



Report

## Perillyl alcohol inhibits human breast cancer cell growth *in vitro* and *in vivo*

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### Summary

The effect of monoterpene perillyl alcohol (POH) on cell growth, cell cycle progression, and expression of cell cycle-regulatory proteins in estrogen receptor (ER)-positive (KPL-1 and MCF-7) and ER-negative (MKL-F and MDA-MB-231) human breast cancer cell lines was examined. POH inhibited cell proliferation in a dose-dependent manner in all cell lines tested. POH at a dose of 500  $\mu$ M had a cytostatic effect, in which growth inhibition was due to accumulation of cells in G1-phase. Cell cycle progression was preceded by a decrease in G1 cyclins (cyclin D1 and E), followed by an increase in p21<sup>Cip1/Waf1</sup> and a decrease in proliferating cell nuclear antigen level. Levels of p53 and cyclin A were unchanged. POH at a dose of 75 mg/kg administered intraperitoneally three times a week throughout the entire 6-week experimental period suppressed orthotopically transplanted KPL-1 tumor cell growth and regional lymph node metastasis in a nude mouse system. POH inhibited both ER-positive and -negative human breast cancer cell growth *in vitro*, and suppressed growth and metastasis *in vivo*.

### Introduction

Monoterpenes are non-nutritive dietary compounds found in the essential oils of many commonly consumed fruits and vegetables. They may function as chemoattractants or chemorepellents, as they are largely responsible for the pleasant fragrances of plants [1]. Limonene, the simplest monocyclic monoterpene, when administered in the diet at mass percentages of 1–5%, inhibits both 7,12-dimethylbenz[ $\alpha$ ]anthracene (DMBA)- and *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in rats [2–5]. Also, dietary limonene can cause regression of both DMBA- and MNU-induced rat mammary carcinomas [6]. However, when limonene is removed from the diet, tumor recurrence is observed [6]; limonene seems to act in a cytostatic fashion.

Perillyl alcohol (POH) is a hydroxylated product of *d*-limonene (*p*-mentha-1,8-diene), which is formed

by the condensation of two isoprene molecules. POH is found naturally in cherries, lavenders, mints, and celery seeds [7]. POH is 5–10 times more potent than limonene in causing regression of DMBA-induced mammary cancer [8]. POH is not only a potent breast cancer chemopreventive agent [9], but is also an effective chemotherapeutic agent against advanced mammary cancers [8]. Moreover, activity of POH is not organ-specific. POH suppresses 4-(methyl-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)-induced mouse lung tumorigenesis [10], inhibits the incidence of azoxymethane (AOM)-induced rat colon carcinogenesis [11], inhibits diethylnitrosamine (DEN)-induced rat liver tumors [12], and shows anti-tumor activity against hamster pancreatic carcinomas [13].

POH inhibits cell proliferation in a variety of cell lines *in vitro*, at a dose within a pharmacologically achievable range [14]. *In vitro* studies have indicated that growth of a murine mammary transformed

epithelial cell line and human breast cancer cell lines is inhibited by POH [15, 16]. Several possible mechanisms of action for the anti-tumor activities of POH have been examined. Putative mechanisms of action include the transforming growth factor  $\beta$  pathway [17] and/or inhibition of p21<sup>ras</sup> signaling [18]. Although the specific mechanism(s) responsible for the anti-cancer activity of POH are not fully known, POH has been found to affect several cell regulatory activities. Anti-proliferative effects of POH on human breast cancer cells have been studied, and growth inhibition induced by POH has been shown to be associated with accumulation of cells in G1-phase, preceded by a decline in cyclin D1 [16].

In light of these previous findings, in order to clarify the mechanisms of action of POH, the present study was designed to investigate the effects of POH on cell proliferation, cell cycle progression and expression of cell cycle regulatory proteins *in vitro*, in estrogen receptor (ER)-positive (KPL-1 and MCF-7) and ER-negative (MKL-F and MDA-MB-231) human breast cancer cell lines. POH has been shown to have a broad range of anti-tumorigenic properties in rodent mammary cancer models, but there have been no studies of its effects on human breast cancer cell growth *in vivo*. Therefore, in the present study, growth inhibitory and anti-metastatic effects of systemic intraperitoneal (ip) administration of POH on orthotopically transplanted KPL-1 human breast cancer cells in nude mice was evaluated. POH exhibited growth inhibitory effects *in vitro* associated with changes in cell cycle-related proteins (decreased cyclin D1 and cyclin E, followed by increased p21<sup>Cip1/Waf1</sup> and decreased proliferating cell nuclear antigen [PCNA] expression), and suppressed KPL-1 tumor cell growth and metastasis in a nude mouse system.

## Materials and methods

### Cell lines and culture conditions

KPL-1 [19] and MCF-7 [20] are human breast cancer cell lines established from malignant pleural effusion of breast cancer patients. They both express ER and progesterone receptor. MKL-F [21] is a transfectant of MCF-7, and is independent of ER. MDA-MB-231 [22] is a human breast cancer cell line established from pleural effusion of a breast cancer patient who had received combined chemotherapy, and is hormone-independent. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM;

Sigma, St Louis, MO) with 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY) in 5% CO<sub>2</sub>/95% humidified air at 37°C.

