

β -Elemene and Taxanes Synergistically Induce Cytotoxicity and Inhibit Proliferation in Ovarian Cancer and Other Tumor Cells

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Abstract. β -Elemene, originally derived from plants, has been recently investigated as a new anticancer agent. The purpose of this study was to explore the efficacy and mechanisms of action of the combined use of β -elemene plus a taxane as an antitumor therapeutic strategy for ovarian cancer and other carcinomas. The interaction of β -elemene with paclitaxel or docetaxel produced additive to moderately synergistic effects against the platinum-resistant ovarian cancer cell line A2780/CP70 and its parental cell line A2780, and showed moderately synergistic activity against PC-3 prostate cancer cells. In addition, the co-administration of β -elemene and a taxane at low-micromolar concentrations dramatically increased the rate of micronucleus formation and the percentage of mitotic arrest in both ovarian cancer cell lines, as compared with treatment with either agent alone. The highest synergy towards the ovarian cancer cells was observed with β -elemene plus docetaxel. Consistent with these data, treatment of A2780/CP70 cells with β -elemene plus a taxane strikingly reduced cell viability and increased cell apoptosis, as assessed by annexin V binding. Moreover, β -elemene plus docetaxel induced elevated levels of caspase-9 and p53 proteins in A2780/CP70 cells, and the combination of β -elemene plus a taxane caused marked cell-cycle arrest at the G₂/M phase in these cells. One possible mechanism to account for the enhanced cytotoxic efficacy of this combination treatment is a β -elemene-induced increase in taxane influx

into cancer cells. These observations indicate that combination therapy with β -elemene and taxanes has synergistic antitumor activity against ovarian and prostate carcinomas *in vitro*. This promising new therapeutic combination warrants further pre-clinical exploration for the treatment of chemoresistant ovarian cancer and other types of tumors.

Ovarian cancer accounts for nearly 4% of cancer cases among women and is the leading cause of gynecological cancer-related death in the United States (1). Taxanes have been used clinically for chemotherapy of patients with ovarian cancer in recent years. The gold standard chemotherapy for previously-untreated ovarian cancer is currently a combination of taxane and platinum. Nevertheless, most patients with ovarian cancer suffer relapse, and fewer than 20% of patients with stage III or IV disease survive long term (2).

Taxanes are natural products derived from trees of the Taxodaceae family (3). Among new chemotherapeutic agents, the taxanes have emerged as one of the most powerful classes of compounds; they exhibit a wide range of antitumor activity *in vitro* and *in vivo* (4-7). Paclitaxel and docetaxel are two of the most studied taxanes. Despite differences in their molecular pharmacology, pharmacokinetics, and pharmacodynamic profiles, paclitaxel and docetaxel share a similar mechanism of antitumor action: the promotion of microtubule assembly and inhibition of microtubule disassembly. The suppression of microtubule dynamics results in cell-cycle arrest at the G₂/M phase (8, 9), which eventually induces cell apoptosis (10). However, side-effects and the development of drug resistance limit the use of taxanes as single agents for treating patients with cancer. One effective strategy to overcome these limitations is to use a combination of drugs that work synergistically or additively. This allows for the use of the lowest possible dose of each drug to achieve certain therapeutic efficacy.

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Combination chemotherapy for treating malignancies is increasingly practiced with greater sensitivity and less toxicity. Drug combinations can exert synergistic, additive, or antagonistic cytotoxic actions. Anticancer drug combinations that include taxanes are especially useful because the anticancer drugs can have non-overlapping side-effect profiles and may work together to be effective against taxane-resistant cancer. Taxanes have been studied in combination with anthracyclines, antimetabolites, alkylating agents, and topoisomerase inhibitors for treating human cancer (11). Although anthracyclines and taxanes exhibit non-overlapping side-effect profiles and lack complete clinical cross-resistance, the combination of these compounds is associated with increased hematological toxicities (12, 13). Vinorelbine acts synergistically with taxanes, but gastrointestinal side-effects limit the use of this combination (11, 14, 15). There is considerable clinical interest in identifying novel agents for combination with taxanes in order to improve clinical outcomes.

β -Elemene (β -1-methyl-1-vinyl-2,4-di-isopropenyl-cyclohexane; Figure 1), derived from an extract of the ginger plant *Rhizoma zedoariae*, has been used as a novel antitumor drug in recent years in China (16, 17). β -Elemene was shown to be effective in the treatment of leukemia and carcinomas of the brain, breast, liver, and other tissues (18-21). The advantages of β -elemene as an antitumor agent include: (i) a broad antitumor spectrum in many types of cancer, (ii) no direct multidrug resistance, (iii) possibility of overcoming resistance to other drugs, and (iv) low toxicity to normal tissues and cells (19, 20). As β -elemene is a novel antitumor drug, the mechanisms by which it leads to cell death are not yet clear. β -Elemene has been shown to induce cell-cycle arrest at G₂/M and trigger apoptosis of brain tumor cells, leukemia cells, and lung and prostate cancer cells (19-27).

The goal of chemotherapy for human malignancies is the inhibition of cell proliferation and/or induction of cell apoptosis. Evidence indicates that antitumor agents exert, at least part, of their cell-killing effects by inducing cell-cycle arrest and apoptotic cell death (28). To assess the ability of different drug combinations to induce apoptosis, several approaches are necessary, including the observation of morphological features of apoptosis, the evaluation of apoptotic parameters such as poly (ADP-ribose) polymerase (PARP) cleavage, and the analysis of annexin-V binding (29). Our previous study demonstrated that concurrent treatment with a taxane and β -elemene markedly increases antitumor activity in human lung cancer cells, compared with the activity of a taxane-alone (30).

We hypothesized that taxanes and β -elemene may also be a useful combination for the treatment of ovarian cancer and other carcinomas. In the present study, the *in vitro* cytotoxicities of β -elemene, paclitaxel, and docetaxel-alone and in two-drug combinations were evaluated in two human ovarian cancer cell

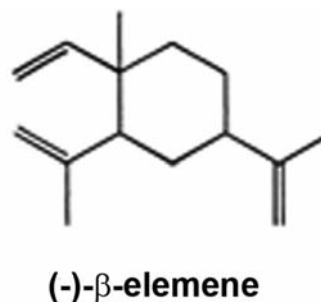


Figure 1. The chemical structure of β -elemene.

lines, A2780 and A2780/CP70. The aim of this study was to address the question of synergism between β -elemene and taxanes against human ovarian cancer. In order to evaluate the anticancer activity of β -elemene in combination with taxanes, synergistic actions, morphological changes, induction of apoptosis, and cell cycle distribution were analyzed.

