

## A Phase 2a Study of Topical Perillyl Alcohol Cream for Chemoprevention of Skin Cancer

Steven P. Stratton<sup>1,8</sup>, David S. Alberts<sup>1,8</sup>, Janine G. Einspahr<sup>1,8</sup>, Paul M. Sagerman<sup>5,8</sup>, James A. Warneke<sup>3,8</sup>, Clara Curiel-Lewandrowski<sup>2,8</sup>, Paul B. Myrdal<sup>4,8</sup>, Kelly L. Karlage<sup>4</sup>, Brian J. Nickoloff<sup>9</sup>, Chris Brooks<sup>8</sup>, Kathylynn Saboda<sup>8</sup>, Michael L. Yozwiak<sup>8</sup>, Mary F. Krutzsch<sup>8</sup>, Chengcheng Hu<sup>7,8</sup>, Maria Lluria-Prevatt<sup>8</sup>, Zigang Dong<sup>10</sup>, G. Timothy Bowden<sup>6,8</sup>, and Peter H. Bartels<sup>8</sup>

### Abstract

The chemopreventive and antitumor properties of perillyl alcohol (POH) that were studied preclinically indicate that topical POH inhibits both UVB-induced murine skin carcinogenesis (squamous cell tumor models) and 7,12-dimethylbenz(*a*)anthracene-induced murine melanoma (transgenic models involving tyrosinase-driven Ras). A previous phase 1 clinical trial in participants with normal-appearing skin showed that topical POH cream was well tolerated at a dose of 0.76% (w/w). Here, we performed a 3-month, double-blind, randomized, placebo-controlled phase 2a trial of two different doses of topical POH in individuals with sun-damaged skin. Participants applied POH cream twice daily to each dorsal forearm. Baseline and end-of-study biopsies were taken from each participant to evaluate whether the topical application of POH was effective in reversing actinic damage as evidenced by normalization of quantitative skin histopathologic scores and change in nuclear chromatin pattern as measured by karyometric analysis. There was a borderline reduction in the histopathologic score of the lower-dose POH group compared with the placebo ( $P = 0.1$ ), but this was not observed in the high-dose group. However, in the high-dose group, a statistically significant reduction in the proportion of nuclei deviating from normal was observed by the use of karyometric analysis ( $P < 0.01$ ). There was no statistical significance shown in the lower-dose group. No changes were observed in p53 expression, cellular proliferation (by proliferating cell nuclear antigen expression), or apoptosis in either treatment group compared with the placebo group. These results suggest that whereas our karyometric analyses can detect a modest effect of POH in sun-damaged skin, improved delivery into the epidermis may be necessary. *Cancer Prev Res*; 3(2); 160–9. ©2010 AACR.

### Introduction

Perillyl alcohol (POH) is a naturally occurring hydroxylated monocyclic monoterpene that is found in the essential oils of a number of plants, including cherries and lavender. POH, administered systemically, has shown both antitumor and chemopreventive properties that have been studied in both human and animal models. In mouse skin carcinogenesis, the mechanism of action of POH includes induction of apoptosis in tumor cells while

leaving normal cells unaffected (1). It has been shown in a UVB mouse skin carcinogenesis study that topical POH inhibits the development of squamous cell carcinomas and also inhibits UVB-induced activator protein-1 activation (2). The inhibition of UVB-induced activator protein-1 activation is significant because it has been shown that activation of activator protein-1 plays a functional role in mouse skin tumor promotion and progression (3). In addition, in an *in vivo* murine model of 7,12-dimethylbenz(*a*)anthracene-induced melanoma, topical administration of POH resulted in a significant reduction in the proportion of new tumors, suggesting that POH may be a promising chemopreventive agent for the prevention of melanoma (4).

Clinical trial experience with oral POH for cancer treatment has been largely unsuccessful due to gastrointestinal toxicity resulting from the extremely high doses required for systemic activity. Gastrointestinal toxicities were observed as dose limiting in a phase 1 trial of 16 participants with refractory cancer (5). Similar toxicities were observed in phase 2 trials of orally administered POH in advanced ovarian, metastatic colorectal, and metastatic breast cancers, leading to early suspension of study accrual (6–8). Thus, topical application may represent a more promising

**Authors' Affiliations:** <sup>1</sup>Section of Hematology and Oncology, Department of Medicine, <sup>2</sup>Section of Dermatology, Department of Medicine, <sup>3</sup>Section of Surgical Oncology, Department of Surgery, <sup>4</sup>Department of Pharmacy Practice and Science, College of Pharmacy, Departments of <sup>5</sup>Pathology and <sup>6</sup>Cell Biology and Anatomy, College of Medicine, <sup>7</sup>Division of Epidemiology and Biostatistics, Mel and Enid Zuckerman College of Public Health, and <sup>8</sup>Arizona Cancer Center, University of Arizona, Tucson, Arizona; and <sup>9</sup>Department of Pathology, Cardinal Bernardini Cancer Center, Loyola University of Chicago, Chicago, Illinois; and <sup>10</sup>Hormel Institute, University of Minnesota, Austin, Minnesota

**Corresponding Author:** Steven P. Stratton, Arizona Cancer Center, University of Arizona, 1515 North Campbell Avenue, P.O. Box 245024, Tucson, AZ 85724. Phone: 520-626-9295; Fax: 520-626-2244; E-mail: sstratton@azcc.arizona.edu.

doi: 10.1158/1940-6207.CAPR-09-0183

©2010 American Association for Cancer Research.

route of administration for this drug, especially in the context of skin cancer chemoprevention.

We have previously shown in a placebo-controlled phase 1 trial that a topical POH formulation was well tolerated (9). A cream-based topical formulation of POH (10) in two strengths was developed, at 0.3% (w/w; 20 mmol/L) and at 0.76% (w/w; 50 mmol/L). No severe cutaneous toxicities, systemic toxicities, or histopathologic abnormalities were observed with a 0.76% (w/w) cream POH formulation (9). Thirty-two percent of the participants reported mild adverse events at least possibly related to topical cream, but there was no significant difference in the number or grade of skin lesions between the POH-treated forearms and the placebo-treated forearm.

