

# Phase I Pharmacokinetic Trial of Perillyl Alcohol (NSC 641066) in Patients with Refractory Solid Malignancies<sup>1</sup>

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## ABSTRACT

Perillyl alcohol (POH) is a monoterpene with anticarcinogenic and antitumor activity in murine tumor models. Putative mechanisms of action include activation of the transforming growth factor  $\beta$  pathway and/or inhibition of p21<sup>ras</sup> signaling, leading to differentiation or apoptosis. In this Phase I trial, 17 patients took POH p.o. three times daily for 14 days of each 28-day cycle. The starting dose of POH was 1600 mg/m<sup>2</sup>/dose, with escalations to 2100 and 2800 mg/m<sup>2</sup>/dose in subsequent cohorts. Chronic nausea and fatigue were dose-limiting toxic effects at 2800 mg/m<sup>2</sup>. Grade 1–2 hypokalemia was common at 2100 and 2800 mg/m<sup>2</sup>. Although POH could not be detected in plasma, two of its metabolites, dihydroperillic acid (DHPA) and perillic acid (PA), were measured in plasma and urine on days 1 and 15 after the first and last doses of POH, respectively. Both area under the concentration versus time curve and peak plasma concentration ( $C_{\max}$ ) values increased with dose and exhibited high intersubject variability. Day 15 DHPA  $C_{\max}$  values ranged from a mean  $\pm$  SD of 22.6  $\pm$  12  $\mu$ M at 1600 mg/m<sup>2</sup>/dose to 42.4  $\pm$  15.24  $\mu$ M at 2800 mg/m<sup>2</sup>/dose. Corresponding mean  $\pm$  SD  $C_{\max}$  values for PA were 433.2  $\pm$  245.8 and 774.1  $\pm$  439.6  $\mu$ M. One patient treated at the 2800 mg/m<sup>2</sup>/dose had markedly prolonged plasma levels of both PA and DHPA and developed grade 3 mucositis. POH treatment did not consistently alter the expression of p21<sup>ras</sup>, rap1, or rhoA in peripheral blood

mononuclear cells obtained from patients treated at the highest dose level. The metabolites PA and DHPA did not change expression or isoprenylation of p21<sup>ras</sup> in MCF-7 breast or DU145 prostate carcinoma cells at concentrations that exceeded those achieved in patient plasma after POH treatment. We conclude that POH at 1600–2100 mg/m<sup>2</sup> p.o. three times daily is well tolerated on a 14-day on/14-day off dosing schedule. Inhibition of p21<sup>ras</sup> function in humans is not likely to occur after POH administration at safe doses of the present oral formulation.

## INTRODUCTION

The monoterpenes are a diverse class of isoprenoid molecules derived from the anabolism of acetate by the mevalonic acid branch biosynthetic pathways of plants. *d*-Limonene, a major component of orange peel oil and the prototype monoterpene in carcinogenesis studies, is formed by the cyclization of the 10-carbon isoprene intermediate geranylpyrophosphate. Interest in *d*-limonene stemmed from the ability of the compound to inhibit carcinogenesis in the murine benzo(*a*)pyrene-induced skin tumor model (1) and inhibition of dibenzpyrene-induced s.c. sarcomas (2). Subsequent studies by Elegbede *et al.* (3, 4) and Haag *et al.* (5) described the inhibitory effects of the compound on the induction of rat mammary tumors by 7,12-dimethylbenz(*a*)anthracene and antitumor activity against established 7,12-dimethylbenz(*a*)anthracene and *N*-nitroso-*N*-methylurea-induced rat mammary tumors. An interesting aspect of these and other preclinical studies was the lack of toxicity in the animals treated with doses of *d*-limonene that produced complete regressions (4, 5). Histological examination of the regressed tumors revealed a lack of gross cytotoxicity, immune cell infiltration, or apoptosis. A remodeling or redifferentiation effect causing tumor regression was hypothesized (5).

The mechanisms by which *d*-limonene and other cyclic monoterpenes inhibit tumor growth have not been firmly established. Geranylpyrophosphate, the isoprene intermediate from which these compounds are derived, is required for synthesis of cholesterol, coenzyme Q (ubiquinone), and substrates used in the isoprenylation of several cellular proteins. Crowell *et al.* (6) found that *d*-limonene and other monoterpenes inhibited isoprenylation of  $M_r$  21,000–26,000 proteins, including p21<sup>ras</sup> and other members of the ras family of GTP-binding proteins that are involved in signal transduction and growth regulation. The posttranslational isoprenylation of these and other proteins is an essential covalent modification that is required for protein localization and function. For example, farnesylation is required for plasma membrane association and signaling function of p21<sup>ras</sup>. Other intracellular proteins require isoprenylation by addition of a farnesyl (15-carbon) or geranylgeranyl (20-carbon)

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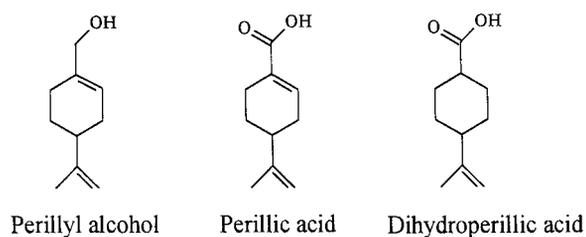


Fig. 1 Structures of POH and the metabolites PA and DHPA.

group to the COOH terminus for localization to a cellular compartment or for interaction with other proteins (7).

In a structure activity study, POH<sup>3</sup> and other 7-monohydroxylated, limonene-derived monoterpenes were more potent inhibitors of protein isoprenylation and cell proliferation than *d*-limonene (8). Inhibition of isoprenylation of  $M_r$  21,000–26,000 proteins by 50% was obtained with 1 mM POH as compared with 5 mM *d*-limonene, and the  $IC_{50}$  of POH against HT29 human colon carcinoma cells was 50  $\mu$ M as compared with an  $IC_{50}$  of >5 mM for *d*-limonene. p.o. administered POH (2% of the diet) was equivalent to limonene (7.5% of the diet) in causing complete regression of >80% of established rat mammary carcinomas with no toxicity to the host (9). Pharmacological studies in rats suggested that the increased potency of POH treatment compared with *d*-limonene was a consequence of 10-fold higher levels of PA and DHPA after chronic POH ingestion (9). PA and DHPA (Fig. 1) are the major metabolites of both *d*-limonene and POH and have antiproliferative and prenylation-inhibitory potencies intermediate between these parent compounds.

