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Research Article

Antiproliferative effect of β-elemene in chemoresistant ovarian carcinoma cells is mediated through arrest of the cell cycle at the G2-M phase

X. Li, G. Wang, J. Zhao, H. Ding, C. Cunningham, F. Chen‡, 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: +1304 293 4667, e-mail: qli@hsc.wvu.edu

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Abstract. Elemene is a natural antitumor plant drug. However, the effect of elemene on cell growth in ovarian cancer is unknown. In this study, we show that β-elemene inhibited the proliferation of cisplatin-resistant human ovarian cancer cells and their parental cells, but had only a marginal effect in human ovary cells, indicating differential inhibitory effects on cell growth between ovarian cancer cells and normal ovary cells. We also demonstrated for the first time that β-elemene markedly enhanced cisplatin-induced growth inhibition in resistant cells compared to sensitive cells. In addition, cell cycle analysis revealed a synergistic effect of β-elemene and cisplatin on the induction of cell cycle G2-M arrest in our resistant ovarian carcinoma cells. Furthermore, we showed that treatment of these cells with both drugs downregulated cyclin B1 and Cdc2 expression, but elevated the levels of p53, $p21^{waf1/cip1}$, $p27^{kip1}$ and Gadd45. Finally, the combination of β-elemene and cisplatin was found to increase the phosphorylation of Cdc2 and Cdc25C, which leads to a reduction in Cdc2-cyclin B1 activity. These novel findings suggest that β-elemene sensitizes chemoresistant ovarian carcinoma cells to cisplatin-induced growth suppression partly through modulating the cell cycle G2 checkpoint and inducing cell cycle G2-M arrest, which lead to blockade of cell cycle progression.

Key words: Ovarian cancer; β-elemene; cisplatin; drug resistance; cell cycle; G2-M arrest.

Ovarian cancer is the leading cause of death from gynecologic malignancies in the United States [1]. Chemotherapy is being used to control and treat ovarian cancer and other tumors but remains relatively non-selective and highly toxic to normal tissues [2–4]. Cisplatin is one of the clinical chemotherapeutic drugs, which plays a major role in the chemotherapy of ovarian carcinomas [5–11]. The activity of cisplatin is believed to be due primarily to its ability to bind covalently to DNA [12–15], which may induce cell cycle arrest and apoptosis [9, 11, 12]. However, the success of cisplatin chemotherapy is limited because of systemic toxicity and acquired resistance to the drug [16–19]. In recent years, a great deal of effort has been directed toward combination chemotherapy to increase the therapeutic potential of compounds and to decrease systemic side effects and drug resistance [20, 21]. Non-cytotoxic agents with high anticancer efficacy and less cellular toxicity to normal tissues have been suggested as possible candidates to be investigated for their synergistic effects in combination with cisplatin or other platinum compounds [22–26].

Elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane) is a novel non-cytotoxic antitumor drug, which is isolated from the traditional Chinese medicinal herb *Rhizoma*

* Corresponding author.
‡ Present address: National Institute for Occupational Safety and Health, Morgantown, West Virginia 26506 (USA)

zedoariae. The extract of elemene is a mixture of β-, δ-, and γ-elemene, with β-elemene as the main component (fig. 1), accounting for 60~72% of the three isoforms. When used as a single agent, elemene was shown to exhibit anticancer effects in human and murine tumor cells in vitro and in vivo and has substantial clinical activity against various tumors without severe side effects [27–29]. No bone marrow suppression and drug resistance have been observed in the clinical studies; on the contrary, patient immunity was improved during the therapy with elemene [30]. In China, elemene has been effective for the treatment of cervical cancer, as well as carcinomas of the liver, brain and other vital tissues [31], and is now in application for clinical studies in the United States. However, the potential application of elemene in conjunction with platinum agents for treatment of resistant ovarian tumor and other human neoplasia has not been explored. Moreover, the mechanisms by which elemene exerts its antitumor effect are not understood. Although previous studies on elemene showed that the induction of cell cycle arrest may account for a mechanism of its action in promyelocytic leukemia HL-60 cells [32], the molecular basis of cell cycle arrest by elemene remains undefined.

The eukaryotic cell cycle is regulated via the sequential activation and inactivation of cyclin-dependent kinases (CDKs) that drive cell cycle progression through the phosphorylation and dephosphorylation of regulatory proteins [33–35]. 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) [36]. The CIP/KIP family proteins, including $p21^{waf1/cip1}$ and $p27^{kip1}$, act as inhibitors of Cdc2 [37, 38]. Initiation of mitosis in human cells requires the activation of M phase-promoting factor (MPF), the complex of Cdc2 and a B-type cyclin [36, 39]. The activity of MPF is increased by dephosphorylation of Cdc2 (Thr-14 and Tyr-15) and nuclear accumulation of cyclin B1 protein [36]. The Cdc2-cyclin B1 complex, which accumulates in the cytoplasm during S and G2 phases, translocates to the nucleus during the prophase of M phase, and thus regulates cell cycle progression [36, 40–45]. Not surprisingly, the loss or deactivation of either component of the MPF will block cellular progression out of G2 [46].