The purpose of this randomized, placebo-controlled, double-blind, phase 2a study was to determine if topical administration of POH cream, applied twice daily to the forearms for 3 months, could reverse actinic damage as evidenced by normalization of quantitative skin histopathologic scores and change in nuclear chromatin pattern as measured by karyometric analysis. In addition, changes in epidermal cell proliferation indices, p53 expression, and apoptosis rates were also measured using immunohistochemistry in skin biopsies.

## Materials and Methods

### Participant eligibility and recruitment

We recruited 89 participants with sun-damaged forearm skin [as evidenced by at least two clinically diagnosed actinic keratoses (AK) in the treatment areas]. Participants were recruited through newspaper, radio and television advertisements, public service announcements, media stories, the Southern Arizona VA Health Care System, the Melanoma/Skin Cancer Screening Program Registry, and the dermatology outpatient clinic at the University Medical Center in Tucson. Participants were screened by the study dermatologists to verify eligibility. All participants provided informed consent and research authorization (Health Insurance Portability and Accountability Act) before initiation of therapy and following a complete explanation of the nature of the study.

Participants  $\geq 18$  y of age were recruited from Pima and adjoining Southern Arizona counties. Female participants were required to be postmenopausal or surgically sterile by hysterectomy. Participants had to have sun-damaged skin as judged by the study physician and have at least two AKs on both of their dorsal forearms. Clinical criteria for determining sun damage included fine wrinkling, coarse wrinkling, mottling, hyperpigmentation, and a global assessment. Participants were eligible if at least 4 wk had passed since the last surgical biopsy, surgical excision, or cryotherapy for AK in the test area. Participants were considered eligible if at least 6 mo had passed since topical treatment for AK or any therapeutic approach for squamous cell carcinoma or basal cell carcinoma in the test area (e.g., 5-fluorouracil, imiquimod, surgical excisions). Participants were required to discontinue all

topical medications to the upper extremities, except for emollients or sunscreens, at least 30 d before study entry. Oral multivitamin intake was limited to Dietary Reference Intake values. Participants also agreed to limit sun exposure as much as possible during the 3-mo study. In addition, participants who used citrus peel topically or consumed citrus peel were excluded.

### Test agent

POH cream formulations were prepared at two strengths [50 mmol/L, 0.76% (w/w) and 20 mmol/L, 0.3% (w/w)], formulated as previously described (10, 11). POH cream and matching placebo creams were packaged in 60 mL plastic ointment tubes and participants were instructed to store tubes at room temperature and avoid exposure to high temperatures. Clinic personnel and study participants were blinded to the specific contents of tubes to ensure the integrity of the double-blinded study. Adherence to the study medication was evaluated through the weighing of returned tubes and self-monitoring via study diaries.

### Trial design

Baseline data (blood, urine, electrocardiogram, and skin biopsies) were obtained before randomization. Participants were randomly assigned by computer to one of three treatment groups (placebo cream, 0.3% POH, or 0.76% POH). Participants were instructed to apply cream twice daily to each dorsal forearm for 3 mo. Additional efficacy evaluations included alterations in patterns of biomarker expression, including p53 expression, cellular proliferation [proliferating cell nuclear antigen (PCNA)], and apoptosis rates (morphologically apoptotic cells; Table 1). Participants were also instructed to maintain a study diary to record daily time of application and any adverse events. Pre-study requirements included dermatologic exam, blood counts, and serum chemistry analysis to rule out any serious underlying disease. In addition, an electrocardiogram was completed at baseline, at steady state (between 4 and 6 wk), and at completion of study to comply with the Food and Drug Administration–mandated cardiac monitoring for new drug products.

### Biopsies

From each participant at baseline, and again after 12 wk (end-of-study), a 4-mm punch biopsy was obtained from

**Table 1.** Schedule of biopsies and end points for analysis

Biopsy	Time	Location	Analysis
1	Baseline	R or L forearm	p53, PCNA, apoptosis, histopathology, karyometry
2	End of study	R or L forearm*	p53, PCNA, apoptosis, histopathology, karyometry

\*Consistent with biopsy #1.

sun-damaged skin on one dorsal forearm, 10 cm distal to the lateral epicondyle, for dermatopathology, karyometry, and immunohistochemical analysis. The matched end-of-study biopsy was collected at a point 2 cm adjacent to the location of the baseline biopsy, oriented using baseline photographs of the forearm. Biopsies were formalin fixed for analyses.

### Histopathologic analysis

Four-millimeter skin punch biopsies were collected from the lateral posterior forearm, approximately 6 cm distal to the elbow joint in all randomized participants at baseline and at the end of study. The end-of-study biopsies for histopathology, karyometry, and immunohistochemical markers were collected 10 to 18 d after the last dose of test article was applied to allow for resolution of any local site reactions because any acute inflammation could confound these results. Biopsies were evaluated in a blinded fashion by one study dermatopathologist (P.S.) and scored according to a histopathologic scoring system previously described (12). Formalin-fixed biopsies were stained by routine H&E. The total histopathologic score was the sum of four criteria including basal or suprabasilar pleomorphism (atypia), inflammation, hyperkeratosis, and parakeratosis. Change in the score for paired biopsies was calculated by subtracting the baseline score from the end-of-study score. The high- and low-dose treatment groups were compared with the placebo-treated group.

### Immunohistochemistry

Immunohistochemical staining was done using peroxidase (p53) or alkaline phosphatase avidin with a fast red chromagen (PCNA) and hematoxylin counterstain (Ventana Medical Systems) on an automated VMS 320 immunostainer (Ventana Medical Systems) at 1:100 dilutions. Anti-PCNA PC10 and anti-p53 (Calbiochem) were used at 1:200 dilution in antibody diluent (Ventana Medical Systems). Negative controls (to ensure lack of nonspecific staining) were run on each sample by substituting antibody diluent for the antibody. Controls included in each run were tonsil tissue for PCNA and T47D human breast cancer cells for p53 to ensure proper staining and to assess variability of staining intensity. Tissue sections were analyzed with ImagePro Plus software (Media Cybernetics), a Leica DMR microscope, and a Sony 3 charge-coupled device color video camera. The percentage positive area per 40× field was determined for each biopsy. Three representative 40× fields were used for image analysis and the entire epidermis was included so that all basal and suprabasal cells were analyzed.

