



Perillyl alcohol and perillaldehyde induced cell cycle arrest and cell death in BroTo and A549 cells cultured in vitro

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Abstract

The role of the monoterpenes, especially limonene and perillyl alcohol, in the treatment of certain cancers is currently being evaluated in clinical trials. In this study, the effects of perillyl alcohol (POH) and its analog, perillaldehyde (PALD), on human carcinoma cell lines (BroTo and A549) cultured in vitro were investigated using proliferation assays (MTT and colony formation) and DNA content analysis by flow cytometry. POH and PALD elicited dose- and time-dependent inhibition of proliferation in both cell lines. Concentrations of POH and PALD that inhibited cell proliferation by 50% (IC₅₀) in 24 hr were 1 and 3 mM, respectively. DNA content analysis revealed that 1 mM of either POH or PALD caused cell cycle arrest in the G₁ phase in both cell lines while POH alone caused increased hypodiploid and annexin V-positive populations in both BroTo and A549 cells. POH induced apoptosis and was more effective than PALD at inhibiting the proliferation of BroTo and A549 cells cultured in vitro.

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Introduction

Monoterpenes are naturally occurring plant compounds that are synthesized via the mevalonate pathway. Physiologically, they function as chemoattractants or chemorepellents (McGarvey and Croteau,

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1995) and are responsible for the distinctive fragrance of many plants. The parent monoterpene compound, d-limonene, is formed by the cyclization of geranylpyrophosphate and serves as precursor to a host of other oxygenated monoterpenes including perillyl alcohol, perillaldehyde, carveol, carvone, menthol and others (McGarvey and Croteau, 1995; Karp et al., 1990). Many of the monoterpenes are components of plants such as citrus (especially in the peel oils), caraway, dill, cherry, spearmint, lemongrass and herbal teas, most of which are found in or used for human foods.

The anti-carcinogenic actions of limonene and perillyl alcohol in several animal tumor models such as breast, liver, colon, and prostate have been reported (Elegbede et al., 1986; Haag and Gould, 1994; Mills et al., 1995; Kelloff et al., 1996). In vitro, perillyl alcohol induced cell cycle arrest and apoptosis (Mills et al., 1995), inhibited the isoprenylation of small G proteins (21–26 kDa) involved in signal transduction (Crowell et al., 1994), and affected differential gene regulation (Jirtle et al., 1993). Both limonene and perillyl alcohol are being evaluated in human clinical trials (Ripple et al., 2000; Vigushin et al., 1998). To our knowledge, there has been no prior study of the effects of perillyl alcohol and perillaldehyde on either human head and neck squamous cell carcinoma or lung adenocarcinoma cell lines. In this study, we investigated the effects of perillyl alcohol (POH) and perillaldehyde (PALD) on human squamous cell carcinoma of the tongue (BroTo) and human lung adenocarcinoma (A549) cell lines cultured in vitro.

Materials and methods

Chemicals and reagents

POH and PALD were obtained from Aldrich Chemical Co. (Milwaukee, WI). Propidium iodide (PI) and ribonuclease A (RNase A) were purchased from Sigma (St. Louis, MO). Hams F12-K and RPMI media, phosphate buffered saline (PBS, lacking Ca^{2+} and Mg^{2+}), penicillin (100 units/ml) and 100 $\mu\text{g}/\text{ml}$ streptomycin (P/S) were obtained from Invitrogen Corporation (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT).

Cells and cell culture

Human lung adenocarcinoma cell line A549 was obtained from ATCC (Manassas, VA) while human tongue squamous cell carcinoma cell line, BroTo, was a gift from J. Truelson, MD, (Department of Otolaryngology, University of Texas-Southwestern Medical Center) and has been characterized as reported earlier (Brown et al., 2000). A549 and BroTo cells were cultured, respectively, in Hams F12-K and RPMI media supplemented with 10% FBS and 1% P/S. Cells were dislodged with trypsin-EDTA, counted, and adequate numbers plated overnight in tissue culture plates to adhere. POH and PALD were dissolved in medium, with shaking in a 37 °C water bath for at least 2 hours before use. Asynchronously growing cells were 70–80% confluent when treated. The culture medium was replaced with either medium only (Control) or medium containing the desired concentration of POH or PALD. Cells were incubated at 37 °C in humidified, 5% CO_2 atmosphere. At designated time points, cells were harvested and analyzed as described below.

