CMLS, Cell. Mol. Life Sci. 62 (2005) 881–893
1420-682X/05/080881-13
DOI 10.1007/s00018-005-5017-3

CMLS Cellular and Molecular Life Sciences

Research Article

Antitumor effect of β-elemene in non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death

G. Wang, X. Li, F. Huang, J. Zhao, H. Ding, C. Cunningham, J. E. Coad, D. C. Flynn, E. Reed and Q. Q. Li*

The Mary Babb Randolph Cancer Center and the Department of Microbiology, Immunology and Cell Biology, West Virginia University Health Sciences Center, Morgantown, West Virginia 26506 (USA), Fax: +1 304 293 4667, e-mail: qli@hsc.wvu.edu

Received 12 January 2005; accepted 5 February 2005

Abstract. β-Elemene is a novel anticancer drug, which was extracted from the ginger plant. However, the mechanism of action of β-elemene in non-small-cell lung cancer (NSCLC) remains unknown. Here we show that β-elemene had differential inhibitory effects on cell growth between NSCLC cell lines and lung fibroblast and bronchial epithelial cell lines. In addition, β-elemene was found to arrest NSCLC cells at G2-M phase, the arrest being accompanied by decreases in the levels of cyclin B1 and phospho-Cdc2 (Thr-161) and increases in the levels of $p27^{kip1}$ and phospho-Cdc2 (Tyr-15). Moreover, β-elemene reduced the expression of Cdc25C, which dephosphorylates/activates Cdc2, but enhanced the expression of the checkpoint kinase, Chk2, which phosphorylates/inactivates Cdc25C. These findings suggest that the effect of β-elemene on G2-M arrest in NSCLC cells is mediated partly by a Chk2-dependent mechanism. We also demonstrate that β-elemene triggered apoptosis in NSCLC cells. Our results clearly show that β-elemene induced caspase-3, -7 and -9 activities, decreased Bcl-2 expression, caused cytochrome c release and increased the levels of cleaved caspase-9 and poly(ADP-ribose) polymerase in NSCLC cells. These data indicate that the effect of β-elemene on lung cancer cell death may be through a mitochondrial release of the cytochrome c-mediated apoptotic pathway.

Key words. Lung cancer, NSCLC, elemene, cell cycle arrest, G2-M arrest, apoptosis.

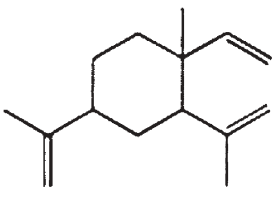
Lung cancer remains the leading cause of mortality among men and women in the United States, with 195,000 deaths every year. This exceeds the sum of the next three leading causes of death due to breast, colon and prostate cancers. There are over one million deaths due to malignant tumors of the lung and 1.2 million new cases of lung cancer occur worldwide annually, making it an epidemic disease [1–3]. Lung cancer is classified into four major types, including squamous cell, adenocarcinoma, large-cell or undifferentiated cell, and small-cell, from a histological point of view. Non-small-cell lung cancer (NSCLC) comprises the first three types and accounts for approximately 75–85% of all cases [4, 5]. Although the best treatment for lung cancer is surgical resection, few patients with lung cancer present with surgically resectable disease and more than 75% of patients with NSCLC prove to be potential candidates for chemotherapy at some point during the course of their disease because of the development of metastases [6]. The most effective systemic chemotherapy for NSCLC is based on cisplatin combinations, and for more than two decades, platinum-based combinations have remained the standard first-line chemotherapy for advanced NSCLC [7–9]. However, chemotherapy for NSCLC seems to have reached a

* Corresponding author.

plateau, and the current prognosis for most patients with this disease remains poor, with an overall survival at 5 years of only 10–15% [7; 8; 10). Therefore, there is a need to develop compounds that can effectively treat this disease.

One candidate drug for this role is elemene. Elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane), an extract from the ginger plant *Rhizoma zeodaria*, is a novel anticancer drug. The extract of elemene is a mixture of *β*-, *δ*- and *γ*-elemene, with *β*-elemene as the main component (fig. 1), which accounts for 60~72% of the three isoforms. *β*-Elemene has been effective in China in the treatment of leukemia and carcinomas of the brain, breast, liver and other tissues [11–13], and is now in application for clinical studies in the United States. The major advantages of *β*-elemene as an anticancer drug are that (*i*) it has a broad-spectrum antitumor effect in many types of cancer, including drug-resistant tumors, (*ii*) it does not direct multidrug resistance and can reverse the resistance to other drugs and (*iii*) it has low toxicity and is therefore well tolerated and accepted by cancer patients [12; 13]. The mechanism of action of *β*-elemene in cancers remains unknown. Recent studies showed that *β*-elemene-inhibited cell proliferation was correlated to G2-M phase arrest in leukemia HL-60 and K562 cells [12, 13]. In addition, *β*-elemene was found to trigger apoptosis in glioma SHG-44 cells and leukemia K562 cells [11–13], and the apoptosis induced by *β*-elemene was associated with reduction of Bcl-2 protein expression. However, the mechanisms underlying G2-M arrest and apoptotic cell death triggered by *β*-elemene are not elucidated.

The goal of the chemotherapy of human malignancies is inhibition of cell proliferation and/or induction of cell apoptosis, and although the primary intracellular targets and the pharmacological mechanisms of action of the anticancer drugs vary, drug-induced cell killing is, at least partially, mediated by cell cycle arrest and apoptotic cell death [14, 15]. The cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) [16]. CDK activity is modulated through three distinct mechanisms: by cyclin binding, by positive and negative phosphorylation events and by interaction with CKIs [17]. CDK1 (Cdc2) and cyclin B are the two major players for control of the G2-M transition in the cell cycle. Chk2 kinase also regulates G2-M transition by phosphorylating Cdc25C [18, 19], and the later activates Cdc2 by removing phosphate from Thr-14 and Tyr-15 on Cdc2 [20]. Cellular apoptosis is mediated through two major pathways in response to different types of stimuli, including genotoxic stresses and anticancer drugs [21, 22]. One pathway is activated by the ligation of death receptors, with the subsequently cleavage of caspase-8 and activated caspase-3 by active caspase-8 [23]. The other apoptosis-mediating route, which is activated by the majority of anticancer drugs, involves the release of cytochrome c from the mitochondria, which can trigger the activation of cytosolic caspase-9 in the complex with Apaf-1 in the presence of ATP [24; 25). Subsequently, effector caspases, such as caspase-3, are activated also, these take care of the execution phase of the apoptotic process, causing the degradation of cellular proteins and the disassembly of the cell [26). Thus caspases play a vital role in the apoptotic process [27).



β-**Elemene**

Figure 1. Chemical structure of *β*-elemene.

To date, little has been known about the effect and mechanism of *β*-elemene in human NSCLC. Therefore, the aims of the present study were to examine the antitumor effect of *β*-elemene in human NSCLC cells and to determine the underlying mechanisms. We investigated whether the regulation of cell cycle control and apoptosis is involved in the mechanisms for the effect and activity of *β*-elemene in the human lung carcinoma cells. Here we report that *β*-elemene inhibits the growth of human NSCLC cells and causes G2-M phase cell cycle arrest and apoptosis in H460 cells, accompanied by marked alterations in the expression of key G2-M phase and apoptosis regulatory components.

Materials and methods

Chemicals and immunoreagents

(-)*β*-Elemenum (98% purity) was obtained from Yuanda Pharmaceuticals (Dalian, China). Propidium iodide (PI), RNase and glycine were purchased from Sigma-Aldrich (St. Louis, Mo.). The primary antibodies against cyclin A, cyclin B1, $p27^{kip1}$, Cdc2, CDK2, Chk2, Cdc25C, caspase 8, caspase-9, Bcl-2, cytochrome c, poly(ADP-ribose) polymerase (PARP) and *β*-actin, and the secondary antibodies HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, as well as nitrocellulose, blotto, and the chemiluminescence luminal reagent were all from Santa Cruz Biotechnology (Santa Cruz, Calif.). Antibodies anti-phospho-Cdc2 (Thr-161), anti-phospho-Cdc2 (Tyr-15), and anti-phospho-CDK2 (Thr-160) were purchased from Cell Signaling Technology (Beverly, Mass.). The CellTiter 96 Aqueous ONE Solution Cell Proliferation Assay Kit was from Promega Corporation (Madison, Wis.). Cell Death Detection $ELISA^{PLUS}$ and TUNEL Label kits were obtained from Roche Diagnos-

tics Corporation (Indianapolis, Ind.). The Vybrant Apoptosis Assay Kit was from Molecular Probes (Eugene, Ore.). Cas-PASE-8, -9, -3, -7 and -10 Assay kits were obtained from Geno Technology (St. Louis, Mo.), and the Mitochondria Isolation Kit was purchased from Sigma-Aldrich.

