β-elemene, a compound derived from *Rhizoma zedoariae*, reverses multidrug resistance mediated by the ABCB1 transporter

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Abstract. In the present *in vitro* study, we examined the effect of the compound β-elemene on the response of KB-C2 cells overexpressing the ABCB1 transporter to specific antineoplastic compounds. The MTT assay was used to determine the effects of β-elemene in combination with other anticancer drugs on ABCB1-overexpressing cancer cell lines. Furthermore, we used [^{3}H]-paclitaxel accumulation, efflux assay, immunofluorescence experiments, western blot assays and docking analysis to ascertain the mechanism of action of β-elemene. The incubation of KB-C2 cells overexpressing ABCB1 transporter with β-elemene (100 µM) significantly augmented the antineoplastic efficacy of colchicine, vinblastine and paclitaxel when compared to KB-C2 cells incubated with these drugs alone. In HEK293 cells overexpressing the ABCB1 transporter, β-elemene significantly increased the cytotoxicity of paclitaxel. In addition, 100 µM of β-elemene significantly increased the accumulation of [^{3}H]-paclitaxel and this was due to a decrease in [^{3}H]-paclitaxel efflux when compared to controls. The incubation of KB-C2 cells with β-elemene (100 µM) for 72 h did not significantly alter the expression of ABCB1 protein levels. Immunofluorescence experiments indicated that β-elemene did not significantly alter the subcellular localization of the ABCB1 transporter. Docking analysis indicated that β-elemene binds to the drug-binding site of ABCB1 transporter. Finally, β-elemene at 100 µM partially (~50%) increased the sensitivity of the BCRP-overexpressing cell line, NCI-H460/MX20, to mitoxantrone, but β-elemene did not significantly alter the resistance of MRP1-transfected HEK293/MRP1 cells to vincristine. Overall, our *in vitro* findings indicated that β-elemene potentiates the cytotoxic effects of various antineoplastic drugs in cell lines overexpressing the ABCB1 transporter and that this is due to the inhibition of the efflux component of the ABCB1 transporter.

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Introduction

One of the primary mechanisms responsible for cancer chemotherapeutic failure is the development of multidrug resistance (MDR), a phenomenon that occurs when cancer cells become resistant to several mechanistically and structurally unrelated antineoplastic drugs (1). One of the major mediators of MDR is the ATP binding cassette (ABC) transporter (1,2). These transporters significantly decrease the intracellular accumulation of antineoplastic compounds by increasing their efflux, thereby inducing MDR (1). Currently, 48 human ABC transporters have been identified and characterized (3). The first identified ABC transporter, ABCB1, is encoded in the human *MDR1* gene (4,5). The ABCB1 protein consists of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) (6). The known substrates of ABCB1 include anthracyclines, vinca alkaloids, taxanes (such as paclitaxel and docetaxel) and epipodophyllotoxins (7). The ABCB1 protein is widely expressed in normal human tissues, including intestinal lining epithelium, bone marrow progenitor cells, hepatocytes, cardiac and striated muscle and blood-brain barrier (8-10).

One pharmacological approach used to increase the response of MDR cancer cells to antineoplastic drugs involves the inhibition of the ABC transporters (11). Research over the past three decades has led to the development of three generations of ABCB1 inhibitors to re-sensitize MDR cells to ABCB1 substrates (12-14). The first generation of ABCB1 inhibitors identified were substrates of ABCB1, such as verapamil and cyclosporine A (CsA) (15,16) and were essentially competitive inhibitors of the efflux activity of different anticancer compounds by the ABCB1 transporter (17). However, the use of these compounds in humans was limited as a

result of significant adverse effects (18). The second generation of ABCB1 inhibitors, such as PSC 833 and biricodar (VX-710) (19,20), were developed and subsequent studies indicated that compared to the first generation, these compounds were superior as they are more potent and less toxic. However, a number of these second generation compounds were inhibitors of CYP3A4 and this led to a significant increase in the levels of the substrate antineoplastic drugs and produced significant adverse and toxic effects (21). The third generation of modulators, such as ONT-093, tariquidar, XR9576, zosuquidar, laniquidar, exhibited high affinity for the ABCB1 transporter but did not significantly alter the metabolism of the antineoplastic substrate drugs and some of them are currently in clinical trials (22-25).

β-elemene, a compound isolated from an extract of traditional Chinese medicinal herb *Rhizoma zedoariae*, is a broad spectrum antineoplastic drug which has been approved by the state Food and Drug Administration of China for the treatment of specific solid and malignant tumors (26,27). However the mechanism of action of β-elemene has not been fully elucidated. It was suggested that β-elemene significantly enhanced the cytotoxicity of cisplatin in treating non-small cell lung cancer cells. This aforementioned effect produced by the β-elemene and cisplatin is due to an increase in the release of cytochrome *c* from mitochondria into the cytoplasm, as well as increasing caspase-3/7/9 activities and caspase-9 cleavage (28). It was previously reported that β-elemene significantly increased the intracellular accumulation of the antineoplastic drug adriamycin (ADM) in MCF/ADM cells (29). Since ADM is a substrate of ABCB1 and breast cancer resistance protein (BCRP/ABCG2), it is possible that the β-elemene-induced increase in ADM levels was due to its inhibition of the ABCB1 and/or BCRP transporters (30,31). However, the present study did not determine which transporter or transporters were affected by β-elemene or the mechanism by which β-elemene increased the intracellular levels of ADM. Therefore, in this *in vitro* study, we examined the effect of the compound β-elemene (see Fig. 1 for structure), on the response of cells to substrates for the ABCB1 (P-glycoprotein/P-gp, MDR), multidrug resistance protein 1 [(MRP1), ABCC1] and BCRP (ABCG2/MXR). In addition, we examined the effect of β-elemene on the efflux of [^{3}H]-paclitaxel and on the expression of the ABCB1 protein in cells overexpressing the ABCB1 transporter. Finally, using docking analysis, the interaction of β-elemene with the ABCB1 transporter was elucidated.