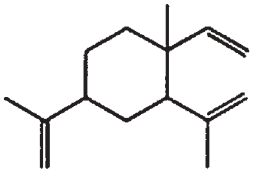

Figure 1. Chemical structure of β-elemene.

p53 plays an important role in the control of the G2-M transition through regulating MPF [41, 47–49]. Cdc2 is inhibited by three transcriptional targets of p53, $p21^{waf1/cip1}$, Gadd45 and 14-3-3σ [41]. Repression of cdc2 and cyclin B1 genes by p53 also contributes to blocking entry into mitosis [41, 50]. In addition, genotoxic stresses also activate p53-independent pathways that regulate the G2-M transition via inhibiting Cdc2 activity [41]. For example, the protein kinases Chk1 and Chk2, activated by the upstream protein kinases ATM and ATR, inhibit Cdc2 by inactivating Cdc25, the phosphatase that normally activates Cdc2 [41]. Moreover, Chk1/2, ATM and ATR also directly or indirectly activate p53 in response to genotoxic stresses and therefore play multiple roles [41]. However, the roles of p53 and other cell cycle regulatory components in elemene-mediated cell growth inhibition and cell cycle arrest are unknown.

The goal of the current study was to investigate the effect of β-elemene on cisplatin-induced growth suppression in human ovarian cancer and the underlying mechanisms. We have examined the effect of β-elemene on cell growth in ovarian cancer and normal ovary cells, and found that β-elemene differentially inhibited cell proliferation between ovarian cancer cells and human ovary cells. We also showed that β-elemene dramatically enhanced the inhibitory effect of cisplatin on cell growth in A2780/CP resistant ovarian tumor cells by blocking cell cycle progression at the G2-M phase, and that the effect of β-elemene and cisplatin on G2-M arrest in these cells is mediated by modulating the levels and activities of cell cycle regulatory proteins. These results support the potential therapeutic applications of β-elemene in combination with cisplatin in chemoresistant ovarian cancer and other tumors.

Materials and methods

Chemicals and immunoreagents

β-Elemenum (98% purity) was obtained from Yuanda Pharmaceuticals (Dalian, China). Cisplatin, dimethylsufoxide (DMSO) and propidium iodide (PI) were purchased from Sigma (St. Louis, Mo.). Antibodies against Cdc2, phospho-Cdc2, cyclin B1, $p21^{waf1/cip1}$, $p27^{kip1}$, p53, Gadd45, Cdc25C, phospho-Cdc25C and β-actin, and the peroxidase-labeled anti-rabbit immunoglobulin G (IgG), Blotto B, and ECL Western blotting system were all purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Cell lines and cell culture conditions

The human ovarian cancer parental cell line A2780 and the cisplatin-resistant human ovarian carcinoma cell line A2780/CP have been described previously [51, 52]. Cells were cultured in a monolayer using RPMI 1640

medium (Invitrogen, Gaithersburg, Md.) supplemented with 10% (v/v) fetal calf serum (FCS), 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen). The human ovary epithelial cell line IOSE-397 was a kind gift of Dr. N. Auersperg (University of British Columbia, Vancouver, Canada), and the cell line was maintained in a 1:1 mixture of Medium 199 and MCDB 105 (Sigma) supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin sulfate (Sigma).

All cell lines were grown in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were routinely tested for mycoplasma infection using a commercial assay system (MytoTect; Invitrogen), and new cultures were established monthly from frozen stocks. All media and reagents contained <0.1 ng/ml endotoxin as determined by *Limulus polyphemus* amebocyte lysate assay (Whittaker Bioproducts, Walkersiville, Md.). Before starting the experiments, cells were grown to 70–80% confluence after subculturing. Cisplatin was initially dissolved in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} at 5 mM. Cisplatin and β-elemene were serially diluted in culture medium to obtain the desired drug treatment concentrations.

Drug treatment and cell survival assays

The effects of cisplatin, β-elemene, or their combination on cell survival were measured by the MTT colorimetric assay. In brief, A2780, A2780/CP or IOSE-397 cells were harvested using 0.25% trypsin-EDTA and resuspended to a final concentration of 5×10^4 cells/ml in fresh medium containing 10% FCS. Aliquots of 0.1 ml from each cell suspension were distributed evenly into 96-well cell culture plates (Corning, Corning, N. Y.). One column from each plate contained medium alone as a blank control, and another column contained cells without drug exposure as an untreated control. After 24 h of incubation with fresh medium, serial dilutions of each drug were added. β-Elemene (20–200 µg/ml), cisplatin (0.5–256 µM), or their combinations were added, and the cells were incubated for an additional 24, 48, 72 and 96 h.

The effects of β-elemene and/or cisplatin on cell growth in the human ovary cells and human ovarian cancer cells were determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wis.) according to the manufacturer's instructions. The absorbance (A) readings were taken using a 96-well Opsys MR Microplate Reader (ThermoLabsystems, Chantily, Va.) and Revelation QuickLink Software at 490 nm. The blank control wells were used for zeroing absorbance. The percentage of cell survival was calculated using the background-corrected absorbance as follows: % cell viability = 100 × (1 – A of experimental well)/A of untreated control well. All experiments were performed at least three times with representative data presented.

To assess the effect of β-elemene on cell growth in ovarian caner cells and normal ovary cells, we also performed colony formation assays using six-well plates. Cells were plated at 250 cells per well, and the procedure and concentrations used for β-elemene treatments were the same (0–200 µg/ml) for each of the two human ovarian carcinoma cell lines A2780 and A2780/CP and the normal ovary cell line IOSE-397. After drug treatment for 24, 48, 72 or 96 h, cells were allowed to grow for a period of 5 days in drug-free medium, at which time colonies were stained with a methylene blue solution of 0.176 % (w/v) in absolute methanol. Visible colonies were counted by hand. Drug treatments were done in triplicate at each dose. The values obtained in wells lacking β-elemene were assigned the value of 100% growth.