Cell culture and antiproliferative assay

The human NSCLC cell lines H460 and A549, the human lung fibroblast cell line CCD-19Lu and the human bronchial epithelial cell line NL20 were obtained from the American Type Culture Collection (ATCC) (Manassas, Va.). Cell culture medium RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin-glutamine (×100) and 0.25% trypsin-EDTA solution were purchased from Invitrogen Corporation (Carlsbad, Calif.). The human NSCLC cell line H460 and the human normal lung fibroblast cell line CCD-19Lu were maintained in RPMI-1640 as described previously [28], supplemented with 10% FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin, and grown at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability or the effects of β-elemene on cell proliferation in human lung cancer cells, human lung fibroblast cells and human bronchial epithelial cells were assessed with an MTT-based colorimetric cell proliferation assay kit following the manufacturer's instructions (Promega). In brief, 5×10^3 of H460, A549, CCD-19Lu and NL20 cells per well were evenly distributed and grown in 96-well plates overnight, and cells were treated with various concentrations of β-elemene (0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) and incubated for 24, 48 or 72 h. At the termination of incubation with β-elemene, 20 µl of CellTiter 96 Aqueous One Solution Reagent was added to each well of a 96-well assay plate containing the samples in 100 µl of culture medium and the plates were incubated at 37 °C, with a 5% CO_2 atmosphere for 1–4 h. Then, the optical density at 590 nm was determined using a 96-well plate reader (ThermoLabsystems, Chantily, Va.). Proliferation rates were determined by calculating the viable percentages of cells from the β-elemene-treated wells compared to those wells where no β-elemene was added (100% control value).

Cell cycle analysis by flow Cytometry

H460 or CCD-19Lu cells were treated with various concentrations of β-elemene (0, 30, 40 and 50 µg/ml) and incubated for 24 or 48 h. At the end of incubation, cells were harvested by trypsinization, then washed with PBS twice and fixed with 70% ethanol at –20 °C overnight, and ethanol was removed by centrifuging the cells and washing them once with PBS. The cells were then incubated in 0.1% Triton X-100-containing 0.2 µg/ml RNase A on ice for 30 min, and then stained with PI dye. Finally, the cells were analyzed for cell cycle perturbation using a FACSCalibur (Becton-Dickinson, San Diego, Calif.), and the CellQuest Pro software (Becton-Dickinson) and the ModFit LT software (Verity Software House, Topsham, Maine) were used to determine the distribution of cells in the various cell cycle compartments: sub-G1 (apoptotic cells), G_1, S and G_2-M.

Cell death detection ELISA assay for apoptosis

This assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This ELISA provides a quantitative in vitro determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cell lysates, determined photometrically at 405 nm. H460 cells (1×10^4/well) were evenly distributed and grown in 96-well plates overnight and then treated with varying concentrations of β-elemene (0, 30, 40 and 50 µg/ml) for 24 or 48 h. Cells were then harvested and assayed for apoptosis using a Cell Death Detection ELISA Kit (Roche Diagnostics) following the manufacturer's instructions.

The enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated as absorbance of sample cells/absorbance of control cells. The enrichment factor was used as a parameter of apoptosis and is shown on the y-axis as mean ± SD of three independent experiments. The apoptotic index was calculated as an enrichment factor, that is, the ratio of the result compared with the control set arbitrarily at 1.0. Each experiment was performed in triplicate, and means and standard deviations were calculated.

TUNEL assay for apoptosis

For the terminal deoxynucleotidyltransferase-mediated deoxy-UTP-fluorescein nick end-labeling (TUNEL) assay, the Apoptosis Cell Detection System (Roche Molecular Biochemicals) was used to detect DNA fragmentation as a marker of apoptotic cell death in situ in β-elemene-treated lung cancer cells. In brief, H460 cells were treated with 0, 30, 40 or 50 µg/ml of β-elemene, and harvested at 24 or 48 h following β-elemene treatment. The collected cell pellets were processed into formalin-fixed paraffin-embedded cell blocks. Sections of the cell blocks were deparaffinized, washed three times with PBS, incubated for 60 min at 37 °C in a humidified atmosphere in the dark with 50 µl of TUNEL reaction mixture containing fluorescein-deoxy-UTP (TUNEL label) and terminal deoxynucleotidyltransferase (TUNEL enzyme), rinsed three times with PBS, incubated with 50 µl Converter-AP in a humidified chamber for 30 min at 37 °C, rinsed again three times with PBS and incubated with 50 µl of substrate solution at 25 °C in the dark for a period of time adjusted for optimal staining. Finally, slides were rinsed three times with PBS and mounted under a glass coverslip for analysis by light microscopy.

Apoptosis assay by annexin V staining

To quantify apoptosis, H460 cells were stained with annexin V and PI using a Vybrant Apoptosis Assay Kit (Molecular Probes) following the step-by-step protocol provided by the manufacturer. Briefly, 1×10^6 cells were treated with 0, 30, 40 and 50 μg/ml of β-elemene, or 30 μg/ml of H_2O_2 as a positive control for 24 or 48 h; cells were then collected by trypsinization and washed once with cold PBS. Cell pellets were resuspended in 100 μl 1 × annexin-binding buffer, 5 μl of FITC-annexin V (component A), and 1 μl of the 100 μg/ml PI working solution was added to each 100 μl of cell suspension. The cells were incubated on ice for 1 h and washed with cold PBS once again, resuspended in 300 μl of 1 × annexin-binding buffer, and the stained cells were analyzed for apoptosis by flow cytometry as soon as the staining finished.

Caspase activity assay

Caspase activities were assayed using CasPASE Apoptosis Assay kits (Geno Technology). In brief, 2×10^7 H460 cells were treated with 0, 30, 40 or 50 μg/ml of β-elemene for 24 or 48 h, collected by trypsinization, washed once with PBS, and cell pellets resuspended in 350 μl lysis buffer. The cells were lysed by freeze and thaw five times. The lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. Supernatants were collected and used for measuring caspase-8, -9, -3, -7 and -10 activities by ELISA-based assay according to the manufacturer's instructions. Caspase-3, -7 and -10 activities were detected using the specific caspases fluorogenic substrate, DEVD peptide conjugated to 7-amino-4-trifluoromethyl coumarin (AFC). Caspase-8 activity was detected using the specific caspase-8 fluorogenic substrate, IETD-AFC, and caspase-9 activity was detected using the specific caspase-9 fluorogenic substrate, LEHD-AFC. Samples were read on a microplate reader at 405 nm (ThermoLabsystems, Chantily, Va.).

Western blot analysis

H460 cells were treated with 0, 30, 40 or 50 μl/ml of β-elemene. Following 24 or 48 h treatment with β-elemene, cells were harvested and lysed with 200 μl RIPA lysis buffer [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] and the amount of protein was quantified by BCA assay. An equal amount of protein from the control and the drug-treated cells was boiled for 5 min in Laemmli's buffer and separated on 12% SDS-PAGE and the resolved gels were blotted on nitrocellulose membrane. The transblots were probed with primary antibodies against cyclin A, cyclin B1, $p27^{kip1}$, Cdc2, CDK2, Chk2, Cdc25C, phospho-Cdc2, phospho-CDK2, caspase-8, caspase-9, Bcl-2, cytochrome c or PARP, followed by secondary HRP-conjugated antibodies. In all cases, blots were stripped using a stripping buffer [62.5 mM Tris (pH 6.7), 2% SDS and 90 mM 2-mercaptoethanol] and reprobed with anti-actin for loading controls. The signals were detected by enhanced chemiluminescence.