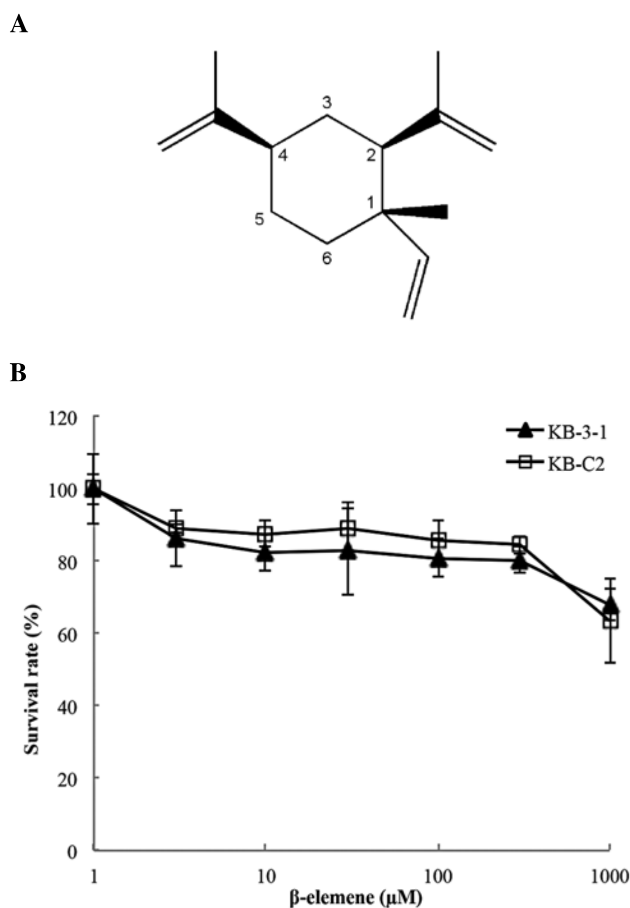

Figure 1. (A) Structure of β-elemene. (B) Cytotoxicity assays with β-elemene. Cell survival percentage after 72 h of incubation with β-elemene. Two cell lines, KB-3-1 and KB-C2, were seeded and cultured for 24 h, various concentrations of β-elemene were added. The cells were cultured for 72 h. The data represent the means and standard deviations of 3 independent experiments.

Materials and methods

Chemicals and equipment. β-elemene was provided by Dalian Jingang Pharmaceutical Co. Ltd. (Dalian, Liaoning, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin/streptomycin and trypsin were purchased from HyClone Thermo Scientific (Logan, UT, USA). The monoclonal mouse antibody C219 (against ABCB1) and the secondary horseradish peroxidase-labeled anti-goat or anti-mouse IgG were purchased from Signet Laboratory (Dedham, MA, USA). Paclitaxel, vincristine, verapamil, vinblastine, cochicine, cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). [^{3}H]-paclitaxel (37.9 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Nilotinib was purchased from Chemie Tek Co. (Indianapolis, IN). Opsys microplate reader was purchased from Dynex Technologies Inc. (Chantilly, VA, USA).

Cell lines. The ABCB1-overexpressing KB-C2 cells were cloned in cell culture medium by a step-wise selection of the parental human epidermoid carcinoma cell line KB-3-1, using colchicine at concentrations up to 2 μg/ml (32). NCI-H460/MX20 and lung cancer cell lines NCI-H460 (parental), HEK293/pcDNA3.1 and wild-type HEK293/ABCB1-transfected cells were kindly provided by Drs Susan Bates and Robert Robey (NCI, NIH, Bethesda, MD, USA). HEK293/ABCB1 cells were obtained by transfecting HEK293 cell lines with a pcDNA3.1 vector containing a full-length cDNA of *ABCB1*. All cell lines were grown in DMEM, supplemented with 10% bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator with 5% CO_2 at 37˚C.

MTT assays. Cell lines were seeded evenly in (160 μl/well) 96-well plates and were cultured for 24 h (33). β-elemene with different concentrations (10, 30, 100 μM) was added into wells 1 h before adding the appropriate antineoplastic

drug colchicine, vinblastine and paclitaxel that were diluted to various concentrations and incubated with cells for 72 h. Subsequently, 20 μl of MTT was added to each well. The plates were placed in an incubator at 37˚C for 4 h. In order to avoid agitating the adhesive cell, the medium and MTT were gently discarded. Subsequently, 100 μl DMSO was added into each well. The absorbance of each sample at 570 nm was determined using an Opsys microplate reader. The magnitude of resistance was calculated by dividing the IC_{50} (concentration required to inhibit cellular proliferation by 50%) for the MDR cells in the absence or presence of inhibitors by the IC_{50} of the parental cells in the absence of the reversal compound.

[^{3}H]-paclitaxel accumulation assays. HEK293/pcDNA3.1 and HEK293/ABCB1 were seeded into two T75 flasks and incubated in DMEM at 37˚C for 24 h (34). Cells were harvested upon reaching 70-95% confluence and two aliquots from each cell line were suspended in DMEM. Subsequently, HEK293/pcDNA3.1 and HEK293/ABCB1 were incubated with 0.1 μM [^{3}H]-paclitaxel in the presence or absence of the β-elemene at 37˚C for 2 h. Cells were washed 3 times with cold phosphate-buffered saline (PBS) and 200 μl of lysis buffer (pH 7.4, containing 1% Triton X-100 and 0.2% SDS). The sample was placed in scintillation fluid and radioactivity was determined using a Packard Tri-Carb 1900CA liquid scintillation analyzer (Packard Instrument Company, Inc. (Downers Grove, IL, USA).

