Chemoprevention of Human Actinic Keratoses by Topical 2-(Difluoromethyl)-dl-ornithine


Abstract
α-2-(Difluoromethyl)-dl-ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, has been shown to suppress skin carcinogenesis in murine models after oral or topical administration. We designed a randomized, placebo-controlled study using a topical hydrophilic ointment formulation with or without 10% (w/w) DFMO. Forty-eight participants with moderate-severe actinic keratoses (AKs) on their forearms (i.e., at least 10 well-circumscribed lesions on the lateral surface) completed a 1-month run-in on placebo ointment. Before randomization, all lateral forearm AKs were circled, counted, photographed, and skin biopsies were obtained for DFMO and polyamine levels. Then participants were randomized to receive DFMO ointment on the right versus the left forearm and placebo hydrophilic ointment on the contralateral forearm twice daily for 6 months. DFMO was not detected in the blood of any subject, and there were no systemic toxicities. None of a subsample of 17 placebo forearms had measurable concentrations of DFMO, whereas 13 of the corresponding DFMO-treated forearms had high DFMO skin levels. As compared with placebo, the 6-month DFMO treatment caused a 23.5% reduction in the number of AKs (P = 0.001) as well as significant suppression of AK biopsy spermidine levels (26%; P = 0.04). Seven of the 48 (14.6%) participants experienced severe (2; 4.2%) or moderate (5; 10.4%) inflammatory reactions on their DFMO-treated arms which required dosing modification. Topical DFMO for 6 months can reduce the number of AK lesions and skin spermidine concentrations in high-risk participants and deserves additional study as a skin cancer chemopreventive agent.

Introduction
It has been estimated that more than 900,000 NMSCs (i.e., squamous cell carcinoma and basal cell carcinoma) were diagnosed in the United States in 1997 (1). The majority of these cutaneous NMSCs develop within or contiguous to areas of preexisting premalignant AKs (2, 3). The presence of AKs represents a major risk factor for NMSC (3, 4). There also is strong evidence that the incidence of NMSC is increasing throughout the United States and other countries, particularly in regions closer to the equator where sunlight is more intense (5–14). Incidence rates for NMSC are expected to increase further as the population ages and larger amounts of UV radiation reach the surface of the earth (15–19). Although the mortality rate for these skin cancers is low, their treatment is associated with considerable morbidity and remarkably high medical costs (20).

The most common treatments of AK continue to be a topical application of 5% fluorouracil cream (21) or liquid nitrogen (22). Both of these methods result in severe inflammation, erythema, and superficial ulceration. There continues to be a need for the development of less toxic drugs which can be applied chronically as chemopreventive agents for patients with severely sun-damaged skin and AKs.

DFMO is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (Fig. 1), which is the rate-limiting enzyme in polyamine synthesis, and decreases intracellular levels of putrescine and spermidine in the skin and other vital tissues (23, 24). In conjunction with the administration of model carcinogens, DFMO significantly reduced tumor incidence in several mammalian in vivo tests for chemopreventive activity (25, 26). Additionally, DFMO chemopreventive activity has been demonstrated in chemical and UV models of mouse skin carcinogenesis (27–29). Gensler has shown that p.o.-administered DFMO reduced UVB-induced skin cancers in C3H/HeN mice from 38% in placebo-treated controls to 9% in treated animals (30). Similarly, topically administered DFMO in an acetone vehicle dramatically reduced UVB-induced skin cancers in BALB/c mice (4). In adult participants with psoriasis, the application of 10% DFMO cream resulted in a 66% reduction in psoriatic lesions in the skin and a marginal improvement in psoriatic lesions (31).

The major purpose of this study was to determine whether topically administered DFMO is associated with a significant reduction in the following: (a) numbers of AKs on the forearms; and (b) polyamine levels in punch biopsies of AKs to assess the effects of DFMO on ornithine decarboxylase activity. The secondary purpose of this study was to determine the

¹ The abbreviations used are: NMSC, non-melanoma skin cancer; AK, actinic keratoses; DFMO, α-2-(difluoromethyl)-dl-ornithine; HPLC, high performance liquid chromatography.
² 2-(Difluoromethyl)-dl-ornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase, an enzyme in polyamine synthesis.
3 H. L. Gensler, personal communication.
long-term tolerance and adherence of participants with AKs to topical DFMO administration.