Detection of cytochrome c release.

Cytosolic and mitochondrial extracts were prepared with a Mitochondria Isolation Kit (Sigma-Aldrich) essentially according to the manufacturer's instructions. Briefly, following 0, 30, 40 or 50 μl/ml of β-elemene treatment and incubation for 24 or 48 h, the cells were collected by centrifugation. The cell pellets were washed twice with cold PBS and 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, 10 μg/ml aprotinin). The cells were homogenized with a glass homogenizer. 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 pellet the mitochondrial fraction. An aliquot of the resulting supernatant was used as the soluble cytosolic fraction. The mitochondrial pellet was washed once and then suspended in buffer A. Protein extracts (equal amounts in the mitochondrial and cytosolic fractions) were subjected to Western blot analysis with an anti-cytochrome c antibody, as described above.

Statistical analysis of data

The quantitative experiments were analyzed by Student's t test. All the ps resulted from the use of two-sided tests were considered significant when < 0.05.

Results

Differential inhibitory effects of β-elemene on cell growth between the human NSCLC cell lines H460 and A549 and the human lung fibroblast cell line CCD-19Lu and the human bronchial epithelial cell line NL20

To understand the antitumor effects of β-elemene in lung cancer as an initiative study, we first established whether β-elemene inhibits lung cancer cell proliferation and whether it has different effects on lung cancer and normal lung cells. The human NSCLC cell lines H460 and A549 were used as models for human NSCLC in this study, and the human lung fibroblast cell line CCD-19Lu and the human bronchial epithelial cell line NL20 were used as controls. The ability of β-elemene to inhibit cellular proliferation was investigated in vitro using the MTT-based colorimetric assay. As shown in figure 2, β-elemene caused a dose-dependent inhibition of H460 and A549 cell proliferation with concentrations ranging from 20 to 140 μg/ml, at all three time points. β-Elemene had a half-maximal (IC_{50}) inhibitory effect on H460 cell growth at

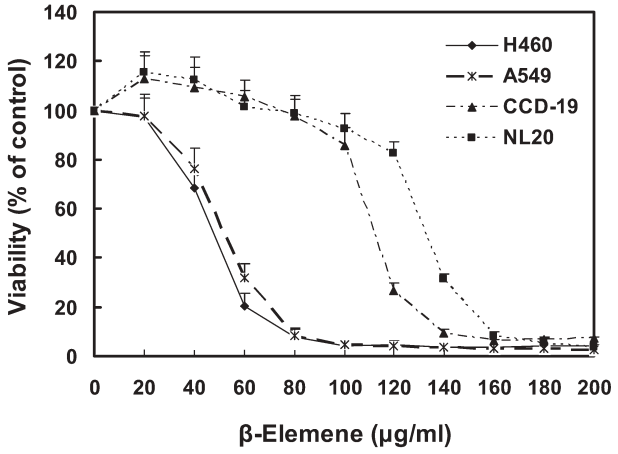


Figure 2. β-Elemene differentially inhibited cell proliferation between human NSCLC H460 and A549 cells and human lung fibroblast CCD-19Lu and bronchial epithelial NL20 cells. H460, A549, CCD-19Lu and NL20 cells (5×10^3 per well) were evenly distributed in 96-well plates and treated with different concentrations of β-elemene for 24, 48 and 72 h (data at 24 and 72 h not shown). The ability of β-elemene to inhibit cell proliferation was determined by the MTT cell survival assay, as described in Materials and methods, and cell viability values were expressed relative to those wells where no β-elemene was added (100% control value). The results represent the mean ± SD of at least three independent experiments.

50, 46 and 42 μg/ml and on A549 cells at 61, 53 and 48 μg/ml at 24, 48 and 72 h, respectively, whereas the IC_{50}s were 108, 100 and 98 μg/ml for the control lung fibroblast CCD-19Lu cells, and 145, 132 and 126 μg/ml for bronchial epithelial NL20 cells at 24, 48 and 72 h, respectively (fig. 2, data at 24 and 72 h not shown), thus showing a more than two-fold difference in IC_{50} values between the lung cancer cells and the control cells. These data indicate that β-elemene is much more potent at inhibiting the proliferation of human NSCLC H460 and A549 cells than human fibroblast CCD-19Lu cells and human bronchial epithelial NL20 cells.

β-Elemene induced cell cycle G2-M phase arrest in human NSCLC H460 cells but not in human fibroblast CCD-19Lu cells

Because β-elemene can effectively inhibit human NSCLC cell growth (fig. 2), we reasoned that this inhibitory activity may be attributable to its ability to interfere with the cell cycle. We therefore analyzed cell cycle perturbation after exposure of the lung carcinoma cells to β-elemene. For these experiments, we treated H460 cells with β-elemene at 30, 40 and 50 μg/ml, and treated CCD-19Lu cells with 40 μg/ml β-elemene for 24 h, and performed cell cycle analysis by flow cytometry. Figure 3 shows that β-elemene arrested H460 cells at the G2-M phase in a concentration-dependent manner, and the cell population in the S phase of the cell cycle was also increased in cells treated with 40–50 μg/ml β-elemene; however, no significant increases in G2-M and S phase cell populations were observed in CCD-19Lu cells. Similar results were observed when cells were treated for 48 h with β-elemene (data not shown). Interestingly, when H460 cells were treated with higher concentrations of β-elemene (50 μg/ml and higher doses) for 24–48 h, cell apoptosis was demonstrated by sub-G1 profile analysis and FACSCalibur flow cytometry (fig. 3). Altogether, the results suggest that the inhibitory effect of β-elemene on proliferation of the human NSCLC cells might be due, at least in part, to a G2-M phase arrest of the cell cycle and apoptotic cell death.

Effects of β-elemene on the levels and activity of cell cycle G2-M regulatory proteins in human NSCLC H460 cells

We showed that β-elemene arrested H460 cells at G2-M and S phases of the cell cycle. To understand the underlying mechanisms, we tested the effects of β-elemene on the expression of selected molecular targets that regulate the S and G2-M transition. The G2-M and S phases of the cell cycle are known to be regulated by cyclin A, cyclin B, Cdc2, CDK2 and CKIs, and the Cdc2 and CDK2 activities are modulated by cyclin B or cyclin A binding, by positive and negative phosphorylation events, and by interaction with CKIs [16, 17]. Activated Chk2 kinase regulates G2-M transition by phosphorylating Cdc25C [18, 19], or regulates S phase by phosphorylating Cdc25A [29], and Cdc25C functions as a mitotic activator by dephosphorylating Cdc2 that forms a complex with cyclin B1 and drives the cell from the G2 to M phase (20). Therefore, we measured the protein levels of $p27^{kip1}$, cyclin A, cyclin B1, Cdc2, CDK2, Chk2 and Cdc25C in the H460 cells treated with β-elemene. As seen in figures 4 and 5, the levels of cyclin A and cyclin B1 were decreased and the levels of $p27^{kip1}$ were increased after exposure of H460 cells to different concentrations of β-elemene. Our results also showed that the protein levels of phospho-Cdc2 (Thr-161), phospho-CDK2 (Thr-160), and Cdc25C were reduced by β-elemene. However, the protein levels of phospho-Cdc2 (Tyr-15) and Chk2 were increased and the protein levels of Cdc2 and CDK2 remained unchanged. These data support the hypothesis that β-elemene modulated the expression of cyclin A, cyclin B, Chk2, Cdc25C and $p27^{kip1}$, and the phosphorylation of Cdc2 (Thr-161 or Tyr-15) and CDK2 (Thr-160), and reduced the activities of cyclin B1-Cdc2, cyclin A-Cdc2 and cyclin A-CDK2 complexes which lead to a cell cycle G2-M block in H460 cells.