[^{3}H]-paclitaxel efflux assays. HEK293/pcDNA3.1- and HEK293/ABCB1-transfected cells were prepared as described for the accumulation experiments (34,35). Cells were exposed to the same procedure as stated for the drug accumulation experiment. The cells were rinsed 3 times with cold PBS and incubated with or without 100 μM β-elemene at 37˚C. The cells were lysed at various time points (0, 30, 60, 120 min) and radioactivity in the lysate was determined using liquid scintillation counter.

Western blotting. NCI-H460/MX20, NCI-H460, KB-3-1 and KB-C2 were incubated with 100 μM of β-elemene for 0, 24, 48 or 72 h (36). The cells were harvested and washed 3 times with PBS. The cell extracts were incubated with radioimmunoprecipitation assay (RIPA) buffer (PBS with 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 100 mg/ml p-aminophenylmethylsulfonyl fluoride) for 30 min and the cells were centrifuged at 12,000 x g for 15 min at 4˚C. The total cell lysate was dissolved in SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in blocking solution containing 5% non-fat milk in TBST buffer [10 mM Tris-HCL (pH 8.0), 150 mM NaCl and 0.1% Tween-20] for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with monoclonal antibodies against ABCB1 protein at 1:200 dilution, and then further incubated for 3 h at room temperature with a horseradish peroxide (HRP)-conjugated secondary anti-mouse antibody (1:1,000 dilution). The presence of the protein-antibody complex was detected by chemiluminescence. The expression level of the protein was measured by determining the area and density of bands, using Scion Image software (Scion Co., Frederick, MD, USA).

Immunofluorescence assays. KB-3-1 and KB-C2 cells were seeded in 24-well plates and 100 μM of β-elemene was added to each well and the samples were incubated for 0 and 72 h at 37˚C. The cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature and rinsed 3 times with PBS. The cells were incubated with BSA (2 mg/ml) for 1 h at 37˚C. Subsequently, cells were incubated with a polyclonal antibody against ABCB1 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 12 h, followed by a goat anti-mouse IgG (1:100; Molecular Probes, Eugene, OR, USA) for 1 h. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (1 ml/well) was applied to counterstain the nuclei. Finally, images were captured by an IX70 microscope with IX-FLA fluorescence and CCD camera (36).

Ligand structure preparation. The structure of β-elemene was constructed using the fragment dictionary of Maestro v9.0 and the energy was minimized by a MacroModel program v9.7 (Schrödinger, Inc., New York, NY, USA, 2009) using the OPLS-AA force-field with the steepest descent followed by truncated Newton conjugate gradient protocol. The low-energy 3D structures of β-elemene were generated by LigPrep v2.3. Ligand structures representing β isomer were further used for generating 100 ligand conformations using the default parameters of mixed torsional/low-mode sampling function. The conformations were filtered with a maximum relative energy difference of 5 kcal/mol to exclude redundant conformers. The output conformational search (Csearch) file containing at most 100 unique conformers of β-elemene were used as input for docking simulations into each of the binding sites of human ABCB1 (37).

Protein structure preparation. The X-ray crystal structure of mouse ABCB1 in the apoprotein state (PDB ID: 3G5U), and in complex with the ABCB1 inhibitors QZ59-*RRR* (PDB ID: 3G60), QZ59-*SSS* (PDB ID: 3G61) (37), were obtained from the RCSB Protein Data Bank and were used as the templates to generate homology models for human ABCB1 for sites 1-4 (38). The protocol for the homology modeling was essentially the same as reported previously (38). The refined human ABCB1 homology model was used to generate different receptor grids for different sites (sites 1-4) through selection of bound QZ59-*RRR* (site-1) and QZ59-*SSS* (site-2) ligands, all amino acid residues known to contribute to verapamil binding (site-3), two residues known to be common to three previously determined sites (site-4) as previously reported (38).

Docking protocol. The conformational library of β-elemene was docked at each of the generated grids (sites 1-4) by using the XP mode of Glide 5.0 (Schrödinger, Inc., 2009) and the default parameters. The top-scoring pose was used for graphical analysis. All computations were performed with a Dell Precision 470n dual-processor computer with the Linux operating system (Red Hat Enterprise WS 4.0).

Statistical analysis. All experiments were performed in triplicate. The data were analyzed using two-tail Student's t-test. The *a priori* significance level was set at $p<0.05$.

