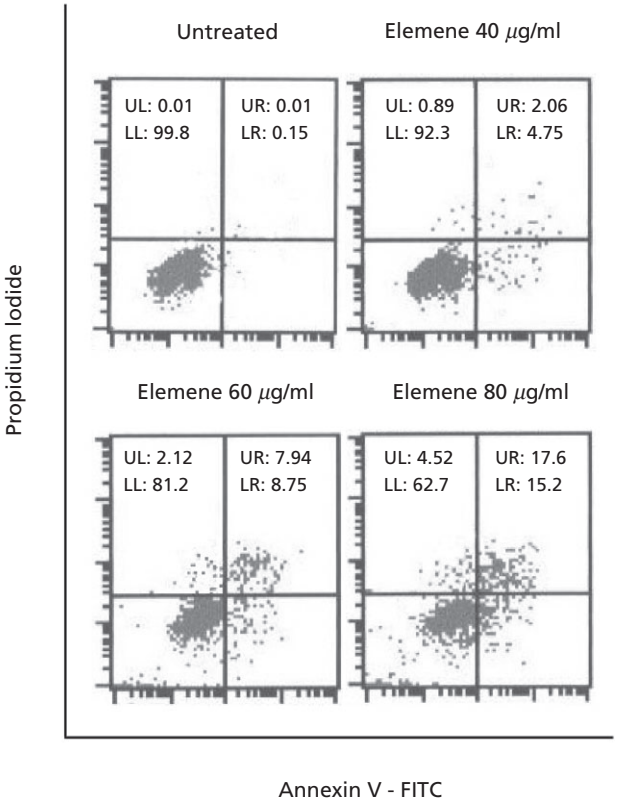
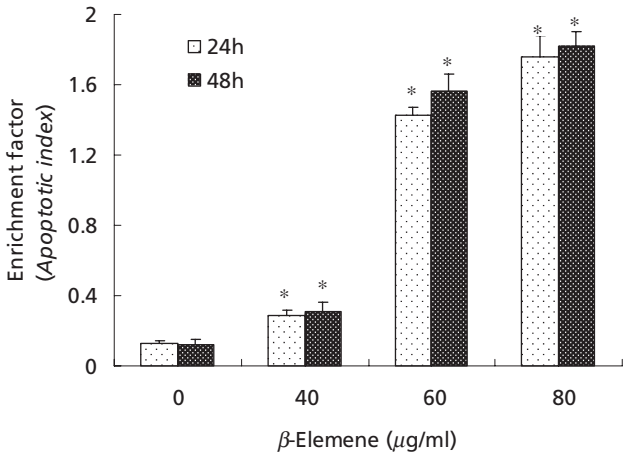


Figure 2 β-Elemene induces apoptosis in prostate cancer cells as analysed by flow cytometry. DU145 prostate cancer cells were exposed to β-elemene at 0, 40, 60 and 80 μg/ml for 24 or 48 h. After exposure, the cells were harvested and stained, and the annexin-V/propidium iodide (PI)-labelled cells were analysed by flow cytometry, as described in the Materials and Methods section. UL, upper left; UR, upper right; LL, lower left; LR, lower right; FITC, fluorescein isothiocyanate. (i) Viable cells (low FITC and low PI signals) in gate LL, (ii) early apoptotic cells (high FITC and low PI signals) in gate LR and (iii) cells that have lost membrane integrity as a result of very late apoptosis (high FITC and high PI signals) in gate UR. The percentages of apoptotic cells (gates LR and UR) are indicated on each cytogram. A representative of three independent experiments is shown (data at 48 h not shown).