β-Elemene triggered apoptosis in human NSCLC H460 cells as determined by ELISA assay, TUNEL assay, and annexin V staining

The major goal of cancer chemotherapy is to commit tumor cells to apoptosis following exposure to anticancer

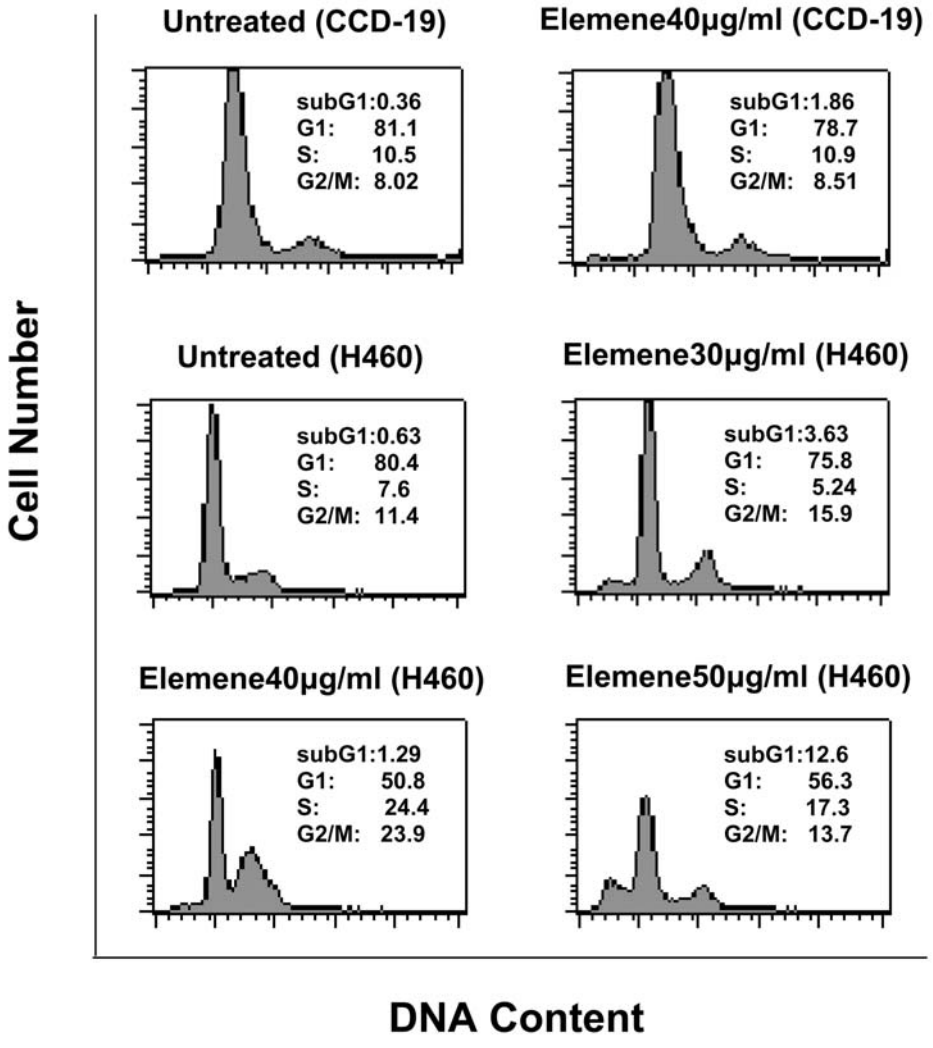


Figure 3. β-Elemene blocked human NSCLC H460 cells at G2-M phase. H460 and CCD-19Lu cells were exposed to varying concentrations of β-elemene (0, 30, 40 and 50 μg/ml) for 24 h. The cells were then harvested, stained, and the PI-labeled cells were analyzed for their cell cycle status by flow cytometry as described in Materials and methods. The results show accumulation of the cells in the G2-M phase and the development of a sub-G1 population in H460 cells, but not in CCD-19Lu cells. The data are representative of three separate experiments.

agents [15], and our results from flow cytometry analysis showed that β-elemene induced apoptosis in H460 cells (fig. 3). Therefore, in this experiment we detected β-elemene-induced apoptosis by ELISA-based assay, TUNEL assay and annexin V staining in H460 cells to confirm the above results. DNA fragmentation is an early and characteristic event of apoptosis [30]. Our ELISA assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, and it provides a quantitative in vitro determination of histone-associated DNA fragments [30, 31]. As seen in figure 6, after exposure to β-elemene at 40 or 50 μg/ml for 24 and 48 h, the apoptotic H460 cells were significantly increased ($P < 0.05$).

The TUNEL technique is classically used to detect apoptotic cells in culture [31, 32]. Here the induction of apoptosis following β-elemene treatment was further confirmed by the TUNEL assay. As can be seen, few apoptotic cells were detected in the untreated control cells (fig. 7). In contrast, apoptosis in the H460 cells treated with β-elemene was increased as measured by the TUNEL assay (fig. 7). Quantitative analysis of the sections revealed that β-elemene induced a significant increase in apoptotic H460 cells at 24 and 48 h in a dose-dependent manner, compared with the untreated controls (fig. 7).

Phosphatidylserine (PS) translocation occurs early in apoptosis when cell membrane integrity is still intact, and annexin V can bind to PS with high affinity. Flow cytometric analysis using FITC-labeled annexin V is therefore useful as a quantitative measure of early apoptosis [33, 34]. β-Elemene-induced apoptosis analysis was per-

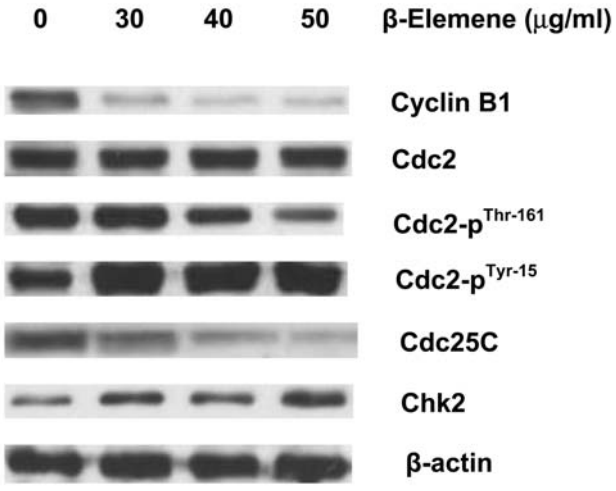


Figure 4. Effects of β-elemene on the levels and activity of key regulators of the cell cycle G2-M transition in human NSCLC H460 cells. H460 cells were treated with 0, 30, 40 or 50 μg/ml β-elemene for 24–48 h. A total 50 μg of cell extract protein isolated from β-elemene-treated H460 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with anti-cyclin B1, Cdc2, phospho-Cdc2 (Thr-161 or Tyr-15), Cdc25C and Chk2 antibodies, or with a β-actin antibody as a control. Western blot data presented are representative of three separate experiments.

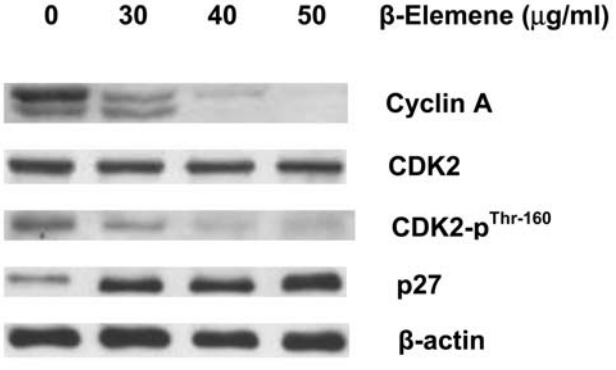


Figure 5. Effects of β-elemene on the levels and activity of cell cycle regulatory proteins in human NSCLC H460 cells. H460 cells were treated with β-elemene at 0, 30, 40 or 50 μg/ml for 24–48 h. A total 50 μg of cell extract protein isolated from β-elemene-treated H460 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with anti-cyclin A, CDK2, phospho-CDK2 (Thr-160) and $p27^{kip1}$ antibodies, or with a β-actin antibody as a control. Western blot data presented are representative of those obtained from three independent experiments.

formed with annexin V/PI staining using the Vybrant Apoptosis Assay Kit, and the results showed that β-elemene induced significant increases in apoptosis in H460 cells in a good concentration-dependent fashion (fig. 8). Together, the results from the above three independent methods suggest that apoptosis may be a major contributor to the biological efficacy of β-elemene in human NSCLC cells.