The mechanism by which monoterpenes inhibit isoprenylation of G proteins is not known. The amount of native p21<sup>ras</sup> protein may be decreased, leading to reduced levels of the unmodified and farnesylated protein (10). POH has been shown to be a weak inhibitor of the prenylating enzymes FPT and geranylgeranyltransferase I *in vitro* (11). However, POH-induced regression of tumors *in vivo* has not been linked to inhibition of protein isoprenylation in tumors or associated normal tissue.

A second putative antitumor mechanism of action has been described for the monoterpenes. Rat tumors that regressed in response to *d*-limonene (12) and POH (13) had increased levels of TGF- $\beta$  protein and had increased expression of the mannose-6-phosphate/insulin-like growth factor type II receptor and TGF- $\beta$  type I, II, and III receptors at the protein and mRNA levels. POH did not affect tumor cell proliferation, but the apoptotic index was increased approximately 10-fold (14).

These findings suggest that the monoterpenes inhibit tumor growth through a TGF- $\beta$ -mediated increase in apoptosis.

The noncytotoxic antitumor effects of POH and related monoterpenes in preclinical models and the interesting possibility that these effects were mediated by inhibition of TGF- $\beta$  or p21<sup>ras</sup>-dependent signaling provided impetus for clinical trials of POH. The primary objectives of this Phase I trial were: (a) to determine the MTD of POH administered p.o. on a TID schedule for 14 consecutive days, every 28 days; (b) to describe and quantify the toxicities of POH on this schedule; and (c) to determine the pharmacokinetics of POH and major metabolites. In addition, toward the goal of defining an optimal biological dose of POH (and agents that target p21<sup>ras</sup>) for further clinical development, we attempted to measure p21<sup>ras</sup> expression in PBMCs of patients treated with POH and to correlate changes in p21<sup>ras</sup> with toxicity and plasma levels of POH metabolites.

## PATIENTS AND METHODS

**Patient Selection.** All patients registered were required to: (a) be at least 18 years of age; (b) have histological proof of a malignant solid tumor and have failed conventional chemotherapy for their particular tumor; or (c) have a disease for which no established therapy existed. All patients had an ECOG performance status of 0 or 1, a minimum life expectancy of 12 weeks, and had recovered from all toxicities of prior treatment. Radiotherapy and chemotherapy were completed at least 4 weeks before registration (8 weeks for nitrosoureas and mitomycin C). Adequate bone marrow function (granulocytes  $\geq$  2,000/ $\mu$ l and platelets  $\geq$  100,000/ $\mu$ l), hepatic function (total bilirubin  $\leq$  1.5 mg/dl and aspartate aminotransferase/alanine aminotransferase  $\leq$  twice the upper limit of normal), and renal function (serum creatinine  $\leq$  1.5 mg/dl) were also required. Patients taking antiepileptic medication and those with a history of hypersensitivity or allergy to citrus fruits or soybean oil were excluded from participation. Patients were required to discontinue cholesterol-lowering agents while on study. A negative pregnancy test was required of women of child-bearing age. All patients registered were informed of the investigational nature of the treatment and the anticipated toxicities. Written consent was obtained from each patient according to state and federal guidelines before beginning treatment. This Phase I trial was approved by the Fox Chase Cancer Center Institutional Review Board.

**Clinical Evaluations.** Pretreatment evaluations included history and physical examination, weight, ECOG performance status, chest X-ray, 12-lead electrocardiogram, complete blood count, and serum electrolytes and chemistry profile including tests of hepatic and renal function, carcinoembryonic antigen, and other tumor markers. Radiographic studies required for tumor measurements were obtained within 2 weeks of registration and repeated every 4 weeks during protocol treatment. History and physical examination and weight, performance status, and tumor marker evaluation were repeated every 4 weeks. Complete blood count, chemistries, and toxicity assessments were performed weekly for 4 weeks and then performed every other week.

The criteria used to grade the severity of toxicity were those of the Cancer Therapy Evaluation Program (Bethesda,

<sup>3</sup> The abbreviations used are: POH, perillyl alcohol; PA, perillic acid; DHPA, dihydroperillic acid; FPT, farnesyltransferase; TGF- $\beta$ , transforming growth factor  $\beta$ ; MTD, maximum tolerated dose; PBMC, peripheral blood mononuclear cell; TID, three times daily; DLT, dose-limiting toxicity;  $C_{max}$ , peak plasma concentration; AUC, area under the concentration *versus* time curve; ECOG, Eastern Cooperative Oncology Group.

MD). Standard solid tumor response criteria were used to measure tumor response (15).

**Formulation and Administration of POH.** POH (NSC 641066) was provided as soft gelatin capsules by the National Cancer Institute, Division of Cancer Treatment and Diagnosis (Bethesda, MD). Each capsule contained 250 mg of POH and 250 mg of soybean oil. POH was taken p.o. TID at standard times of 8 a.m., 4 p.m., and 12 a.m. for 14 consecutive days. Patients were instructed to take each dose of POH either 1 h before or 2 h after meals, food, or medications such as antacids to minimize potential effects on drug absorption. For cycle 1, one additional dose of POH was administered at 8 a.m. on day 15 to enable a prolonged blood sampling period for pharmacokinetic analysis.

**Dose Escalation and Definition of MTD.** Based on safety data from the initial clinical trial of POH conducted at the University of Wisconsin (Madison, WI), which used a continuous oral TID dosing schedule, the starting total dose of POH was 4800 mg/m<sup>2</sup>/day or 1600 mg/m<sup>2</sup>/dose TID × 14 days. Subsequent planned dose levels of 2100, 2800, and 3700 mg/m<sup>2</sup> per dose were 30% increases from the preceding level. The total number of capsules taken for each dose was determined by dividing the calculated dose (in milligrams) by 250 and rounding to the nearest integer. Patients were instructed to keep diaries to document the time and number of capsules taken daily.

Four patients were treated initially at each dose level. If none of these patients experienced DLT, which was defined as hematological or nonhematological toxicity (excluding alopecia) of grade 3 or 4, then the dose was escalated to the next higher level for four subsequent patients. If one or two of the initial four patients had a DLT, then four additional patients were accrued to the same dose. The MTD was exceeded if DLT occurred in three of the initial four patients or in three or more of eight total patients at a given dose level. The probability of exceeding the MTD (defined as escalation above a dose that would be toxic to at least 50% of patients) with this design was ≤0.20.