Materials and Methods

Chemicals and reagents. The (-)- β -elemene (98% purity) was provided by Dalian Yuanda Pharmaceutical Co., Ltd. (Dalian, China). Paclitaxel, docetaxel, propidium iodide (PI), and other chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC) was purchased from Caltag Laboratories (Burlingame, CA, USA). Stock solutions of 1 mM paclitaxel, 1 mM docetaxel, and 4.89 mM β -elemene [dissolved in dimethyl sulfoxide (DMSO)] were stored at -80°C . Desired concentrations of each drug were prepared by dilution with RPMI-1640 culture medium (Invitrogen, Life Technologies, Inc., Gaithersburg, MD, USA) before use. Antibodies were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Cell lysis buffer was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cells and cell culture. The human ovarian cancer cell lines A2780 and A2780/CP70 have been described previously (16, 24). The human prostate cancer cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA, USA). Prior to the experiment, cells were kept in logarithmic growth in a 75-cm² cell culture flask in RPMI-1640 medium (Invitrogen, Life Technologies, Inc.) supplemented with 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin, and 10% (v/v) heat-inactivated fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells in mid-logarithmic growth (50% confluence) were used for the experiments. Cultures were repeatedly evaluated for the presence of mycoplasma and had greater than 95% viability.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The inhibition of ovarian cancer cell proliferation was assayed using a commercial MTT assay kit (CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay; Promega Corp., Madison, WI, USA). Cells were harvested during logarithmic growth and plated at 1000 cells/well in 100 μl of medium in 96-well flat-

bottomed microtiter plates. After incubation overnight, the medium was discarded, and the cells were incubated in the presence or absence of the drug. After treatment for 24, 48, or 72 h, 20 μ l of MTT assay solution were added to each well. The plates were incubated for 2 h at 37°C, the absorbance of each well was measured at 690 nm using a microplate reader (Thermo Labsystems, VA, USA). Eight replica wells were measured for each treatment group, and each experiment was repeated three times. Appropriate solvent controls were included. The data were analyzed by converting the dose–response curves into log-linear regression curves. For each drug alone, and for the two drugs in combination, the 50% inhibitory concentration (IC₅₀) was calculated from plots of the percentage growth inhibition *versus* the log of the drug concentration (linear regression).

Micronucleus assay. Briefly, cells were grown on a sterilized glass slide (25 mm \times 75 mm \times 0.1 mm) in a cell culture dish (100 mm \times 20 mm). After incubation in the presence or absence of a drug, the cells were gently washed one time with phosphate buffered saline (PBS) at 4°C, incubated in a hypotonic solution containing 0.075 M KCl for 8 min, and fixed in a solution of methanol and glacial acetic acid (3:1, v/v) for 1 h. The cells were stained using TREND Giemsa Plus Stain (Alexon Treny, Ramsey, MN, USA) according to the manufacturer's protocol and were examined under a light microscope. Morphological changes were analyzed on photomicrographs. One thousand cells per slide were scored to calculate the micronucleus (MN) rate, and 300 nuclei per slide were counted to determine the percentage of cells in mitotic arrest.

Cell-cycle analysis. After treatment with β -elemene and/or taxanes, A2780/CP70 cells were harvested, washed once with cold PBS, and fixed in 75% ethanol/PBS. After washing twice more with PBS, the cells were suspended in PBS containing 10 μ g/ml propidium iodide (PI), 20 μ g/ml RNase A, 0.1% sodium citrate, and 0.1% Triton X-100. Labeled cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) using CellQuest Pro software (Becton Dickinson). Cell-cycle distributions were analyzed with ModFit v3.0 software (Verity, Topsham, ME, USA).

Western blot analysis. A2780/CP70 cells were incubated with β -elemene and/or taxanes for 48 h, harvested by trypsinization, and washed with ice-cold PBS. The cells were lysed on ice for 30 min in a mammalian cell lysis buffer (Quality Biological, Inc., Gaithersburg, MD, USA) containing 10 μ l/ml of 200 mM phenylmethylsulfonyl fluoride, 10 μ l/ml of 100 mM sodium orthovanadate, and 10 μ g/ml aprotinin. Cell extracts were clarified by centrifugation at 13,000 \times g for 30 min at 4°C, and protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA, USA). Fifty micrograms of proteins from whole-cell lysates were mixed with an equal volume of 2 \times gel loading solution (Quality Biological, Inc.), heated for 5 min at 95°C, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience, Inc., Keene, NH, USA). After blocking by incubation in Blotto B (Santa Cruz) for 1 h at room temperature, the membranes were incubated with specific primary antibodies (diluted 1:100 to 1:300) overnight at 4°C. The membranes were washed with Tris-buffered saline/0.1% Tween 20, incubated with anti-rabbit peroxidase-conjugated secondary antibody (diluted 1:10,000), and washed again. Immunoreactive

bands were detected using enhanced chemiluminescence substrate (Santa Cruz) according to the manufacturer's instructions and visualized on X-ray film (Eastman Kodak, Rochester, NY, USA). All blots are representative of three independent experiments.

Annexin-V binding assay. A2780/CP70 cells were grown in 25-cm² cell culture flasks and incubated for 48 h with different dosages of each individual drug or two-drug combinations. The cells were harvested by trypsinization, gently washed with 4 ml of PBS at 4°C, collected by centrifugation at 56 \times g for 6 min at 4°C, and suspended in 100 μ l of 2 μ g/ml annexin V-FITC in HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, pH 7.4). Subsequently, cells were incubated for 1.5 h on ice in the dark, followed by incubation for 20 min at 4°C in PBS supplemented with 0.2 μ g PI per sample. The cells were washed once with 4 ml of HEPES buffer and analyzed within 30 min by flow cytometry using a FACSCalibur dual laser cytometer (Becton Dickinson). Data analysis was performed with the CellQuest Pro software (Becton Dickinson). The number of apoptotic cells is expressed as a percentage of the total number of events.

Statistical data analysis. Cytotoxic efficacy of each drug individually or in combination is expressed as the IC₅₀ value (*i.e.*, the drug concentration causing 50% inhibition of cell viability). The IC₅₀ values, expressed as mean \pm SD, were obtained from dose–response curves after fitting the data by log-linear regression. Differences in values between groups were evaluated by Student's *t*-test. The level for statistically significant differences was set at *p*<0.05.

