Phase I Chemoprevention Study of Difluoromethylornithine in Subjects with Organ Transplants[1]

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Abstract

Individuals who receive life-saving organ transplants and the required immunosuppression often develop secondary cancers. One of the most common secondary cancers is nonmelanoma skin cancer in sun-exposed areas. Attempts to prevent these cancers have not been successful. Difluoromethylornithine (DFMO), a suicide inhibitor of ornithine decarboxylase (ODC), is a known experimental cancer prevention agent that is being evaluated in a number of human cancer prevention trials. This report describes a Phase I trial in 18 organ transplant recipients, randomized to 1.0 and 0.5 g of DFMO or a placebo, designed to look at short-term toxicities over 28 days as well as the impact of DFMO on two biological parameters, skin polyamines and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ODC activity. Blood levels of DFMO were also measured. The results indicate that DFMO was well tolerated over the 28-day period. The TPA-induced ODC activity in 3-mm skin biopsies was significantly lowered by 80 and 67% at the two dose levels. Polyamine levels were not affected significantly except for putrescine at the 0.5-g level. Blood levels of DFMO were about two times higher than expected, based on our prior pharmacokinetic studies. Our studies indicate that DFMO is a reasonable agent that should be tested further in larger Phase 2b trials in this population as a chemopreventive agent. TPA-induced ODC activity appears to be a relevant intermediate biological assay.

Introduction

OTRs[3] have a 100-fold increase in cutaneous neoplasms compared with age-matched controls (1). In Australia, the prevalence of skin cancers in kidney transplant recipients has risen to 57% by 20 years after transplant (2). The data from Penn *et al.* (3, 4) suggest that the prevalence in the United States is 18%. The incidence curve rises linearly, and calculations suggest that kidney transplant recipients who survive 33 years may all develop some form of cancer, of which 75% would be skin cancers (5).

The University of Wisconsin Transplant Service has a long history of doing renal and other transplants. It is the second largest organ transplant service in the United States. Between 1968 and 1998, a survey of a sample of 96 patients with organ transplants revealed 108 skin cancers. Of the 96 patients, 21 have had more than one cancer. The median time for development of skin cancers was 2.7 years after transplant. More recently, the onset of cancers in our patients seems to be occurring earlier, usually within 2 years. In 1991 and 1992, 40 cancers were diagnosed. These figures are likely to be low estimates because careful assessments by dermatologists were not done on a regular basis. These patients are on a variety of immunosuppressive agents including steroids, cyclosporine, and anti-lymphocyte immunoglobulin.

DFMO, a chemoprevention agent, has a broad spectrum of antipromotion effects in many experimental systems (6, 7). DFMO is being tested in two Phase III trials in nonmelanoma skin cancers and superficial bladder cancers. DFMO has its primary effect on ODC and subsequently the production of putrescine from ornithine. The use of DFMO in preventing skin cancers in OTRs would be important. The impact of DFMO on TPA-induced ODC activity and skin polyamines, as well as on organ survival in this clinical environment is not known.

This pilot study attempted to assess the short-term biological and clinical effects of DFMO, a known experimental chemopreventive agent, in this population. This modified Phase I study was done to obtain some experience with DFMO in this population and to assess the utility of our biological markers, TPA-induced ODC, and skin polyamine levels. The study used a simple dosage administration by allocating subjects to 0.5 or 1.0 g/day. In nontransplant subjects, this dosage administration was well tolerated (8). This report describes the toxicities, results of TPA-induced ODC activity, and polyamines as measured in skin biopsies. Serum levels of DFMO were also obtained.

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[3] The abbreviations used are: OTR, organ transplant recipient; DFMO, difluoromethylornithine; ODC, ornithine decarboxylase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Materials and Methods

Subject Selection. Subjects over the age of 21 and at high risk of developing skin cancer, with organ transplants (renal, liver, or heart), ≥1 year after transplantation were referred to the research team for consideration. High risk was defined as skin types I-IV (9), photoaging (Glogau) criteria II–IV (10), a prior history of blistering sunburns, outdoor occupations, or living in Florida, Hawaii, California, Arizona, New Mexico, and Texas. In addition, subjects with a history of actinic keratosis, Bowen's disease, basal cell carcinoma, squamous cell carcinoma, or keratoacanthomas were also eligible. Eligible subjects were approached by study coordinators and investigators who explained the study purpose and design. Subjects who were lactating or pregnant were not eligible for randomization. A simple clinical hearing test was done in all subjects. Any subject with a hearing aid or had clinical evidence of hearing loss was not considered eligible. Also, the use of concomitant topical medications for skin cancer, such as retin-A, Accutane, psoralens ultraviolet A, and Efudex (5-fluorouracil), was not allowed for the subjects on study.