**Pharmacokinetic Sampling.** All patients were admitted to the Mary S. Schinagl Clinical Studies Unit of the Fox Chase Cancer Center for pharmacokinetic studies on days 1 and 15 of the first cycle of treatment. Blood samples were obtained just before and after the first dose of POH at 15 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 5 h, 6 h, and 8 h (just before the second dose) and then at 16 h and 24 h (before the second and third doses, respectively) on day 1. A second intensive blood sampling schedule was begun after a single dose of POH was administered at 8 a.m. on day 15 of the first cycle. Samples were obtained between hours 0 and 8 as described for the first dose and at 12, 18, and 24 h to permit more accurate estimation of the terminal elimination phase. Each blood sample was centrifuged to separate plasma. Plasma from each sample was transferred to labeled tubes and stored at -80°C until assay. Urine was obtained in three 8-h collections on day 1, coinciding with the three doses of POH, and as a single 24-h collection on day 15 after the last dose of POH was given. Aliquots from each collection period were transferred to labeled tubes and frozen at -80°C until assay.

Table 1 Patient characteristics

Total no.	17
Male/Female	10/7
Median age (range) (yrs)	56 (43–70)
ECOG performance status	
0	5
1	12
Primary site of cancer	
Colon	9
Renal	3
Lung	2
Parotid	2
Unknown primary site	1
No. of prior chemotherapy regimens	
0–1	6
2	5
3 or more	6

**Measurement of POH and Metabolites.** Analysis of plasma and urine for POH and the metabolites PA and DHPA was performed according to the method of Phillips *et al.* (16), with a slight modification. Plasma samples (50 µl) were combined with an acid buffer (50 µl) consisting of 0.2 M phosphoric acid plus 1.0 M potassium chloride and 10 µl of an internal standard solution containing  $\gamma$ -terpinene (1 mg/ml in acetonitrile) diluted with tert-butyl methyl ether to yield a final internal standard concentration of 10 µg/ml. The mixture was vortexed vigorously for 1 min, followed by centrifugation (15,000 × g, 20 min). The upper organic phase was transferred to a borosilicate glass conical insert, placed within an autosampler vial, and sealed with a silicone/Teflon-line septum closure. Analyses were performed on a model 5890 series II gas chromatograph and a 5791A mass selective detector (Hewlett-Packard). The detector was operated in the selected ion-monitoring mode for quantitative determinations by measuring the ions at *m/z* 121 and 122. Limits of quantitation were 1.71, 1.55, and 1.57 µM for POH, DHPA, and PA, respectively. The assay was linear over a range from 1.55 to 620 µM for all three compounds. Interday (*n* = 3) variations of the assay were 7.5%, 7.0%, and 12.4% for POH, DHPA, and PA, respectively.

**Pharmacokinetic Analysis.** Noncompartmental analysis (17) was used to obtain estimates of the AUC and terminal half-lives for PA and DHPA. For the day 15 data, two AUC values were calculated: (a) the time infinity value; and (b) the day 1 equivalent AUC. The latter was normally based on an 8-h time interval and allowed an assessment of drug accumulation and time-dependent alterations in pharmacokinetics. The total amounts of POH, PA, and DHPA excreted in urine were measured in a few patients on day 15, and these were expressed as a percentage of the dose administered. The observed *C*<sub>max</sub> and time of peak plasma concentration (*T*<sub>max</sub>) were recorded. Dose- and time-dependent changes in the pharmacokinetics of PA and DPA were based on comparison of AUC values obtained on day 15 and on day 1.

**Measurement of p21<sup>ras</sup> and ras-related Proteins in PB-MCs.** Blood was obtained from patients on days 1 (before the first dose of POH), 8, and 15 of treatment. The samples were collected in lymphocyte separation vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged for 20 min

Table 2 Dose levels, number of capsules per dose/day, and first course toxicity

Dose level (mg/m <sup>2</sup> /dose)	No. of patients	Capsules per dose (range)	Capsules per day (range)	Toxicities $\geq$ grade 2 (grade for each person with toxicity)
1600	4	13 (12–13)	39 (36–39)	Hypokalemia (2)
2100	4	16 (14–18)	48 (42–54)	Nausea (2 <sup>a</sup> ) Vomiting (2 <sup>a</sup> ) Pyrosis (2 <sup>a</sup> )
2800	9 <sup>b</sup>	22 (17–30)	66 (51–90)	Nausea (2, 2, 2 <sup>a</sup> ) Vomiting (2, 2, 2, 2 <sup>a</sup> ) Hypokalemia (2, 2, 2, 2) Fatigue (3 <sup>a</sup> ) Diarrhea (2 <sup>a</sup> ) Stomatitis (3 <sup>a</sup> ) Anorexia (3 <sup>a</sup> )

<sup>a</sup> Denotes one patient with multiple toxicities.

<sup>b</sup> Includes two patients who withdrew before day 14 due to toxicity.

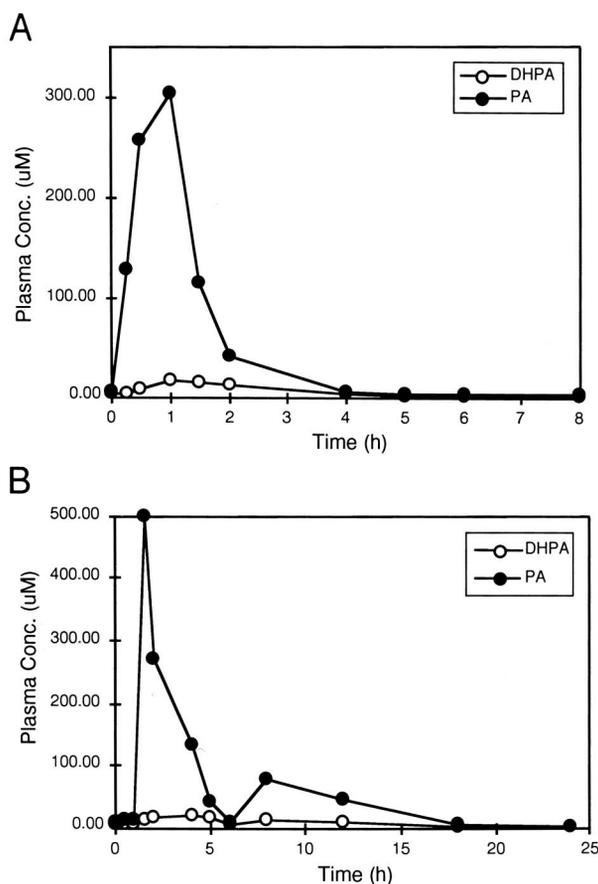


Fig. 2 Representative plasma concentration versus time curves for PA and DHPA for a patient treated at 2800 mg/m<sup>2</sup>. A, day 1, after first dose, with pharmacokinetic sampling until the second 8-h dose; B, day 15, final dose of cycle 1.