Results

IC₅₀ value of each drug determined individually by the MTT assay. Before studying the effect of β -elemene combined with paclitaxel or docetaxel, the IC₅₀ of each drug was evaluated individually. Dose–response and time-dependent studies were conducted by incubation with different concentrations of each drug alone for 24, 48, and 72 h, respectively, and the IC₅₀ values of β -elemene, paclitaxel, and docetaxel were calculated from the MTT assay results at three different time points. Each anticancer drug inhibited the growth of the platinum-resistant ovarian cancer cell line A2780/CP70 and its sensitive parental cell line A2780 (Table I; Figure 2). β -Elemene was less toxic than paclitaxel and docetaxel under the same experimental conditions in each ovarian cancer cell line, and docetaxel exhibited the strongest cytotoxicity among the three drugs. With increasing β -elemene concentration or prolonged incubation time, the percentage of cell viability decreased in a dose- and time-dependent manner. The IC₅₀ of β -elemene did not differ significantly between the two ovarian cancer cell lines. However, marked differences in cytotoxic susceptibility to paclitaxel and docetaxel were observed between the cell lines (Table I). After 24 h of incubation with paclitaxel or docetaxel, an IC₅₀ could not be calculated for A2780 cells, even at drug concentrations up to 1280 nM. These results imply that compared with β -elemene, a longer exposure time is needed for taxanes to penetrate the cell membrane or exert toxic effects in A2780 cells.

Table I. IC_{50} ^a values of β -elemene, paclitaxel, and docetaxel against two human ovarian carcinoma cell lines, as assessed by the MTT assay^b.

Drug treatment	Cell line	IC_{50}		
		24 h	48 h	72 h
β -Elemene (μ M)	A2780	305.9	307.7	265.3
	A2780/CP70	398.6	353.3	307.4
Paclitaxel (nM)	A2780	>1280.0 ^c	8.5	4.6
	A2780/CP70	86.1	8.8	7.4
Docetaxel (nM)	A2780	>1280.0 ^c	2.0	1.9
	A2780/CP70	64.5	1.7	1.3

^a IC_{50} is the drug concentration required to inhibit cell growth by 50% relative to untreated controls. Data are the means of eight simultaneous replicates repeated three times. ^bPercentage cell viability = [(OD with drug - blank) ÷ (OD without drug - blank)] × 100. Means of three independent experiments are provided. ^cValues listed as >1280.0 nM indicate that the IC_{50} was not determinable in the treated ovarian cancer cells. MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Effects of β -elemene plus taxanes as determined by synergistic analysis. To determine the nature of the combined effects of β -elemene and taxanes, the combination index (CI), as defined by Chou and Talalay (32), was calculated assuming both the case of mutually-exclusive drugs and the case of mutually non-exclusive drugs (Table II). A2780 and A2780/CP70 ovarian cancer cells, and the prostate cancer cell line PC-3 were incubated for 72 h with 10 different fixed combinations of two drugs at concentrations above and below their respective IC_{50} , *i.e.*, within a range of 0.1- to 2- times a value near the IC_{50} of each individual drug. The dose-effect curves were plotted for each drug and for the 10 combinations, and the CI was determined for mutually exclusive (i) and non-exclusive drugs (ii) as below (31-33), where El is β -elemene; Tax, taxane; and comb, combined:

(i) $CI = [IC_{50} \text{ El comb} \div IC_{50} \text{ El alone}] + [IC_{50} \text{ Tax comb} \div IC_{50} \text{ Tax alone}]$

(ii) $CI = [IC_{50} \text{ El comb} \div IC_{50} \text{ El alone}] + [IC_{50} \text{ Tax comb} \div IC_{50} \text{ Tax alone}] + [IC_{50} \text{ El comb} \times IC_{50} \text{ Tax comb}] \div [IC_{50} \text{ El alone} \times IC_{50} \text{ Tax alone}]$

It is generally considered that a CI <1.0 indicates synergism; a CI=1.0, an additive effect; and a CI >1.0, antagonism (15). However, the more conservative classification method of Soriano *et al.* (34), which takes into account the confidence interval or standard deviation of the CI values, classifies the effects of drug combinations according to CI values as follows: >1.3, antagonism; 1.1-1.3, moderate antagonism;