Mitochondrial dehydrogenase activity

The effect of POH or PALD on cell proliferation was studied using the MTT protocol, an assay based on the reduction of the yellow dye, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT), to purple formazan crystals by mitochondrial dehydrogenase activity (Mosmann, 1983). The crystals were dissolved and the optical density of the solution was measured at 570 nm against a reference at 630 nm using SpectraCount microplate reader (Packard Instruments, Meriden, CT). Enzyme activity in treatment group was calculated relative (%) to the Control activity.

Colony formation assay

The toxicity of the compounds on treated cells was assessed using colony formation assay. Cells (200 cells/well) were plated overnight in 6-well plates. The culture medium was replaced with medium containing varying concentrations of POH or PALD and the cells were exposed to the agents for 12 or 24 hr. Following the exposure, treatment medium was removed and the cells washed twice with sterile PBS and once with the appropriate medium. Fresh medium was then added to each well and the cells incubated for 12–14 days at 37 °C. Plates were viewed under the microscope every other day. At termination, the culture medium was decanted and the cells rinsed with PBS. The cells were then fixed and stained with crystal violet (0.5% in 95% ethanol) for 5 min and the dye gently rinsed off. Colonies, containing at least 50 cells, were counted.

Cell cycle analysis

DNA contents of Control and treated cells were assessed by flow cytometry following propidium iodide (PI) staining (Darzynkiewicz et al., 1994). Cells were cultured in 6-well plates and treated with different concentrations of POH or PALD for varying time periods. Cells were harvested, stained with PI and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). PI fluorescence was linearly amplified and both the area and width of the fluorescence pulse were measured. Ten thousand events were acquired and the proportion of hypodiploid (sub- G_0/G_1) events and cells in G_0/G_1 , S and G_2 -M phases of the cell cycle were determined using the DNA analysis software ModfitLT, version 2.0 (Verity Software, Topsham, Maine).

Apoptosis Assay

Fadok et al. (1992) reported that cells undergoing apoptosis lose membrane phospholipid asymmetry and expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Annexin V, a phospholipid-binding protein, preferentially binds to negatively charged phospholipids such as PS (Andre et al., 1990). The shift in fluorescence of fluorescein isothiocyanate (FITC) conjugated to annexin V upon binding to PS was used to confirm apoptosis (Boersma et al., 1996). BroTo or A549 cells were treated with 0, 0.5, or 1 mM POH or PALD for 24 hr. Harvested cells were labeled with annexin V-FITC according to the manufacturer's instructions (Clontech, Palo Alto, CA) and analyzed on a flow cytometer using a single beam laser emitting excitation light at 488 nm. All color assessments were logarithmically amplified and 10,000 events were acquired and analyzed using Cell Quest analysis software program (Becton Dickinson, San Jose, CA).

Results

Cytotoxicity

The effects of POH and PALD on cell proliferation were assessed using the mitochondrial dehydrogenase (MTT) assay (Mosmann, 1983). Both POH and PALD inhibited proliferation of BroTo and A549 cell lines in a dose-dependent fashion (Fig. 1). The concentrations that elicited 50% inhibition of proliferation (IC_{50}) after 24-hr exposure were determined. The IC_{50} values for POH and PALD respectively were 1.0 and 3.2 mM in BroTo cells and 1.2 and 3.0 mM in A549 cells (Fig. 1).

Dose-response and kinetic studies using colony formation assays were also performed to determine whether these compounds exhibited delayed toxicity. Exposure of both cell lines to POH or PALD for 24 hr resulted in no colony formation in the treatment groups compared to Controls. When exposed for 12 hr POH elicited a dose-dependent inhibition of colony formation in both BroTo and A549 cell lines (Fig. 2A). The calculated IC_{50} values for 12-hr POH exposure were 1 mM for BroTo and 1.4 mM for