at 1,500  $\times$  g. PBMC fractions were removed, washed with PBS, pelleted, and stored immediately at  $-80^{\circ}\text{C}$  until preparation of protein lysates. Day 1, 8, and 15 samples were analyzed together for each patient. Cytosolic extracts were prepared in the following manner. PBMCs were lysed for 15 min in PBS containing

1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, and protease inhibitors and centrifuged at 10,000  $\times$  g for 15 min. Protein concentrations of the supernatants were estimated by the Bio-Rad Protein Assay (Bio-Rad, Melville, NY). Cytosolic proteins (200  $\mu\text{g}$ ) from each sample were separated on 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes as described previously (18). Blots were blocked with 3% nonfat milk in TBS and incubated with Ab-3 anti-pan-ras primary antibodies (Oncogene Science, Cambridge, MA) at a 1:500 dilution overnight. Blots were incubated with horseradish peroxidase-conjugated antimouse secondary antibodies (Amersham, Arlington Heights, IL) at a 1:3000 dilution, and proteins were visualized by chemiluminescence detection (Amersham). Protein intensities were quantitated by densitometry and analyzed.

PBMCs were also analyzed for total expression of the *ras*-related proteins rhoA, rap2, and rab4. rhoA, rap2, and rab4 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The resulting bands in samples obtained for each patient on days 1, 8, and 15 were quantitated by densitometry and compared. Changes in protein expression were correlated with plasma concentrations and AUC values for PA and DHPA.

**Treatment of Cell Lines with POH, PA, and DHPA.** Human prostate carcinoma cells (DU145) and breast carcinoma cells (MCF-7) were treated for 48 h with POH (0.25, 0.5, or 0.75 mM), PA and DHPA (0.5, 1.0, or 2.0 mM), ethanol alone, or FPT3, an inhibitor of FPT (Calbiochem, La Jolla, CA). After a 48-h drug exposure, cells were isolated as pellets, and cell lysates were prepared and analyzed for p21<sup>ras</sup> expression as described above. The primary antibody used was a pan-ras antibody (1:500 dilution) from Transduction Laboratories (Lexington, KY).

## RESULTS

**Patient Demographics.** A total of 17 patients were registered, all of whom were evaluable for toxicity. Pretreatment characteristics of the patients are summarized in Table 1. Nine of the 17 patients (53%) had metastatic colorectal cancer that was refractory to at least two separate chemotherapy regimens. All of the patients had excellent performance status and were highly motivated to comply with the oral treatment regimen. A

Table 3 Summary of  $C_{\max}$  and AUC values for PA and DHPA

	Dose level (mg/m <sup>2</sup> /dose)					
	1600		2100		2800	
	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
$C_{\max}$ ( $\mu\text{M}$ )						
PA	672.8 $\pm$ 337.3	433.17 $\pm$ 245.8	630.20 $\pm$ 476.0	507.52 $\pm$ 114.7	698.1 $\pm$ 431.9	774.1 $\pm$ 439.6
DHPA	20.18 $\pm$ 6.3	22.5 $\pm$ 12.1	21.0 $\pm$ 8.9	26.55 $\pm$ 3.6	35.2 $\pm$ 19.9	42.4 $\pm$ 15.2
AUC infinity ( $\mu\text{M}\cdot\text{h}$ )						
PA		904.6 $\pm$ 553.0		914.71 $\pm$ 99.6		2340.4 $\pm$ 1264.7
DHPA		96.17 $\pm$ 49.8		152.1 $\pm$ 22.1		297.2 $\pm$ 85.4
AUC T <sub>last</sub> ( $\mu\text{M}\cdot\text{h}$ )						
PA	1586.7 $\pm$ 917.6	865.48 $\pm$ 577.9	1496.82 $\pm$ 850.6	883.0 $\pm$ 88.1	2021.6 $\pm$ 1240.7	2116.7 $\pm$ 1279.3
DHPA	79.22 $\pm$ 41.5	73.55 $\pm$ 52.3	113.11 $\pm$ 36.3	102.90 $\pm$ 9.0	148.4 $\pm$ 73.6	182.9 $\pm$ 74.7

total of 29 courses were administered, with a median of 2 courses/patient (range, 1–4 courses/patient). The dose levels, the number of capsules/dose, and the total number of capsules/day for each dose level are summarized in Table 2. Aside from the three exceptions (due to early discontinuation of three patients on study) described below, compliance with taking POH was essentially 100%.

**Toxicity, Response, and Determination of MTD.** Toxicity by dose level is presented in Table 2. Mild (grade 1) fatigue and nausea were common at all dose levels but were dose limiting at the 2800 mg/m<sup>2</sup>/dose level. Four of the nine patients treated at the 2800 mg/m<sup>2</sup>/dose level had grade 2 nausea, pyrosis, or vomiting. These were significant toxicities that were poorly tolerated on a continuous dosing schedule, were not ameliorated by standard antiemetic medications, and led to the withdrawal of two patients at the 2800 mg/m<sup>2</sup> dose level due to intolerance of the medication. A third patient described below experienced acute DLT and refused further POH treatment.

Hypokalemia of mild to moderate severity was observed in 10 of the 17 patients (58.8%). Paired urine potassium levels taken from the urine collections on days 1 and 15 were not significantly different, suggesting that renal loss was unlikely. Similarly, there was no evidence of significant acid-base change as judged from plasma electrolyte levels before and during cycle 1 of treatment. In each case, plasma potassium was easily corrected by modest oral potassium supplementation.

One patient treated at the 2800 mg/m<sup>2</sup> dose level developed grade 3 stomatitis and grade 2 diarrhea beginning on day 4 of treatment. This patient also experienced grade 3 fatigue, anorexia, and grade 2 nausea and vomiting. These toxicities improved within 2 days of stopping POH and resolved by 1 week. The patient refused additional drug level measurements to determine the relationship between toxicity and metabolite levels, and she also declined additional treatment with POH at a reduced dosage. Myelosuppression did not occur in this patient or in any of the other patients.

There were no objective tumor regressions observed in the 15 patients (9 of whom had colon cancer) who completed at least one cycle of therapy. Seven patients had tumor progression after one cycle of POH. Eight patients had stable disease; of these patients, four progressed after cycle 2 of POH, one progressed after cycle 3 of POH, and three progressed after cycle 4 of POH. Three of the nine patients with colon cancer had stable disease for at least 2 months.