Cell cycle analysis by flow cytometry

The effect of the drugs on cell proliferation was evaluated by measuring the distribution of the cells in the different phases of the cell cycle by flow cytometry. This analysis was based on the measurement of the DNA content of nuclei labeled with PI. Cells were cultured in 75-cm^2 flasks and treated with β-elemene alone, cisplatin alone, or the combination of both drugs for 48 h at the indicated concentrations. Cell suspensions from either control cultures or drug-treated cultures were prepared by trypsinization. Approximately 1×10^6 cells/condition were aliquoted, washed twice with ice-cold PBS, and pelleted in a centrifuge. Cell specimens were fixed in 70% ethanol overnight at –20 °C. The cells were then washed twice in PBS, permeabilized with 0.1% Triton X-100 in PBS containing 0.2 µg/ml RNase A (Boehringer Mannheim, Indianapolis, Ind.) for 30 min on ice, and washed twice in PBS. A solution containing 10 µg/ml PI and 0.1% Triton X-100 in PBS was added. Cells were then incubated for 30 min at room temperature to allow maximum labeling of DNA. 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 as G1, S and G2-M.

Protein extraction and Western blot analysis

Cells treated with β-elemene alone, cisplatin alone, or their combinations at the indicated concentrations were harvested by trypsinization following 48 h incubation. After washing with ice-cold PBS, the cells were lysed on ice for 30 min in a mammalian cell lysis buffer (Quality Biological, Gaithersburg, Md.), containing 10 µl/ml 200 mM phenylmethylsulfonyl fluoride (PMSF), 10 µl/ml 100 mM sodium orthovanadate and 10 µg/ml apro-

tinin. Cellular extracts were clarified by contrifugaton at 12,000 rpm at 4 °C for 30 min, and protein concentration were determined using the Bradford assay (Bio-Rad, Richmond, Calif.). Proteins (60 µg) from whole-cell lysates were mixed 1:1 with 2 × sodium dodecyl sulfate (SDS) protein gel solution (Quality Biological), heated for 5 min at 95 °C, separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass.), or nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, N. H.). After blocking in Blotto B for 1 h at room temperature, membranes were incubated overnight at 4 °C with the specific primary antibodies (dilutions were 1:100–1:300). Filters were washed with TBS/0.1% Tween 20 solution, incubated with anti-rabbit peroxidase-conjugated secondary antibody (dilution 1:1000), washed again, and developed with enhanced chemiluminescence substrate (Santa Cruz) according to the manufacturer's instructions. The protein bands were visualized using X-ray films (Eastman Kodak, Rochester, N. Y.). All blots are representative of three independent experiments.

Statistical analysis of data

Data are presented as mean ± SD. Student's t test was used to analyze the difference between the means of the treatment and the control groups. Differences with a p value of less than 0.05 were considered statistically significant.

Results

β-Elemene differentially inhibited the growth of human ovarian cancer and human normal ovary cells

We first examined the effects of *β*-elemene on the proliferation of the cisplatin-resistant human ovarian cancer cell line A2780/CP and its parental cell line A2780 using the MTT colorimetric assay. Significant inhibition of cell growth was observed in both cell lines following treatment with *β*-elemene for 24, 48, 72 or 96 h. The IC_{50} values ranged from 60 to 68 µg/ml for A2780 cells and from 65 to 80 µg/ml for A2780/CP cells (fig. 2). These data show that *β*-elemene has similar inhibitory effects on cell proliferation in both resistant and sensitive ovarian carcinoma cells ($p > 0.05$), and indicate that cisplatin-resistant ovarian tumor cells are still sensitive to *β*-elemene. These experiments demonstrated high antitumor activity of *β*-elemene in human ovarian cancer cells. However, the clinical use of this compound will be linked to its safety, particularly the lack of toxicity in normal cells. Therefore, we evaluated the effect of *β*-elemene on cell viability in a human ovary epithelial cell line, IOSE-397. The cell survival results indicated that the IC_{50}s of *β*-elemene

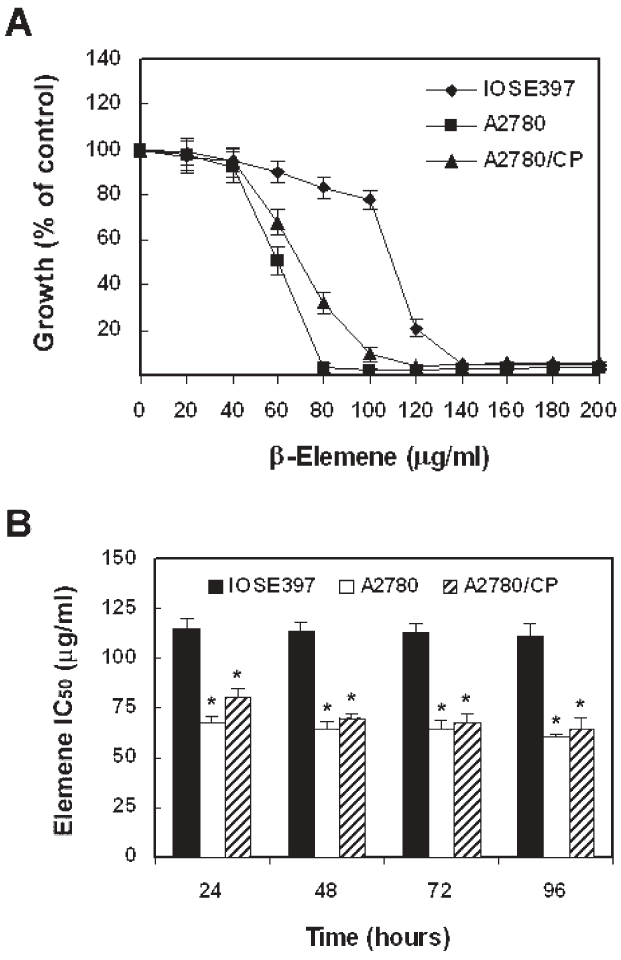

Figure 2. Effects of *β*-elemene on cell proliferation in the human ovary epithelial cell line IOSE-397 and the cisplatin-resistant human ovarian cancer cell line A2780/CP and its parental cell line A2780. Exponentially growing cells were treated with *β*-elemene at the indicated concentrations (20–200 µg/ml) for 96 h (*A*). Cell survival was assessed by MTT assay as described in Materials and methods, and cell growth values were expressed relative to those of the untreated cells (100% control value). The IC_{50}s of *β*-elemene were 110~115 µg/ml for IOSE-397 cells, 60~68 µg/ml for A2780 cells and 65~80 µg/ml for A2780/CP cells (*B*). The results represent the mean of at least three independent experiments. * $p < 0.01$, as compared with IOSE-397; $p > 0.05$, in comparison between A2780 and A2780/CP.