β-Elemene activated caspases in prostate cancer cells

Because the mitochondrial pathway involves cytochrome *c* release into cytosol, which forms complexes with apoptotic protease activating factor-1 (Apaf-1) and further results in cleavage of procaspase-9 into active caspase-9, the latter in turn recruits and activates caspase-3 and/or -7 and engages a series of proteolytic events that will culminate in a coordinated disintegration of the cell; hence it is indicated as the primary pathway in anticancer drug-mediated cell killing.[16,33,34] We therefore examined the effect of β-elemene on the activity of caspase-3, -7, -9 and -10 by an ELISA-based assay and investigated whether β-elemene-induced apoptosis is via the intrinsic signals. As shown in Figure 5, the activities of caspase-3, -7 and -10 (Figure 5a) and caspase-9 (Figure 5b) were markedly increased in the prostate cancer cells following exposure to 40, 60 and 80 μl/ml of β-elemene



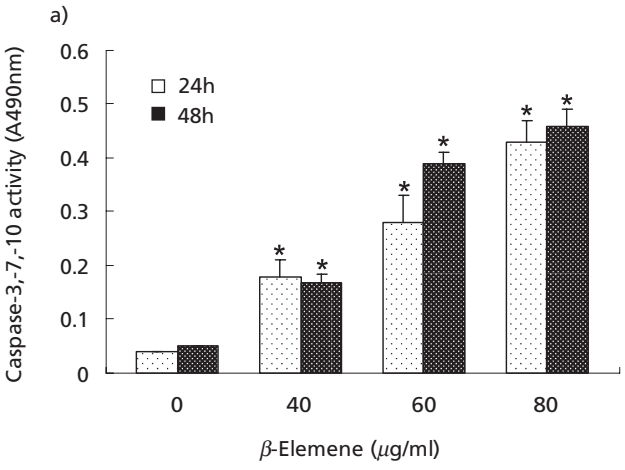


Figure 3 β-Elemene induces apoptosis in prostate cancer cells as determined by an ELISA-based assay. DU145 prostate cancer cells were treated with β-elemene for 24 or 48 h at the indicated doses, and apoptosis was determined with an ELISA-based cell death detection kit, as described in the Materials and Methods section. The results are expressed as the mean ± SD of three separate experiments. *$P < 0.05$ vs untreated control; n = 6.

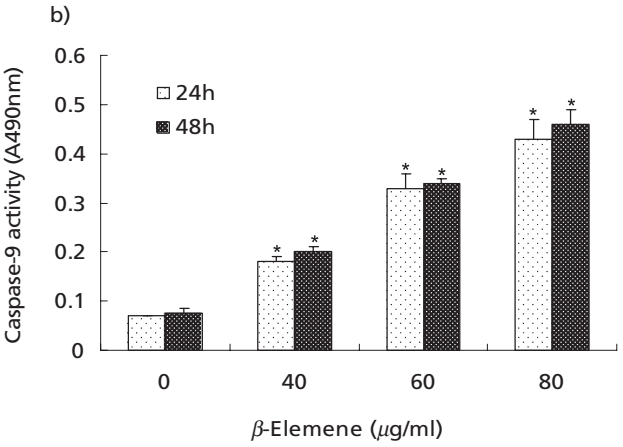


Figure 5 β-Elemene activates caspase activity in prostate cancer cells. DU145 prostate cancer cells were treated with β-elemene at the indicated concentrations for 24 or 48 h. Caspase-3, -7 and -10 (a) and caspase-9 (b) activities were measured with an ELISA-based assay, as described in the Materials and Methods section. The results are expressed as the mean ± SD of three separate experiments. *$P < 0.05$ vs untreated controls.