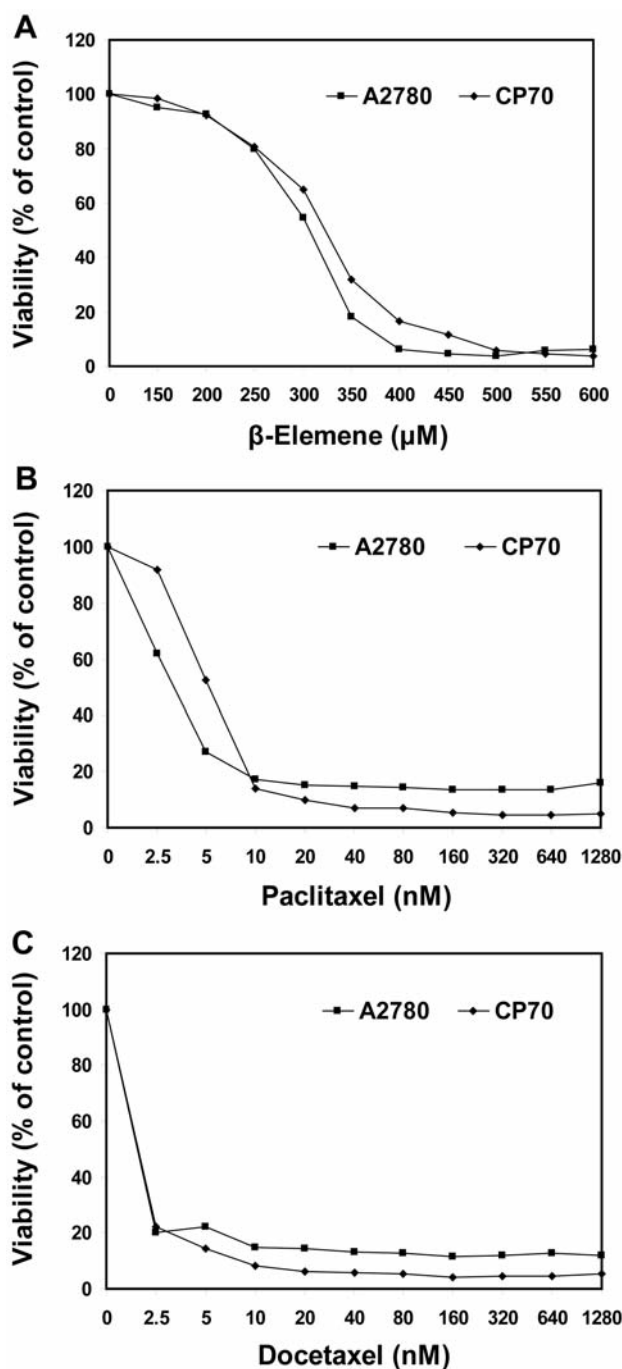


Figure 2. Cytotoxicity of β -elemene, paclitaxel, and docetaxel towards two human ovarian cancer cell lines, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ovarian carcinoma cells (A2780 and A2780/CP70 cells) were evenly distributed in 96-well plates (5×10^3 per well) and treated with different concentrations of β -elemene (A), paclitaxel (B), or docetaxel (C) for 24, 48, and 72 h (data at 24 and 48 h not shown). Each concentration was assayed in triplicate. The ability of the drugs to inhibit cell proliferation was determined by the MTT cell survival assay, as described in Materials and Methods. Cell viability values are expressed relative to cell viability in the absence of a drug (100% control value). The results represent the mean of at least three independent experiments.

Table II. Synergistic analysis of the combined effect of β -elemene with paclitaxel or docetaxel in human ovarian and prostate cancer cells following treatment for 72 h^a.

Cancer cell line	Combination of β -elemene plus	CI ^b (mean \pm SD)	CI ^c (mean \pm SD)
A2780	Paclitaxel	0.64 \pm 0.17	0.76 \pm 0.24
	Docetaxel	0.75 \pm 0.13	0.90 \pm 0.18
A2780/CP70	Paclitaxel	0.77 \pm 0.04	0.92 \pm 0.06
	Docetaxel	0.79 \pm 0.07	0.95 \pm 0.09
PC-3	Paclitaxel	0.65 \pm 0.08	0.77 \pm 0.12
	Docetaxel	0.71 \pm 0.07	0.86 \pm 0.09

^aSynergistic effect of β -elemene with paclitaxel or docetaxel was evaluated in two ovarian cancer cell lines (A2780 and A2780/CP70) and one prostate cancer cell line (PC-3). CI values are shown for Fa50 (Fa is the fraction of cells affected; Fa50 is the point at which 50% of the cells were inhibited). Data are means \pm SD of three independent experiments for each cell line. ^bCI calculated as for mutually exclusive drugs. ^cCI calculated as for mutually non-exclusive drugs.

0.9-1.1, additive effect; 0.8-0.9, slight synergism; 0.6-0.8, moderate synergism; 0.4-0.6, synergism; and 0.2-0.4, strong synergism.

The CIs of β -elemene combined with paclitaxel or docetaxel at high levels of cytotoxicity (at IC₅₀) for the inhibition of human ovarian and prostate cancer cell proliferation are shown in Table II. Under both assumptions, *i.e.*, mutually exclusive and mutually non-exclusive, the CIs for the combinations of β -elemene with paclitaxel and with docetaxel revealed cytotoxic effects ranging from additive to moderately-synergistic in ovarian cancer cells, even when the CIs were evaluated using the classification method of Martin *et al.* (31). Similarly, the CIs of the combinations of β -elemene with paclitaxel or docetaxel exhibited moderately synergistic activity in prostate cancer cells (Table II). Furthermore, the CIs of β -elemene with paclitaxel or docetaxel varied depending on the cell type or cell line. These variations may reflect differences in the way in which ovarian and prostate cancer cells handle drug-induced damage. However, within each cell line tested, the CI did not significantly differ between the combination of β -elemene and paclitaxel and the combination of β -elemene and docetaxel.

Effects of β -elemene plus taxanes as demonstrated by micronucleus formation and cell cycle arrest. Morphological changes in the ovarian cancer cells treated with the combinations of β -elemene and paclitaxel or docetaxel were examined by observing micronucleus formation. The results were consistent with those of the synergistic analysis. The cytotoxic efficacy of the two-drug combinations was stronger than that of each drug alone (Table III and Figure 3). β -

Table III. Micronucleus rate (MN) and frequency of mitotic arrest (M) in human ovarian cancer cells following a 48-h treatment with β -elemene, paclitaxel, and docetaxel, alone and in two-drug combinations^a.

Drug treatment	Ovarian cancer cell line			
	A2780		A2780/CP70	
	MN ^b (%)	M ^c (%)	MN ^b (%)	M ^c (%)
Untreated control	0	2	1	3
90 μ M β -Elemene	2	2	3	1
4.2 nM Paclitaxel	50**	15**	60*	49*
2.1 nM Docetaxel	20**	17**	80*	30*
180 μ M β -Elemene	0	2	4	20*
8.4 nM Paclitaxel	2	6	30*	19*
4.2 nM Docetaxel	30**	6	40*	18*
90 μ M β -Elemene + 4.2 nM paclitaxel	12**	8**	50*	50*
90 μ M β -Elemene + 2.1 nM docetaxel	150**	10**	120*	65*

^aData are means of three independent experiments for each cell line, with three slides per treatment group for each experiment. Synergistic antitumor activity can be seen for the combination of β -elemene plus paclitaxel or docetaxel, especially in A2780/CP70 cells. ^bMN represents the number of cells with a micronucleus (or micronuclei) among 1,000 cells counted under a light microscope. ^cM represents the number of cells in mitotic interphase among 100 cells counted under a light microscope. * p <0.05 and ** p <0.01 vs. untreated control groups.

Elemene treatment alone did not cause a significant increase in the rate of micronucleus formation or the percentage of cell-cycle arrest compared with the rates in untreated control cells (p >0.05 for each), whereas treatment with paclitaxel or docetaxel alone induced significant increases in both rates (p <0.05 for each), especially in the A2780/CP70 cells. After 48-h treatment with the combinations of β -elemene and taxanes, especially β -elemene plus docetaxel, both the micronucleus formation rate and mitotic arrest percentage were obviously enhanced compared with the rates for each drug alone (p <0.05 for each). Thus, the morphological observations supported the results of the synergistic analysis. These results indicate that β -elemene significantly increases the susceptibility of ovarian cancer cells to the cytotoxic effects of paclitaxel and docetaxel. This may be attributable to a β -elemene-induced increase in intracellular uptake of paclitaxel and docetaxel, or competition between β -elemene and taxanes for the efflux pump.