for IOSE-397 cells ranged from 110 to 115 µg/ml (fig. 2), nearly twofold higher than those for the ovarian carcinoma cells ($p < 0.01$).

To confirm the inhibitory effect of *β*-elemene on the growth of the three cell lines, we performed colony formation assays. As seen in figure 3, a proportion of human ovary cells treated with *β*-elemene at 40, 60 and 80 µg/ml began to proliferate again 5 days after *β*-elemene had been removed from the medium. However, regrowth in human ovarian cancer cells treated with *β*-elemene at 40, 60 and 80 µg/ml was significantly diminished (fig. 3). The IC_{50}s of *β*-elemene for IOSE-397 cells ranged from

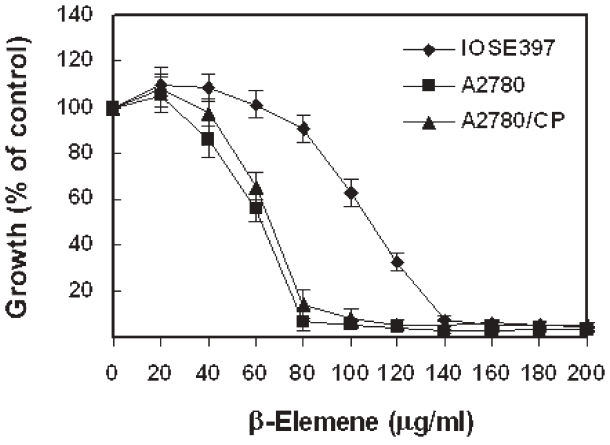

Figure 3. Effects of β-elemene on cell growth in cultured human ovary cells and ovarian cancer cells as determined by the colony growth assay. A total of 250 cells per well of IOSE-397, A2780, and A2780/CP were plated in six-well plates. Cells were treated with 0–200 μg/ml β-elemene for 96 h. The drug-containing medium was then changed, and cells were replenished with fresh drug-free medium and grown for 5 days. The surviving fraction of cells (the visible colonies) was counted manually, and values were expressed relative to those wells where no β-elemene was used (100% control value). Each data point is the mean ± SD from three separate determinations.

110 to 114 μg/ml, and the IC_{50}s of β-elemene for human ovarian cancer cells ranged from 62 to 66 μg/ml (fig. 3). Together, these data indicate that β-elemene differentially inhibited cell growth between human ovarian cancer cells and human normal ovary cells.

β-Elemene augmented cisplatin-induced growth inhibition in resistant human ovarian cancer cells

To determine the potential combinatorial effects of β-elemene and cisplatin in ovarian cancer, we performed experiments to examine the effect of β-elemene on cisplatin activity in resistant A2780/CP cells and their parental A2780 cells. The cells were treated with cisplatin alone or cisplatin plus β-elemene at its IC_{10} doses for 24, 48, 72 or 96 h. β-Elemene was found to enhance cisplatin-induced growth inhibition dramatically in the resistant ovarian cancer cells. As seen in figure 4, the IC_{50}s of cisplatin for A2780/CP cells ranged from 60 to 95 μM when cells were exposed to cisplatin alone, while the IC_{50}s of cisplatin dropped to 1.0–2.5 μM when cells were treated with cisplatin in combination with β-elemene, showing 30- to 60-fold differences between the two treatment groups. In contrast, the IC_{50} values of cisplatin for A2780 cells were 1.5–6.2 μM when cells were exposed to cisplatin alone, and the IC_{50}s of cisplatin were 0.6- to 3.8 μM when cells were exposed to cisplatin plus β-elemene, showing only 2- to 3-fold differences between the two IC_{50} values. These data indicate that β-elemene differentially augments cisplatin activity between resistant A2780/CP cells and their parental A2780 cells. In other words, β-elemene has much greater activity in enhancing growth suppression by cisplatin in chemoresistant ovarian tumor cells than in chemosensitive ovarian tumor cells.

β-Elemene and cisplatin induced ovarian cancer cell cycle arrest at the G2-M phase

Uncontrolled cell proliferation is the hallmark of cancer, and β-elemene was shown to inhibit ovarian cancer cell proliferation and increase cisplatin activity in resistant A2780/CP ovarian carcinoma cells. To probe the mechanism of β-elemene-mediated cell growth inhibition, as well as the mechanism underlying the combination effect of β-elemene and cisplatin in chemoresistant ovarian tumor cells, we examined cell cycle perturbation after exposure of A2780/CP cells to β-elemene, cisplatin, or their combination. The proportions of cells in different phases of the cell cycle were analyzed by flow cytometry using PI staining. As shown in figure 5, there were substantial increases in the fraction of cells in the G2-M phase at 48 h after treatment with the indicated concentrations of β-elemene alone, cisplatin alone, or their combination. Consistent with our cell growth studies, the combination of both agents synergistically induced increases in cell accumulation at G2-M. These results indicate that growth inhibition by β-elemene and cisplatin was associated with significant cell cycle arrest in the G2-M phase and suggest that β-elemene and cisplatin suppress cell proliferation by controlling the G2 checkpoint and inducing a specific block in cell cycle progression.