Table I. The reversal effect of β-elemene on ABCB1-mediated drug resistance.[a]

Compounds	IC_{50} ± SD[b] (μM)	
	KB-3-1	KB-C2
Paclitaxel (μM)	0.0050±0.0007 (1.0)[c]	3.240±0.901 (648.0)
+β-elemene (10)	0.0058±0.0015 (1.2)	0.261±0.019[e] (52.2)
+β-elemene (30)	0.0051±0.0011 (1.0)	0.084±0.014[e] (16.8)
+β-elemene (100)	0.0047±0.0021 (0.9)	0.023±0.005[e] (4.6)
+Verapamil (10)	0.0035±0.0007 (0.7)	0.010±0.003[e] (2.0)
Colchicine (μM)	0.0054±0.0015 (1.0)[c]	3.891±0.531 (720.6)
+β-elemene (10)	0.0052±0.0014 (1.0)	0.612±0.201[e] (113.3)
+β-elemene (30)	0.0048±0.0021 (0.9)	0.152±0.050[e] (28.1)
+β-elemene (100)	0.0047±0.0018 (0.9)	0.029±0.013[e] (5.4)
+Verapamil (10)	0.0036±0.0011 (0.7)	0.025±0.008[e] (4.6)
Vinblastine (μM)	0.0411±0.0012 (1.0)[c]	0.482±0.111 (11.7)
+β-elemene (10)	0.0442±0.0113 (1.1)	0.138±0.073[e] (3.4)
+β-elemene (30)	0.0418±0.0087 (1.0)	0.087±0.009[e] (2.1)
+β-elemene (100)	0.0370±0.0096 (0.9)	0.046±0.011[e] (1.1)
+Verapamil (10)	0.0313±0.0003 (0.8)	0.045±0.008[e] (1.1)
Cisplatin (μM)	2.444±0.192 (1.0)[c]	2.628±0.196 (1.1)
+β-elemene (100)	2.023±0.157 (0.8)	2.140±0.287 (0.9)
+Verapamil (10)	2.361±0.087 (1.0)	2.639±0.061 (1.0)

[a]Cell survival was determined by the MTT assay as described in Materials and methods. [b]Data are means ± SD of at least 3 independent experiments performed in triplicate. [c]Fold-resistance was determined by dividing the IC_{50} value for paclitaxel, colchicine, vinblastine and cisplatin in KB-3-1 and KB-C2 cells by the IC_{50} value for paclitaxel, colchicine, vinblastine and cisplatin in KB-3-1 and KB-C2 cells in the absence or presence of β-elemene or verapamil. [d]$p<0.05$, [e]$p<0.01$, significantly different from IC_{50} of KB-C2 cells in the absence of the reversal drug.

Results

Effect of β-elemene on the sensitivity of ABCB1-, MRP1- and BCRP-overexpressing cells to antineoplastic drugs. In order to determine if β-elemene alone produces significant *in vitro* cytotoxicity, we examined its effect on the survival of KB-3-1 and KB-C2 cell lines using the MTT assay. The results indicated that up to a concentration of 100 μM, β-elemene did not significantly alter the survival rate of either KB-3-1 or KB-C2 cells (Fig. 1B). Based on these results, the highest concentration of β-elemene we used was 100 μM. Subsequently, we determined the effect of β-elemene at concentrations of 10, 30 or 100 μM on the response of KB-3-1 or KB-C2 cells to the antineoplastic drugs paclitaxel, vinblastine and colchicine. As shown in Table I, at a concentration of 10 μM, β-elemene significantly decreased the IC_{50} values for the ABCB1 substrates paclitaxel, vinblastine and colchicine in the ABCB1-overexpressing cell line KB-C2. However there was no significant change in the cytotoxicity of these drugs for parental cell line KB-3-1. The response to paclitaxel, vinblastine and colchicine in KB-C2 cells was 648-, 11.7- and 720-fold lower, respectively, than that for the parental KB-3-1 cell line (Table I). The incubation of KB-C2 cells with β-elemene significantly increased their sensitivity to paclitaxel, vinblastine and colchicine. For example, in the presence of 10 μM β-elemene, the response of KB-C2 to paclitaxel, vinblastine and colchicine was 52.2-, 3.4- and 113.3-fold lower, respectively (Table I). The magnitude of drug resistance in the presence of 30 μM of β-elemene to paclitaxel, vinblastine and colchicine was 16.8-, 2.1- and 28-fold lower, respectively, which was significantly lower than that for the 10 μM β-elemene concentration (Table I). The 100 μM concentration of β-elemene significantly lowered the magnitude of resistance to paclitaxel, vinblastine and colchicine (4.6-, 5.4- and 1.1-fold, respectively), when compared to the 10 and 30 μM concentrations (Table I). In fact, the 100 μM concentration of β-elemene reduced resistance to a level comparable to that for verapamil (5 μM), a known inhibitor of the ABCB1 transporter (Table I). β-elemene, at 100 μM, did not significantly alter the response of KB-C2 to cisplatin, a compound that is not a substrate for ABCB1.

We also determined the response of HEK293 cells overexpressing ABCB1 transporters to paclitaxel in the presence of β-elemene. β-elemene, at 100 μM, did not significantly alter the sensitivity of the empty vector-transfected cell line HEK293/pcDNA3.1 to paclitaxel (Table II). In contrast, 30 and 100 μM of β-elemene significantly increased the sensitivity of HEK293/ABCB1 cells to paclitaxel and the effect produced by 100 μM of β-elemene was almost identical to that produced by verapamil (10 μM) (Table II).

We further determined if β-elemene significantly reverses multidrug resistance in cell lines that overexpress BCRP or MRP1. In the parental NCI-H460 and the mitoxantrone-resistant cells NCI-H460/MX20, which overexpress BCRP, 100 μM of β-elemene did not significantly alter the sensi-

Table II. The effect of β-elemene and verapamil on the response of ABCB1-transfected cells to paclitaxel and cisplatin.[a]