### Apoptosis by morphology

The number of apoptotic cells was assessed based on morphology (i.e., condensed and/or pyknotic nuclei, eosinophilic cytoplasm, formation of apoptotic bodies) on H&E per 100 basal keratinocytes. The entire biopsy was used for each apoptosis assessment.

### Karyometric analysis of topical drug effects

For the 27 participants treated with the higher dose of POH, 1,330 nuclei were recorded at baseline and 1,945 nuclei at the end of study. For the 17 fully evaluable participants receiving the placebo cream, 1,318 nuclei were recorded at baseline and 1,394 nuclei at the end of study. Tissue sections were cut at 5 μm and stained with H&E under carefully controlled conditions, as previously reported (13). A high-resolution digitized imagery of the histopathologic sections was recorded on a Sony 3 charge-coupled device video-microphotometer, equipped with a 100:1, numerical aperture 1.4, plan apochromatic oil immersion objective (NIKON). Sampling density was adjusted to 6 pixels per linear micron. A 620-nm interference filter was used to enhance contrast. The nuclei in the digitized imagery were segmented by a semiautomated procedure with manual correction where needed. For each nucleus, features descriptive of the texture of the nuclear chromatin pattern were computed. In the feature selection, a statistical difference for two data sets at a significance level of  $P < 0.005$  was chosen. This was done in view of the multiple comparisons—for 95 different features—to keep the selection of a spuriously significant feature at a chance of less than 1 in 200, and as a compromise to not increase the chances of a type II error (i.e., unnecessarily rejecting a useful feature). For a skin biopsy to be evaluable, a target of 100 stained nonoverlapping nuclei per biopsy was set for the analysis, randomly selected after pathologist review and demarcation of the areas to be imaged.

### Statistical analysis

Histopathologic scoring and biomarker analyses were conducted using Stata version 10. To determine if histopathologic scores varied by treatment group at baseline, the Kruskal-Wallis test was used. Changes in histopathologic scores for treated sun-damaged skin were compared between any treatment group and the placebo group using the Wilcoxon rank sum test.

The values for p53 and PCNA expression were derived by averaging the three highest-staining 40× fields for each biopsy. Apoptosis measures were calculated by counting the number of apoptotic cells and basal cells and then standardizing the results to the number of apoptotic cells per 100 basal cells. The Wilcoxon rank sum test was used to compare the change in each measure between the treatment groups and the placebo group. Proportions of adverse events in different groups were compared using Fisher's exact test.

In the karyometric analyses, to determine differences for the karyometric features, the Kruskal-Wallis test (14) and ambiguity function of Genchi and Mori (15) were used. Wilks' lambda was used to evaluate the difference between the four major features selected from the stepwise linear discriminant algorithm. A reduction in the proportion of nuclei assigned to the baseline data set was used as a measure of efficacy of the drug. The statistical significance of such a reduction was established based on the Fleiss tables (16).

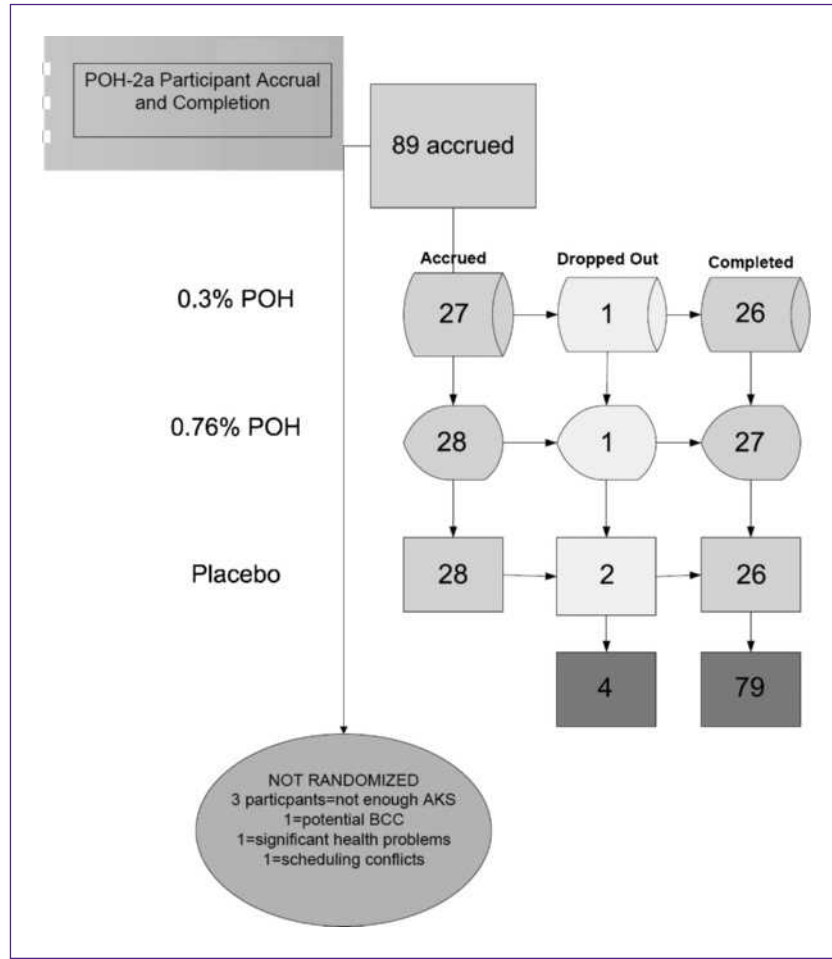


Fig. 1. Flowchart of study accrual.

The Kolmogorov-Smirnov statistic was used to determine the deviation from the “normal” in the end-of-study biopsy nuclei.