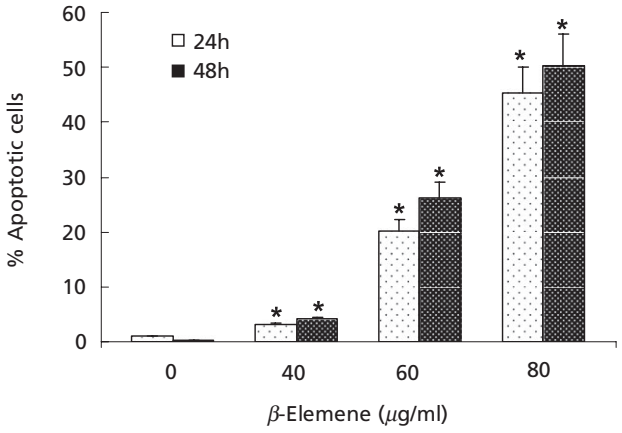


Figure 4 β-Elemene induces apoptosis in prostate cancer cells as assessed by TUNEL assay. DU145 prostate cancer cells were treated with β-elemene for 24 or 48 h at the indicated doses, and apoptosis was determined by TUNEL assay, as described in the Materials and Methods section. The results are expressed as the mean ± SD of three independent counts by experts. *$P < 0.05$ vs untreated control.

for 24 or 48 h. These results suggest that β-elemene induces apoptosis in prostate carcinoma cells, at least in part, through a caspase-9 involved intrinsic pathway.

β-Elemene altered the levels of apoptosis regulators in prostate cancer cells

To further investigate the mechanism of β-elemene-induced apoptosis, the protein level of Bcl-2, the release of cytochrome *c*, and the cleavage of caspases and PARP were examined following β-elemene exposure *in vitro*. After exposure of DU145 cells to β-elemene at 0, 40, 60 and 80 μg/ml for 24 or 48 h, the protein levels of Bcl-2, mitochondrial and cytosolic cytochrome *c*, and the cleaved forms of caspase-9, caspase-3 and PARP were analysed by Western blotting, as described in the Materials and Methods section. Figure 6 shows that the protein expression of Bcl-2 was downregulated, the level of mitochondrial cytochrome *c* was decreased, and the level of cytosolic cytochrome *c* was increased. Additionally, the levels of cleaved caspase-9, caspase-3 and PARP were increased by β-elemene, in a dose-dependent manner (data at 48 h not shown). These data suggest that β-elemene modulates Bcl-2 expression to induce apoptosis through a pathway involving the release of cytochrome *c* from mitochondria.

Discussion

Although testosterone depletion remains the gold standard for advanced-stage hormone-sensitive disease, castration-

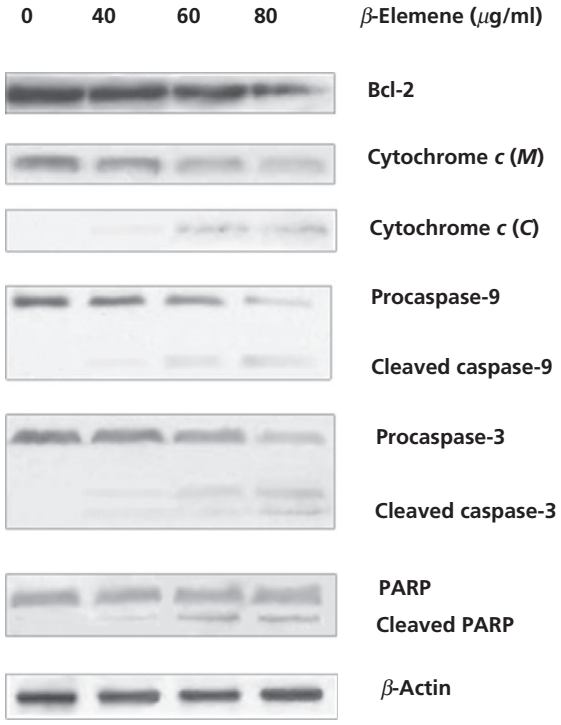


Figure 6 Effect of β-elemene on protein levels of apoptotic regulators in prostate cancer cells. DU145 prostate cancer cells were exposed to β-elemene at 0, 40, 60 and 80 μg/ml for 24 or 48 h (data at 48 h not shown). A total of 50 μg of cellular proteins extracted from the β-elemene-treated cells were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with antibodies against Bcl-2, cytochrome *c*, caspase-9, caspase-3 and PARP. β-Actin antibody was used as a loading control. Western blot data are representative of three independent experiments. Cytochrome *c* (*M*), mitochondrial cytochrome *c*; cytochrome *c* (*C*), cytosolic cytochrome *c*.