β-Elemene and cisplatin reduced cyclin B1 and Cdc2 expression and Cdc2 activity in human ovarian carcinoma cells

Eukaryotic cell cycle progression is regulated by a series of CDKs, their activity being positively regulated by cyclins and negatively regulated by cyclin-dependent kinase inhibitors (CKIs). Because β-elemene and cisplatin blocked cell cycle progression and arrested A2780/CP cells at the G2-M phase, we assessed whether inhibition of cell cycle progression was associated with alterations in cell cycle regulatory molecules in the resistant ovarian cancer cells. To examine the expression of intracellular proteins regulating cell cycle progression at the G2-M boundary, cyclin B1 and Cdc2 expression were analyzed. When cells were incubated for 48 h with 30–50 μg/ml β-elemene and/or 10–20 μM cisplatin, dose-dependent inhibition of cyclin B1 and Cdc2 levels was seen (fig. 6A). Since the Cdc2-cyclin B1 complex is maintained in an inactive form by negative phosphorylation of the residue Tyr-15 on Cdc2, and Cdc2-cyclin B1 activity is increased by phosphorylation of Cdc2 at Thr-161, we examined Cdc2 phosphorylation by Western blotting and found that the phosphorylation of Cdc2 at Tyr-15 was increased but the phosphorylation of Cdc2 at Thr-161 was de-