**Results**

**Participant characteristics**

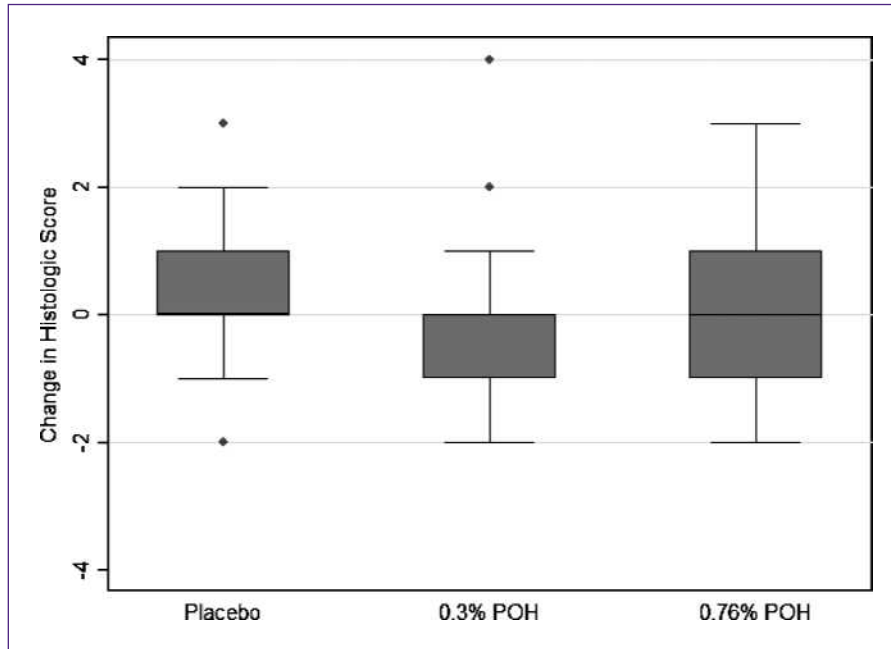
Eighty-nine participants were accrued and 83 participants were randomized (Fig. 1), with a total of 79 participants completing the study. Table 2 shows the age and gender distribution of participants within the three groups (placebo, 0.3% POH, and 0.76% POH). There was no statistically significant difference in gender (Pearson  $\chi^2 = 0.95, P = 0.62$ ) or age [ $F_{(3, 79)} = 0.49, P = 0.69$ ] between the three groups. All participants had sun-damaged skin with at least two clinically diagnosed and visible AKs on each of their dorsal forearms. Baseline variables including age, gender, histologic score, and number of AKs were all well balanced (no statistically significant differences) across the three study groups. To determine adherence, the number of drug applications actually administered to the skin was calculated as a percentage of the total pre-

scribed number of applications, and the weight of the drug actually consumed was calculated as a percentage of the total weight of drug expected to be consumed under the protocol. No statistically significant difference in either

**Table 2. Gender and age of participants**

Treatment group	Female	Male	Total
<b>Placebo</b>			
Average age (SD), y	63.9 (8.4)	68.1 (10.4)	66.9 (9.9)
n (%)	8 (29)	20 (71)	28 (100)
<b>0.3% POH</b>			
Average age (SD), y	66.1 (11.6)	69.7 (10.4)	68.8 (10.6)
n (%)	7 (26)	20 (74)	27 (100)
<b>0.76% POH</b>			
Average age (SD), y	68.0 (7.4)	67.6 (11.6)	67.6 (10.9)
n (%)	5 (18)	23 (82)	28 (100)
<b>Total</b>			
Average age (SD), y	65.7 (9.1)	68.4 (10.7)	67.7 (10.4)
n	20	63	83

Downloaded from <http://aacrjournals.org/cancerpreventiononresearch/article-pdf/3/2/160/2336864/160.pdf> by guest on 07 February 2025



**Fig. 2.** Box plots of change in histopathologic score for sun-damaged skin by treatment. A borderline significant difference in score was detected between the low-dose POH group and the placebo group ( $P = 0.1005$ ), whereas the score change in the high-dose group was not statistically significantly different from that in the placebo group ( $P = 0.4124$ ).

of these two adherence measures was observed across the three study groups (data not shown).

### Histopathologic scoring

Baseline and end-of-study biopsies of sun-damaged skin were evaluated by the study dermatopathologist and scored according to a histopathologic scoring system previously described (12). This score reflects the presence or absence of two criteria (hyperkeratosis and parakeratosis) and the severity (absent, mild/moderate, or severe) of two more criteria (pleomorphism and inflammation). Scoring of the biopsies taken at baseline did not vary by treatment ( $P = 0.42$ ). Figure 2 shows the box plot of distribution of the change in histopathologic score changes for biopsies taken from sun-damaged skin by treatment group. Table 3 contains the statistical analyses of these data. The score change in the lower-dose POH group approached a statistically significant difference versus the placebo group ( $P = 0.10$ ), which may be meaningful given the relatively small sample size. However, there was not a statistically significant difference between the high-dose POH group and the placebo group ( $P = 0.41$ ).

### Biomarker expression

No statistically significant differences by treatment group were apparent in the sun-damaged skin for change in p53 expression, cellular proliferation (as measured by PCNA expression), or apoptosis (data not shown).

### Karyometric analysis

For the samples in the high-dose POH group, the results from the analysis using the Kruskal-Wallis test (14) and the ambiguity function of Genchi and Mori (15, 17) indicated the presence of statistically significant differences for

more than 20 karyometric features, at  $P < 0.005$ . The ambiguity function values, however, suggested poor discrimination potential for nearly all of them. Table 4 lists the best of these features, with their mean values, the percent difference between the baseline and end-of-study data sets, and the ambiguity function value. An ambiguity value of 1.0 indicates no discrimination. All features are given in relative arbitrary units. All of the features listed in Table 4 had a  $P$  value of  $<0.005$ .