Materials and Methods

Eligibility Criteria
Males or postmenopausal females, at least 30 years of age, with at least 10 discrete, clinically diagnosable AKs on the lateral, sun-exposed surface of each forearm (determined by a dermatologist) were eligible for this study. The participants had not received topical or systemic therapy for AK for the past 3 months or any other topical medications on their arms for at least 30 days (excluding emollients and sunscreens). They also were free of medication or any disease that would cause even minor immunosuppression.

DFMO Formulation
DFMO was supplied as a white powder of the monohydrate, monochloride (MW = 236.65) from Marion-Merrell Dow Pharmaceutical Company (Lot No. 71,782a; Kansas City, MO). The drug was weighed and mixed by blender into a 10% w/w concentration in hydrophilic ointment USP (Lot No. 0210; East Fougera & Company, Melville, NY). Once mixed, the ointment was transferred to polyethylene-lined, 30-g metal ointment tubes which were then crimp-sealed to preclude exposure to light and air. A HPLC assay, as described below, was used to determine the chemical stability of DFMO in hydrophilic ointment over a 6-month period during storage at 4°C in the sealed metal tubes. There was <1% loss of DFMO noted at the 6-month time point.

When applied to full thickness skin samples in vitro using a standard skin chamber apparatus, uptake after 24 h at 37°C was considerable in mouse skin (6.8 mg/g wet weight), but there was negligible transdermal penetration of human skin (32).

On the basis of data from toxicological and pharmacological experiments, the Food and Drug Administration approved our Investigational New Drug application for a Phase IIIB clinical study of DFMO in hydrophilic ointment (data not shown).

Study Design
To obtain baseline adherence data, a 1-month run-in was performed during which participants used a placebo formulation (hydrophilic ointment) twice daily on both right and left forearms. Participants who signed University of Arizona Human Subjects Committee-approved informed consents applied ~1 inch (in length) of the formulation to the exposed area of the forearm from the elbow to the knuckles of the hand in the morning and at night. Before randomization, participants were stratified on the basis of gender and numbers of AKs on the forearms. Participants were then randomly assigned, in a double-blind fashion, to treatment with hydrophilic DFMO ointment on the right versus the left forearm and placebo hydrophilic ointment on the contralateral forearm twice daily for 6 months. The weighed tubes were color coded and had large “left” and “right” labels. At the end of each month, the tubes were returned to the clinic and reweighed to provide a measure of adherence. Participants also maintained a daily diary of ointment usage.

Before the first application of the placebo ointment, at randomization, and at each succeeding monthly visit, a clinical toxicity assessment was completed. Data were collected on the presence of any redness (erythema), dryness, burning, itching or pain that had been experienced on each forearm. Each toxicity measure was rated for frequency and severity. Severity of each symptom was assessed using a scale in which level “0” was indicative of no symptoms, “1” indicated mild symptoms (easily tolerated), “2” indicated moderate symptoms (caused some discomfort and inconvenience), and “3” indicated severe symptoms (caused considerable discomfort and interference with activities and required some degree of treatment modification). Symptoms were also rated in terms of perceived severity on a 3-point scale of mild, moderate, or severe. Moderate or severe symptoms were assessed by the study dermatologist and appropriate measures taken (dose reduction or the additional use of moisturizers).

During the placebo run-in and at the end of 6 months there was a clinical evaluation of AKs (individual AK lesions were circled and counted on each arm by a dermatologist and then photographed using a Nikon N5005 camera with a 60-mm Micro Nikkor lens, SB-21 Macro Speedlight, and Kodachrome ASA 64 film) and skin punch biopsies (3 mm) were obtained for polyamine levels. Additionally, serum samples were obtained from all participants who completed the study for measurement of DFMO levels, and skin punch biopsies were obtained on a subset of 17 participants for DFMO and ornithine concentrations at baseline and after 6 full months of treatment. Punch biopsies were taken from an area of clinically apparent AK located on the lateral surface of forearms before and after treatment with placebo or DFMO. Complete blood counts and serum chemistry panels (SMA20s) were performed during the run-in and at end of study.
Laboratory Methods

Tissue Preparation. The area of skin to be biopsied was anesthetized with 1% lidocaine HCL with epinephrine (Elkins-Sinn, Incorp. Cherry Hill, NJ). Two 3-mm punch biopsies were obtained for polyamines, DFMO, and ornithine concentrations from AKs on the lateral surfaces of each of the two forearms and transported on ice to the analytical chemistry laboratory.