Effects of β -elemene plus taxanes as evaluated by cell-cycle analysis. To investigate how β -elemene and taxanes effectively inhibit human ovarian cancer cell growth (Table I and Figure 2), the cell-cycle distributions of ovarian cancer cells after treatment with β -elemene and taxanes, alone and

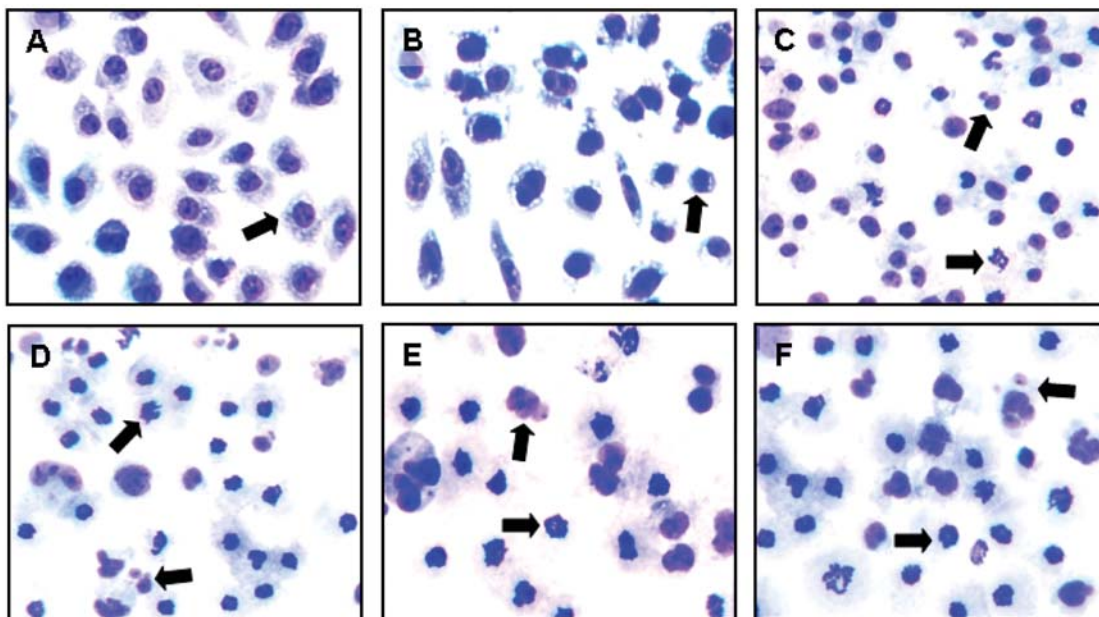


Figure 3. Effect of β -elemene and taxanes on cell morphological changes in human ovarian cancer cells. A2780/CP70 cells were treated with β -elemene or a taxane alone, a two-drug combination, or no drugs (control). Photographs were taken after a 48-h treatment. A: Untreated control cells. The arrow shows a normal cell. B: Treatment with 90 μ M β -elemene alone. Cells show shrinkage and nuclear chromatin condensation (see arrow), similar to the morphological changes of cells undergoing apoptosis. C: Treatment with 4.2 nM paclitaxel alone. The upper arrow shows nuclear damage-related micronucleus (MN) formation. The lower arrow indicates a cell arrested in a post-mitotic interphase state, which is consistent with our results demonstrating cell-cycle arrest at the G_2/M phase. D: Treatment with 2.1 nM docetaxel alone. The upper arrow shows a post-mitotic interphase cell. The lower arrow indicates nuclear damage-related micronucleus formation. E: Treatment with the combination of 90 μ M β -elemene and 4.2 nM paclitaxel. The upper arrow shows a multinucleated cell with disassembled and fragmented nuclei, resembling the apoptotic bodies of apoptotic cells. The lower arrow indicates a post-mitotic interphase cell. F: Treatment with the combination of 90 μ M β -elemene and 2.1 nM docetaxel. The arrows show a multinucleated cell and a post-mitotic interphase cell, respectively. These results demonstrate that combination treatment exerted stronger cytotoxic efficacy than did any of the three drugs used individually.

in combinations, were analyzed by flow cytometry. The flow cytometric profiles revealed substantial increases in the fraction of A2780/CP70 cells in the G_2/M phase of the cell cycle (Figure 4), with 27, 30, and 29% of A2780/CP70 cells in G_2/M after treatment with paclitaxel (4.2 nM), docetaxel (2.1 nM), and β -elemene (90 μ M), respectively, compared with 19% of untreated cells. Following treatment with the two-drug combinations, the percentages of A2780/CP70 cells at G_2/M increased markedly from 19% of untreated cells to 43% and 50% of cells treated with paclitaxel plus β -elemene and docetaxel plus β -elemene, respectively. Western blot analysis showed that the levels of p53 protein were higher in cells treated with β -elemene plus docetaxel than in cells treated with either agent alone at the same doses (Figure 5). These results suggest that the combination of β -elemene plus taxanes suppresses ovarian cancer cell proliferation by inducing cell-cycle blockade specifically at the G_2/M phase and that the inhibitory effect of the two-drug combinations is mediated through a p53-dependent regulatory mechanism in cisplatin-resistant ovarian cancer cells.

Effects of β -elemene plus taxanes demonstrated by apoptotic induction as assessed by annexin-V binding assay. Cells arrested at G_2/M very often enter apoptosis, and an annexin-V binding assay was used to detect the induction of apoptosis by β -elemene plus paclitaxel or docetaxel in A2780/CP70 cells. The percentage of apoptotic cells induced by 48-h treatment with β -elemene plus paclitaxel, and β -elemene plus docetaxel was greater than that induced by each drug alone (Figure 6). After treatment with the combination of 90 μ M β -elemene plus 2.1 nM docetaxel, 70.5% of the cancer cells were in an early stage of apoptosis, compared with 1.9% after treatment with 90 μ M β -elemene alone and 11.7% after treatment with 2.1 nM docetaxel alone. A similar synergistic effect was observed for the induction of apoptosis by the combination of β -elemene plus paclitaxel. Cells treated with 2000 μ M H_2O_2 (positive control) showed strong induction of apoptosis, with about 50% early apoptotic cells and 7% late apoptotic or necrotic cells. Furthermore, treatment with β -elemene plus docetaxel for 48 h induced an increase in the caspase-9