The data sets treated with the higher dose were each divided into two halves by assigning alternate nuclei to the first or the second halves. The first-half data sets from the baseline and the end-of-study nuclei were used as a training set, with 668 and 995 nuclei. These data were submitted to a stepwise linear discriminant algorithm. It selected four features from the set listed in Table 4. The values following the feature ID number represent the standardized

**Table 3.** Average change in histopathologic score by treatment group

Treatment group	Type of skin	n	Change in histopathologic score				SD
			Mean*	Median	Min	Max	
Placebo	SD skin	26	0.4	0	-2.0	3.0	1.3
0.3% POH	SD skin	26	-0.1	0	-2.0	4.0	1.3
0.76% POH	SD skin	27	0.1	0	-2.0	3.0	1.4
Total	SD skin	79	0.1	0	-2.0	4.0	1.3

Abbreviation: SD, sun-damaged.

\*Mean change from baseline.

**Table 4.** Karyometric features

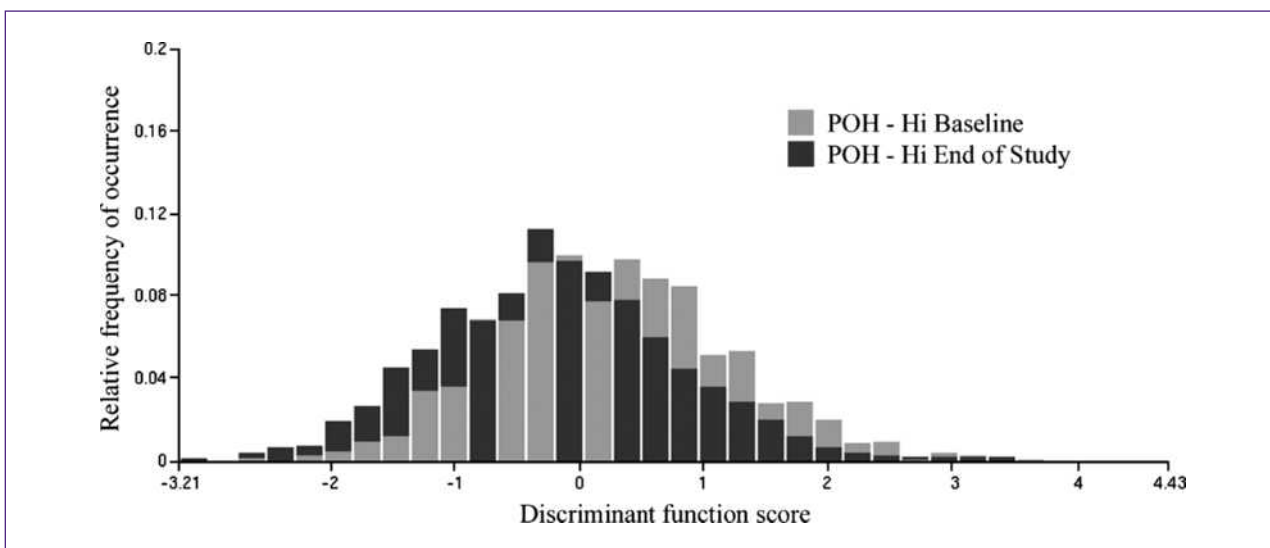
Feature ID	Mean value		%Difference	Ambiguity value
	Baseline	End of study		
#307 run percentage	783.4	695.3	-11.2	0.981
#001 total absorbance	0.740	0.665	-10.2	0.981
#002 rel. nuclear area	35.2	31.5	-10.5	0.988
#275 run length 5-6	4.2	3.6	-14.3	0.987
#321 no. dense-staining pixels	603.3	548.7	-9.1	0.990
#303 short run emphasis	0.51	0.52	+2.0	0.986
#014 pixel absorbance Int. 0.7	13.8	12.4	-10.1	0.981
#314 pixel absorbance clumpiness	0.72	0.71	-1.4	0.995

coefficients in the discriminant function. These selected features include the run percentage (#307, 1.196), short run emphasis (#303, -0.823), the relative frequency of occurrence  $dn/n$  in the pixel absorbance histogram interval from absorbance 0.70 to 0.79 (#014, 0.271), and the relative nuclear area (#002, 0.662). Wilks'  $\lambda$  was reduced to 0.9595 (i.e., only by 4.1%). The proportion of nuclei assigned to the baseline data set (i.e., the set with presumably greater deviation from normal) was reduced by 15.3%. For the sample size of 668 nuclei in the smaller data set, this is a significant reduction at  $P < 0.01$  and a power of test of 0.99. The same discriminant function was next applied to the second-half data sets. The reduction in the number of nuclei assigned to the baseline data set here was 19.1%.

For the combined data sets, of 1,330 and 1,945 nuclei, the classification resulted in a reduction from 55.9% to 39.5% (i.e., by 16.4%). For the sample sizes involved, this reduction is highly significant at  $P < 0.01$  and a power of test of 0.99. The distribution of discriminant function

scores is shown in Fig. 3. There is a notable shift to the left on the score axis, indicating less deviation from normal in the end-of-study biopsy nuclei. The two distributions were statistically significantly different, as shown by the Kolmogorov-Smirnov statistic. The  $D_{max}$  value was 0.179288 and the  $D_{\alpha}$  value was 0.0546, which resulted in a  $P$  value of  $<0.0001$ . Application of the discriminant function to the placebo-treated end-of-study data set (of 1,394 nuclei) resulted in a reduction of the proportion of nuclei assigned to the baseline data set from 55.9% to 53.4% (i.e., by only 1.5%).

Discriminant function scores for the three treatment groups at baseline and at the end of study are shown in Table 5. As indicated above, only one discriminant function was used throughout this study, and the same function was applied in all comparisons. The function compounds, as a linear combination, the values of the four features listed above (Table 4). It is evident that there is a significant shift for the group treated with the high dose of POH, with no overlap of confidence limits

**Fig. 3.** Distribution of discriminant function scores for the high-dose POH samples at baseline and at the end of study.



**Table 5.** Discriminant function scores determined by karyometric analysis of biopsies from sun-damaged skin

	Baseline, mean (95% CI)	End of study, mean (95% CI)
POH (high)	0.2492 (0.1982-0.3002)	-0.1705 (-0.2145 to -0.1265)
POH (low)	0.2367 (0.1786-0.2948)	0.2747 (0.2200-0.3293)
Placebo	0.2392 (0.1822-0.2962)	0.1498 (0.0998-0.1997)

Abbreviation: 95% CI, 95% confidence interval.

between baseline and the end of study. The baseline and end-of-study values for the low-dose group are statistically indistinguishable, as are the values for the placebo-treated group. Also, there are no statistically significant differences between the baseline groups.