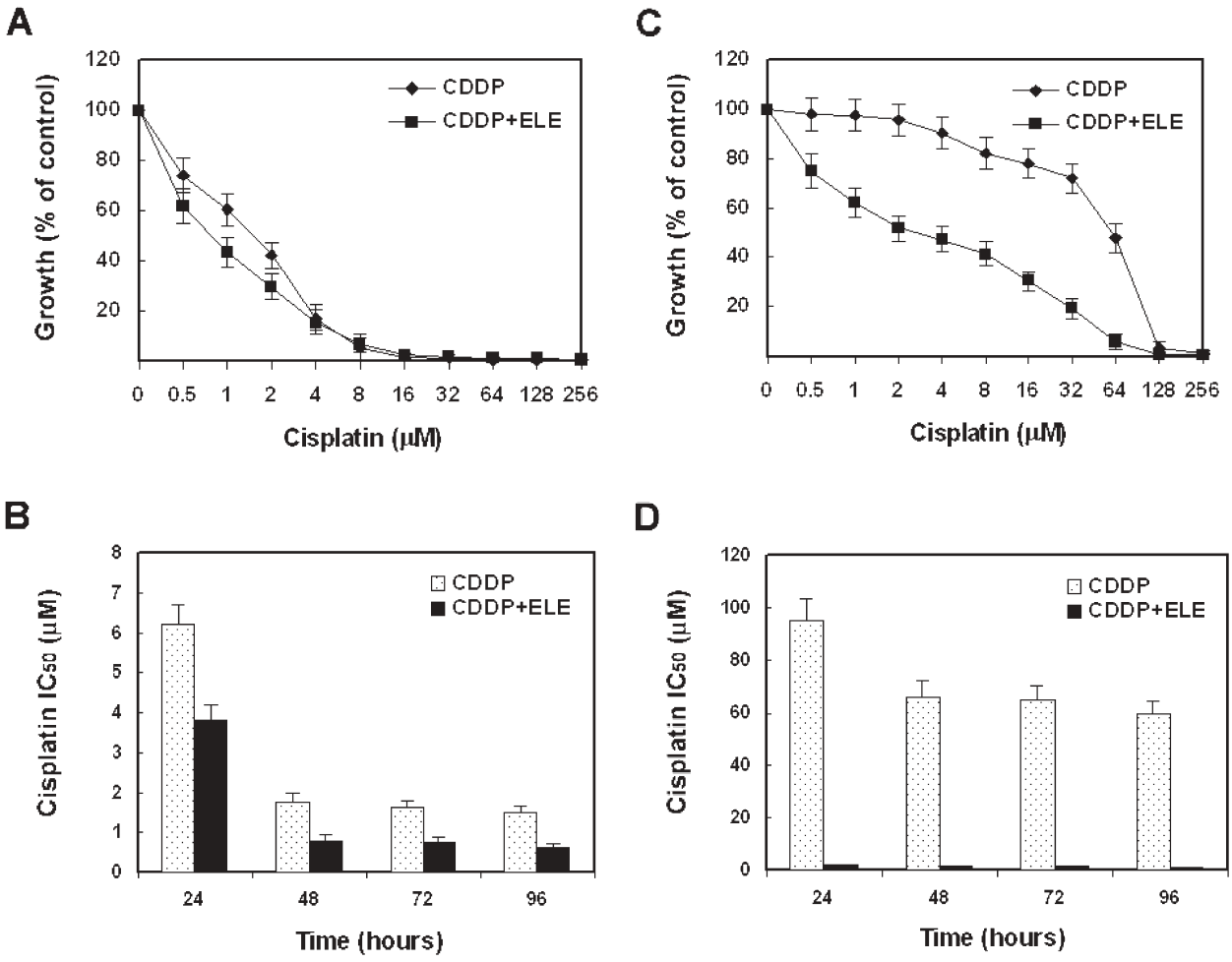

Figure 4. Effects of β-elemene on cisplatin-induced growth suppression in chemoresistant ovarian carcinoma A2780/CP cells and their parental A2780 cells. Exponentially growing cells were exposed to varying concentrations of cisplatin alone (0.5~256 μM), or cisplatin (0.5~256 μM) plus β-elemene at its IC_{10} doses for 24, 48, 72 and 96 h. Cell viability was determined by MTT assay as described in Materials and methods. Shown are the growth of A2780 cells exposed to the drugs for 72 h (*A*) and of A2780/CP cells exposed to the drugs for 72 h (*C*). The cisplatin IC_{50} values were 1.5~6.2 μM for A2780 cells (*B*) and 65~95 μM for A2780/CP cells (*D*) in cells treated with cisplatin alone. In contrast, the IC_{50} values of cisplatin for cells exposed to cisplatin plus β-elemene were reduced to 0.6~3.8 μM for A2780 cells (2- to 3-fold decrease) and to 1.0~2.5 μM for A2780/CP cells (30- to 60-fold decrease) (*B*, *D*). ELE, β-elemene; CDDP, cisplatin.

creased after 48 h of β-elemene and/or cisplatin treatment (fig. 6A). These data suggest that reduced Cdc2-cyclin B1 activity by β-elemene and cisplatin is responsible for the G2-M arrest in our cell model system.

β-Elemene and cisplatin increased the expression of p53, p21$^{waf1/cip1}$ and Gadd45 protein in human ovarian tumor cells

p53 not only directly represses the cyclin B1 and cdc2 genes, but also indirectly inhibits Cdc2-cyclin B1 complex activity through induction of several downstream targets, including p21$^{waf1/cip1}$ and Gadd45. To determine whether a p53-associated mechanism was involved in the β-elemene- and cisplatin-mediated reduction in cyclin B1 and Cdc2, the expression of p53, p21$^{waf1/cip1}$ and Gadd45 protein was analyzed. Western blot analysis of extracts of A2780/CP cells after 48-h treatment with varying concentrations of β-elemene and/or cisplatin showed increases in p53, p21$^{waf1/cip1}$ and Gadd45 protein levels (fig. 6B), which were positively correlated with the G2-M cell proportion and negatively correlated with cyclin B1 and Cdc2 expression (compare with figs 5 and 6A). These results suggest that the induction of p53, p21$^{waf1/cip1}$ and Gadd45 expression by β-elemene and cisplatin might account for a large part of the reduction in Cdc2-cyclin B1 activity and the control of the G2 checkpoint in the ovarian cancer cells.

Effect of β-elemene and cisplatin on p27^{kip1} and Cdc25C expression and Cdc25C activity in human ovarian cancer cells

The protein tyrosine phosphatase Cdc25C activates Cdc2 by dephosphorylation of the residue Tyr-15, which is phosphorylated by Wee1 kinase. Since Cdc2 phosphoryl-

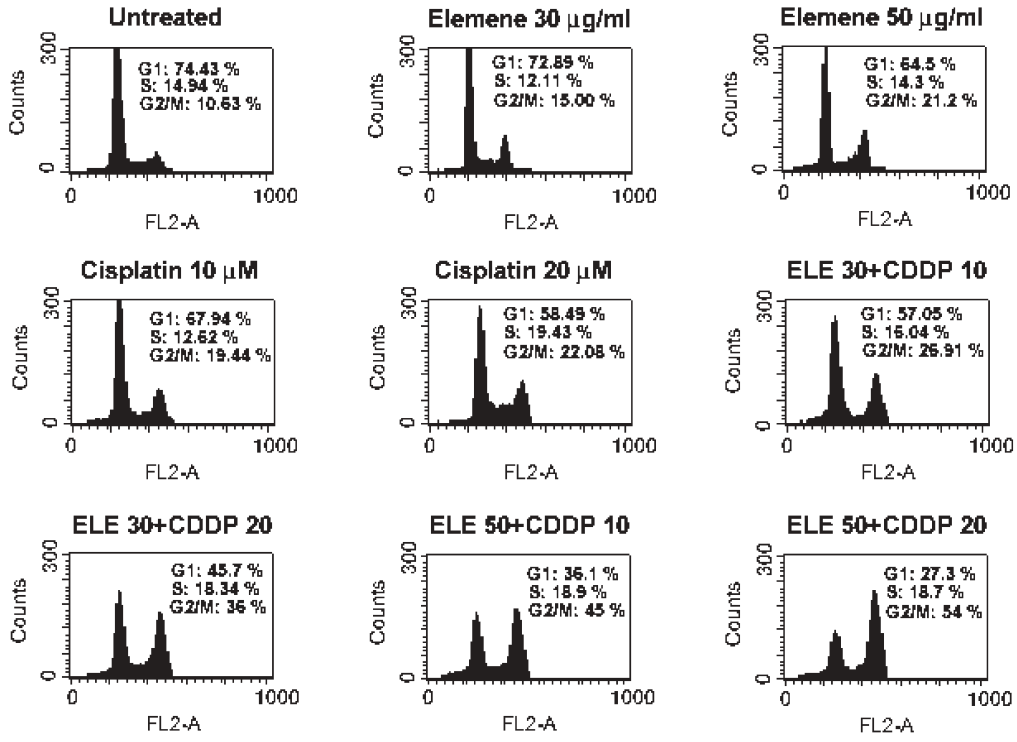

Figure 5. Effects of β-elemene and cisplatin on cell cycle distribution in human A2780/CP ovarian carcinoma cells. Asynchronously growing cells were treated with cisplatin, β-elemene, or cisplatin + β-elemene at the indicated concentrations and harvested at 48 h following exposure to the drugs. DNA content was analyzed by flow cytometry using PI staining, as described in Materials and methods. The percentage of cells in each phase of the cell cycle was calculated using CellQuest Pro and is indicated in the top right of each cell cycle profile. A representative profile is shown for each treatment. As can be seen, cisplatin or β-elemene independently caused cells arrest at the G2-M phase in a concentration-dependent manner, and there was an additive/synergistic effect when cells were treated with both cisplatin and β-elemene. ELE, β-elemene; CDDP, cisplatin.