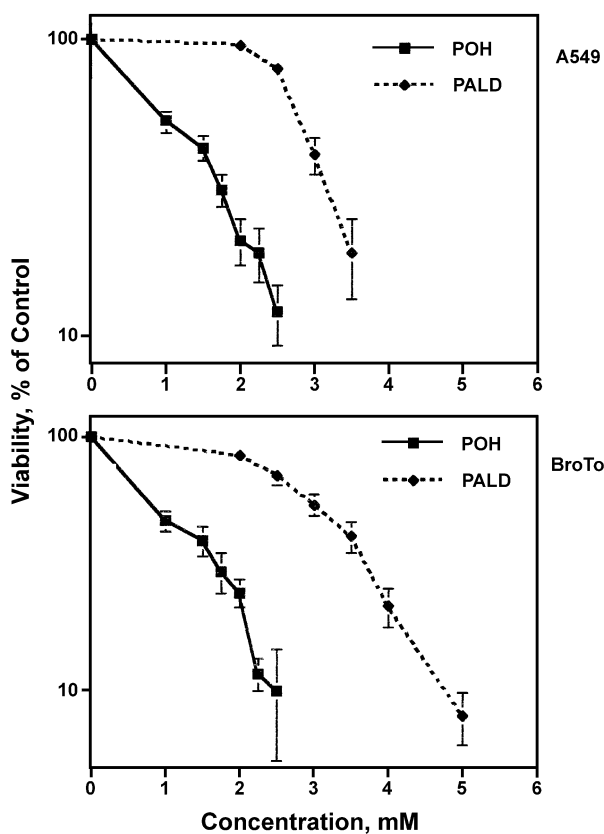


Fig. 1. Effect of monoterpenes on the viability of BroTo and A549 cells cultured in vitro for 24 hr. BroTo and A549 cells were treated with POH or PALD and cell viability was determined by mitochondrial dehydrogenase activity assay. Proportions of viable cells in the treatment groups were calculated relative to the Controls (100%). Log values of percent viable cells (Mean \pm SD) were plotted against monoterpene concentration (mM). Figure is representative of at least three separate experiments.

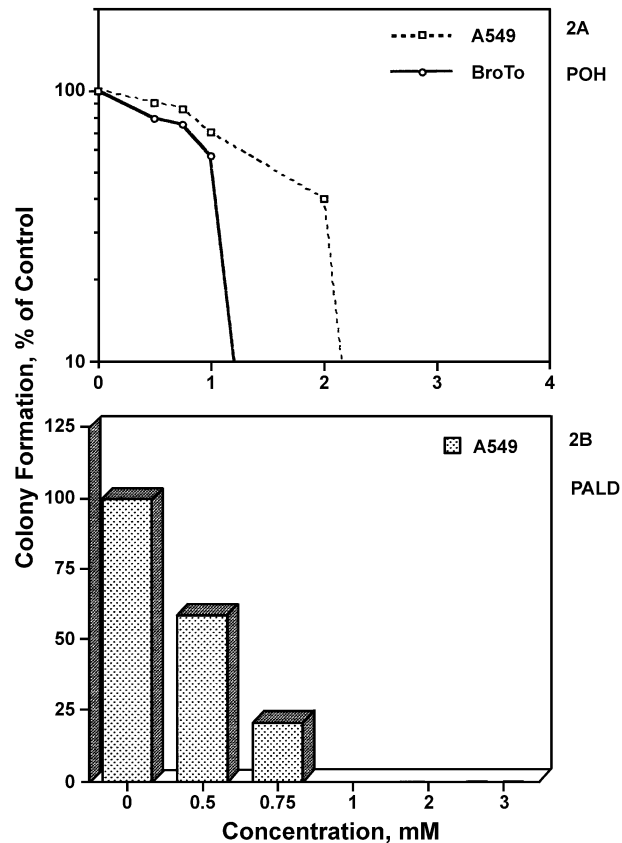


Fig. 2. Inhibition of cancer cell proliferation measured by colony formation assay. BroTo and A549 cells were treated with monoterpenes for 12 hr. Cells were washed and incubated with fresh medium for 12–14 days and then stained with crystal violet. The number of colonies formed was counted, calculated as percentage of Controls (Mean \pm SD) and plotted against monoterpene concentrations. Fig. 2A is a plot of the log of percent viability (colonies formed as a proportion of the Control) in A549 and BroTo cells versus POH concentration; Fig. 2B shows the effect of PALD on colony formation in A549 cells. BroTo cells treated with PALD did not produce colonies compared to the Control. Values are representative of triplicate analyses and Figure is representative of two separate experiments.

A549 cells. These values were in close agreement with those obtained for the 24 hr MTT assays (Fig. 1). Exposure of both cell lines to PALD for 12 hr elicited a dose-response effect in the proportion of colonies formed in A549 cells compared to the Control (Fig. 2B). With BroTo cells, colony formation was not observed in PALD-treated cells compared to the untreated Control.