#### Response in individual participants

For the high-dose POH group, a total of 3,275 nuclei were recorded, 1,330 at baseline and 1,945 at the end of study. There were 27 cases, but a paired comparison was available for only 18 cases. For most of these cases, the full target sample size of 100 nuclei had not been reached. The average number of nuclei per case was 78 nuclei. Figure 4 shows the histogram of the relative number of cases plotted over a scale of proportion of nuclei, decreased or increased, at the end of study. The large peak around the zero mark reflects case-to-case sampling error. In the lower half of Fig. 4, the same plot is shown for the placebo-treated group. Here, no paired comparison was possible, as the high-dose baseline cases were different from the placebo-treated cases. The differences were computed from a scrambled list and represent an estimate of the difference distribution, adjusted to the same percentage as presented in the top portion of Fig. 4. In this study, the difference in proportion of nuclei assigned to the baseline data set was defined as a measure of efficacy. The large sample size of nuclei available for establishing the overall efficacy of the intervention provided significance at  $P < 0.01$  and a power of test of 0.99. For the average sample size of 75 nuclei per case, a reduction in the proportion of nuclei from 55% to 30% would be needed to establish statistical significance at  $P < 0.05$  and a power of test of 0.80 for efficacy in an individual case.

#### Low-dose topical administration of POH

In the low-dose group, no statistically significant effect could be established. The discriminant function derived in the high-dose group was applied to the end-of-study data set from the low-dose treated samples. It resulted in a decrease of nuclei assigned to the baseline data set by only

0.2%, from 55.9% to 55.7%, with sample sizes of 1,330 and 1,657 nuclei, respectively. In the low-dose POH baseline and the low-dose POH end-of-study data sets, the percentage of nuclei applied to the baseline data set increased by 0.6%, from 55.1% to 55.7%, with sample sizes of 1,346 and 1657 nuclei, respectively.

#### Clinical response and safety evaluation

Participants kept a daily diary during the study, which included time of application and any adverse events. Each subject recorded the time of onset and resolution of any such events, and events were rated as mild, moderate, or severe. Diaries were reviewed by clinic personnel at each visit.

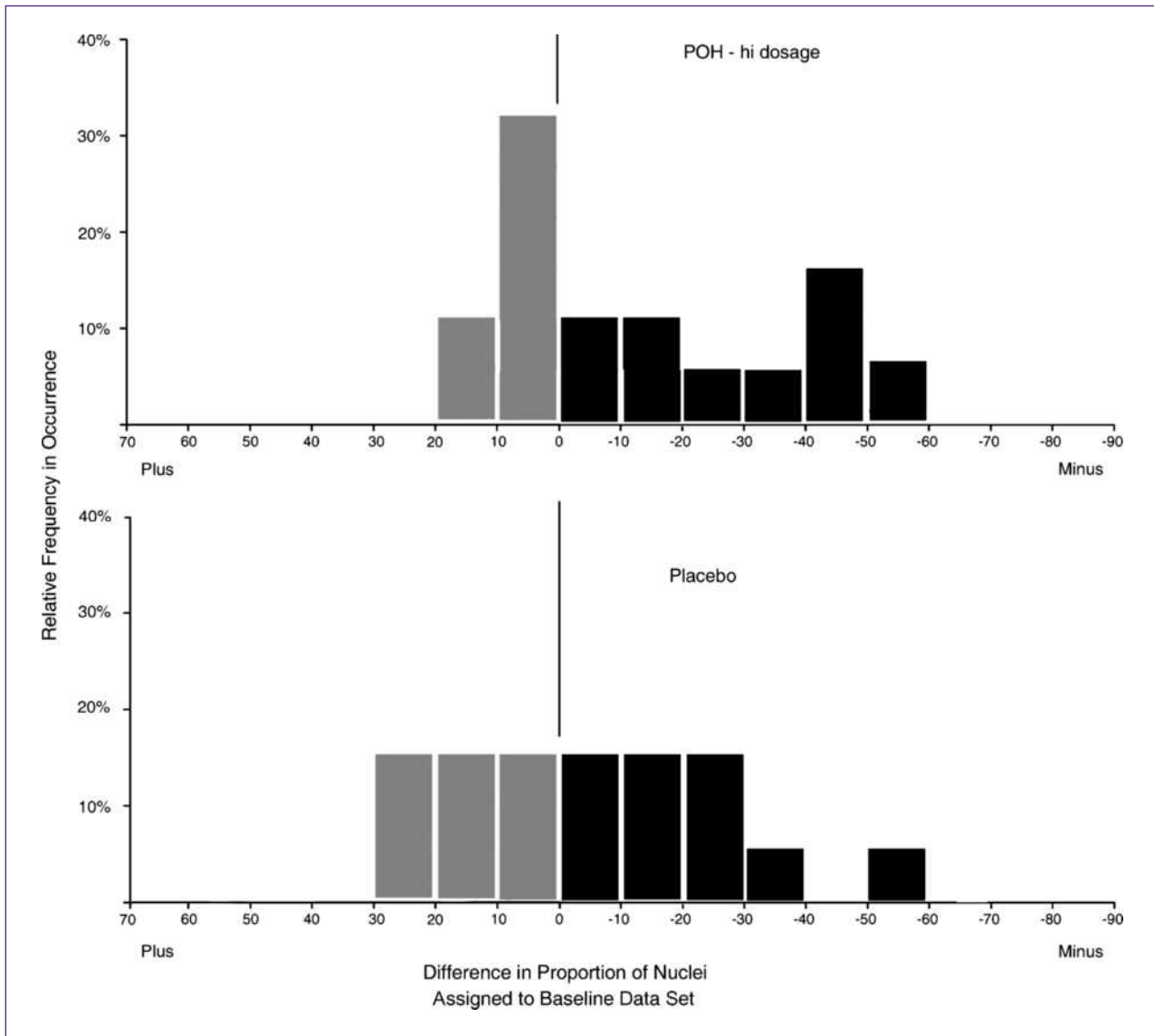
Of the total events reported in the study, 66% were "possibly" or "probably" related to study treatment, as rated by the study physician. Only one event in the high-dose group was rated "definitely" related to study treatment. This event was tenderness in the test area, which was rated as mild in severity. Table 6 shows the skin-related events by participant. The most frequent event occurring in the treatment groups was "rash, redness, erythema" rated mild or moderate in 54% of participants in the high-dose group and in 56% of participants in the low-dose group. The placebo group reported the same type of event in 43% of the participants. There was no statistically significant difference in the proportion between the treatment and the placebo groups.