### Reagents

(+)-POH was purchased from Fluka (Buchs, Switzerland). The purity was ~99% by gas chromatography. Polyclonal antibody against cyclin E (M-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal antibodies against cyclin D1 (P2D11F11), cyclin A (6E6) and PCNA (PC10) were obtained from Novocastra (Newcastle, Upon Tyne, UK). p53 (DO7) was obtained from DAKO (Glostrup, Denmark), p21<sup>Cip1/Waf1</sup> (SX-118) was obtained from PharMingen (San Diego, CA), and Ki-67 (MIB-1) was obtained from Immunotech (Marseille, France). Tricaprylin (1,2,3-trioctanoylglycerol) was purchased from Sigma.

### Cell growth experiments *in vitro*

The effect of POH on growth of human breast cancer cell lines was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [23]. Cells were seeded in 96-well and/or 24-well plates at a density of  $2 \times 10^3$ /well, and allowed to sit overnight to adhere to the bottom of the plates. Next, the culture medium was replaced with the experimental medium containing 0–1000  $\mu$ M POH dissolved in ethanol, followed by incubation under the same conditions. The final concentration of ethanol in the culture medium (<0.1%) had no anti-proliferative effect on any of the cell lines. Following culturing with POH in a 96-well plate for 24, 48 or 72 h, and in a 24-well plate for 0, 3, 5 or 7 days, MTT was added, and the plates were analyzed by ImmunoMini NJ-2300 (Nalge Nunc Int., Rochester, NY). In the 24-well plate assay, the experimental medium was changed after culturing for 3 days. In the 96-well plate assay, each data point represents the mean of eight wells, and the percentage of live cells cultured with POH to untreated controls (medium alone) was calculated. In the 24-well plate assay, cells were treated with 0, 500 or 1000  $\mu$ M POH for up to 7 days. Each data point represents the mean of viable cell numbers from four wells.

### Cell cycle analysis

Flow cytometry was used to measure the DNA content of individual cells, with or without POH treatment.

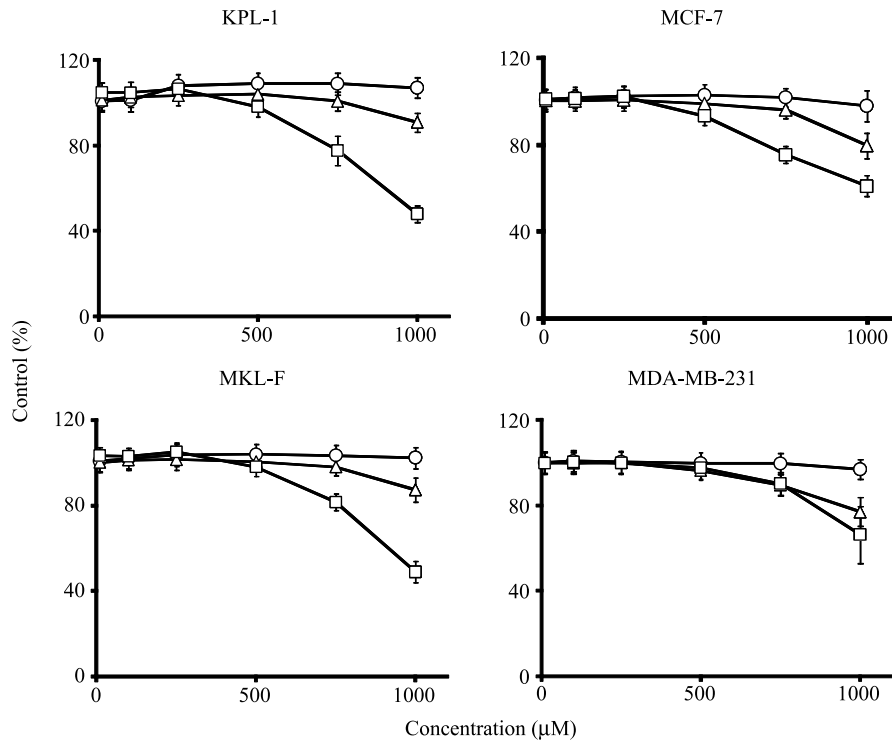


Figure 1. Colorimetric assay. Dose- and time-dependent inhibition of human breast cancer cells by POH. Each cell line was cultured with 0–1000  $\mu\text{M}$  POH for 24 (○), 48 (△) or 72 h (□). The data are mean  $\pm$  SE of eight wells.