Treatment	IC$_{50}$ ± SD[b] (μM)	
	HEK293/pcDNA3.1	HEK293/ABCB1
Paclitaxel (μM)	0.0222±0.0008 (1.0)[c]	2.5981±0.1583 (117.2)
+β-elemene (10)	0.0212±0.0003 (1.0)	0.2431±0.0721[e] (11.0)
+β-elemene (30)	0.0203±0.0039 (0.9)	0.0628±0.0838[e] (2.8)
+β-elemene (100)	0.0206±0.0021 (0.9)	0.0315±0.0040[e] (1.4)
+Verapamil (10)	0.0187±0.0020 (0.8)	0.0323±0.0030[e] (1.5)
Cisplatin (μM)	1.9972±0.1916 (1.0)[c]	1.9918±0.2714 (1.0)
+β-elemene (100)	1.9187±0.1446 (1.0)	1.8500±0.0687 (0.9)
+Verapamil (10)	1.8445±0.0662 (0.9)	1.8326±0.2062 (0.9)

[a]Cell survival was determined by the MTT assay as described in Materials and methods. [b]Data are means ± SD of at least 3 independent experiments performed in triplicate. [c]Fold-resistance was determined by dividing the IC$_{50}$ value for paclitaxel and cisplatin in HEK293/PcDNA3.1 cells by the IC$_{50}$ value for paclitaxel and cisplatin in HEK293/PcDNA3.1 and HEK293/ABCB1 cells in the absence or presence of β-elemene and verapamil. [d]$p<0.05$, [e]$p<0.01$, significantly different from IC$_{50}$ of HEK293/ABCB1 cells without reversal drug.

Table III. The reversal effect of β-elemene on HEK293/pcDNA3.1, HEK293/MRP1, NCI-H460 and NCI-H460/MX20 cells.[a]

Treatment	IC$_{50}$ ± SD[b] (μM)	
	NCI-H460	NCI-H460/MX20
Mitoxantrone (μM)	0.15±0.06 (1.0)[c]	18.8±1.30 (125.0)
+β-elemene (100)	0.10±0.01 (0.8)	9.20±1.40[e] (61.3)
+Nilotinib (5)	0.13±0.04 (0.9)	0.24±0.07[f] (1.6)
Treatment	IC$_{50}$ ± SD[b] (μM)	
	HEK293/pcDNA3.1	HEK293/MRP1
Vincristine (μM)	0.004±0.0003 (1.0)[d]	0.055±0.0120 (13.8)
+β-elemene (100)	0.004±0.0006 (1.0)	0.050±0.0009 (12.7)
+ONO-1078 (5)	0.0037±0.0006 (0.9)	0.006±0.0011[f] (1.6)

[a]Cell survival was determined by the MTT assay as described in Materials and methods. [b]Data are means ± SD of at least 3 independent experiments performed in triplicate. [c]Fold-resistance was the value of the IC$_{50}$ value for mitoxantrone of NCI-H460 cells divided by the IC$_{50}$ value for mitoxantrone of NCI-H460 and NCI-H460/MX20 cells in the absence or presence of β-elemene and nilotinib. [d]Fold-resistance was determined by dividing the IC$_{50}$ value for vincristine in HEK293/pcDNA3.1 cells by the IC$_{50}$ value for vincristine in HEK293/pcDNA3.1 and HEK293/MRP1 cells in the absence or presence of β-elemene and ONO-1076. [e]$p<0.05$, [f]$p<0.01$, significantly different from the IC$_{50}$ of resistant cells in the absence of the reversal drug.

tivity of the parental NCI-H460 cells (Table III). In contrast, 100 μM of β-elemene significantly enhanced the sensitivity of cells overexpressing the BCRP transporter to mitoxantrone, a known substrate of BCRP (Table III). In addition, β-elemene (100 μM) did not significantly alter the sensitivity of HEK293 cells overexpressing MRP1 (HEK293/MRP1) to the MRP1 substrate, vincristine (Table III).

Effect of β-elemene on the accumulation of [^{3}H]-paclitaxel in HEK293/ABCB1 cells. In order to understand the mechanism of β-elemene-induced sensitization of MDR cell lines to antineoplastic drugs, we examined the effect of β-elemene on the intracellular accumulation of [^{3}H]-paclitaxel in HEK293 cells overexpressing the ABCB1 transporter. Overall, in the absence of β-elemene, the accumulation of [^{3}H]-paclitaxel was 88% lower in the HEK293/ABCB1 cells when compared to the HEK293/pcDNA3.1 cells that do not overexpress ABCB1 (Fig. 2A). However, the incubation of HEK293/ABCB1 cells with β-elemene (100 μM) completely reversed the accumulation of [^{3}H]-paclitaxel to a level comparable to that of the parental cell line (Fig. 2A). These results suggested that β-elemene may potentiate the cytotoxicity of paclitaxel by increasing the intracellular accumulation of antineoplastic drugs.