#### Discussion

We performed a phase 2a dose-finding and efficacy trial of topically administered POH in a cream formulation having previously documented suitable skin tolerability in a phase 1 study (9). Trial participants had sun-damaged skin with at least two clinically diagnosed AKs on each dorsal forearm to document at least a moderate level of risk for nonmelanoma skin cancer. There were multiple primary end points (i.e., normalization of quantitative skin histopathologic scores and skin nuclear chromatin pattern) and secondary end points (i.e., skin biopsy PCNA, apoptosis, and p53 biomarker indices).

Our interest in POH as a topical chemopreventive agent for skin cancer was fueled by positive preclinical studies, documenting considerable activity in both UVB-induced murine squamous cell carcinoma and melanoma (2, 4). There is increasing interest in the development of topically administered agents that could slow the progression of moderately or severely sun-damaged skin to nonmelanoma skin cancer, and the strong preclinical chemopreventive activity and excellent tolerability of topically administered POH make it a strong candidate for further clinical development.

As reported, there was a borderline difference that approached statistical significance in the change in sun-damaged dorsal forearm biopsy histopathologic score between the study participants treated with placebo cream and those treated with 0.3% (low-dose) POH; however, the high-dose (0.76%) POH group did not show a similar



**Fig. 4.** Histograms showing relative frequency of occurrence of cases with specific differences in the proportion of nuclei assigned to the baseline data sets, comparing the high-dose POH group (top) with the placebo group (bottom).

trend. This was surprising, given the strong preclinical results, and the apparent lack of dose dependence indicates that either the drug is not effective or the pharmacokinetic profile (i.e., the residence time of the drug at the site of action) was not suitable. However, in contrast to the results obtained using the histopathologic scoring system, a dose-dependent reduction in nuclear abnormalities was observed by karyometric analysis. This supports the hypothesis that the negative histopathologic results were due to inadequate exposure of the drug to its target, most likely due to the suboptimal pharmacokinetic properties of the formulation.

Using karyometric analysis, we observed that the higher dose of POH (but not the lower dose of POH) was asso-

ciated with a statistically significant reduction in the proportion of skin biopsy nuclei deviating from normal (i.e.,  $P < 0.01$  with a power of test of 0.99). In case-by-case comparisons, it was difficult in this study to establish the statistical significance of the high-dose POH versus the placebo, due to the limited sample sizes of nuclei recorded for each participant. Of those participants treated with topically administered higher-dose POH, 31% had a statistically significant reduction in proportions of nuclei deviating from normal, versus just 17% in the placebo group. This trend to less deviation from normal may very well represent a beneficial cancer-preventive effect that will continue to be studied in future clinical trials using karyometric analysis as a biomarker end point.



**Table 6.** Skin-related events by participants

Symptom/ sign	Severity	Placebo (n = 28), n (%)	0.30% POH (n = 27), n (%)	0.76% POH (n = 28), n (%)
Rash, redness, erythema	No rash	16 (57)	12 (44)	13 (46)
	Mild	11 (39)	14 (52)	15 (54)
	Moderate	1 (4)	1 (4)	0 (0)
Flaking, crusting	No flaking	23 (82)	20 (74)	23 (82)
	Mild	5 (18)	7 (26)	4 (14)
Burning or stinging	Moderate	0 (0)	0 (0)	1 (4)
	No burning	25 (89)	24 (89)	27 (96)
	Mild	2 (7)	3 (11)	1 (4)
Pruritus	Moderate	1 (4)	0 (0)	0 (0)
	No pruritus	22 (79)	23 (85)	23 (82)
	Mild	6 (21)	3 (11)	4 (14)
	Moderate	0 (0)	1 (4)	1 (4)

p53, PCNA, and apoptosis were not affected by the high- or low-dose POH concentrations administered topically twice daily over 3 months. These negative biomarker results match those observed in our prior positive skin cancer chemoprevention studies of oral vitamin A and topical difluoromethylornithine (18–20). On the other hand, karyometric analysis of nuclear chromatin abnormality in baseline and end-of-study sun-damaged skin once again has proved useful as an “integrating biomarker” of response to chemopreventive agent activity (13, 18–23).

The only adverse event that occurred frequently was a mild or moderate local reaction (termed rash, redness and/or erythema) that occurred at a rate of 56% and 54% for the low- and high-dose POH, respectively. However, this reaction may very well be due to the vehicle cream because the placebo group reported this same type of event in 43% of participants, and this proportion is not statistically significantly different from that observed in the treatment groups. Similarly, the phase 1 trial also showed a lack of any serious adverse events (9) and no significant differences in the mild,

study-related adverse events between the treatment and the placebo groups.

Obviously, topical POH cannot be effective as a skin cancer chemoprevention agent if it penetrates poorly into epidermal skin, or (possibly) if it rapidly penetrates the skin transdermally. An ongoing direction in our research program includes both *in vivo* preclinical and clinical projects to compare the skin penetration of the parent POH topical formulation and a novel prodrug topical formulation, engineered specifically to concentrate POH in the epidermis (24, 25). Development of true pharmacokinetic profiles (concentration versus time curves) of percutaneous absorption and transdermal penetration of topically administered drugs in the context of a trial such as this one is impractical due to the excessive numbers of biopsies that would be required. Development of a prodrug of POH engineered with appropriate lipophilicity will allow for *ex vivo* human skin models to be developed that can compare the skin pharmacokinetics of parent POH and POH prodrugs to ensure adequate residence time of the active form of the drug.