HPLC Methods. Polyamines were quantitated in skin biopsies by measurement of dansyl derivatives (33). Skin biopsy samples were homogenized with 0.2 M perchloric acid containing 10 μM 1,7-diaminoheptane as an internal standard. The homogenate was then centrifuged, and 100 μl of the supernatant were transferred to a new 1.5-ml Eppendorf tube which contained 100 μl of 1 M sodium carbonate. One hundred μl of 1% dansyl-chloride in acetone was added to the sample tube, and the sample was placed at 60°C for 1 h to allow for derivatization to occur. Fifty μl of 10% glycine was then added to remove excess dansyl-chloride. After incubation for an additional 30 min, dansyl-polyamines were extracted with hexane. The extract was dried under nitrogen and redissolved with 250 μl of acetonitrile. A 50-μl aliquot of the resulting solution was injected onto the HPLC column. An Ultrasphere ODC 5-μm reversed-phase column (4.6 × 250 mm; Beckman Instruments, Inc., San Ramon, CA) was used for analysis at room temperature with a gradient of acetonitrile-dilisodium phosphate (1.2 mM; pH 5.49), a flow rate of 2.5 ml/min, and a 7-min sample time. Detection was provided by a Kratos Spectroflow 980 fluorescence detector (ABI Analytical, Inc., Remsey, NJ) with excitation at 340 nm and emission at 550 nm. The detection limit was <1 pmol, with linearity of ≤250 pmol for each polyamine injected. Recoveries for putrescine, spermidine, and spermine were 105%, 99%, and 81%, respectively. Additionally, all analytes were stable in skin stored at −80°C for at least 2 months.

HPLC analysis of DFMO required precolumn derivitization with 6-aminoquinolinol-N-hydroxysuccinimidyl to produce highly stable derivatives (34). Two-hundred μl of the plasma samples were mixed with 200 μl of 0.4 μM perchloric acid containing 120 μM dl-2,4-diamino-n-butyric acid (as internal standard) to precipitate the proteins. A 3-mm skin punch biopsy sample was homogenized with 0.2 M perchloric acid (1.67%, w/v) in the presence of 60 μM dl-2,4-diamino-n-butyric acid. After centrifugation, 20 μl of the supernatant was transferred to a microcentrifuge tube containing 80 μl of saturated sodium tetraborate solution (pH 9.1). Derivatives were formed via the addition of 20 μl of 10 mM 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate in acetonitrile and then by vortexing for 5 s. A 5-μl volume of the derivatives was directly injected onto the HPLC column. A Nova-Pak C18 column (3.9 × 300 mm; Waters Chromatography Division, Millipore, Millford, MA) with a gradient elution of sodium acetate-acetonitrile, a flow rate of 1.2 ml/min, and detection with a Kratos Spectroflow 980 fluorescence detector (excitation at 245 nm and emission at 370 nm). The complete cycle/sample was 30 min with a retention time of 7.9 min for DFMO. The detection limit (signal-to-noise ratio = 3) was 90 fmol for DFMO. Two skin samples were used for reproducibility studies, where samples were homogenized and the aliquots of supernatant were stored at −80°C for the analysis of DFMO on 5 different days. The results indicated good precision with the coefficient of variation for each compound being <8%. For recovery studies, known amounts of mixed standards were added to plasma or tissue samples. Recovery was calculated by dividing the peak height of the standard in tissue sample to the peak height of the standard in aqueous solution. In the case of skin samples, recovery for DFMO was 94–99%.

Statistical Methods

The DFMO effects were assessed by fitting regression models for change data. The outcome measure was the difference in the change (of a factor) on the DFMO-treated arm minus the difference on the untreated arm. The explanatory factor was the change on the untreated arm, centered at its mean so that the regression intercept had the interpretation as the (adjusted) DFMO effect (35). Initial values were used to adjust for regression to the mean, whereas the change on the untreated arm was used to adjust for person-specific effects. Additionally, all models initially included dummy variables for whether the right or left arm was treated.