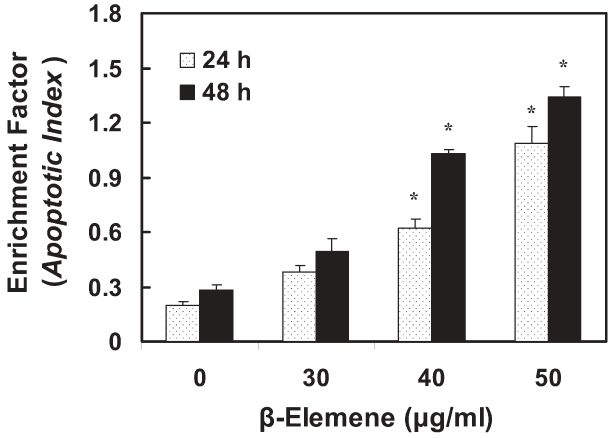


Figure 6. β-Elemene induced apoptosis in human NSCLC H460 cells as measured by ELISA assay. H460 cells were treated with β-elemene for 24 or 48 h at the indicated concentrations and cellular apoptosis was determined with an ELISA-based cell death detection kit as described in Materials and methods. The results are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ versus untreated controls.

β-Elemene induced increases in caspases activities in human NSCLC H460 cells

We showed that β-elemene induced apoptosis in H460 cells as determined by ELISA-based assay, TUNEL assay and flow cytometry analysis. In mammalian cells, two major apoptosis pathways have been proposed [32, 35, 36]. Caspases are the central executors of the apoptotic process, especially caspase-3, -7 and -10, and caspase-8 and caspase-9 are considered markers for different pathways [27, 36, 37]. To understand the mechanism of β-elemene-induced apoptosis and the apoptotic pathway involved, we assessed the effects of β-elemene on the activities of caspase-3, -7, -10, -8 and -9 by ELISA-based assay. As shown in figure 9, the activities of caspase-3, -7 and -10 (fig. 9C) and caspase-9 (fig. 9B) were increased significantly following treatment of H460 cells with 40 and 50 μl/ml β-elemene for 24 and 48 h ($p < 0.05$). However, no significant changes in caspase-8 activity were detected in cells exposed to various concentrations of β-elemene (fig. 9A). These results suggest that β-elemene may induce apoptosis in H460 cells via a caspase-9-dependent pathway.

Effects of β-elemene on the levels and activity of apoptosis regulatory proteins in human NSCLC H460 cells

To further elucidate the mechanisms of β-elemene-induced H460 cell apoptosis, we investigated the apoptosis regulatory proteins in this model system. We chose to study some key apoptosis targeting genes in this project. For instance, PARP is an early marker of chemotherapy-induced apoptosis [38]; caspase-8 and caspase-9 are the marker genes for the extrinsic pathway and intrinsic path-

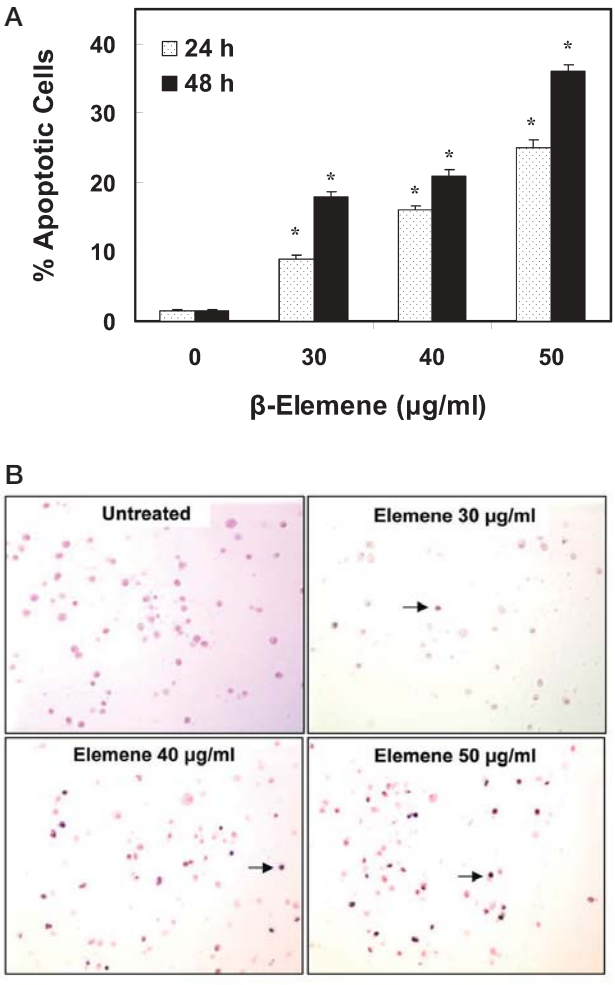


Figure 7. β-Elemene induced apoptosis in human NSCLC H460 cells as assessed by TUNEL assay. H460 cells were treated with β-elemene for 24 or 48 h at the indicated concentrations. Following the treatment period, the cells were collected, processed into paraffin-embedded cell blocks, sectioned at 5 μm and cell apoptosis was determined with a TUNEL assay kit as described in Materials and methods. Positive and negative staining were observed by light microscopy. Apoptotic cells were counted, and reported as a percentage of the total cells present for evaluation (*A*). The results are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ versus untreated controls. (*B*) Under light microscopy, apoptotic cells are stained brown or dark brown (indicated by arrows). As can be seen, β-elemene induced DNA fragmentation consistent with apoptotic cell death in H460 cells, in a dose-dependent manner.

way of apoptosis, respectively, and the release of cytochrome c to the cytosol initiates caspase activation and apoptotic cell death in the intrinsic apoptotic pathway [24, 25], while Bcl-2 plays a critical role in regulation of cytochrome c release from the mitochondrial intermembrane space into the cytosol [39–41]. H460 cells were treated with various concentrations of β-elemene as indicated for 24–48 h, and Bcl-2, cytochrome c, and the cleavages and protein levels of caspase-8, caspase-9 and PARP were analyzed by Western blotting as described in Materials and methods. Figure 10 shows that Bcl-2 expression was reduced, while the levels of cytochrome c in the cytosol as well as the levels of cleaved caspase-9 and PARP were increased in a concentration-dependent manner. Moreover, no significant changes in the levels of procaspase-8 and procaspase-9 were measured. Taken together with our caspase data, these findings suggest that β-elemene may induce apoptosis in human NSCLC H460 cells through a mitochondrial cytochrome c release-dependent pathway.

Discussion

Elemene, an extract from the ginger plant *R. zeodaria*, is a promising novel anticancer drug [11–13]. Although elemene has been effective for the treatment of human tumors and is currently pending approval by the Chinese FDA (the State Food and Drug Administration) for clinical use in China, the mechanisms underlying its antineoplastic activity are not understood. In the present study, we used H460 and A549 cell lines as models for NSCLC and found that β-elemene inhibited the growth of the human NSCLC cells in vitro. The IC_{50} values of β-elemene for H460 and A549 cells ranged from 42 to 61 μg/ml compared to 98–145 μg/ml, the IC_{50}s for human lung fibroblast CCD-19Lu and human bronchial epithelial NL20 cells; i.e. there are twofold differences between the two group IC_{50}s indicating that human lung carcinoma cells are more sensitive to β-elemene than lung fibroblast and bronchial epithelial cells. These results are consistent with the effects of elemene in several other types of human cancer cells [11–13]. In addition, our cell cycle analysis revealed that β-elemene arrested NSCLC H460 cells at S phase and G2-M phase. Furthermore, we demonstrated that β-elemene induced apoptosis in this model system, as assessed by four different methods. Together, these results suggest that the inhibitory effect of β-elemene on cell proliferation in NSCLC is mediated through induction of cell cycle arrest and apoptotic cell death.