Each cell line was starved (treated with FCS-free medium) for 24 h for synchronization of cell cycle, and was then either exposed to 500  $\mu\text{M}$  POH or left untreated, during culturing in DMEM supplemented with 10% FCS for 0, 7, 24 or 72 h. Cells were then trypsinized, and floating cells were washed in cold phosphate-buffered saline (PBS) (–), centrifuged and fixed with 70% ethanol. The samples were then treated with RNase, diluted with PBS (–), stained with 50  $\mu\text{g}/\text{ml}$  propidium iodide, and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Cell cycle distribution was quantified using ModFit LT software (Verity Software House, Topsham, ME). Rate of G<sub>0</sub>/G<sub>1</sub>-phase was compared between cells cultured for 72 h with 500  $\mu\text{M}$  POH and cells cultured with medium alone. In all cases, proper gating was used to exclude doublets and other cell aggregates for analysis.

#### Western blot analysis

Synchronized cells were exposed to 500  $\mu\text{M}$  POH for 3 or 24 h. After washing with cold PBS (–), cells were trypsinized and pelleted by centrifugation. Cell pellets were homogenized by lysis buf-

fer (50 mM Tris-HCl [pH 6.8], 2% SDS, 5 mM  $\beta$ -mercaptoethanol, 10% glycerol). Cell lysates were clarified by centrifugation at  $13,500 \times g$  for 40 min. Protein concentrations were measured by Bio-Rad assay (Bio-Rad, Richmond, CA), and 50  $\mu\text{g}$  of protein from each sample was mixed with loading buffer, electrophoresed on 12.5 or 15% SDS-PAGE gel, and electroblotted onto transfer membrane (GeneScreen Plus, PerkinElmer Life Sciences, Boston, MA). The membranes were blocked with 5% dry milk in Tris buffered saline-Tween (TBST) for 1 h at room temperature, and incubated with each primary antibody (p53, p21<sup>Cip1/Waf1</sup>, cyclin D1, cyclin E, cyclin A and PCNA) at 4°C overnight, followed by a secondary antibody (DAKO Envision Peroxidase, mouse or rabbit, Carpinteria, CA). Protein bands were visualized using an ECL chemiluminescent system and hyperfilm (Amersham Biosciences, Buckinghamshire, UK). Intensity of protein bands was quantified using Scion Image software (Scion Corporation, Frederick, ML).

#### KPL-1 tumor growth in vivo

Thirty 4-week-old female BALB/c nude mice purchased from Clea Japan (Osaka, Japan) were used as