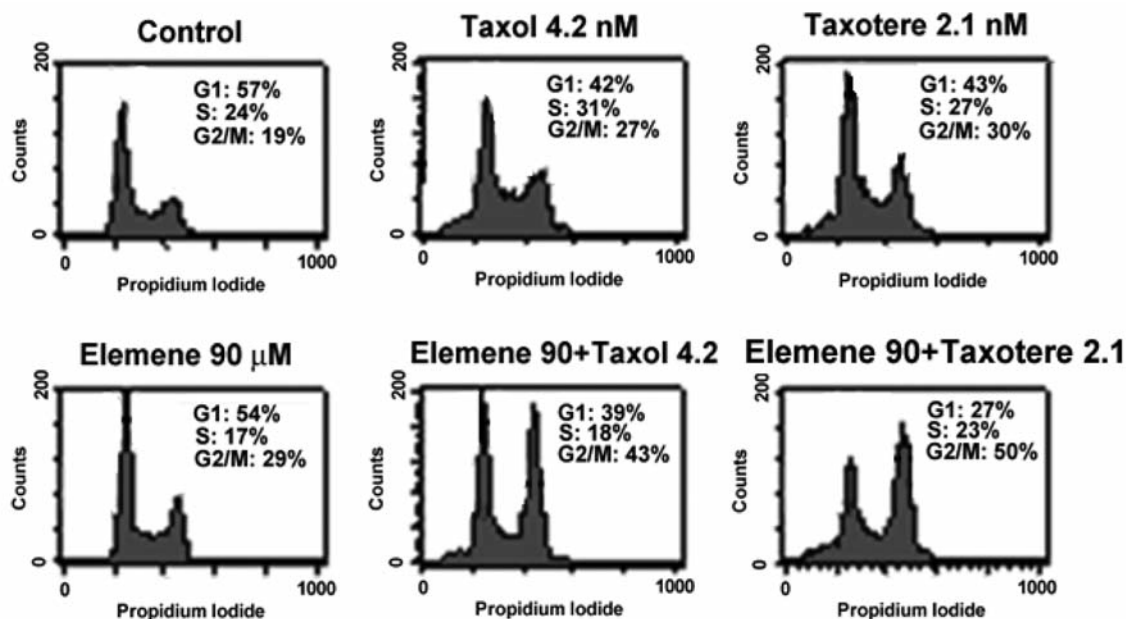


Figure 4. Combination effect of β -elemene and taxanes on cell-cycle distribution of human ovarian cancer cells. Asynchronously growing A2780/CP70 cells were treated with β -elemene, paclitaxel, or docetaxel alone, or with a two-drug combination at the indicated concentrations for 48 h and harvested. DNA content was analyzed by flow cytometry using propidium iodide staining, as described in the Materials and Methods. The percentage of cells in each cell cycle phase was calculated using CellQuest Pro software and is indicated at the upper right of each profile. A representative profile for each treatment is shown. β -Elemene, paclitaxel, and docetaxel alone, and in two-drug combinations caused cell-cycle arrest at the G₂/M phase. Control, untreated; Taxol, paclitaxel; Taxotere, docetaxel.

protein levels in A2780/CP70 cells (Figure 5). These data suggest that the combined effect of β -elemene plus taxanes on ovarian cancer cell death may occur through the intrinsic mitochondrial apoptosis pathway.

Discussion

Combination chemotherapy was first applied successfully in the treatment of leukemia and lymphomas (35) and is now routinely used for most malignancies. The objectives of combination chemotherapy are to kill a maximum number of tumor cells while avoiding excessive toxicity, to deliver cytotoxic drugs that are active against potentially resistant heterogeneous tumor populations, and to prevent selection for resistant cell lines. Two drugs are thought to act synergistically when the effect of their combined use exceeds the sum of their individual effects (31). However, the determination of the synergistic action of chemotherapeutic combinations is hampered by the lack of a single established methodology for examining synergy and antagonism. Several methodologies have been used, including the CI method (32), the CI-isobologram method (36), a modified fixed-ratio isobologram method (37), and the percentage of granulocyte/macrophage-colony forming units compared with control levels (38).

To investigate the effects of β -elemene and taxane combination treatments against human ovarian cancer cells, in the current study, we first conducted a synergistic analysis by determining the CI based on the IC₅₀ values. The interaction between β -elemene and paclitaxel or docetaxel demonstrated CI values that indicated additive effects to moderately-synergistic effects against two ovarian cancer cell lines and one prostate cancer cell line. Similar synergistic interactions were revealed by micronucleus formation assays, annexin-V binding studies, and cell-cycle analyses. In A2780/CP70 cells, combinations of β -elemene plus taxanes exerted a markedly synergistic action in the induction of apoptosis. The synergy between β -elemene and taxanes may be related to a β -elemene-induced alteration of cell membrane permeability with the potential to enhance cellular uptake of taxanes.

The broad antitumor spectra of taxanes make them ideal candidates for combination with other agents to treat many different types of cancer (39). Over the last few years, the combination of a platinum compound, such as cisplatin or carboplatin, with a taxane has emerged as standard chemotherapy for advanced ovarian cancer, as it prolongs the time-to-cancer progression and improves the average duration of survival (1, 16, 40-42). However, patients treated with paclitaxel plus cisplatin have experienced side-effects