**Pharmacokinetics of POH Metabolites.** The pharmacokinetic analysis of PA and DHPA focused on two properties, dose-dependent and time-dependent changes in the measured parameters. Dose-dependent changes were based on the day 15 PA and DHPA AUC<sub>∞</sub> values, whereas time-dependent changes were based on comparisons of day 1 and day 15  $C_{\max}$  values and time-equivalent AUC (AUC<sub>Tlast</sub>) values. The latter AUCs were based on the day 1 sampling after the first dose and normally consisted of an 8-h sampling period. Fig. 2 shows a representative patient's PA and DHPA concentration-time curves after the initial dose on day 1 and after the final dose on day 15. Consistent with previous studies in rats and dogs and with an earlier Phase I trial using the same formulation (19), POH was not detected in plasma. Table 3 summarizes  $C_{\max}$  and AUC values for PA and DHPA over the doses studied. Individual patient day 15 PA and DHPA  $C_{\max}$  values are depicted in Fig. 3, A and B, respectively. Day 15 AUC values of PA and DHPA are shown in Fig. 4, A and B.

$T_{\max}$  was approximately 2 h after dosing for PA and 3.5 h after dosing for DHPA. Day 15 harmonic mean elimination half-lives (averaged over all dose levels) were 2.13 h for PA and 3.96 h for DHPA. Day 15 PA AUC<sub>∞</sub> values (mean  $\pm$  SD) were 904.6  $\pm$  553, 914.7  $\pm$  99.6, and 2340.4  $\pm$  1264.7  $\mu\text{M}\cdot\text{h}$  at the 1600, 2100, and 2800 mg/m<sup>2</sup>/dose levels, respectively. Analogous day 15 DHPA AUC values were 96.17  $\pm$  49.9, 152.1  $\pm$  22.1, and 297.2  $\pm$  85.4  $\mu\text{M}\cdot\text{h}$ . Although the day 15 AUC values for PA and DHPA were generally consistent with dose-independent pharmacokinetics, there were two inconsistencies. Day 15 DHPA AUC values showed a disproportional increase as the dose increased, and the differences approached statistical significance ( $P = 0.063$ , Kruskal-Wallis test). Also, PA AUC values did not increase in proportion to dose at the 1600 and 2100 mg/m<sup>2</sup> levels, but when compared with the AUC values at 2800 mg/m<sup>2</sup> and the large interpatient variability (Fig. 4), statistical differences in the dose-normalized AUC values were not found ( $P = 0.30$ , Kruskal-Wallis test).

Comparisons of PA and DHPA day 1 and day 15  $C_{\max}$  and time-equivalent AUC values revealed only a few differences. At the 2800 mg/m<sup>2</sup> dose level, DHPA  $C_{\max}$  values were elevated on day 15 (42.4  $\pm$  15.2  $\mu\text{M}$ ) compared with day 1 (35.2  $\pm$  19.9  $\mu\text{M}$ ). There were decreases in the mean PA and DHPA time-equivalent AUC values from day 1 to day 15 at the 1600 and 2100 mg/m<sup>2</sup> dose levels (Table 3). However, interpatient variability was great, and the differences were

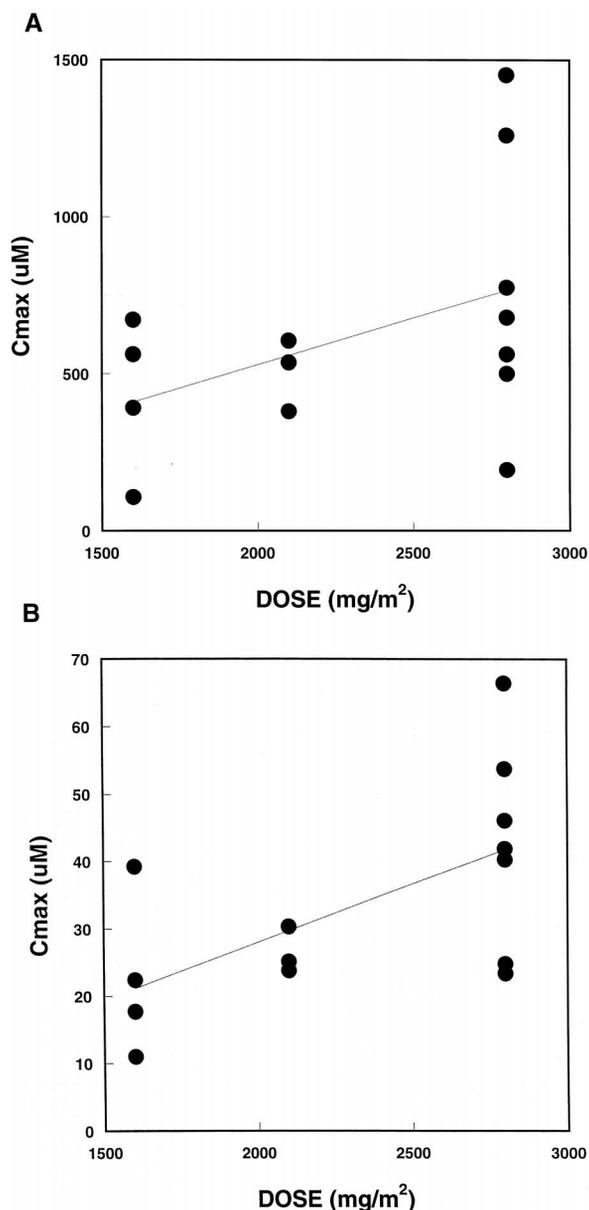


Fig. 3 Scatterplots showing individual day 15  $C_{\max}$  values according to the dose level of POH for PA (A) and DHPA (B).

not statistically significant. Because all other comparisons between day 1 and day 15 parameters revealed no significant difference, the presence of a uniform time-dependent change in DHPA and PA pharmacokinetics is unlikely.

Urinary excretion of PA was most prominent with  $9.96 \pm 10.12\%$  ( $n = 13$ ) of the POH dose excreted in 24 h on day 15, whereas only  $1.40 \pm 1.66\%$  ( $n = 11$ ) of the dose was excreted as DHPA. POH was not consistently found in urine, and in the few patients in whom it could be measured, the fraction of the administered dose excreted was less than 1%. There was no apparent dose dependency in urinary excretion of DHPA and PA.