The eukaryotic cell cycle is regulated via the sequential activation and inactivation of CDKs that drive cell cycle progression through the phosphorylation and dephosphorylation of regulatory proteins [42–44]. Cells pass through several checkpoints as they proceed through the cell cycle, and certain criteria must be met to pass each of these checkpoints. In the G2 checkpoint, cyclin B1 regulates cell cycle progression and provides substrate specific to its partner, CDK1 (Cdc2) [45]. The loss or deactivation of either cyclin B1 or Cdc2 will block cellular progression out of G2 [46]. The Cip/Kip family proteins including $p27^{kip1}$ act as inhibitors of Cdc2 [47, 48]. In this study, we showed that although the levels of Cdc2 protein were not changed in the H460 NSCLC cells treated with β-elemene, the levels of phospho-Cdc2 (Thr-161) and cyclin

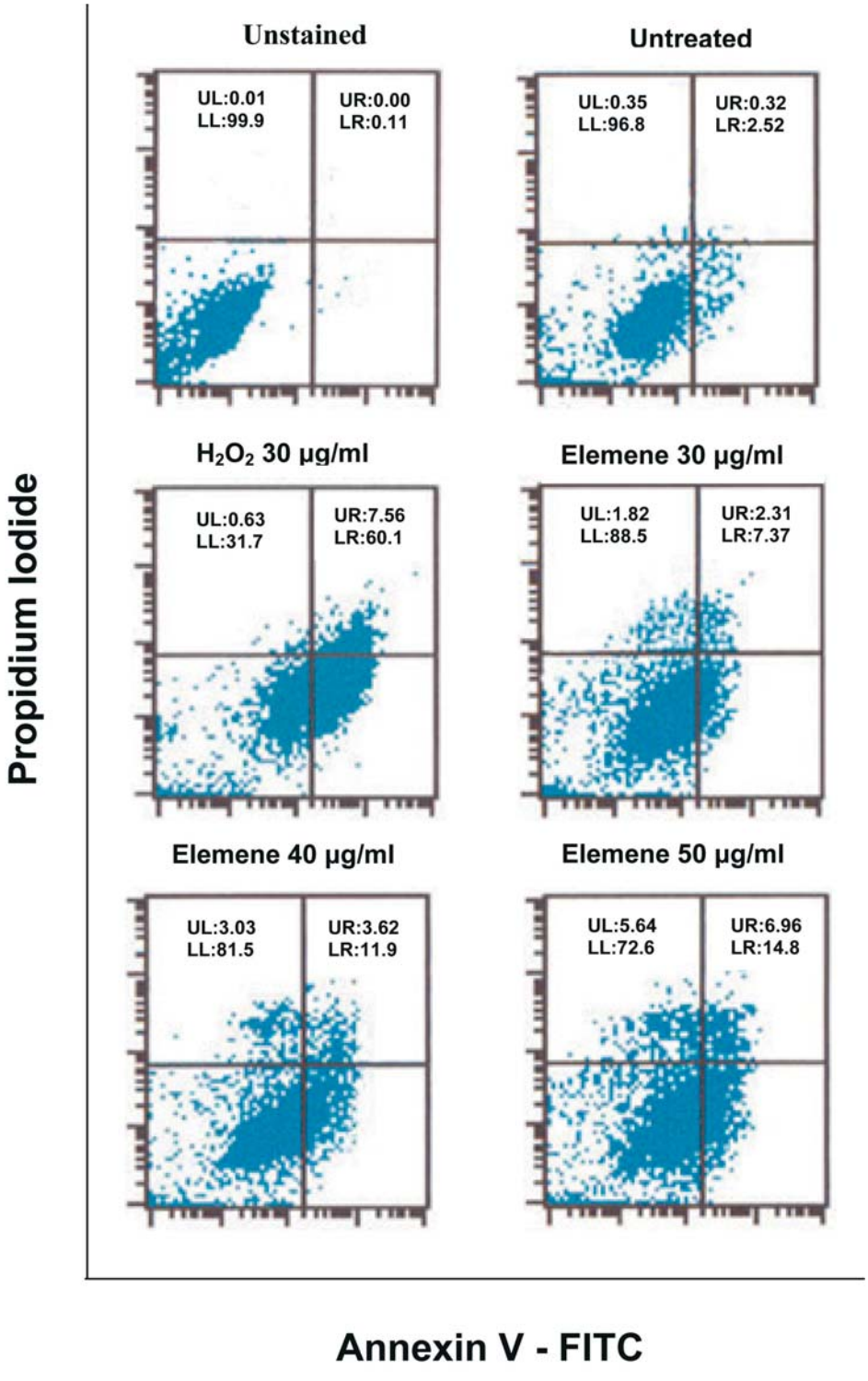


Figure 8. β-Elemene induced apoptosis in human NSCLC H460 cells as assayed by annexin V staining. H460 cells were exposed to various concentrations of β-elemene (0, 30, 40, and 50 µg/ml) and to 30 µg/ml H_2O_2 for 24 or 48 h (data at 24 h not shown). The cells were then harvested and stained using a Vybrant Apoptosis Assay Kit. Apoptosis was determined by flow cytometry for annexin-V-FITC and PI dual labeling, as described in Materials and methods. Cytograms of annexin-V-FITC binding (abscissa) *versus* PI uptake (ordinate) show three distinct populations: (*i*) viable cells (low FITC and low PI signal) in gate LL; (*ii*) early apoptotic cells (high FITC and low PI signal) in gate LR and (*iii*) cells that have lost membrane integrity as a result of very late apoptosis (high FITC and high PI signal) in gate UR. Percentages of apoptotic cells (gate LR and gate UR) are indicated on each cytogram. A representative of three separate experiments is shown.

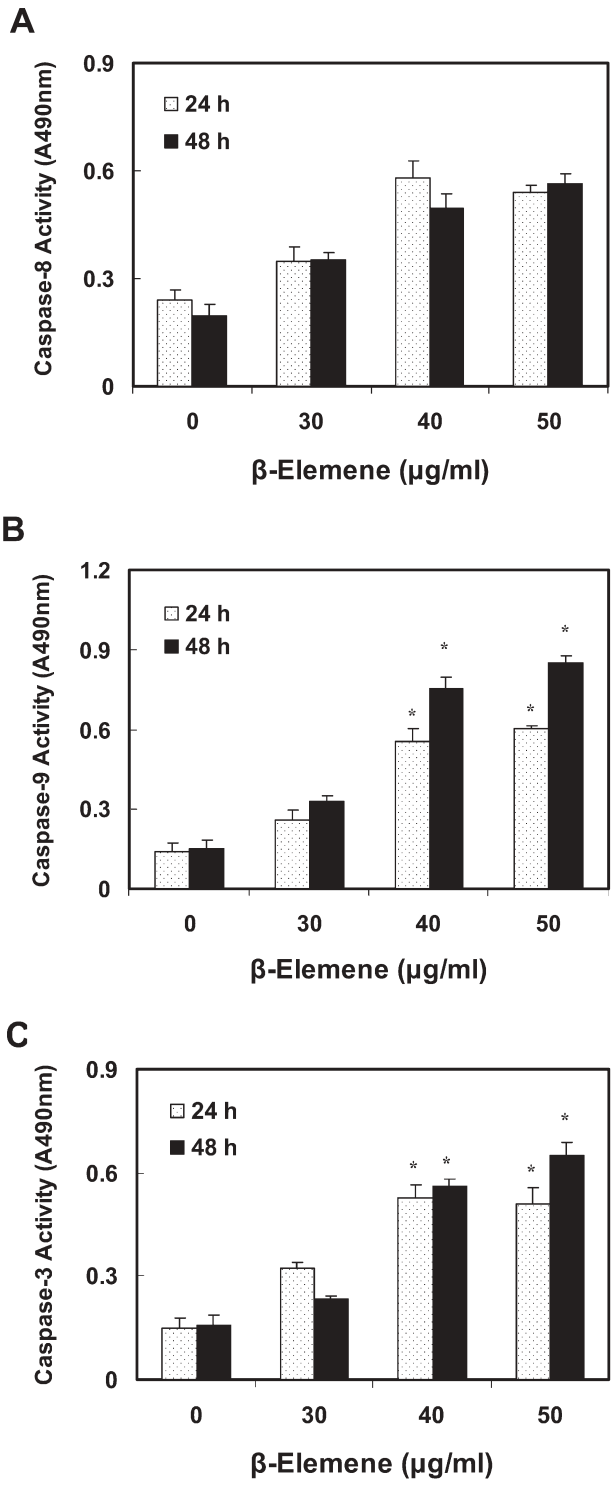


Figure 9. β-Elemene induced increases in caspase activities in human NSCLC H460 cells. H460 cells were treated with different concentrations of β-elemene as indicated for 24 or 48 h. Caspase-8 (*A*), caspase-9 (*B*), and caspase-3, -7 and -10 activities (*C*) were measured by ELISA-based assay as described in Materials and methods. The results are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ versus untreated controls.