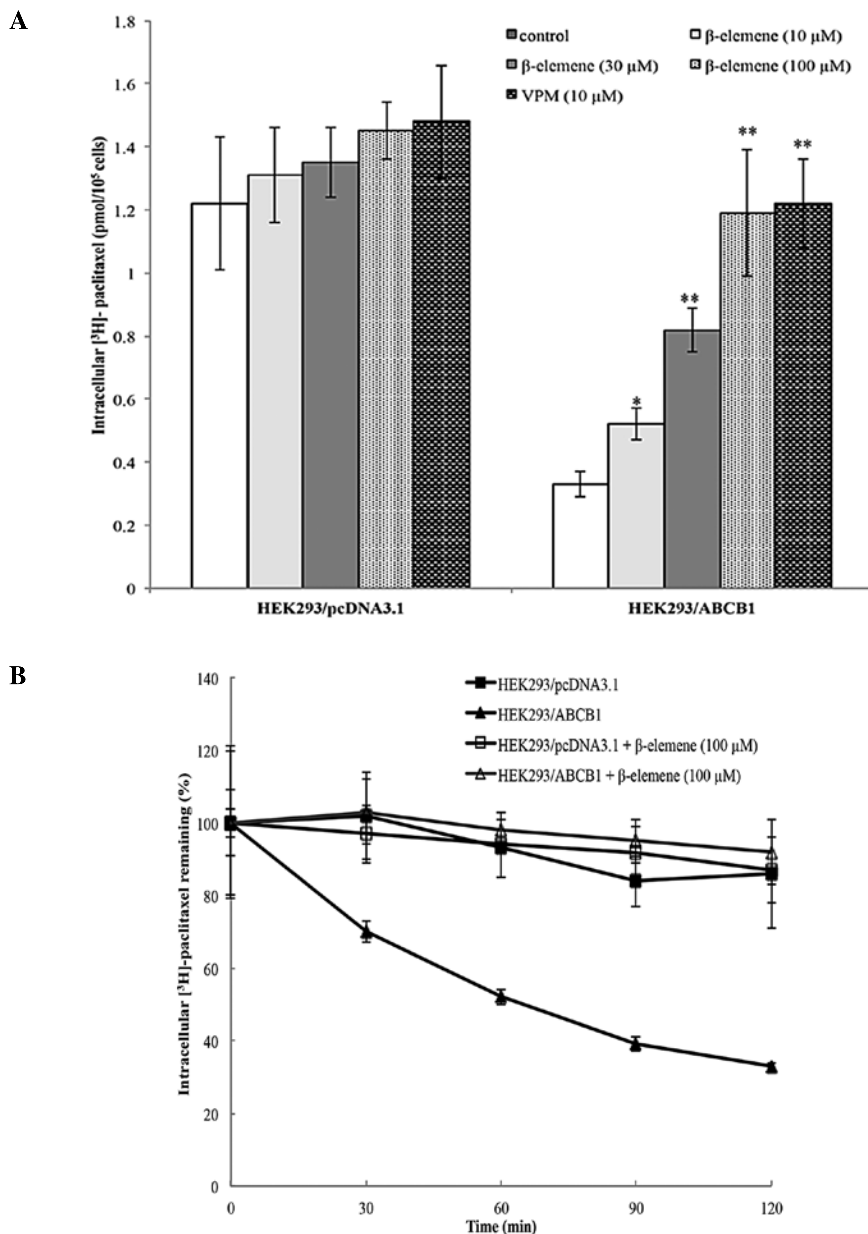

Figure 2. (A) Effect of β-elemene on the accumulation of [^{3}H]-paclitaxel. The intracellular [^{3}H]-paclitaxel accumulation in HEK293/pcDNA3.1 and HEK293/ABCB1 cells was measured after the incubation with 0.1 μM [^{3}H]-paclitaxel for 2 h at 37˚C. Each column represents the means (± SD). All experiments were performed in triplicate. $^*p< 0.05$, $^{**}p< 0.01$ significantly different from the intracellular [^{3}H]-paclitaxel accumulation of HEK293/ABCB1 cells in the absence of the reversal drug. (B) Effect of β-elemene on the efflux of [^{3}H]-paclitaxel. The percentage of the [^{3}H]-paclitaxel released was plotted as a function of time. After 1 h of incubation with β-elemene, HEK293p/cDNA3.1 cells were incubated with [^{3}H]-paclitaxel. Cells were washed and re-incubated in the [^{3}H]-paclitaxel-free medium. At the time points of 0, 30, 60, 120 min, cells were collected and the level of [^{3}H]-paclitaxel was determined by scintillation counting. Each point represents the means (± SD) of 3 separate experiments performed using triplicate samples.

Effect of β-elemene on the efflux of [^{3}H]-paclitaxel in HEK293/ABCB1 cells. In these experiments, we determined the effect of β-elemene on the efflux of [^{3}H]-paclitaxel in the parental HEK293/pcDNA3.1 and the ABCB1-overexpressing cell line HEK293/ABCB1. The efflux of [^{3}H]-paclitaxel from the ABCB1-overexpressing cells was significantly greater than that of the non-overexpressing parental cells (Fig. 2B). However, in the presence of 100 μM of β-elemene, the efflux of [^{3}H]-paclitaxel from the HEK293/ABCB1 cells was significantly reduced when compared to controls (Fig. 2B). These results indicated that β-elemene sensitized ABCB1-overexpressing cells to paclitaxel by blocking its efflux via the ABCB1 transporter.

Effect of β-elemene on the expression of ABCB1 and BCRP protein levels. It is possible that β-elemene increases the intracellular accumulation of [^{3}H]-paclitaxel by downregulating the expression of the ABCB1 protein as opposed to simply blocking efflux activity of ABCB1. To further delineate the mechanism of action of β-elemene, we examined its effect on the level of ABCB1 protein expression in KB-3-1 and KB-C2 cells. The incubation of the cell lines with 100 μM of β-elemene for either 24, 48 or 72 h did not significantly alter the expression level of the ABCB1 protein (Fig. 3A). Thus, it is unlikely that the β-elemene-induced increase in the intracellular levels of [^{3}H]-paclitaxel is due to the downregulation of expression of the ABCB1 transporter.