Results

A total of 50 participants with moderate to severe (≥10) clinically diagnosed AKs on their lateral forearms began the 1-month placebo cream run-in period. Two of these participants did not complete the 1-month run-in period and were not randomized. Forty-eight participants were randomized to apply DFMO cream to either their right (24) or left (24) posterior forearms, with the placebo cream to be used on the contralateral arm. Ultimately, 42 (87.5%) of 48 randomized participants completed the full 6 months of treatment and underwent analysis. Six of 48 randomized participants did not complete the study protocol before the 6-month completion date because of the development of a rash (2 participants; see description below) or for personal reasons (4 participants). As shown in Table 1, there was an even distribution of age and gender on the two treatment arms among the 42 participants who completed 6 months of treatment. The majority of participants were male (76%). Among the 42 participants included in this analysis, adherence during the 1-month run-in was 99%. Overall adherence to the study protocol was ≥95%.

On the basis of clinical observations and the review of complete blood count and SMA20 data, there was no evidence of topical DFMO-induced systemic toxicities. Of 48 randomized participants, 2 (4.2%) experienced a severe inflammatory reaction on only one forearm subsequent to the administration of ointment after an average of 88 days (range, 55–168 days) and could only be included in toxicity analyses. One developed a severe reaction that began on day 54. A second participant continued to apply the topical ointment despite the onset of redness and scaling after 115 days of treatment. This participant presented with confluent erythema, crusting, and scaling on one forearm at the final clinic visit, resulting in an inability to quantitate the number of AKs or to obtain skin punch biopsies for polyamine content analysis. Both of these severe reactions occurred on the DFMO-treated arm only, and these participants were excluded from the efficacy end point analysis.
Topical DFMO Suppresses AKs and Skin Polyamines

Table 2  Mean (± SD) DFMO and ornithine concentrations in AKs after 6 mo of DFMO or placebo topical treatment in a subset of 17 study participants

<table>
<thead>
<tr>
<th>DFMO arm (ng/g wet skin)</th>
<th>Ornithine (ng/g wet skin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFMO arm</td>
<td>Placebo arm</td>
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<tr>
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</tr>
<tr>
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<td>0</td>
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<tr>
<td>1719.7</td>
<td>0</td>
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<tr>
<td>962.7 ± 885.2</td>
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*P = 0.28 (paired t test).

Five (10.4%) additional participants, who were included in the efficacy analysis, experienced moderate, localized skin toxicities and were managed as follows. One of these participants experienced symptoms after 145 days, was taken off study for one week, rechallenged with treatment, experienced rash after 2 days of rechallenge, and discontinued treatment. A second participant experienced erythema, redness, and scaling after 54 days of treatment. Treatment was discontinued for 43 days, and the subject was rechallenged. Symptoms returned after 22 days and treatment was discontinued. The three other participants experienced localized skin toxicities of moderate severity and discontinued topical treatment within 2 days of completing the study. These three participants were not rechallenged before final biopsies were taken.

The skin DFMO and ornithine concentrations after 6 months of DFMO and placebo topical administration are shown in Table 2 for the subsample of 17 participants who underwent skin punch biopsies. As shown in the table, none of the punch biopsies obtained from the forearms assigned to the placebo topical treatment contained DFMO, whereas, 13 of the 17 biopsies from DFMO-treated forearms had high levels of DFMO, whereas none of the biopsies from placebo-treated forearms showed evidence of DFMO uptake into skin, documenting reasonably high-frequency hearing loss, which is reversible when drug treatment is discontinued (36, 37).

We formulated DFMO in a hydrophilic ointment at a 10% (w/w) concentration for studies of stability, tolerance, and penetration through human and mouse skin in supplied tubes. We documented that the topical administration of this formulation to human skin in vitro was associated with substantial uptake after 24 h at 37°C, with little evidence of transdermal penetration (36, 37).

Participants in this Phase Ib study were randomly assigned to topical DFMO treatment of the right versus left forearm and placebo treatment of the contralateral arm. A subset of 17 participants was randomly selected to undergo biopsies of AK lesions on the forearms for measurement of DFMO and ornithine levels. Thirteen of 17 biopsies from DFMO-treated forearms had high levels of DFMO, whereas none of the biopsies from placebo-treated forearms showed evidence of DFMO uptake into skin, documenting reasonably good adherence to the randomized right- versus left-arm topical DFMO and placebo daily treatments.