Apoptosis induction

Analyses of the DNA content of cells following PI staining was used to evaluate whether the observed inhibition of proliferation was due to programmed cell death (apoptosis). A characteristic feature of apoptosis is the activation of endonucleases, resulting in degradation of DNA at nucleosomal linker regions (Arends et al., 1990). During subsequent fixation and permeabilization, DNA fragments are lost

Table 1
Cell cycle distribution in A549 and BroTo cells treated with monoterpenes

Drug	Conc.	Cell cycle distribution (%) in cancer cell lines							
		A549				BroTo			
		Apop	G ₀ /G ₁	S	G ₂ -M	Apop	G ₀ /G ₁	S	G ₂ -M
POH	0 mM	0.3 ± 0.28	58.4 ± 1.42	32.3 ± 0.68	9.3 ± 1.40	0.8 ± 0.31	71.7 ± 1.54	17.8 ± 2.10	10.5 ± 2.49
	1 mM	0.1 ± 0.08	76.7 ± 2.55	12.6 ± 1.14	10.7 ± 1.53	1.9 ± 1.43	83.2 ± 1.07	6.1 ± 0.76	10.6 ± 0.80
	2 mM	4.3 ± 1.19	53.4 ± 1.50	28.8 ± 1.2	17.9 ± 2.19	28.2 ± 2.69	57.8 ± 4.52	12.6 ± 14.7	29.6 ± 10.3
	3 mM	28 ± 1.47	56.4 ± 0.90	34.7 ± 4.53	9.0 ± 4.0	49.2 ± 1.98	63.7 ± 6.90	34.9 ± 9.12	1.4 ± 2.42
PALD	0 mM	0.5 ± 0.75	56.6 ± 0.75	33.2 ± 0.76	10.2 ± 0.26	0.4 ± 0.23	68.8 ± 1.32	24.8 ± 6.38	6.4 ± 5.87
	1 mM	0.2 ± 0.16	73.3 ± 0.61	17.8 ± 1.11	8.9 ± 0.62	2.6 ± 0.78	48.3 ± 1.59	26.6 ± 2.97	25.2 ± 4.21
	2 mM	17.4 ± 5.31	61.8 ± 0.25	11.6 ± 6.66	26.6 ± 6.69	11 ± 4.03	49.2 ± 2.31	29.5 ± 3.48	21.3 ± 2.60
	3 mM	25.7 ± 3.84	62.7 ± 0.90	21.3 ± 0.81	16.0 ± 1.28	17.3 ± 1.94	53.8 ± 6.76	28.8 ± 25.0	17.4 ± 18.2

Values are Mean ± SD (n = 3). Results are representative of 2 separate experiments. (Apop = hypodiploid population).

Table 2
Externalization of phosphatidylserine (PS) in A549 and BroTo cells exposed to POH or PALD for 24 hr

Cell line	Drug	Cells (%) showing positive annexin V staining		
		Concentration, mM		
		0	0.5	1.0
A549	POH	5.5	28.4	66.6
	PALD	7.5	8.0	9.4
BroTo	POH	5.8	33.6	72.2
	PALD	3.5	16.8	22.1

Data represent the sum of cells showing positive annexin V-FITC staining (sum of cells in apoptotic (P^+A^+) and pre-apoptotic (P^-A^+) quadrants).

Values are the average of two determinations and representative of two experiments.

and apoptotic cells are recognized by their diminished DNA content when stained with PI and analyzed by flow cytometry (Darzynkiewicz et al., 1992, 1994).

Table 1 shows the proportion of cells in different phases of the cell cycle following 24-hr exposure to the monoterpenes. Responses to the monoterpenes appeared to be cell line specific. In A549 cells, 1 mM POH induced cell cycle arrest in G_0/G_1 phase while cell accumulation was observed in the G_2 -M phase with 2 mM and no specific accumulation in any phase with 3 mM POH. PALD elicited cell cycle arrest at G_0/G_1 phase at all concentrations (Table 1). In BroTo cells, POH appeared to induce cell cycle arrest in G_0/G_1 (1 mM), G_2 -M (2 mM), and S phase (3 mM). PALD on the other hand caused cell cycle arrest in the G_2 -M phase at all concentrations compared to the Control (Table 1). Furthermore, the proportion of hypodiploid or apoptotic populations (Apop), as measured by propidium iodide uptake, increased as the concentration of monoterpenes increased (Table 1). Further work is in progress to address the apparent inconsistency.

To confirm the mode of cell death, a dual color (PI and annexin V-FITC) flow cytometric technique was used to study the effects of the monoterpenes. Cells in the early stages of apoptosis usually bind annexin V-FITC (positive fluorescence, A^+) in the absence of PI uptake (negative fluorescence, P^-), while those in the later stages of apoptosis bind annexin V-FITC in the presence of PI uptake. Based on the fluorescence of P or A, cells were assessed as live (P^-A^-), pre-apoptotic (P^-A^+), apoptotic (P^+A^+) or necrotic (P^+A^-). Table 2 shows the sum of cells in the pre-apoptotic and apoptotic stages following exposure to 0, 0.5, or 1.0 mM of POH or PALD for 24 hr.