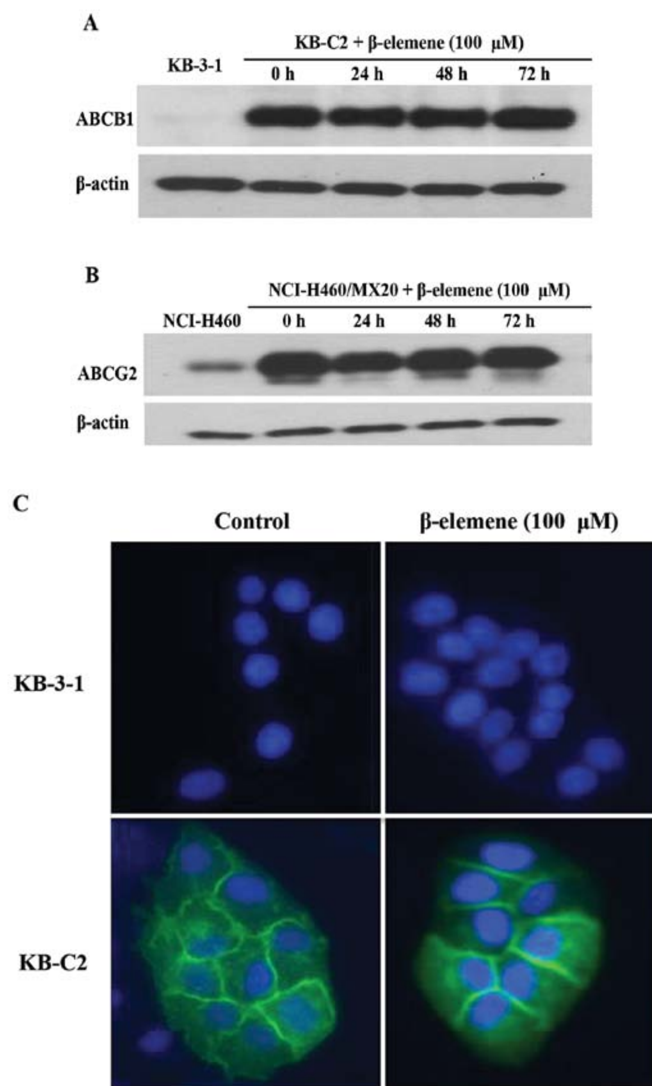

Figure 3. The effect of β-elemene on the expression of ABCB1 and BCRP proteins. (A) The effect of 100 μM of β-elemene on the protein expression of ABCB1 in KB-C2 cell line, at 0, 24, 48, 72 h. (B) The effect of 100 μM of β-elemene on the protein expression of BCRP in NCI-H460/MX20 cell line, at 0, 24, 48, 72 h. (C) Effect of β-elemene on the subcellular localization of ABCB1. KB-C2 and KB-3-1 cell lines were incubated with 100 μM of β-elemene for 72 h. The subcellular localization of ABCB1 was analyzed by immunofluorescence. ABCB1 staining is shown in green. DAPI (blue) counterstains the nuclei.

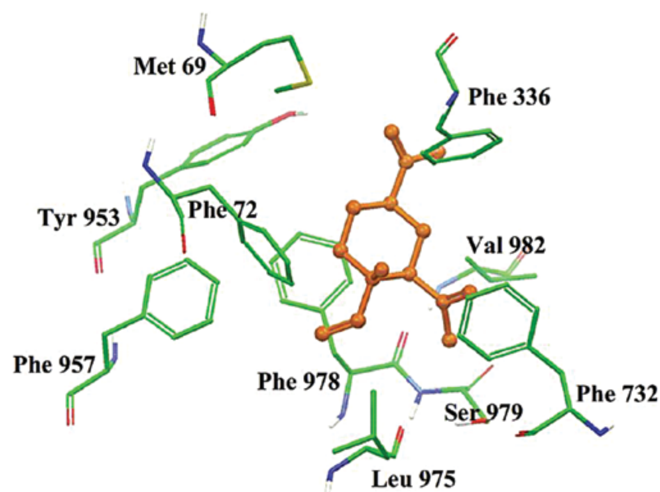

Figure 4. XP docking pose of β-elemene on human ABCB1. XP-Glide predicted binding mode of β-elemene with homology modeled ABCB1. The docked conformation of β-elemene as ball and stick model is shown within the large hydrophobic cavity of ABCB1. Important amino acids are depicted as sticks with the atoms colored as carbon, green; hydrogen, white; nitrogen, blue; oxygen, red; sulfur, yellow; whereas β-elemene is shown with the same color scheme as above except carbon atoms are represented in orange.

Effect of β-elemene on the subcellular localization of the ABCB1 transporter. In these experiments, we sought to ascertain if β-elemene altered the cellular localization of the ABCB1 transporter. The incubation of KB-3-1 and KB-C2 cells with 100 μM of β-elemene for 72 h did not alter the cellular distribution of the ABCB1 transporter when compared to cells exposed only to incubation solution (Fig. 3C).

Calculation of the interaction between β-elemene and the ABCB1 protein using docking analysis. The interaction between β-elemene and the ABCB1 transporter was determined using the docking analysis technique. A docking score was calculated for each of the 4 possible binding sites on the ABCB1 transporter for β-elemene. The docking score (site-1, -7.4 kcal/mol; site-2, -3.7 kcal/mol; site-3, -4.8 kcal/mol and site-4, -5.5 kcal/mol) clearly indicated that site-1 of ABCB1 is the best docking or binding site for β-elemene. Therefore, the XP-Glide predicted binding model for β-elemene at site-1 of ABCB1 is discussed below and shown in Fig. 4.

Since β-elemene contains only hydrocarbon atoms, the primary factor contributing to the binding of β-elemene to the ABCB1 protein is via hydrophobic interactions. The 3 carbon-carbon double bonds located at the C_4, C_2 and C_1 (atom nos. shown in Fig. 1A) side chains of β-elemene are stabilized by π-π interactions with the phenyl rings of Phe336, Phe732 and Phe978, respectively. The side chain at the C_1 atom is stabilized by Leu975, Phe978 and Ser979, whereas the side chain at the C_2 atom is stabilized by Phe732, Ser979 and Val982. The cyclohexyl ring of β-elemene is positioned in the large hydrophobic cavity formed by the side chains of Met69, Phe72, Tyr953, Phe957 and Val982.