In conclusion, the results of this study suggest that topically administered POH at 0.76% twice daily for 3 months has a modest effect in reducing nuclear chromatin abnormality in moderately to severely sun-damaged skin. Further studies of prodrug formulations of POH may result in a more active formulation of this novel topical agent that could be used in future nonmelanoma skin cancer chemoprevention studies.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Grant Support

National Cancer Institute/NIH grants CA027502 and CA023074.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 8/27/09; revised 9/24/09; accepted 9/25/09; published OnlineFirst 1/26/10.

### References

- Mills JJ, Chari RS, Boyer IJ, Gould MN, Jirtle RL. Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. *Cancer Res* 1995;55:979–83.
- Barthelman M, Chen W, Gensler HL, Huang C, Dong Z, Bowden GT. Inhibitory effects of perillyl alcohol on UVB-induced murine skin cancer and AP-1 transactivation. *Cancer Res* 1998;58:711–6.
- Cooper SJ, MacGowan J, Ranger-Moore J, Young MR, Colburn NH, Bowden GT, et al. Expression of dominant negative c-jun inhibits ultraviolet B-induced squamous cell carcinoma number and size in an SKH-1 hairless mouse model. *Mol Cancer Res* 2003;1:848–54.
- Lluria-Prevatt M, Morreale J, Gregus J, et al. Effects of perillyl alcohol on melanoma in the TPras mouse model. *Cancer Epidemiol Biomarkers Prev* 2002;11:573–9.
- Ripple GH, Gould MN, Arzooonian RZ, et al. Phase I clinical and pharmacokinetic study of perillyl alcohol administered four times a day. *Clin Cancer Res* 2000;6:390–6.
- Bailey HH, Levy D, Harris LS, et al. A phase II trial of daily perillyl alcohol in patients with advanced ovarian cancer: Eastern Cooperative Oncology Group Study E2E96. *Gynecol Oncol* 2002;85:464–8.
- Meadows SM, Mulkerin D, Berlin J, et al. Phase II trial of perillyl alcohol in patients with metastatic colorectal cancer. *Int J Gastrointest Cancer* 2002;32:125–8.
- Bailey HH, Attia S, Love RR, et al. Phase II trial of daily oral perillyl alcohol (NSC 641066) in treatment-refractory metastatic breast cancer. *Cancer Chemother Pharmacol* 2008;62:149–57.
- Stratton S, Saboda K, Myrdal PB, et al. Phase 1 study of topical perillyl alcohol cream for chemoprevention of skin cancer. *Nutr Cancer* 2008;60:325–30.

10. Gupta A, Myrdal PB. Development of a perillyl alcohol topical cream formulation. *Int J Pharm* 2004;269:373–83.
11. Gupta A, Stratton SP, Myrdal PB, Gupta A, Stratton SP, Myrdal PB. An HPLC method for quantitation of perillyl alcohol in a topical pharmaceutical cream formulation. *J Pharm Biomed Anal* 2005;37:447–52.
12. Bozzo P, Saboda K, Einspahr JG, et al. Reliability and validity of a histologic score as a marker for skin cancer chemoprevention studies. *Anal Quant Cytol Histol* 2003;25:285–92.
13. Bartels PH, Krouse RS, Prasad AR, et al. Actinic damage in histopathologically normal skin. *Anal Quant Cytol Histol* 2008;30:316–22.
14. Kruskal W, Wallis W. Use of ranks on one-criterion variance analysis. *J Am Stat Assoc* 1952;47:583–621; addendum 48:907–11.
15. Genchi H, Mori K. Evaluation and feature extraction on automatic pattern recognition system. *Denki Tsuchin Gakkai Pari* (in Japanese) 1965;1:1–6.
16. Fleiss J. *Statistical methods for rates and proportions*. New York: Wiley; 1981.
17. Bartels P, Olson G. Computer analysis of lymphocyte images. In: Catsimpoilas N, editor. *Methods of cell separation*. 1980. New York: Plenum Press; 2005, p. 1–99.
18. Alberts D, Ranger-Moore J, Stratton S, et al. Safety and efficacy study of dose intensive oral vitamin A in participants with sun damaged skin. *Clin Cancer Res* 2004;10:1875–80.
19. Alberts DS, Dorr RT, Einspahr JG, et al. Chemoprevention of human actinic keratoses by topical 2-(difluoromethyl)-dl-ornithine. *Cancer Epidemiol Biomarkers Prev* 2000;9:1281–6.
20. Bartels P, Yozwiak M, Einspahr JG, et al. Chemopreventive efficacy of topical difluoromethylornithine and/or triamcinolone in the treatment of actinic keratoses analyzed by karyometry. *AQCH* 2009;31:355–66.
21. Bartels PH, Yozwiak ML, Bartels HG, et al. Limits of detection of chemopreventive efficacy: karyometry of skin biopsies. *Cancer Epidemiol Biomarkers Prev* 2008;17:1689–95.
22. Frank DH, Kimler BF, Fabian CJ, et al. Digital image analysis of breast epithelial cells collected by random periareolar fine-needle aspirates (RPFNA) from women at high risk for breast cancer taking hormone replacement and the aromatase inhibitor, letrozole, for six months. *Breast Cancer Res Treat* 2009;115:661–8.
23. Bartels PH, Fabian CJ, Kimler BF, et al. Karyometry of breast epithelial cells acquired by random periareolar fine needle aspiration in women at high risk for breast cancer. *Anal Quant Cytol Histol* 2007;29:63–70.
24. Jacobson EL, Kim H, Kim M, et al. A topical lipophilic niacin derivative increases NAD, epidermal differentiation and barrier function in photodamaged skin. *Exp Dermatol* 2007;16:490–9.
25. Jacobson EL, Kim M, Kim H, Wondrak GT, Jacobson MK. Developing topical prodrugs for skin cancer prevention. In: Alberts D, Hess L, editors. *Fundamentals of cancer prevention*. Berlin-Heidelberg: Springer-Verlag.