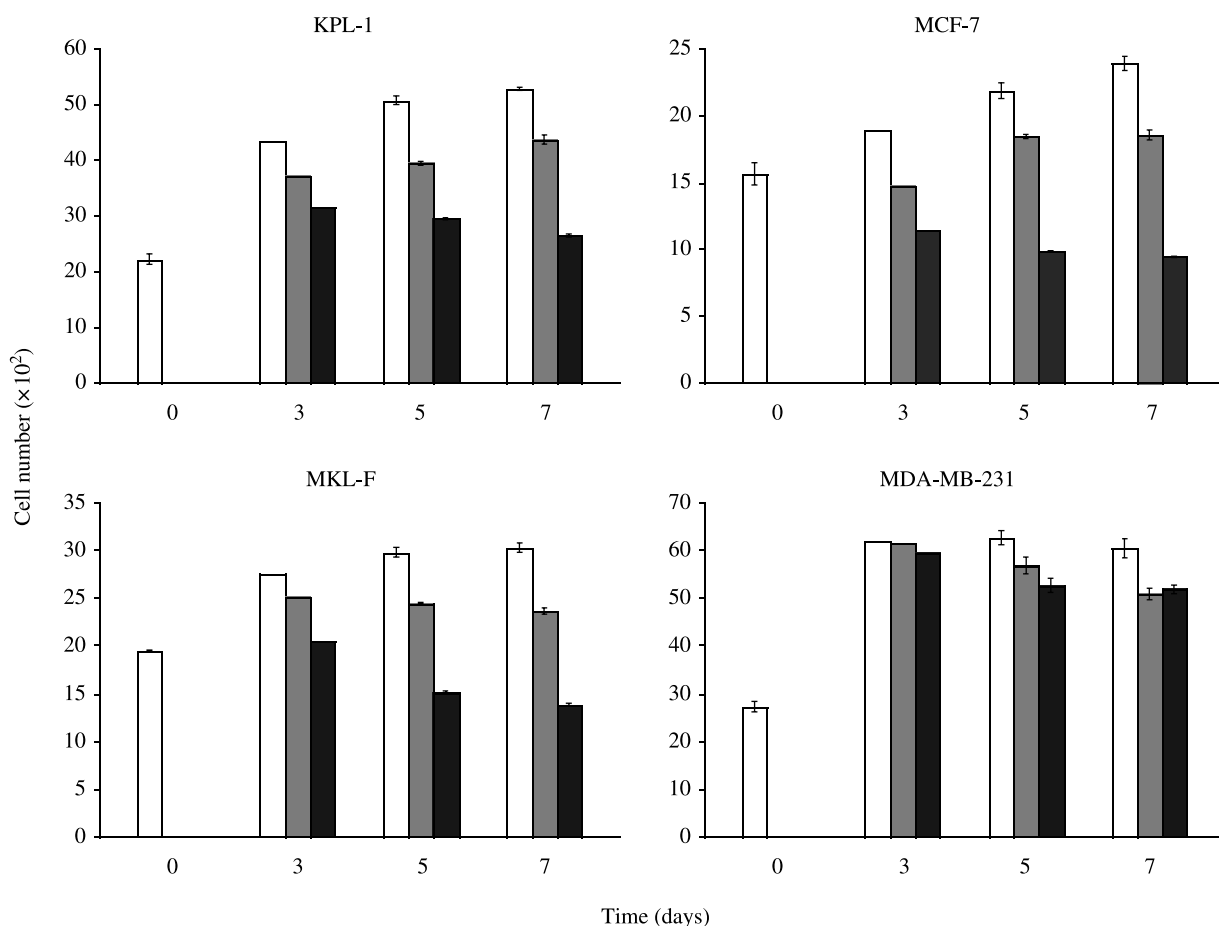


Figure 2. Colorimetric assay. Open bar, medium alone (control); gray bar, treated with 500 μM POH; black bar, treated with 1000 μM POH in DMEM for 3, 5 or 7 days. Each bar represents the mean ± SE of four wells.

host animals. During the experiment, the animals were housed five per cage in plastic cages with sterilized white pine chips as bedding. The animal room was kept specific pathogen-free, and was controlled for temperature ( $22 \pm 2^\circ\text{C}$ ), light (12 h light/dark cycle) and humidity ( $60 \pm 10\%$ ). Following 2 weeks acclimatization (i.e., at 6 weeks of age),  $2 \times 10^7$  viable KPL-1 cells/0.25 ml DMEM supplemented with 10% FCS were injected into the right thoracic mammary fat pad with a 26-gauge needle. Mice were randomly divided into two groups: POH-treated ( $n = 15$ ), and untreated ( $n = 15$ ). Immediately after tumor cell inoculation, the POH-treated group received an intraperitoneal injection of freshly prepared 75 mg/kg POH dissolved in tricaprilyn. The POH was dissolved in tricaprilyn immediately before injection, and 0.1 ml of the solution was injected. After this initial injection, POH was injected three times per week until

the termination of the experiment. During the experiment, the mice had free access to laboratory chow (CMF; Oriental Yeast, Chiba, Japan) and water. The mice were weighed, and locally growing tumors were checked once a week until the termination of the experiment. Tumor volume was calculated using the standard formula:  $\text{width}^2 \times \text{length} \times 0.5$  [24]. The experiment was terminated 6 weeks after inoculation of KPL-1 tumor cells. At the termination of the experiment, all mice were weighed and then sacrificed by cervical dislocation. At autopsy, the locally growing tumors were weighed and all organs were examined macroscopically. The primary tumors and regional axillary lymph nodes (KPL-1 cells occasionally undergo regional lymph node metastasis [19]) were fixed in 10% neutral buffered formalin and stained with hematoxylin–eosin (H–E) for histological examination. All procedures were approved by the Animal