Discussion

One of the major findings of the present *in vitro* study is that in cell lines overexpressing the ABCB1 transporter, β-elemene significantly increased the sensitivity to the ABCB1 substrates paclitaxel, colchicine and vinblastine. This effect was specific, as β-elemene did not significantly alter the response of cells to cisplatin, which is not a substrate for ABCB1. Furthermore, β-elemene did not significantly affect the sensitivity of the ABCB1-negative cell lines KB-3-1 and HEK/293pcDNA3.1.

In contrast to the result of a previous study (29), β-elemene produced a significant reversal of MDR by overexpression of the ABCB1 transporter (see Tables I and II). The discrepancy between the present study and that of Hu *et al* may be due to use of different cell lines (MCF-7/ADM and MCF vs. KB-C2 and HEK293 overexpressing ABCB1). In the present study, 100 μM of β-elemene significantly reduced the IC_{50} value of paclitaxel to 0.23 μM, a result that is comparable to that of the TKI lapatinib (IC_{50} of 0.18 μM), an established ABCB1 inhibitor (39). However, β-elemene moderately enhanced the

sensitivity or response to mitoxantrone in cell lines overexpressing the BCRP transporter (NCI-H460/MX20) and did not significantly reverse MRP1-mediated vincristine resistance. Therefore, β-elemene selectively increases the response of cell lines overexpressing the ABCB1 transporter to certain antineoplastic drugs.

Our results indicated that at 10 μM, β-elememe was less potent than vardenafil (a PDE5 and ABCB1 inhibitor) in decreasing the fold-resistance value of paclitaxel (12.4 vs. 54.1) and the colchicine (184 vs. 6.4) in KB-C2 cells overexpressing the ABCB1 transporter (40). However, in HEK293-transfected cells overexpressing the ABCB1 transporter, the fold-resistance value of paclitaxel for 10 μM of β-elememe (10.6) was greater than that for vardenafil (8.7) (40). In contrast, in KB-C2 cells, the fold-resistance for paclitaxel for 10 μM of tadalafil (a PDE5 inhibitor) was lower than that for 10 μM of β-elememe (4.3 vs. 12.4) and there was no difference in the fold-resistance value for colchicine between 10 μM of vardenafil (6.38) (42) and 10 μM of β-elememe (6.36).

Another finding of the present study was that 100 μM of β-elememe significantly enhanced (3.6-fold) the intracellular accumulation of [^{3}H]-paclitaxel in HEK293 cells transfected with the *ABCB1* gene (Fig. 2A). These findings are similar to those reported for HEK293/ABCB1 cells incubated with other ABCB1 inhibitors, such as PD173074, pargueren (5.6-fold increase) and vardenafil (3.5-fold increase) (40-42). Additional experiments indicated that the intracellular increase in [^{3}H]-paclitaxel levels is due to β-elemene's inhibition of the efflux function of the ABCB1 transporter.

Previously, it was reported that 30 μM of β-elemene significantly downregulated the levels of the ABCB1 protein *in vitro* in the MCF-7 cell line (43). In contrast, our results indicated that β-elemene (100 μM for 24, 48 and 72 h) did not significantly alter the level of ABCB1 protein expression. The exact explanation for this discrepancy is uncertain but it may be due, in part, to differences in the cell lines used in the present study (KB-C2 and HEK293/ABCB1) when compared to that of Xu *et al* (MCF-7/DOX) (43). In addition, the results of immunofluorescence assays indicated that the incubation of KB-3-1 and KB-C2 cells with 100 μM of β-elemene for 72 h did not significantly alter the cellular distribution of the ABCB1 transporter when compared to cells exposed only to the incubation solution (Fig. 3C). Overall, our results indicated that β-elemene significantly increased the sensitivity of the cell lines we used by inhibiting the efflux function of the ABCB1 transporter.

In order to characterize the binding interaction of β-elemene with ABCB1, docking study was carried out to describe the site on which β-elemene functioned. The docking simulations of β-elemene on ABCB1 were performed on a homology model of human ABCB1 due to the lack of the co-crystal structures of human ABCB1. To identify the predicted binding site of β-elemene, docking studies were performed at 4 possible binding sites of ABCB1 as described by Aller *et al* (37). The most favorable binding site is identified as site-1 by exploring docking scores of β-elemene at all of the binding sites. The drug-binding pocket located in transmembrane domains of human ABCB1 contains predominantly hydrophobic and aromatic residues as illustrated by Aller *et al* (37). Then, the relatively high affinity of the docking pose showed 3 terminal located double bonds of β-elemene forming π-π interactions with aromatic residues. These interactions may be critical in restricting the ligand at the correct position of binding cavity of ABCB1. Overall, these results suggest that β-elemene directly interacts with the substrate binding pocket of the ABCB1 transporter. However, further studies, using other approaches, must be conducted to confirm that β-elemene directly binds to the ABCB1 protein.

In conclusion, in the present *in vitro* study, β-elemene significantly increased the cytotoxicity of paclitaxel, colchicine and vinblastine in cell lines overexpressing the ABCB1 transporter. β-elemene's reversal of MDR is most likely due to its inhibition of the efflux activity of the ABCB1 transporter. The *in vivo* effect of β-elemene on the action of paclitaxel remains to be determined.

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