ation at Tyr-15 was enhanced by β-elemene and cisplatin treatment, we investigated the effect of β-elemene and cisplatin on Cdc25C expression by Western blotting. As shown in figure 6C, the exposure of the ovarian tumor cells to β-elemene and/or cisplatin for 48 h markedly reduced Cdc25C protein levels. Additionally, Cdc25C is also kept inactive and the level of Cdc25C in the nucleus is reduced through negative phosphorylation at serine 216 by upstream Chk1/2 kinases [53]. Therefore, we assessed Cdc25C activity and found that the levels of Cdc25C phosphorylation were increased in A2780/CP cells treated with β-elemene and cisplatin (fig. 6C). Since there was a decrease in Cdc25C expression and activity at 30–50 μg/ml β-elemene and 10–20 μM cisplatin, the concentrations leading to cell cycle arrest, this suggests that Cdc2 inactivation is due not only to increased phosphorylation by Wee1, but also to decreased dephosphorylation by Cdc25C in our model system.

$p27^{kip1}$ is another key inhibitor of Cdc2 in the cell cycle. To determine the role of $p27^{kip1}$ in G2-M arrest in our system, we examined the effect of β-elemene and cisplatin on $p27^{kip1}$ expression. When A2780/CP cells were exposed to 30–50 μg/ml β-elemene and/or 10–20 μM cisplatin for 48 h, the protein levels of $p27^{kip1}$ showed a dose-dependent increase (fig. 6C). Taken together with the Cdc25C data, these findings suggest that a p53-independent mechanism may also be involved in G2-M arrest in β-elemene- and cisplatin-treated A2780/CP ovarian carcinoma cells.

Discussion

Cisplatin is widely used for the treatment of many solid tumors, including bladder, cervical, head and neck, lung, testicular and ovarian cancers [2, 3, 6, 7, 18, 54]. One of the greatest limitations to the successful treatment of these malignancies is the development of clinical resistance to cisplatin [16, 17, 19, 55]. To overcome this obstacle, combination chemotherapy has attracted attention for the purposes of lowering doses of cisplatin, reducing side effects and increasing treatment efficacy by complementing with a non-toxic agent [22, 56, 57]. Based on this rationale, in this study we tested β-elemene, a non-cytotoxic compound, in combination with cisplatin for their effectiveness against chemoresistant human ovarian cancer cells and their parental cells in vitro. The central finding in the present study is that β-elemene strikingly sensitizes ovarian tumor cells to cisplatin-induced growth

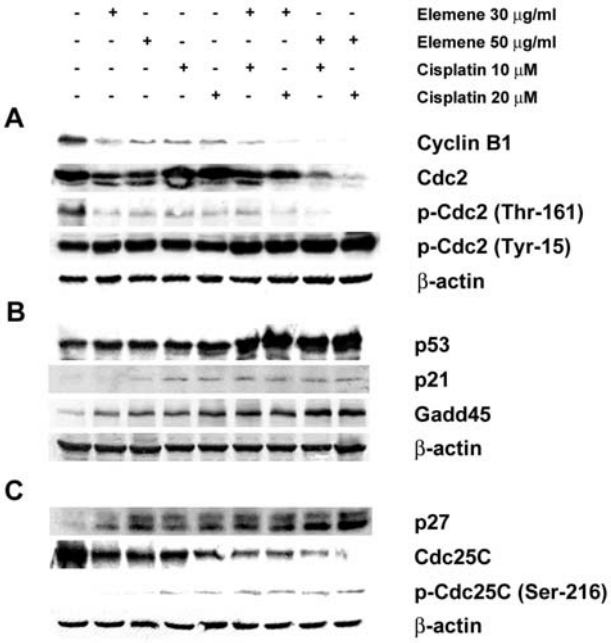

Figure 6. Effects of *β*-elemene and cisplatin on the levels and activity of cell cycle regulatory proteins in A2780/CP ovarian cancer cells. Cells were treated with *β*-elemene alone, cisplatin alone, or their combination, at the indicated concentrations. Cellular proteins were extracted 48 h following drug exposure. A total of 60 μg cell extract protein isolated from the drug-treated and untreated A2780/CP cells was subjected to SDS-PAGE and immunoblotted with antibodies against cyclin B1, Cdc2, phospho-Cdc2 (Tyr-15), phospho-Cdc2 (Thr-161), p53, $p21^{waf1/cip1}$, Gadd45, $p27^{kip1}$, Cdc25C and phospho-Cdc25C (Ser-216). *β*-Actin was used as a loading control. Western blot data presented are representative of those obtained from three separate experiments.

suppression and reverses drug resistance to cisplatin in resistant human ovarian cancer cells. This finding is significant because tumors develop resistance to cisplatin during treatment, and *β*-elemene and cisplatin may be a potential combinatorial therapy to circumvent cisplatin drug resistance in patients with ovarian cancer and other malignancies. In fact, we have found that *β*-elemene also reversed cellular resistance and sensitized the cells to cisplatin in several other types of human carcinoma cells (data not shown).