resistant prostate cancer is a conundrum. Thus, the development of promising novel chemotherapeutic agents for castration-recurrent prostate cancer is a high priority. Recently there has been a shifting focus towards finding natural compounds that may prevent and treat prostate cancer.[35] For example, Albrecht and colleagues found that proliferation and invasion of LNCaP, DU145 and PC-3 prostate cancer cells *in vitro* could be significantly reduced in the presence of polyphenols extracted from various parts of the pomegranate fruit.[36] Malik and coworkers evaluated a crude pomegranate fruit extract (PFE) containing ellagic acid, the main polyphenol in pomegranate, for its anti-proliferative and pro-apoptotic properties and found that it caused both cell growth inhibition and apoptosis in a dose-dependent manner in androgen-insensitive PC-3 cells.[37,38] Oral administration of PFE to mice implanted with androgen-sensitive prostate cancer cells resulted in inhibition of tumour growth, with a significant decrease in serum PSA levels. A number of clinical trials are ongoing, focusing on the potential of other phytochemicals, such as green tea catechins, curcumin, resveratrol and genistein, in the treatment of prostate cancer and other tumours.[39–43]

In the present study, we demonstrate for the first time that β-elemene, a natural compound, inhibits the in-vitro growth and proliferation of the human androgen-insensitive prostate cancer cell lines DU145 and PC-3 in a dose-dependent manner. β-Elemene treatment of prostate cancer cells resulted in down-regulated Bcl-2 expression, enhanced cytochrome *c* release, activated caspase-3, -7, -9 and -10, and cleaved PARP, suggesting that β-elemene triggers cell death in prostate cancer cells, at least in part, through an apoptotic pathway mediated by the release of mitochondrial cytochrome *c*. We also show that β-elemene exhibits strong antitumour efficacy in brain, breast, cervical, colon and lung carcinoma cells. These results are consistent with previous findings that β-elemene is a potent inhibitory agent against several types of solid tumours, but has only moderate effects on normal and non-cancerous cells.[22–28]

β-Elemene (98% preparation formulation) has been shown to be a potent inhibitor of cell growth and has been approved for phase II clinical investigation for treatment of secondary brain tumours in China. Nevertheless, the molecular mechanisms of β-elemene-induced cell death are not well established. It has become clear during the past decade that aberrations in the initiation or execution of the apoptotic programme at various levels are associated with most human malignancies, including prostate cancer, underscoring the importance of pharmacological therapies that target apoptosis regulators. Indeed, most chemotherapeutic agents act via the induction of apoptosis.[16,44]

In the current study, β-elemene induced apoptosis in prostate cancer cells, as demonstrated by flow cytometry, ELISA-based assay and TUNEL assay. These results indicate that β-elemene inhibits DU145 and PC-3 cell growth and triggers cell death by inducing apoptosis, suggesting the potential of β-elemene as an effective agent for the treatment of castration-resistant prostate cancer.