Discussion

POH and PALD inhibited the proliferation of A549 and BroTo cells cultured in vitro. POH ($IC_{50} = 1$ mM) was more effective at inhibiting proliferation than PALD ($IC_{50} = 3$ mM) (Fig. 1). Both compounds inhibited colony formation in both cell lines. POH elicited a dose-dependent reduction in colony formation compared with Controls (Fig. 2).

When cells were treated with PALD for 12 hr, a dose-dependent effect was observed in A549 cells while no colony was formed in treated BroTo cells, compared to its Control group and the 12-hr POH

groups. However, examination of the culture plates under the microscope immediately after washing off the PALD treatment medium showed that there were live cells still attached to the plates. It was therefore concluded that PALD elicited delayed toxicity on the BroTo cells.

From DNA content analysis, dose-dependent increases in the proportion of hypodiploid populations were observed in cells treated with POH or PALD, an indication that the cells might be undergoing apoptosis. POH and PALD, depending on the concentration, appeared to cause cell cycle arrest at different phases of the cell cycle (Table 1). The reason for this observation is not clearly understood. However, at higher concentrations, the agents might be affecting cells at different stages thus halting cell cycle progression. Alternatively, it is possible that the apparent arrest at different phases was due to the death of cells arrested in G₁ phase. Upon redistribution of live cells, a shift towards lower proportions of cells in G₁ phase and higher proportions in other phases were observed. Data obtained with annexin V-FITC (Table 2) confirm the suggestion that POH induced apoptosis in both cell lines while PALD was, at best, a weak apoptosis inducer in BroTo cells and a non-inducer in A549 cells. Consequently, the hypodiploid (apop) DNA events observed when cells were treated with PALD (Table 1) were probably not as a result of apoptosis.

Several important anti-tumor activities of POH have been reported. POH up-regulated the expression of mannose 6-phosphate (M6P)/insulin-like growth factor II (IGF-II) receptor that binds a variety of ligands leading to direct inhibition of cyclin D1 (Jirtle et al., 1993). Ariazi et al. (1999) also showed that in regressing rodent mammary tumors, POH-induced apoptosis in the cancer cells within 48 hr was associated with increased expression of TGF- β -related genes that suppress cyclins D1 and E activities. The expressed proteins include: c-jun and c-fos signaling proteins that were induced within 12 hr; TGF- β 1 that was induced within 24 hr, and M6P/IGF-II receptor and TGF- β types I and II receptors induced within 48 hr (Ariazi et al., 1999).

In vitro, it has been reported that at pharmacologically achievable levels, POH inhibited cell proliferation and induced apoptosis in mammary and hepatic tumor cell lines (Mills et al., 1995; Shi and Gould, 2002). Shi and Gould (2002) also reported that in murine mammary transformed cell line TM6, POH caused an early G₁ cell cycle block and slowed the G₂-M transition. POH reduced cyclin D1 mRNA and protein levels and inhibited cyclin D1- and cyclin E-associated kinases, which are important targets in the G₁-S transition in cell cycle (Shi and Gould, 2002). In similar studies using leukemia cell lines, Sahin et al. (1999) reported that POH selectively induced G₀/G₁ arrest followed by apoptosis in *Bcr/Abl*-transformed, but not in control, non-transformed FDC.P1 and 32D myeloid cell lines. In leukemia cells, expression of the *Bcr/Abl* tyrosine kinase by the Philadelphia chromosome stimulates proliferation and activates anti-apoptotic pathways that are associated with a G₂-M delay, thereby protecting the cells from apoptosis caused by cytotoxic agents and ionizing radiation (Zhu et al., 1996; Bedi et al., 1995). In later investigations of the action of POH on leukemia cell lines, Clark et al. (2002) reported that POH-induced cell cycle arrest at G₀/G₁ phase preceded apoptosis in *Bcr/Abl*-transformed cell lines. It was therefore concluded that the primary effect of POH was to induce G₀/G₁ arrest with apoptosis being a consequence of the growth arrest (Clark et al., 2002). Our data on the effects of POH on head and neck squamous cell carcinoma and human lung adenocarcinoma cell lines are in agreement with these reports (Shi and Gould, 2002; Clark et al., 2002).

In this report, data have been presented indicating that POH elicited dose-dependent induction of apoptosis in both BroTo and A549 cells cultured in vitro. At the concentrations studied, PALD weakly induced apoptosis in BroTo and A549 cells compared to POH (Table 2). Based on the annexin V-FITC results (Table 2), it was concluded that POH caused cell death via apoptosis in both BroTo and A549

cells while PALD induced cell death via a different pathway, probably necrosis. The mechanism of action of POH in head and neck and lung carcinoma cell lines is being investigated.

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