β-Elemene is a novel plant anticancer drug with a broad spectrum of antitumor activity in different types of cancer. Despite the demonstration of impressive antiproliferative activity *in vivo*, very little is known about the mechanism of its action. In the present study, we provided the first evidence showing that *β*-elemene has differential inhibitory effects on cell proliferation between ovarian tumor cells and normal ovary cells, but has similar suppressive effects on cellular growth in both sensitive and resistant ovarian carcinoma cells, suggesting that *β*-elemene may be effective for the treatment of cisplatin-resistant ovarian cancer. In addition, we showed that *β*-elemene has a greater activity in increasing cisplatin sensitivity in chemoresistant ovarian cancer cells than in their parental cells, and that the effect of *β*-elemene and cisplatin on growth control in resistant ovarian carcinoma A2780/CP cells is associated with cell cycle arrest at the G2-M phase. Furthermore, we found that the treatment with both *β*-elemene and cisplatin decreased cyclin B1 and Cdc2 levels and enhanced the expression of p53, $p21^{waf1/cip1}$ and Gadd45. From these results, we conclude that *β*-elemene may exert its augmenting effect on cisplatin growth inhibition activity in resistant ovarian cancer cells by controlling the G2 checkpoint and inducing cell cycle G2-M phase arrest.

p53 protein plays a critical role in regulating cell cycle progression in response to stressful stimuli, including DNA damage and chemotherapeutic agents [41, 47, 58–61]. The mechanism by which p53 mediates cell cycle arrest at the G1 checkpoint involves transactivation of the cyclin-dependent CKI $p21^{waf1/cip1}$, which is a universal CDK inhibitor [37, 62–64]. Recently, a novel form of $p21^{waf1/cip1}$ protein was shown to inhibit growth by acting not at G_1, but at G2-M [62, 65–68]. In addition, $p21^{waf1/cip1}$ can associate with the activated Tyr-15 dephosphorylated form of Cdc2, and this complex is devoid of Cdc2 kinase activity, indicating that $p21^{waf1/cip1}$ plays a role in Cdc2 inhibition and G2 arrest [65–67, 69, 70]. Gadd45, another transcriptional target of p53, is also implicated in regulating the G2-M transition. The effect of Gadd45 on the G2-M transition is due to its ability to dissociate complexes of cyclin B1 and Cdc2 [71, 72]. Addition of recombinant Gadd45 released Cdc2 from the Cdc2-cyclin B1 complex, suggesting that Gadd45 inhibits Cdc2 by blocking its binding to cyclin B [71, 72]. Gadd45 may also cooperate with other downstream targets of p53, such as $p21^{waf1/cip1}$, to cause G2-M arrest. Since our results showed that *β*-elemene and cisplatin induced p53 accumulation and upregulated $p21^{waf1/cip1}$ and Gadd45 expression, but downregulated cyclin B1 and Cdc2 levels in A2780/CP cells, we speculate that a p53-associated pathway might occur in *β*-elemene- and cisplatin-treated ovarian carcinoma cells, and that p53 may contribute to blocking entry into mitosis directly or indirectly through (i) repression of the cyclin B1 and cdc2 genes and (ii) upregulation of Gadd45 and $p21^{waf1/cip1}$, which interact with and inhibit Cdc2 and cause G2-M phase arrest.

Additionally, we found that *β*-elemene and cisplatin induced increases in $p27^{kip1}$ levels and Cdc2 phosphorylation (Tyr-15) in A2780/CP ovarian cancer cells. $p27^{kip1}$ is a KIP family inhibitor of Cdc2 kinase, but is not a target gene of p53; phosphorylation of Cdc2 at the Tyr-15 residue leads to a decrease in Cdc2 activity. Therefore, increased $p27^{kip1}$ levels and Cdc2 phosphorylation may contribute to the effect of *β*-elemene and cisplatin in blocking cell cycle progression and arresting ovarian tumor cells at the G2-M phase. Finally, we demonstrated in the current study that *β*-elemene and cisplatin downregulated Cdc25C expression and enhanced Cdc25C phosphoryl-

ation at the Ser-216 residue in our cell model system. The activity of Cdc25C, which is essential for progression into mitosis, is regulated by changes in protein level, subcellular localization and phosphorylation state [73]. For example, G2-M cell cycle-blocking agents lead to inhibition of Cdc25C activity through phosphorylation of the Ser-216 by upstream Chk1/2 kinases [74, 75]. Therefore, the effect of β-elemene and cisplatin on G2-M arrest in A2780/CP cells may be mediated by Chk1/2 activation through a p53-independent mechanism as well. However, further investigations are necessary to determine (i) how p53 transcriptionally regulates cyclin B1, Cdc2, $p21^{waf1/cip1}$ and Gadd45 gene expression in our model system and (ii) whether β-elemene and cisplatin exert their effects by activating ATM and ATR kinases and, if so, how these activations link to p53 or Chk1/2, and whether a p53-independent pathway is also involved in the regulation of the G2-M transition in β-elemene- and cisplatin-treated ovarian cancer cells.

In summary, we have shown in this work that β-elemene differentially inhibits cell proliferation between human ovarian cancer cells and normal human ovary cells. We have also demonstrated for the first time that β-elemene markedly augments cisplatin sensitivity in chemoresistant ovarian carcinoma A2780/CP cells. This enhanced chemosensitivity can be attributed to increased capacity to block cell cycle progression at the G2-M phase. Furthermore, our data reveal that the cell cycle arrest at G2 by β-elemene and cisplatin is mediated via regulating the levels and activities of cell cycle regulatory components in A2780/CP cells. Although more studies are required to elucidate whether the enhanced sensitivity by cisplatin-resistant tumor cells to β-elemene is through modulation of apoptotic signaling, this report represents a pioneer study on the mechanistic actions to β-elemene against chemoresistant ovarian carcinoma cells. These findings also provide a scientific basis for developing the combination of β-elemene and cisplatin as a potentially more effective chemotherapy regimen for treatment of patients with resistant ovarian tumors, and suggest that further in vivo studies in preclinical ovarian cancer models with this combinatorial therapy are warranted.

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