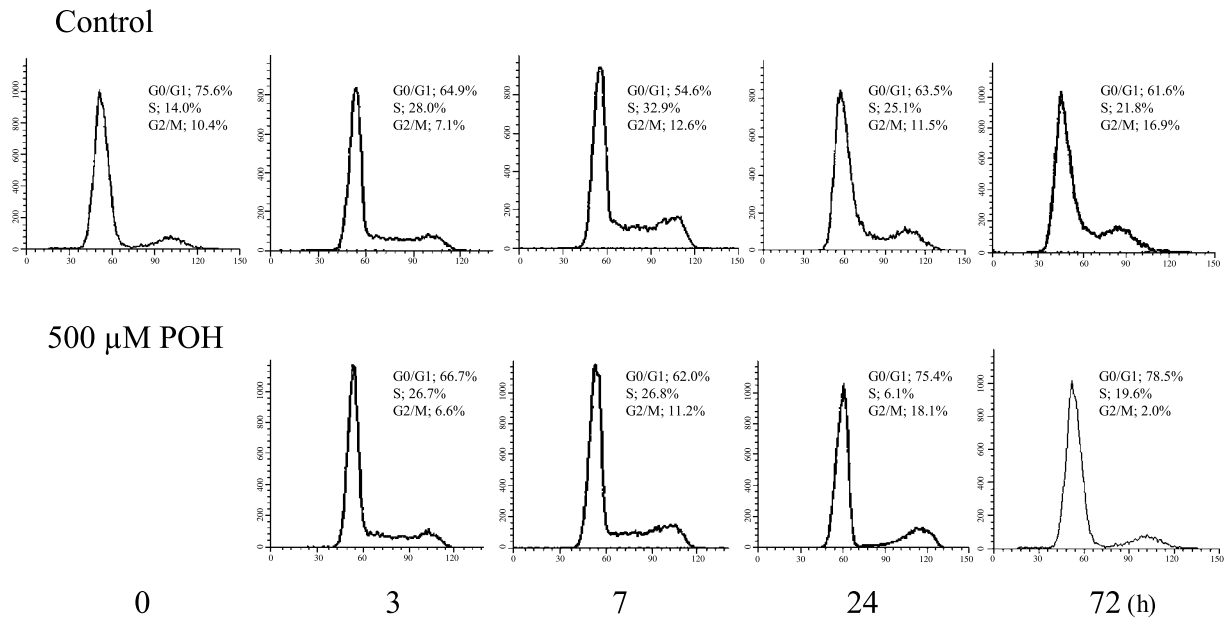


Figure 3. Flow cytometry. Changes in cell cycle fraction of synchronized KPL-1 cells cultured with medium alone (control) or with 500  $\mu$ M POH in DMEM for 3, 7, 24 or 72 h.

Experimentation Committee, Kansai Medical University.

#### *Ki-67 immunohistochemistry and TUNEL staining*

Tumor growth is a balance between cell proliferation and cell death. Accordingly, cell proliferation and cell death were evaluated by Ki-67 labeling and TUNEL index, respectively. First, 4- $\mu$ m-thick formalin-fixed, paraffin-embedded sections from primary tumors in nude mice were deparaffinized and washed in distilled water, and antigen was retrieved by microwave heating for 5 min in a high pressure cooker. Then, Ki-67 immunohistochemistry with anti-Ki-67 antibody (MIB-1) was performed by DAKO autostaining using a DAKO LSAB II-Kit according to the manufacturer's instructions. Nuclear antigens were visualized using 3, 3'-diaminobenzidine-4HCl (DAB) (Wako, Pure Chemical, Osaka, Japan), and were counterstained with hematoxylin. The average percentage of Ki-67-positive cells was determined for the sections, which contained approximately 1000 cells each. To detect apoptotic cells, we performed terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end-labeling (TUNEL) reaction using the ApopTag Kit (Intergen, Purchase,

NY) [25]. Deparaffinized slides were washed with PBS, and pretreated with 20  $\mu$ g/ml proteinase K for 15 min. Equilibration buffer was applied after quenching of endogenous peroxidase with 2% hydrogen peroxide, and terminal deoxynucleotidyl transferase (TdT) reaction was performed for 60 min at 37°C in a moist chamber. Sections were treated with anti-digoxigenin-peroxidase for 30 min after the TdT reaction was suspended by stop/wash buffer. TdT-labeled cells were detected by DAB, and sections were counterstained with hematoxylin. Apoptotic index represents the average percentage of TUNEL-positive cells per section; each section contained approximately 1000 cells. To detect apoptotic cells after culturing with 500  $\mu$ M POH for 72 h, TUNEL staining was performed after cells were fixed in neutral buffered formalin for 10 min.

#### *Data analysis*

All results are expressed as the mean  $\pm$  standard error (SE). In all statistical analyses, the significance of differences was determined using the unpaired, two group *t*-test or Mann-Whitney's *U*-test, after assuring homogeneity of variance. In all analyses, differences with probability values <0.05 were considered significant.

## Results

### *Anti-proliferative effect of POH on human breast cancer cell lines in culture*

Actively proliferating human breast cancer cell lines were cultured with 0–1000  $\mu\text{M}$  POH, and viable cells were quantified after 24, 48 or 72 h of culture. As shown in Figure 1, proliferation of each cancer cell line was inhibited by culture with  $\geq 500$   $\mu\text{M}$  POH for 48 or 72 h, in a dose- and time-dependent manner. No inhibition was observed at 24 h of culture or at POH concentrations  $< 500$   $\mu\text{M}$ . For up to 7 days, POH at a dose of 500 or 1000  $\mu\text{M}$  inhibited cell proliferation in a dose-dependent manner (Figure 2). Among the four cell lines tested, MDA-MB-231 cells showed relatively rapid growth; that is, their growth was less inhibited by POH. In all cell lines, 500  $\mu\text{M}$  POH did not induce a decrease in viable cell number. Also, TUNEL-positive signals were not detected in cells cultured with 500  $\mu\text{M}$  POH for 72 h (data not shown). Thus, the growth inhibitory effect of 500  $\mu\text{M}$  POH was due to cytostatic effect.