The plasma concentration *versus* time curves for the patient

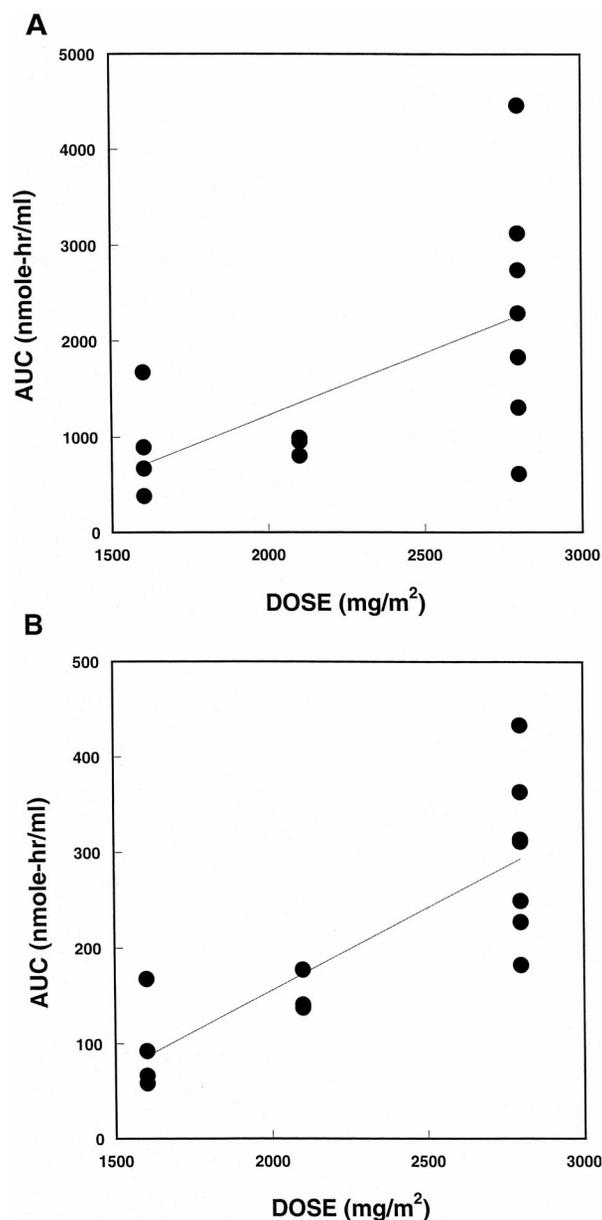


Fig. 4 Scatterplots showing individual day 15  $AUC_{0-\infty}$  values according to the dose level of POH for PA (A) and DHPA (B).

who experienced grade 3 diarrhea and fatigue and grade 2 mucositis are shown in Fig. 5. Unlike other patients, PA and DHPA plasma levels for this patient demonstrated major secondary peaks and continued to rise at 8 h after the initial dose. In addition, day 1 AUC values were relatively high compared with the means ( $3056.4 \mu\text{M}\cdot\text{h}$  for PA and  $238.9 \mu\text{M}\cdot\text{h}$  for DHPA). This patient refused further POH therapy, and no additional drug levels were obtained.

**Effects of POH Therapy on PBMC p21<sup>ras</sup> and Ras-related Proteins.** Of the nine patients enrolled at the 2800 mg/m<sup>2</sup> level, six completed at least one full cycle of POH therapy and provided serial blood samples for PBMC studies. Western blot analyses of p21<sup>ras</sup> from PBMCs obtained from

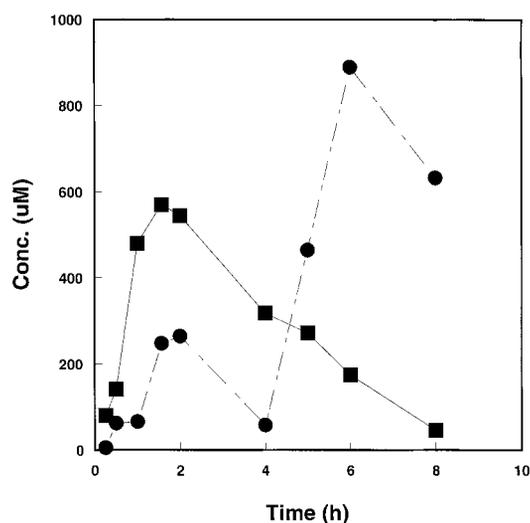


Fig. 5 Plasma PA concentration versus time plot for patient SK (●) and mean plasma concentrations at each sampling time after the first dose of POH for the other patients (■) treated at 2800 mg/m<sup>2</sup>. Patient SK had large and prolonged secondary peaks for both PA and DHPA (data not shown) and experienced unique toxicity.

these six patients before treatment and on days 8 and 15 of the first treatment cycle are shown in Fig. 6. Relative change in p21<sup>ras</sup> expression during treatment is provided in Table 4, together with each patient's day 1 and day 15 C<sub>max</sub> and AUC values. The amount of p21<sup>ras</sup> in PBMCs obtained on days 8 and 15 is expressed as a percentage of the day 1 pretreatment level determined by densitometric scanning of the bands in Fig. 6.

As can be seen in Fig. 6 and Table 4, the effect of POH treatment on lymphocyte p21<sup>ras</sup> was variable. Two of the patients had overall decreases from baseline, whereas four patients had increases over the course of treatment. Interestingly, the patient with the most pronounced decline in p21<sup>ras</sup>, a 76% decrease from baseline, had the highest day 15 AUCs for PA and DHPA. However, one of the patients with increased p21<sup>ras</sup> also had high day 15 AUCs for the two POH metabolites. We did not observe changes in the ras-related proteins rhoA, rap1, or rab4.

**Effects of POH and Metabolites on p21<sup>ras</sup> in Human Tumor Cell Lines.** The effects of POH, PA, and DHPA on p21<sup>ras</sup> expression and isoprenylation were studied in human tumor cell lines to anticipate the dose range of the metabolites that could be expected to alter p21<sup>ras</sup> in human tissues. As shown in Fig. 7, treatment of MCF-7 breast carcinoma cells with an inhibitor of the enzyme FPT resulted in a shift of p21<sup>ras</sup> from the farnesylated form of the protein (lower bands in Fig. 7) to the more slowly migrating unprocessed form. The total amount of the protein appears unchanged. In contrast, the same cells treated with 250–750 μM POH for 48 h, concentrations associated with a 20–40% cell kill, demonstrate an overall decrease in p21<sup>ras</sup> levels without a change in isoprenylation. Treatment with 500 μM POH reduced levels of p21<sup>ras</sup> by 82.5%, whereas exposure of MCF-7 cells to 500 μM PA (<10% inhibition) or 500 μM DHPA (38% inhibition) was significantly less potent than exposure

to POH. Identical results were seen in DU145 human prostate carcinoma cells (data not shown). The combined mean C<sub>max</sub> values for PA and DHPA in patient plasma at the highest dose (2800 mg/m<sup>2</sup>) studied were below the concentrations (1–2 mM) required for inhibition of cell growth and p21<sup>ras</sup> expression in the two cell lines (Fig. 7).