The most clinically relevant DFMO effect with respect to study end points involved a significant 23.5% reduction in the number of AKs after 6 months of DFMO treatment. Results of regression analysis of DFMO treatment on the study end points are presented in Table 4. The change in number of AKs is negatively related to the initial number, which presumably represents the regression to the mean effect. The change on the treated arm is negatively related to the change on the matched placebo arm, suggesting that those participants who had the largest improvement on the treated arm tended to have the least improvement on the placebo arm. The DFMO effect was estimated to be a reduction of 6.1 AK lesions on the treated arm (P = 0.001).

DFMO treatment was effective in significantly suppressing (P = 0.037) spermidine concentrations in the skin (Tables 3 and 4). The relative percent of reduction in skin spermidine concentrations was 26%, which was similar to the 23.5% reduction observed for AK number.

In the process of examining other potential effects, it was discovered that the DFMO effect on AKs was essentially restricted to the case in which the right arm was treated. In Table 5, a simplification of the regression analysis is presented for all study end points. There was a 10.8 AK reduction on treated right arms (P for the difference = <0.001) with no effect on treated left arms. A similar effect was observed for the polyamine measurements, with spermidine concentrations being significantly reduced (P = 0.034) and a nonsignificant reduction in putrescine concentrations (P = 0.098) on the right arm only; however, there were no statistically significant differences in participant age, compliance, number of AKs, or in polyamine concentrations at baseline between the right and left arms.

Discussion

When administered p.o., DFMO is a potent inhibitor of carcinogenesis in experimental animal models and is especially active in preventing carcinogen-induced epithelial cancers, including those of the skin (26 –30). At p.o.-administered doses of up to 0.5 g/m²/day for 6 months, DFMO can induce ototoxicity, a high-frequency hearing loss, which is reversible when drug treatment is discontinued (36, 37).

The graphs of the right lateral forearm of one of the DFMO-treated study participants. As demonstrated by inked circles, there was substantial clearing of the AKs after 6 months of DFMO treatment.

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The most clinically relevant DFMO effect with respect to study end points involved a significant 23.5% reduction in the number of discrete AK lesions from baseline to the 6-month follow-up evaluation. Of importance mechanistically, there also
was a 26% reduction in spermidine concentrations in skin biopsies obtained from the DFMO-treated arms. Suppression of polyamines by DFMO can down-regulate the expression of genes demonstrated to play a role in transformation (i.e., c-myc) and in non-melanoma skin cancer (i.e., c-fos and c-jun) in vitro (38). The ability of the topical DFMO formulation used in the present study to cause alterations in the expression of these genes or other genes important in skin epithelial cell transformation in vivo is currently under study in our research program.

Although our clinical study model, which uses a within-participant randomization of chemoprevention agent and placebo topical applications to opposite arms appears efficient and effective, it was discovered that the topical DFMO effect on AKs was essentially restricted to the treated right arm. There was a reduction of \( \frac{26}{10.8} \) AKs on the DFMO-treated right arms, but no reduction on the DFMO-treated left arms. This right-arm efficacy effect of topical DFMO correlated well with right-sided suppression of skin punch biopsy spermidine concentrations. There is no obvious reason for this disparity in DFMO effect. It is possible that the left arm tends to receive considerably more Arizona sun exposure than the right (related to the amount of time spent driving automobiles) and thus was less likely to respond to topical DFMO. Also, the small participant sample size may have contributed to a disproportionate chance for a DFMO effect on the right forearm. Similarly, there were no significant differences in the number of AKs or polyamine levels at baseline between left and right arms that could explain this finding.

Although the DFMO topical treatment was associated with both a large and a significant reduction in the mean skin spermidine concentration, it is possible that the effect of topically administered DFMO on polyamines was washed out by our insistence on biopsying AK lesions that persisted through 6 months of DFMO treatment. In other words, these were AKs

![Image 64x162 to 287x546](cancer-epidemiology-biomarkers-prevention)
that survived the DFMO treatment and could be considered DFMO-resistant.

There was a 4.2% rate of severe and a 10.4% rate of moderate local skin toxicity requiring treatment modification associated with long-term administration of topical 10% DFMO. On the basis of this rate of local inflammatory reactions, we suggest that future topical DFMO clinical trials in participants with forearm AKs evaluate both lower topical concentrations (e.g., 5%) and/or the addition of topical steroids.

The present study results suggest efficacy for topically administered DFMO in the treatment of participants with severe AK. We believe that future randomized studies of topical DFMO, using standard trial designs, perhaps in participants with less severe AKs, are indicated in an attempt to reproduce and extend the results of the present clinical trial.

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References


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