### *Effect of POH on cell cycle progression*

To determine if inhibition of cell growth by POH is due to specific cell cycle arrest, breast cancer cell lines, untreated or treated with 500  $\mu\text{M}$  POH, were analyzed by flow cytometry. Cell cycle was synchronized by starvation for 24 h, followed by re-feeding. Cell cycle fraction was recorded after 3, 7, 24 or 72 h of treatment with 500  $\mu\text{M}$  POH, and was compared with POH-untreated cells. All four cell lines that were tested showed similar results. Representative data (for KPL-1) are shown in Figure 3. In POH-untreated cells, after 24 h starvation, 75.6% of cells were in G0/G1 and 14.0% cells in S-phase. At 3 h after re-feeding, percentage of cells in G0/G1 decreased to 64.9%, and percentage of cells in S-phase increased to 28.0%. After 7 h, percentage of G0/G1-phase cells further decreased to 54.6%, and percentage of S-phase cells increased to 32.9%. At 24 and 72 h, percentage of G0/G1 cells was 61.6 and 63.5%, and percentage of S-phase cells was 21.8 and 25.1%, respectively. In cells treated with 500  $\mu\text{M}$  POH, percentage of G0/G1-phase cells gradually increased, with 75.4 and 78.5% of cells remaining in the G0/G1-phase at 24 and 72 h, respectively, whereas percentage of cells in S- and/or G2/M-phase decreased. A transient increase in G2/M-phase was seen, but subG1 fraction was not detected.

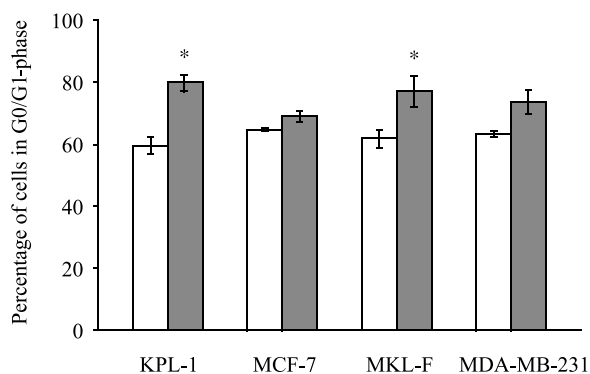


Figure 4. Changes in percentage of G0/G1-phase in breast cancer cell lines cultured with medium alone (control; open bar) or treated with 500  $\mu\text{M}$  POH for 72 h (black bar). \* $p < 0.05$ .

Treatment with 500  $\mu\text{M}$  POH for 72 h caused accumulation of cells in G1-phase in all cell lines tested (Figure 4). Thus, POH seems to cause G1 arrest in these human breast cancer cell lines.

### *Effect of POH on cell cycle regulators*

To assess effects of POH on cell cycle regulatory proteins, western blot analysis of total proteins was performed for hormone-dependent (KPL-1) and -independent (MKL-F) human breast cancer cells, with or without treatment with 500  $\mu\text{M}$  POH. Cell cycle regulatory proteins including p53, p21<sup>Cip1/Waf1</sup>, cyclin D1, cyclin E, cyclin A and PCNA were examined. Representative western blotting results for KPL-1 cells are shown in Figure 5, and KPL-1 and MKL-F data

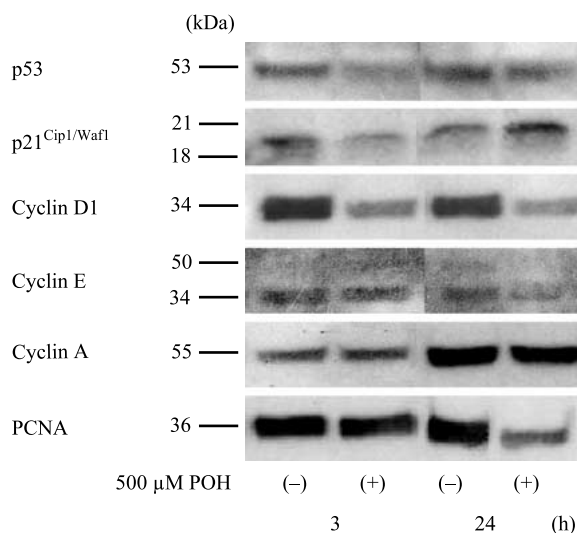


Figure 5. Expression of G0/G1 cell cycle-related proteins in synchronized KPL-1 cells cultured with medium alone or treated with 500  $\mu\text{M}$  POH for 3 or 24 h.

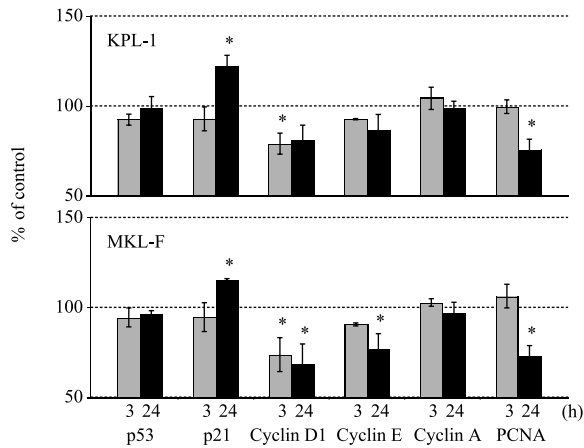


Figure 6. Quantification of G0/G1 cell cycle-related protein expression, with controls designated as 100%. All results were calculated from three independent samples. \*  $p < 0.05$ , compared with controls.

from three independent experiments are summarized in Figure 6. In both cell lines, 500  $\mu\text{M}$  POH caused no change in p53 level, increased p21<sup>Cip1/Waf1</sup> protein levels, and decreased cyclin D1 and cyclin E, compared with POH-untreated cells. Cyclin A was unchanged, and PCNA levels were decreased. In the time course experiment, with 500  $\mu\text{M}$  POH treatment, cyclin D1 and cyclin E were found to be down-regulated at 3 h, and up-regulation of p21<sup>Cip1/Waf1</sup> and down-regulation of PCNA were observed at 24 h. However, p53 and cyclin A levels were not altered at these time points.