## DISCUSSION

We did not define a MTD for POH by the usual criteria of grade 3 or 4 toxicity in a significant proportion of patients. However, the chronic nausea and fatigue associated with continuous treatment over a 14-day period proved to be dose limiting at 2800 mg/m<sup>2</sup> per dose (total daily dose of 8400 mg/m<sup>2</sup>). Thus, even grade 2 toxicity was not tolerable when it was chronic and unremitting. Only one patient had grade 3 toxicity that would be considered dose limiting by conventional criteria.

Although many patients found ingesting a large number of capsules three times a day to be annoying, it is uncertain that the number of capsules *per se* was the sole cause of nausea and pyrosis. The large amount of soybean oil ingested with each dose may have contributed to the nausea and bloating observed. Because the monoterpene menthol is known to impair gastroesophageal sphincter function (19) and could thus potentiate gastric reflux, it is possible that POH might have a similar effect. POH may be directly toxic to gut mucosa, a possibility supported by animal toxicology. In these studies, Fisher 344 rats given POH in soybean oil by gavage TID for 28 days developed forestomach hyperplasia and inflammation by day 3 of treatment at doses below the MTD, suggesting a direct mucosal irritant effect. In dogs given POH TID for 14 days by gavage at the MTD of 4,000 mg/m<sup>2</sup>/dose (12,000 mg/m<sup>2</sup> total daily dose), emesis, diarrhea, and renal toxicity were the principal toxicities (20).

Hypokalemia was a common metabolic toxicity, occurring more frequently at the 2800 mg/m<sup>2</sup> dose level. Urine potassium levels obtained from day 1 and day 15 urine collections did not differ, and renal function was not impaired in any patient, suggesting that decreased absorption or bioavailability of dietary potassium was the underlying reason for the low serum levels during POH therapy. Potassium deficits were uniformly corrected with modest oral potassium supplementation.

Although the day 15 PA and DHPA AUC values fit the criteria for dose-independent pharmacokinetics, there were inconsistencies in the data. For DHPA, there was a more than proportional increase in AUC and C<sub>max</sub> in the dose range of 1600–2800 mg/m<sup>2</sup>/dose, suggesting a saturable process. It cannot be determined whether this might represent saturable elimination because the oral bioavailability of POH is unknown. In light of the large intra- and interpatient variability, it would be difficult to delineate POH and metabolite pharmacokinetics without investigation of a broader dose range, preferably with an i.v. formulation.

In all but one patient, plasma concentrations of PA and DHPA declined after reaching peak levels at approximately 2 and 3.5 h after the oral dose. One patient at the 2800 mg/m<sup>2</sup> dose level had metabolite concentrations that continued to increase between doses and developed unique toxicities consisting of

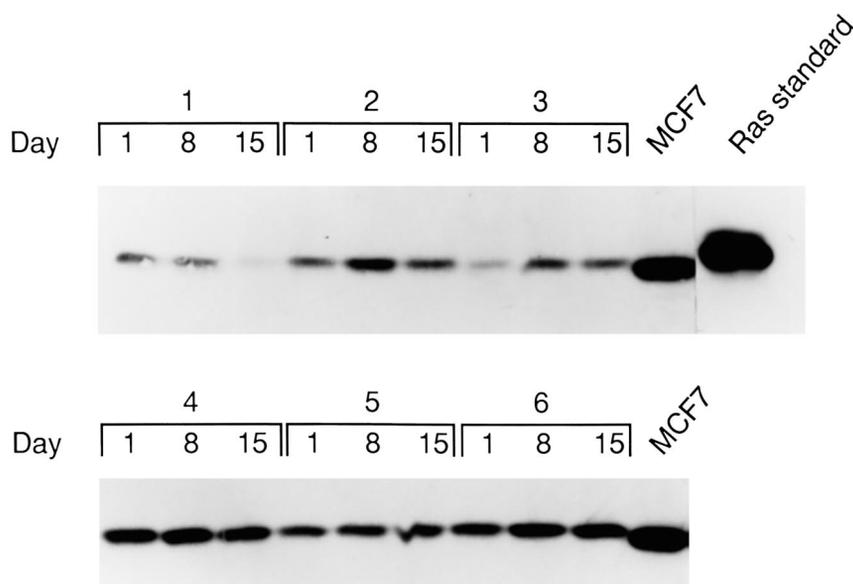


Fig. 6 Proteins obtained from patient PBMC lysates and separated by 12% SDS-PAGE followed by Western blot using anti-pan-ras antibodies. Ras standard and MCF-7 cell proteins serve as positive controls for the unprocessed and processed forms of p21<sup>ras</sup>. Samples from day 1 (pre-treatment) and days 8 and 15 of treatment are shown for six patients treated with POH at the 2800 mg/m<sup>2</sup> dose level. Decreased p21<sup>ras</sup> signal is seen on day 15 for patient 1. Increased signal is seen for patients 2, 3, 5, and 6.

Table 4 p21<sup>ras</sup> levels in PBMCs from patients treated with 2800 mg/m<sup>2</sup> POH: correlation with plasma levels of POH metabolites

Patient no.	p21 <sup>ras</sup> levels <sup>a</sup>			PA, day 15		DHPA, day 15	
	Day 1	Day 8	Day 15	C <sub>max</sub>	AUC	C <sub>max</sub>	AUC
1	1.00	0.92	0.24	1258.9	4287.0	53.8	263.80
2	1.00	1.98	1.39	194.5	423.0	24.8	102.20
3	1.00	2.40	2.52	1451.6	2701.8	40.3	158.80
4	1.00	1.20	0.75	562	1731.0	46.1	161.71
5	1.00	1.21	1.34	774	3067.8	66.4	276.34
6	1.00	1.64	1.85	500	881.0	23.4	118.77

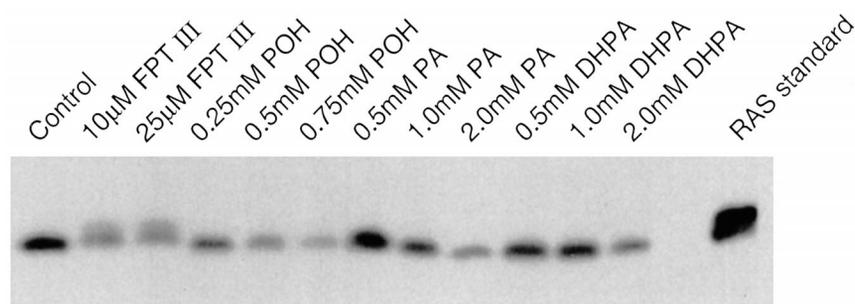
<sup>a</sup> Quantitated by densitometric scanning of protein bands in Fig. 5; day 8 and day 15 levels were normalized to the day 1 value for each patient.