Survival pathways for prostate cancer cell growth include deregulated expression and/or sequence variations of the *PTEN* gene that occur with high frequency in advanced prostate cancer, leading to aberrant action of Akt kinase activity, which in turn promotes tumour growth.[45] Loss of *PTEN* also permits activated Akt to phosphorylate the intracellular protein Bad, resulting in the release of the anti-apoptotic protein Bcl-2, which then leads to cancer cell survival.[45] Thus, recent therapeutic strategies have been directed at suppressing the anti-apoptotic function of Bcl-2.[46–48] The most successful targeting of the Bcl-2 axis in prostate cancer, however, has been achieved using chemotherapy. For instance, success of docetaxel chemotherapy in prolonging survival of patients with castration-resistant prostate cancer was first demonstrated in 2004. Docetaxel is a taxoid that inhibits the depolymerisation of microtubules, leading to disruption of the mitotic process and cell cycle arrest at the G2/M phase and apoptosis. In addition to its apoptotic effect via microtubule stabilisation, docetaxel also induces apoptosis by inhibiting Bcl-2, a key mechanism for cancer cell survival achieved by the effect of *PTEN* sequence variations on Akt signalling. Androgen-insensitive cells may overexpress *Bcl-2* independent of the PTEN–Akt signalling effects.[45] Docetaxel phosphorylates the serine residues of Bcl-2, resulting in its

inactivation and consequent activation of the caspase cascade and apoptosis.

To understand the molecular mechanisms of β-elemene actions in prostate carcinoma cells, we investigated its effects on several apoptotic regulators. Bcl-2, a protein on the mitochondrial outer membrane, prevents apoptosis by suppressing the release of the caspase-activating protein cytochrome *c* from mitochondria.[49,50] Despite controversy regarding the role of Bcl-2 expression in the induction of apoptosis in prostate cancer cells,[51,52] there is general agreement that Bcl-2 is involved in the mechanisms of prostate cancer initiation and progression, as well as the emergence of androgen-insensitive prostate cancer cells.[53,54] Furthermore, Bcl-2 expression has been shown to be associated with metastatic stages in prostate cancer.[55] Studies have also demonstrated that high Bcl-2 expression levels and low Bax expression levels are correlated with a poor therapeutic response of prostate cancer to radiotherapy,[56,57] indicating that Bcl-2 overexpression is an adverse prognostic indicator. In the present study, we demonstrated that β-elemene reduces the expression of Bcl-2 in a dose-dependent manner, suggesting that β-elemene induces apoptosis in human prostate cancer cells by modulating Bcl-2 expression.

To date, 14 distinct mammalian caspases have been identified and classified as initiator or effector caspases. The initiator caspases such as caspase-2, -8, -9 and -10 are activated by various apoptotic signals, after which they cleave and activate downstream effector caspases. In response to some death stimuli, cytochrome *c* is released from mitochondria into the cytosol, where it promotes the formation of a caspase-activating complex that includes cytochrome *c*, Apaf-1 and procaspase-9. This apoptosome complex triggers the activation of caspase-9, which leads to a proteolytic cascade that activates downstream effector caspases.[58,59] The effector caspases, which include caspase-3, -6 and -7, target specific cellular protein substrates for either activation or inactivation.[33,60] Accumulating evidence demonstrates that caspase cascades, especially via caspase-3, -7 and -9, are involved directly or indirectly in the execution of apoptosis in response to diverse stimuli in prostate cancer cells.[42,61–65] For example, caspase-3 cleaves and thereby inactivates PARP, which is generally considered to be an early marker of chemotherapy-induced apoptosis.[66] The current study revealed that β-elemene treatment of prostate cancer cells resulted in the activation of caspase-3, -7, -9 and -10 and the cleavage of PARP, suggesting that β-elemene induces apoptotic cell death through a caspase-dependent mitochondrial pathway in prostate carcinoma cells.

Conclusions

In summary, we have demonstrated that β-elemene suppresses the growth and proliferation of prostate cancer cells and other types of tumour cells *in vitro*, indicating that β-elemene has broad-spectrum antitumour activity. We have also shown that β-elemene exerts its antitumour effect in prostate carcinoma cells by inducing apoptosis via a pathway involving the down-regulation of Bcl-2 expression, enhancement of cytochrome *c* release, activation of caspase-3, -7, -9 and -10, and cleavage of PARP. Although the clinical treatment and management of castration-resistant prostate cancer remains a challenge, β-elemene holds promise as a new potentially effective therapeutic agent for prostate cancer and other types of solid tumours.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts on interest to disclose.

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