*Effect of POH on KPL-1 cell growth in nude mouse*

Two animals in the POH-treated group died after POH injection, due to intraabdominal hemorrhage. Death was determined to be due to procedural error at the time of injection. These two mice were excluded from calculations. POH caused no observable toxicity in the mice. Although average body weight of the POH-treated mice was lower than that of the controls throughout the experiment, all mice gained weight, and the difference in body weight between control and POH-treated mice was not statistically significant (Figure 7). Locally growing tumor volume was always smaller in the POH-treated group (Figure 8). There were significant differences in average tumor volume and average tumor weight at the termination of the experiment between POH-treated and -untreated

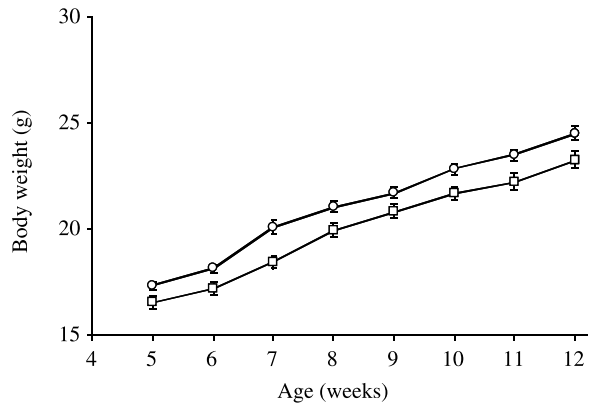


Figure 7. Effects of POH on body weight change in female BALB/c nude mice (○, control; □, 75 mg/kg POH  $\times$  3/week).

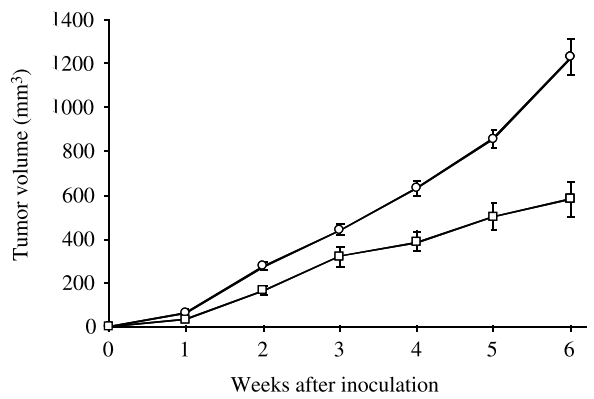


Figure 8. Tumor growth of KPL-1 cells injected into the right thoracic mammary fat pad of female BALB/c nude mice at 6 weeks of age (○, control; □, 75 mg/kg POH  $\times$  3/week).

mice ( $p < 0.05$ , respectively); there was a 36% reduction in tumor weight in the POH-treated group (Table 1). Moreover, axillary lymph node metastasis was seen in 3 (20%) of the 15 mice in the POH-untreated group, whereas no metastasis was seen in the POH-treated group (0/13). Cell kinetics data are shown in Figure 9. Cell proliferation, as indicated by Ki-67 labeling, was significantly lower in the POH-treated group ( $p < 0.05$ ). Cell death, as indicated by TUNEL index, tended to decrease after POH treatment, but this decrease was not statistically significant. POH treatment did not cause abnormality in major organs (data not shown). *In vivo* results indicate that POH suppressed KPL-1 tumor cell growth and metastasis when tumor cell inoculation and POH injection were begun concurrently.

Table 1. Effects of POH on KPL-1 cell growth and metastasis after transplanted to female BALB/c nude mice

POH treatment	Effective no. of mice	Tumor volume (mm <sup>3</sup> )	Tumor weight (mg)	Axillary lymph node metastasis (%)
None	15	1230 ± 83	898 ± 52	3/15 (20)
75 mg × 3/week	13	582 ± 79*	575 ± 77*	0/13 (0)

Values are mean ± SE; \**p*-value < 0.05 compared with control.