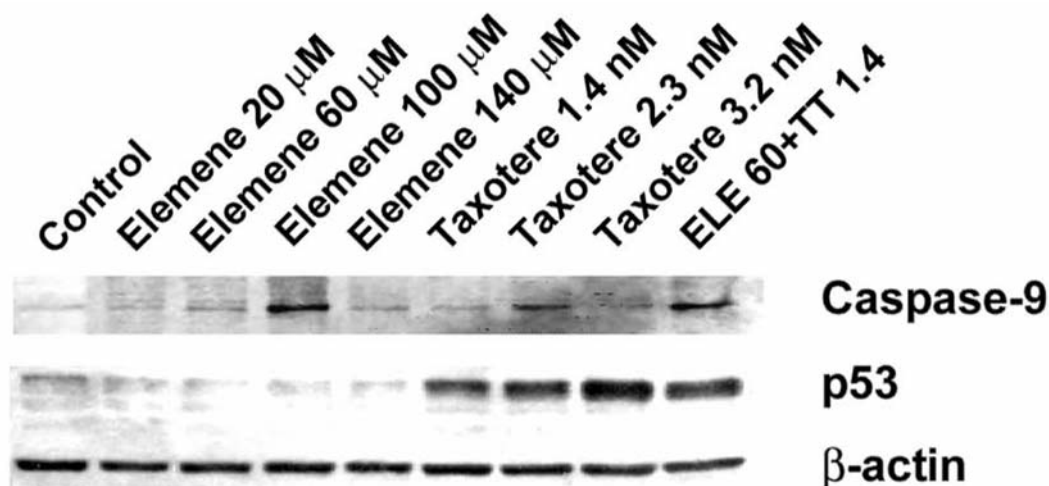


Figure 5. Effect of β -elemene and taxane on the levels of apoptosis regulator proteins in human ovarian cancer cells. A2780/CP70 cells were treated with β -elemene or taxotere (docetaxel) alone or in combination at the indicated concentrations for 48 h. The levels of caspase-9 and p53 proteins were analyzed by western blotting as described in the Materials and Methods. Equal protein loading was verified with an anti- β -actin antibody. Control, untreated; ELE, β -elemene; TT, taxotere.

such as hair loss, bone marrow suppression, and fever (43, 44). Of particular note, neurotoxicity is the major drawback to the combined use of these drugs (1). Interest has recently focused on enhancing the antitumor activity of taxanes by combining them with agents with lower toxicity.

β -Elemene, a novel plant-derived anticancer drug, has been shown to be effective for treating cervical carcinoma and cancers of the lung, liver, brain, and other tissues (45-47). An acute toxicity study in the mouse indicated that the lethal dose, 50% (LD_{50}) of β -elemene was 279 mg/kg when administered intraperitoneally and more than 5000 mg/kg when administered gastrointestinally (48). Used alone, β -elemene may present fewer or different side-effects compared with more commonly used antitumor drugs (18). The principal side-effects of β -elemene include slight fever, gastrointestinal reactions, allergic reactions, local pain, and phlebitis. In an MTT assay analysis of β -elemene effects, the IC_{50} values for growth inhibition of promyelocytic leukemia HL-60 cells and erythroleukemia K562 cells were 135 μ M and 397 μ M, respectively, whereas the IC_{50} for peripheral blood leukocytes was 1247 μ M, indicating differential inhibitory effects on cell growth between leukemia cells and normal leukocytes (49). Tumor cell apoptosis induced by β -elemene has been confirmed by DNA ladder formation, down-regulation of Bcl-2 (B-cell lymphoma-2) expression (21, 50, 51), and ultrastructural alterations (21). In this regard, β -elemene was proposed as a candidate for possible synergistic activity in combination with taxanes.

In the present study, MTT assay results indicated that docetaxel was more toxic than β -elemene or paclitaxel,

which is in agreement with a previous report that docetaxel is more toxic than paclitaxel (52). β -Elemene was less toxic than paclitaxel or docetaxel. Although the IC_{50} of β -elemene did not appear to differ between the cisplatin-sensitive and -resistant ovarian cancer cell lines, the IC_{50} values of paclitaxel and docetaxel were strikingly different between the two ovarian cancer cell lines. In addition, no IC_{50} value could be determined for the A2780 cells after 24 h of treatment with paclitaxel or docetaxel, even at concentrations as high as 1280 nM. These results indicate that compared with β -elemene, taxanes require a longer exposure time to penetrate the cell membrane and exert toxic effects in human ovarian cancer cells.

To evaluate synergism, we used the CI value for the point at which 50% of the cells were inhibited because it represents a linear approximation of a higher order equation demonstrating an inhibitory effect (15). In the present study, the CIs for the combinations of β -elemene plus a taxane revealed additive to moderately-synergistic effects ($0.64 < CI < 0.95$) and differed between the two ovarian cancer cell lines, although no significant difference in the CI was seen between the β -elemene plus paclitaxel combination and the β -elemene plus docetaxel combination. However, the molecular mechanism underlying the synergism between β -elemene and taxanes cannot be determined based only on a synergistic analysis.

Genetic toxicology assays such as the micronucleus formation assay (53) can determine whether an agent interacts with DNA and leads to a gene mutation or chromosomal breakage. During the metaphase/anaphase