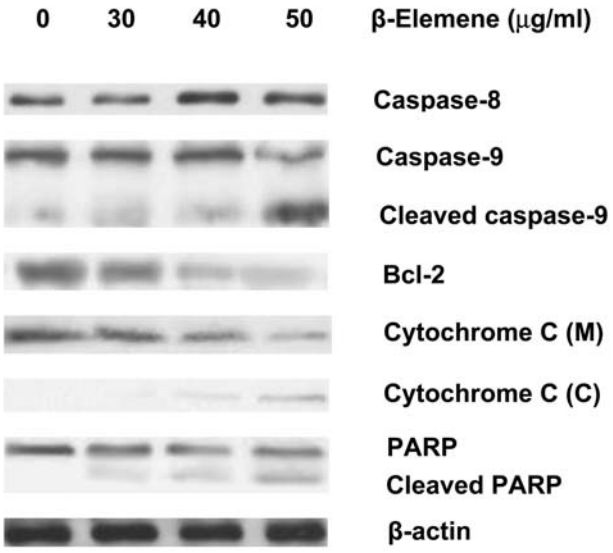


Figure 10. Effects of β-elemene on the levels and activity of apoptosis regulatory proteins in human NSCLC H460 cells. H460 cells were exposed to 0, 30, 40 or 50 μg/ml β-elemene for 24–48 h. A total 50 μg of cell extract protein isolated from β-elemene-treated H460 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with the antibodies against caspase-8, caspase-9, Bcl-2, cytochrome c or PARP. β-Actin was used as a loading control. Western blot data presented are representative of those obtained from three separate experiments. M, mitochondrial cytochrome c; C, cytosolic cytochrome c.

B1 protein were reduced, while the levels of phospho-Cdc2 (Tyr-15) and $p27^{kip1}$ were increased, all these leading to decreased activity of the Cdc2-cyclin B1 complex. These data suggest that the G2-M arrest by β-elemene in our model is due to a decrease in Cdc2 phosphorylation on Thr-161 by CAK, an increase in Cdc2 phosphorylation on Tyr-15 by Wee1 and Myt1, and a reduction in Cdc2 activity as a result of augmented $p27^{kip1}$ expression. Additionally, we found that β-elemene reduced expression of Cdc25C, which dephosphorylates/activates Cdc2, but enhanced the expression of the checkpoint kinase, Chk2, which phosphorylates/inactivates Cdc25C. These findings suggest that the effect of β-elemene on G2-M arrest in NSCLC cells may be mediated partly through a Chk2-dependent mechanism.

Cyclin A is a key cell cycle regulator which starts to accumulate during the S phase of the cell cycle and is abruptly destroyed before metaphase. It can activate two different cyclin-dependent kinases (CDK1 and CDK2), contributes to the control of cyclin B stability and functions in both S phase and mitosis [49]. Cyclin A associates with CDK2 to drive cells through the S phase, and CDK2-cyclin A phosphorylates a large number of target proteins, including pRb, transcription factors and regulators of transcription factors. Inhibition of CDK2-cyclin A results in S phase arrest and apoptosis [50]. Cyclin A is present throughout G2, and associates with Cdc2 (CDK1)

during the transition from the G2 to M phase [50]. CDK2 is also highly regulated [51]. In addition to the dependence on the association with cyclin, CDK2 requires activating phosphorylation on Thr-160 by CAK, and CDK2 activity is inhibited by CDK inhibitors of the Cip/Kip family, such as $p27^{kip1}$, and by inhibitory phosphorylation of CDK2 proteins by Wee1 and Myt1 [50]. In the current study, we observed that the levels of cyclin A protein and CDK2 phophorylation on Thr-160 were dramatically reduced in H460 cells following exposure to various concentrations of β-elemene, these alterations may contribute to β-elemene-induced S and G2-M arrest in H460 NSCLC cells.

Programmed cell death or apoptosis is mediated by different mechanisms and regulated through multiple pathways. One pathway depending on the ligation of death receptors is activated through cleavage and activation of caspase-8, and the other mitochondria-dependent apoptotic route needs the cleavage and activation of caspase-9 [23–27]. Therefore, caspase-8 and caspase-9 serve as markers of different pathways, and caspases are considered to be the central executors of the apoptotic process, especially caspase-3, -6 and -7 [27, 36, 37]. Bcl-2, an antiapoptotic protein which prevents apoptosis by suppressing the release of the caspase-activating protein cytochrome c from mitochondria, can prevent the activation of caspases in the effective stage of apoptosis [39–41]. Here, we showed that β-elemene triggered the apoptosis of H460 cells, as determined by sub-G1 profile analysis, cell death detection ELISA assay, TUNEL assay, annexin V staining and Western blot analysis. The results of our experiments clearly demonstrated that β-elemene induced increases in caspase-9, caspase-3 and caspase-7 activities, decreased Bcl-2 expression, caused cytochrome c release into the cytosol from mitochondria and increased the protein levels of cleaved caspase-9 and PARP in H460 cells. These data indicate that the effect of β-elemene on cell death may be mediated via a mitochondrial cytochrome c release-dependent apoptotic pathway. Interestingly, caspase-8 activity and protein level was also elevated by β-elemene in our system; therefore, the role of caspase-8 in the anticancer activity of β-elemene cannot be ruled out at this point. Further investigation is necessary to define and clarify whether caspase-8 or a caspase-8-associated cell death signaling pathway is involved in the effect and mechanism of action of β-elemene in human NSCLC cells.

In this study, we showed that β-elemene blocked H460 cells at the G2-M phase and induced apoptosis with a decrease in Bcl-2 expression. However, the mechanism accounting for the association of G2-M cell cycle arrest with apoptosis is unclear. Recent evidence demonstrated that Bcl-2 modulates CDK2 activation during apoptosis, which links cell cycle control to apoptotic cell death [52]. Several studies suggest that G2-M phase cells proved more susceptible to death signals because stress response kinases phosphorylate Bcl-2 during cell cycle progression as a physiologic process to inactivate Bcl-2 at G2-M [53]. On the other hand, others suggest that Bcl-2 phosphorylation may be a signal to delay apoptosis in cells arrested at the G2-M phase [54]. In our recent studies using human ovarian carcinoma cells, we also found that β-elemene induced both cell cycle G2-M arrest and apoptosis. However, the link of G2-M arrest to cell death by Bcl-2, and the relationship between β-elemene-induced G2-M arrest and β-elemene-induced apoptosis remain elusive.

In conclusion, we provide here the first evidence that β-elemene has differential inhibitory effects on cell proliferation between human NSCLC cells and human lung fibroblast and bronchial epithelial cells. Furthermore, we show that β-elemene-induced cell growth inhibition in H460 cells is associated with cell cycle G2-M arrest and apoptotic cell death. Our results obtained by means of various biochemical assays and molecular and cell biology assessments demonstrate that β-elemene treatment of H460 cells causes marked changes in the levels of key G2-M-regulating and apoptosis-modulating proteins, including cyclin B1, Cdc2, Cdc25C, Chk2, Bcl-2, cytochrome c, and cleaved caspase-9 and PARP, suggesting that Chk2-mediated G2-M arrest and mitochondrial cytochrome c release-dependent apoptosis are involved in the process. These observations clearly indicate that the antiproliferative effect and antineoplastic activity of β-elemene in human NSCLC cells are mediated through induction of cell cycle arrest and apoptotic cell death. Studies are under way to determine how β-elemene transcriptionally downregulates the expression of cyclin A, cyclin B1, cdc25c and bcl-2 genes, how β-elemene-induced G2-M arrest links to apoptosis, and to elucidate the molecular basis for the signaling pathways by which β-elemene triggers cell cycle arrest and apoptosis.

Acknowledgements. This work was supported by grants from the National Center for Research Resources (No. P20RR16440-010003) and West Virginia University School of Medicine, and by a fund from Long Range International Limited USA, Inc.