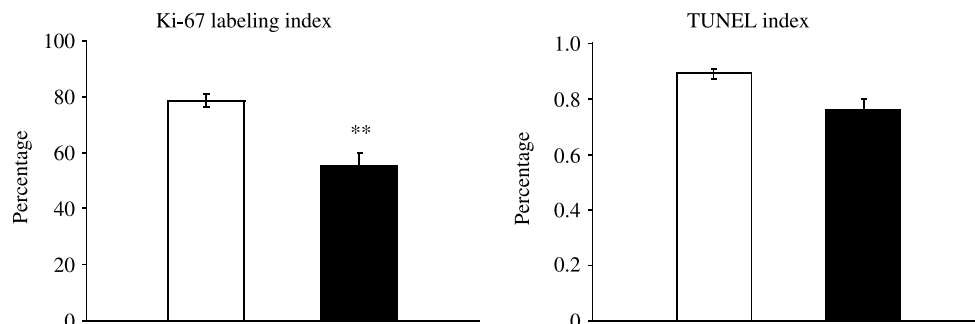


Figure 9. Changes in cell proliferation (Ki-67 labeling) and cell death (TUNEL index) of KPL-1 cells grown in nude mice with (black bar) or without (open bar) POH treatment. Values are mean ± SE; \*\**p*-value < 0.01, compared with POH-untreated control.

## Discussion

In human breast cancer cells in culture, POH is a more potent cell proliferation inhibitor than limonene [16]. In the present study, POH directly inhibited proliferation of cultured human breast cancer cell lines in a dose- and time-dependent manner. In a previous study, treatment of cultured human pancreatic carcinoma cells with 800 μM POH significantly increased the percentage of cells undergoing apoptosis [13]. In the present study, after exposure to 500 μM POH for 72 h, evidence of apoptosis was not detected by flow cytometry or TUNEL staining. Thus, the effect of POH at this dose on human breast cancer cells is cytostatic. Although the concentration of POH (500 μM) used in the present experiment was fairly high, it is well within the range of serum monoterpene levels that can be reached in rats, mice and humans [8, 14, 26]. To elucidate the growth inhibitory mechanism of POH on human breast cancer cells, we assessed the effect of POH on cell cycle progression. As in previous studies [15, 16], POH induced an increase in the G0/G1 fraction of the cell cycle, suggesting G1 arrest.

Passage through the cell cycle is determined by the function of cyclin/cyclin-dependent kinase (CDK) complexes [27, 28]. p21<sup>Cip1/Waf1</sup>, a Cip/Kip family member, act as a broad specific inhibitor of cyclin D, E and A [29]. G1-phase progression is mediated by the combined activity of cyclin D1/CDK 4 and 6, and cyclin E/CDK 2 complexes [27]. Up-regulation

of p21<sup>Cip1/Waf1</sup> inhibits cell cycle via inhibition of cyclin/CDK complexes, or via inhibition of PCNA function resulting in G1 arrest [30]. Changes in cell cycle distribution are related to alterations of expression of cell cycle-related proteins. In the present study, POH caused down-modulation of cyclin D1 and cyclin E, followed by up-regulation of p21<sup>Cip1/Waf1</sup> and down-regulation of PCNA, in both ER-positive and -negative human breast cancer cell lines. p53 level was not altered by POH treatment. Up-regulation of p21<sup>Cip1/Waf1</sup> by POH is independent of function of p53 protein [15]. Cyclin A functions in late S- to G2/M-phase [31], which may explain the lack of alteration of cyclin A level. It has previously been reported that POH inhibits cyclin D1 and cyclin E and increases p21<sup>Cip1/Waf1</sup> in a murine transformed mammary epithelial cell line [15] and a human colon cancer cell line [32]. These molecular changes preceded effects on cell cycle progression and cell growth.

In a previous study, rats with DMBA-induced mammary carcinoma fed a 1%-POH diet (880 mg/kg/day) had a tumor regression rate of 55% [5]. Topical application of POH delays the appearance of DMBA-induced melanoma in mice, and reduces its incidence [33]. Intraperitoneal administration of POH has significant *in vivo* chemopreventive activity against mouse lung tumorigenesis [10]. Apparently, all routes other than the oral route are effective for delivery of POH to target tissue. The anti-tumor effect of monoterpenes is attained with little or no host tox-

icity; in a previous study, differences in body weight of tumor-bearing animals between POH-treated and paired controls were not significant [13]. In the present study, intraperitoneal administration of 75 mg/kg POH significantly suppressed growth of primary tumor and metastasis, in KPL-1 human breast cancer cells. Several mechanisms of action *in vivo* may be involved in the chemopreventive activities of POH. In a liver carcinogenesis model, the suppressive activity of POH was associated with a marked increase in tumor cell apoptosis, with no effect on tumor cell proliferation [12]. In the present study, marked decrease in tumor proliferation was seen. Mechanisms of the effects of POH may differ among tumor types or doses. The present cell culture and *in vivo* data indicate that POH has anti-tumor activity against human breast carcinomas.

In conclusion, POH can affect growth of human breast cancer cells in culture, with correlation between the anti-proliferative action of POH and accumulation of cells in the G1-phase. The molecular factors involved were reduced cyclin D1 and cyclin E expression, followed by increased p21<sup>Cip1/Waf1</sup> and decreased PCNA expression; p53 and cyclin A levels were unchanged. POH also exhibited anti-proliferative and anti-metastatic activity in the present nude mice system.

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