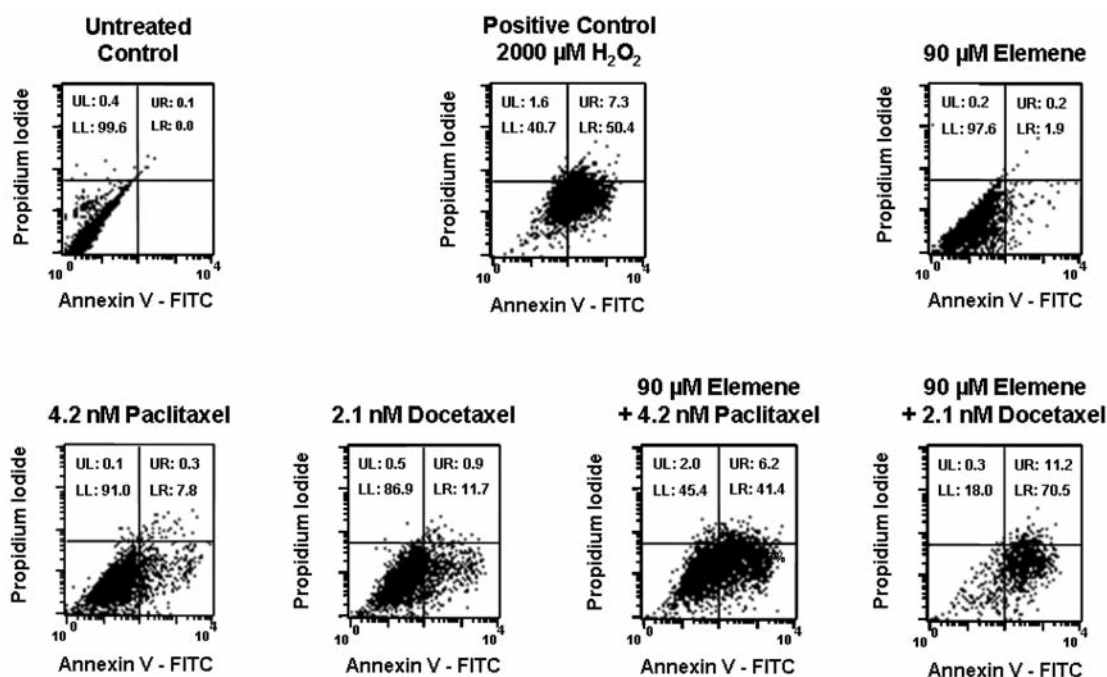


Figure 6. Combination effect of β -elemene and taxanes on apoptosis in human ovarian carcinoma cells. A2780/CP70 cells were treated with β -elemene, paclitaxel, or docetaxel alone, or with a two-drug combination for 48 h. Cells that bind annexin-V (AV) but not propidium iodide (PI) (lower right quadrant, LR) are thought to be in an early stage of apoptosis. Cells that bind both AV and PI (upper right quadrant, UR) are thought to be in a late stage of apoptosis or necrosis. The upper left quadrant (UL) represents cells that bind only PI. The results represent one of three independent experiments. Compared with each drug used alone, the combination drug treatments (β -elemene plus paclitaxel or β -elemene plus docetaxel) induced apoptosis of more cells.

transition of mitosis, a micronucleus can form as a result of a lagging chromosome (aneugenic event), leading to chromosome loss, or an acentric chromosome fragment (clastogenic event), which detaches after breakage and does not integrate into the daughter nucleus (54). In the present study, β -elemene treatment alone did not result in a significant increase in micronucleus formation or mitotic arrest, whereas treatment with paclitaxel or docetaxel alone markedly increased both of these parameters. A 48-h treatment with either two-drug combination, especially β -elemene plus docetaxel, enhanced the rates of both micronucleus formation and mitotic arrest compared with the rates for individual drug treatments alone. These findings are consistent with the results of our synergistic analysis. Thus, β -elemene induces a significantly enhanced susceptibility of ovarian cancer cells to the antitumor activity of paclitaxel or docetaxel. This enhancement may occur *via* a β -elemene-induced increase in the intracellular uptake of paclitaxel or docetaxel, or as a result of competition between β -elemene and taxanes for the efflux pump. The mechanism remains to be elucidated in further studies.

Uncontrolled cell proliferation is the hallmark of cancer, and β -elemene and taxanes were shown to inhibit cell

proliferation in our ovarian carcinoma models. To probe the mechanism underlying the combined effects of β -elemene and taxanes in chemoresistant ovarian tumor cells, we examined cell-cycle perturbations after exposure of A2780/CP70 cells to β -elemene and taxanes. The two-drug combination synergistically induced an accumulation of cells in the G_2/M phase, consistent with our cell growth studies. Western blot analysis revealed up-regulation of the p53 protein levels in A2780/CP70 cells treated with β -elemene plus docetaxel. G_1 and G_2 checkpoint proteins such as p53 have been major targets of anticancer drug development. Taken together, the present results suggest that β -elemene and taxanes produce cell-cycle arrest by controlling the G_2 checkpoint *via* activation of p53, which in turn activates p21^{Cip1/Waf1} to inhibit the cyclin B1-cell division control protein-2 homolog (CDC2) complex and other proteins controlling the G_2 checkpoint, leading to the suppression of ovarian cancer cell proliferation.

Apoptosis is an important process in tissue homeostasis and a key cytotoxic mechanism of anticancer therapies. The number of apoptotic cells after treatment with a drug combination compared with the number after treatment with each individual drug can indicate the combined effect of

multidrug treatments. The central cytotoxic activity of taxanes and β -elemene in tumor cells is thought to be the induction of apoptosis (21, 50, 51, 55, 56), although the molecular basis for the effects of β -elemene and taxanes is not clearly defined. Apoptosis is characterized by many biological and morphological changes, including relocation of phosphatidylserine (PS) to the outer cell membrane, change of mitochondrial membrane potential, activation of caspases, and fragmentation of DNA. Based on these changes, various assays have been designed to detect or quantitate apoptotic cells. The collapse of lipid asymmetry and exposure of PS on the cell surface is an early event in apoptosis (57) and is critical for effective recognition and phagocytosis of target cells (58). Annexin-V, a 35- to 36-kDa Ca^{2+} -dependent phospholipid-binding protein, has a high affinity for PS (59), and the flow cytometric detection of annexin-V binding to cells can distinguish among cells in early apoptosis, late apoptosis, and necrosis. In the present study, an annexin-V binding assay showed that treatment with a two-drug combination for 48 h significantly enhanced the number of apoptotic A2780/CP70 cells. This result is consistent with the results of the synergistic analysis, micronucleus formation assay, and cell-cycle distribution analysis in the present study. Our results indicate that treatment with β -elemene plus paclitaxel, and with β -elemene plus docetaxel dose-dependently induced an increase in the number of apoptotic cells. Furthermore, the percentage of early apoptotic cells was greater after treatment with each two-drug combination than after treatment with each drug alone. These data suggest that β -elemene acts synergistically with paclitaxel and docetaxel to induce apoptosis of ovarian cancer cells.

In summary, the use of drug combinations is the accepted standard for chemotherapy of most human malignancies, but little attention has been paid to drug interactions. In the present study, a synergistic analysis indicated that the combination of β -elemene with paclitaxel and with docetaxel produces additive to moderately-synergistic effects against human ovarian cancer cells and prostate cancer cells *in vitro*. Synergistic antitumor activity was further demonstrated by changes in cell morphology observed microscopically, cell-cycle G_2/M arrest analyzed by flow cytometry, and apoptotic induction detected by annexin V binding. β -Elemene may interact with the cell membrane to trigger the apoptotic process at an early stage, while taxanes tend to initiate cell death cascades through chromosome-damaging events. The mechanism of the synergistic action of β -elemene with taxanes is unclear, but a few possibilities are suggested by the present findings. Firstly, the synergistic antitumor activity of β -elemene plus a taxane may result from enhanced cell-cycle arrest and apoptotic induction. Secondly, the change in cell membrane permeability induced by β -elemene may increase the cellular influx of taxanes, thereby producing a synergistic effect. Thirdly, β -elemene may compete with taxanes for sites

on the efflux pump, thus enhancing the intracellular accumulation and activity of taxanes. The demonstrated synergism between β -elemene and taxanes implies that when they are used in combination, these agents can be administered at reduced dosages to achieve antitumor efficacy in clinical treatment. This promising drug combination warrants further preclinical exploration for the treatment of chemoresistant ovarian cancer and other tumor types.

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