1 Jemal A., Thomas A., Murray T. and Thun M. (2002) Cancer statistics. CA. Cancer J. Clin. **52:** 23–47
2 Parkin D., Bray F., Ferlay J. and Pisani P. (2001) Estimating the world cancer burden: Globocan 2000. Int. J. Cancer **94:** 153–156
3 Weir H., Thun M., Hankey B., Ries L., Howe H., Wingo P. et al. (2003) Annual report to the nation on the status of cancer, 1975–2000, featuring the uses of surveillance data for cancer prevention and control. J. Natl. Cancer Inst. **95:** 1276–1299
4 Beadsmoore C. and Screaton N. (2003) Classification, staging and prognosis of lung cancer. Eur. J. Radiol. **45:** 8–17
5 Cortes-Funes H. (2002) New treatment approaches for lung cancer and impact on survival. Semin. Oncol. **29:** 26–29

6 Bunn P. J., Vokes E., Langer C. and Schiller J. (1998) An update on North American randomized studies in non-small cell lung cancer. Semin. Oncol. **25:** 2–10
7 Group N-SCLCC-O (1995) Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomized clinical trials. BMJ **311:** 899–909
8 Johnson D. (2000) Evolution of cisplatin-based chemotherapy in non-small cell lung cancer: a historical perspective and the eastern cooperative oncology group experience. Chest **117:** 133S–137S
9 Fossella F., Lynch T. and Shepherd F. (2002) Second line chemotherapy for NSCLC: establishing a gold standard. Lung Cancer **38:** 5–12
10 Gridelli C., Rossi A. and Maione P. (2003) Treatment of non-small-cell lung cancer: state of the art and development of new biologic agents. Oncogene **22:** 6629–6638
11 Zou L., Liu W. and Yu L. (2001) beta-elemene induces apoptosis of K562 leukemia cells (in Chinese). Zhonghua Zhong Liu Za Zhi **23:** 196–198
12 Zhou H., Shen J., Hou J., Qiu Y. and Luo Q. (2003) Experimental study on apoptosis induced by elemene in glioma cells (in Chinese). Ai Zheng **22:** 959–963
13 Yang H., Wang X., Yu L. and Zheng S. (1999) Study on the anticancer mechanism of elemene (in Chinese). Chin. Clin. Cancer **26:** 4–7
14 Swanton C. (2004) Cell-cycle targeted therapies. Lancet Oncol. **5:** 27–36
15 Fisher D. (1994) Apoptosis in cancer therapy: crossing the threshold. Cell **78:** 539–542
16 Nasmyth K. (1996) Viewpoint: putting the cell cycle in order. Science **274:** 1643–1645
17 McDonald Er. and El-Deiry W. (2000) Cell cycle control as a basis for cancer drug development. Int. J. Oncol. **16:** 871–886
18 Singh S., Herman-Antosiewicz A., Singh A., Lew K., Srivastava S., Kamath R. et al. (2004) Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. J. Biol. Chem. **279:** 25813–25822
19 Matsuoka S., Huang M. and Elledge S. (1998) Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science **282:** 1893–1897
20 O'Connell M., Walworth N., Carr A. (2000) The G2-phase DNA-damage checkpoint. Trends Cell. Biol. **10:** 296–303
21 Sun X., MacFarlane M., Zhuang J., Wolf B., Green D. and Cohen G. (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem. **274:** 5053–5060
22 Green D. (2000) Apoptotic pathways: paper wraps stone blunts scissors. Cell **102:** 1–4
23 Ashkenazi A. and Dixit V. (1999) Apoptosis control by death and decoy receptors. Curr. Opin. Cell Biol. **11:** 255–260
24 Li P., Nijhawan D., Budihardjo I., Srinivasula S., Ahmad M., Alnemri E. et al. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91:** 479–489
25 Liu X., Kim C., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:** 147–157
26 Earnshaw W., Martins L. and Kaufmann S. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. **68:** 383–424
27 Thornberry N. and Lazebnik Y. (1998) Caspases: enemies within. Science **281:** 1312–1316
28 Wang H., Quan T., He T., Franke T., Voorhees J. and Fisher G. (2003) Epidermal growth factor receptor-dependent, NF-kappaB-independent activation of the phosphatidylinositol 3-kinase/Akt pathway inhibits ultraviolet irradiation-induced caspases-3, -8, and -9 in human keratinocytes. J. Biol. Chem. **278:** 45737–45745
29 Bartek J., Falck J. and Lukas J. (2001) CHK2 kinase – a busy messenger. Nat. Rev. Mol. Cell Biol. **2:** 877–886
30 Kim H., Ingermann A., Tsubaki J., Twigg S., Walker G. and Oh Y (2004) Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. Cancer Res. **64:** 2229–2237
31 Negoescu A., Lorimier P., Labat-Moleur F., Drouet C., Robert C., Guillermet C. et al. (1996) In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J. Histochem. Cytochem. **44:** 959–968
32 Cryns V. and Yuan J. (1998) Proteases to die for. Genes Dev. **12:** 1551–1570
33 Homburg C., Haas M. de, Borne A. von dem, Verhoeven A., Reutelingsperger C. and Roos D. (1995) Human neutrophils lose their surface Fc gamma RIII and acquire annexin V binding sites during apoptosis in vitro. Blood **85:** 532–540
34 Koopman G., Reutelingsperger C., Kuijten G., Keehnen R., Pals S., Oers M. van (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood **84:** 1415–1420
35 Johnstone R., Ruefli A. and Lowe S. (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell **108:** 153–164
36 Zou H., Li Y., Liu X. and Wang X. (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. **274:** 11549–11556
37 Senderowicz A. (2003) Cell cycle modulators for the treatment of lung malignancies. Clin. Lung Cancer **5:** 158–168
38 Falck J., Mailand N., Syljuasen R., Bartek J. and Lukas J. (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature **410:** 842–847
39 Gross A., McDonnell J. and Korsmeyer S. (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev. **13:** 1899–1911
40 Kroemer G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. **3:** 614–620
41 Kluck R., Bossy-Wetzel E., Green D. and Newmeyer D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science **275:** 1132–1136
42 Morgan D. (1995) Principles of CDK regulation. Nature 374
43 Sherr C. (1996) Cancer cell cycles. Science **274:** 1672–1677
44 Dynlacht B. (1997) Regulation of transcription by proteins that control the cell cycle. Nature **389:** 149–152
45 Choi Y., Lee W., Park K. and Zhang L. (2000) p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. Jpn. J. Cancer Res. **91:** 164–173
46 Heller J., Kuo J., Wu T., Kast W. and Huang R. (2001) Tetra-O-methyl nordihydroguaiaretic acid induces G2 arrest in mammalian cells and exhibits tumoricidal activity in vivo. Cancer Res. **61:** 5499–5504
47 Sherr C. and Roberts J. (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. **9:** 1149–1163
48 Niculescu A. III, Chen X., Smeets M., Hengst L., Prives C., Reed S. (1998) Effects of p21Cip1/Waf1 at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol. Cell. Biol. **18:** 629–643
49 Yam C., Fung T. and Poon R. (2002) Cyclin A in cell cycle control and cancer. Cell Mol. Life Sci. **59:** 1317–1326
50 Johnson L., De Moliner E., Brown N., Song H., Barford D., Endicott J. et al. (2002) Structural studies with inhibitors of the cell cycle regulatory kinase cyclin-dependent protein kinase 2. Pharmacol. Ther. **93:** 113–124
51 Shapiro G. and Harper J. (1999) Anticancer drug targets: cell cycle and checkpoint control. J. Clin. Invest. **104:** 1645–1653

52 Gil-Gomez G, Berns A and Brady H (1998) A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. EMBO J. **17:** 7209–7218

53 Yamamoto K., Ichijo H. and Korsmeyer S. (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol. Cell Biol. **19:** 8469–8478

54 Vantieghem A., Xu Y., Assefa Z., Piette J., Vandenheede J., Merlevede W. et al. (2002) Agostinis P.: Phosphorylation of Bcl-2 in G2/M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal and delays the onset of apoptosis. J. Biol. Chem. **277:** 37718–37731