grade 2 diarrhea and grade 3 stomatitis. Although her AUCs for PA and DHPA were among the highest observed on day 1 of treatment, other patients had comparable values on day 1 and did not develop severe toxicity. After C<sub>max</sub>, secondary peaks of PA and DHPA concentrations could be detected for most patients and, with the exception of one patient, were invariably minor. The elevated secondary metabolite peaks for this patient might be most consistent with a prolonged and pulsatile absorption phase for POH; however, we have no direct evidence to rule out the possibility of enterohepatic recycling of POH metabolites. No concomitant medications or clinical characteristics were apparent to explain the unusual pharmacokinetics of PA and DHPA in this patient.

The clinical toxicities and pharmacokinetics observed in the present Phase I trial are similar to those reported by Ripple *et al.* (21) in a Phase I trial of POH performed at the University of Wisconsin. Oral doses of 800, 1600, and 2400 mg/m<sup>2</sup> TID were investigated in the initial Wisconsin study, but with continuous daily treatment rather than the 14 day-on/14 day-off schedule used in the present study. Nausea and other gastrointestinal toxicity were dose limiting at 2400 mg/m<sup>2</sup>/dose (total daily dose of 7200 mg/m<sup>2</sup>) in the Wisconsin trial. Mild fatigue was common in both studies. Two

patients in the Wisconsin trial developed treatment-related, reversible, grade 3–4 granulocytopenia with continuous dosing at 1600 or 2400 mg/m<sup>2</sup> TID during their second and third months of treatment. In contrast, neither granulocytopenia nor thrombocytopenia occurred with the intermittent schedule, although only eight patients in our trial received two or more months of treatment. Thus, the contribution of intermittent *versus* continuous POH treatment on myelosuppression cannot be determined from these small Phase I studies. Individual C<sub>max</sub> and AUC values for PA and DHPA varied greatly in both the present trial and the study of Ripple *et al.* (21). Considering differences in the range of doses studied and the interpatient variation in the data of each trial, mean values of C<sub>max</sub> and AUC were comparable at similar dose levels.

POH and other monoterpenes have been reported to alter p21<sup>ras</sup> expression either by decreasing overall levels of p21<sup>ras</sup> or by inhibiting farnesylation of the protein (6, 8, 10). Lacking easily accessible tumor tissue for such studies, PBMCs are an easily accessible surrogate tissue to gauge the effects of POH on signaling proteins. By Western blot analysis, we found no consistent change in p21<sup>ras</sup> levels or in the levels of several



**Fig. 7** Effects of POH and metabolites on p21<sup>ras</sup> protein in MCF-7 breast carcinoma cells. Cells were treated with FPT inhibitor (FPT3), POH, PA, or DHPA for 48 h. Untreated (control) cells were treated with ethanol only. RAS standard (*right*) was authentic, nonprenylated p21<sup>ras</sup>. FPT3-treated cells demonstrate a partial shift of p21<sup>ras</sup> from the processed form of the protein (*bottom*) to the nonprenylated form (*top*) compared with untreated cells (*Control, left*). POH (250–750  $\mu$ M) treatment decreases the p21<sup>ras</sup> signal. PA and DHPA are approximately 4-fold less potent than POH in decreasing p21<sup>ras</sup> levels.

ras-related proteins in PBMCs obtained from six patients during treatment at the 2800 mg/m<sup>2</sup> dose level.

In agreement with Hohl and Lewis (10), we observed decreased expression of p21<sup>ras</sup> in tumor cells treated with POH. The decrease in p21<sup>ras</sup> was proportional to drug concentration and associated with cytotoxicity, occurring at POH concentrations from 250–750  $\mu$ M. POH has been shown to be a weak inhibitor of the enzymes FPT and geranylgeranyltransferase, with IC<sub>50</sub>s in the millimolar range (12), and has been reported to inhibit isoprenylation of p21<sup>ras</sup> in tumor cells grown in culture. We did not observe changes in the isoprenylation of p21<sup>ras</sup> after POH treatment at concentrations that were toxic to the cells, whereas treatment of the cells with a known specific inhibitor of FPT resulted in shift of p21<sup>ras</sup> from the isoprenylated to the unprocessed form of the protein. These results are also consistent with an earlier study in which monoterpene (including POH and PA)-induced growth inhibition of H-*ras*-transformed rat liver epithelial cells was not related to altered p21<sup>ras</sup> membrane association (22).

Of importance for the present clinical trial, PA and DHPA were much weaker than POH in decreasing p21<sup>ras</sup> expression *in vitro*. In contrast to POH, significant effects on p21<sup>ras</sup> levels were observed only in tumor cells treated with 2 mM concentrations of PA or DHPA, levels and cumulative exposures that exceed the C<sub>max</sub> and AUC values of PA and DHPA found in the plasma of patients treated with POH. These data, combined with lack of uniform effect on p21<sup>ras</sup> expression in patient PBMCs, indicate that p21<sup>ras</sup> function may be neither a relevant target for POH nor a suitable intermediate end point in the dose range tolerated by humans. Another putative mechanism, *e.g.*, up-regulation of the TGF- $\beta$  pathway via the receptor for mannose-6-phosphate/insulin-like growth factor type II (13, 14), may be more relevant at these concentrations of PA and DHPA.

Our experience with the present oral formulation of POH suggests that doses of 1600 and 2100 mg/m<sup>2</sup> taken TID for 14 consecutive days are tolerable in highly motivated patients with refractory solid tumors. Nausea, pyrosis, and fatigue, although of moderate severity, were unremitting and became dose limiting at 2800 mg/m<sup>2</sup> TID for 14 days. Goals of future clinical trials should include: (a) determination of bioavail-

ability for POH and the impact of bioavailability on the variable pharmacokinetics of the metabolites; (b) investigation of a potentially more tolerable POH formulation and/or schedule (for example, a schedule that incorporates a rest period such as 4–5 days on/2–3 days off might be better tolerated at higher doses of POH); and (c) identification of the relevant POH metabolite(s) and intermediate end point(s) for evaluating drug biological activity. If a relevant metabolite and biological end point can be determined, then development of analogues with less gastrointestinal toxicity may be possible.

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