Pretreatment Evaluations. Baseline blood studies within the past 8 weeks for eligible subjects included hemoglobin, white blood count, platelet count, a general chemistry panel plus electrolytes, and a liver panel as well as any other clinically indicated assessments of transplant function done as part of the routine follow-up. A history and physical were done at the start of the study. Suspicious skin lesions were checked by one of the specialists in the University of Wisconsin Hospital and Clinics Mohs or Dermatology Clinics. An audiogram was done in all randomized subjects. Randomization was performed by the University of Wisconsin Comprehensive Cancer Center Clinical Trials Data Unit.

Treatment Plan. Eighteen subjects were randomized to three arms, a placebo, or two dosage levels of DFMO. The dosages were 0.5 or 1.0 g/day for 4 weeks. Skin biopsies and measurements of skin polyamines and TPA-induced ODC activity levels were done at start of study and day 28. Serious adverse events were to be reported to ILEX Oncology, Inc. and the Food and Drug Administration as per regulations.

Clinical Monitoring. In step 1, a physical exam, assessment of weight, blood pressure, pulse, respiratory rate, and temperature were done before the study.

Hearing Loss Monitoring. A baseline audiogram was done on all subjects. During the 28-day study, hearing was monitored clinically and confirmed by audiometry as needed. Repeat audiograms at day 28 were done only if the subject reported a hearing loss. The criteria used for hearing loss and toxicity were based on criteria defined by the National Cancer Institute Common Toxicity Criteria, version 2.0 (11).

Intermediate Markers. Inhibition of TPA-induced ODC activity and skin polyamine levels were measured in all subjects in step 1 pre-drug and day 28.

Human Skin TPA-induced ODC Activity. The method for *in vitro* induction of human skin ODC activity by TPA has been reported by Verma *et al.* (12). Three-mm punch biopsies were obtained from the volar surface of the forearm and transferred to a flask containing serum-free MEM at 37°C. The medium was gassed with 95% oxygen and 5% CO_2 for 1 min; the appropriate additions (TPA or ethanol) were made, and the flasks were sealed and incubated in a shaking water bath at 37°C. At appropriate times after incubation, the skin biopsies were removed to ice-cold distilled water. ODC activity in the whole biopsy was determined by measuring the release of $^{14}CO_2$ from [1-^{14}C]DL-ornithine hydrochloride. The assay mixture contained 20 mM Tris-HCl (pH 7.5), 0.32 mM pyridoxal phosphate, 4 mM DTT, 0.4 mM EDTA, 20 μM L-ornithine, 0.02% Brij 35 containing 0.25 μCi [1-^{14}C]DL-ornithine hydrochloride in a total volume of 0.25 ml. After incubation at 37°C for the indicated time in 15-ml Corex centrifuge tubes equipped with rubber stoppers and center well assemblies, the reaction was stopped by addition of 0.5 ml of 2 M citric acid. The incubation was continued for at least another hour to ensure complete absorption of $^{14}CO_2$ by the ethanolamine and methoxyethanol (0.2 ml; 2:1 vol/vol) contained in the center well. Finally, the center well containing the ethanolamine:methoxyethanol mixture was transferred to a vial containing 10 ml of toluene-based scintillation fluid and 2 ml of ethanol, and the associated radioactivity was determined in a liquid scintillation counter. The results are expressed as pmol/h/biopsy.

DFMO Plasma Concentrations. DFMO was measured using the method of Smithers (13). DFMO was extracted from plasma with methanol and derivatized with *o*-phthalaldehyde. Derivatized DFMO was quantitated by reverse-phase high-performance liquid chromatography with fluorometric detection (elution, 335 nm; emission, 450 nm). Bloods were drawn 12 h after the evening dose.

Tissue Polyamines. For the study, 3-mm skin punch biopsy samples (weight ~3 mg) were obtained at days 0 and 28. The samples were immediately placed in ice-cold 2% perchloric acid and homogenized in a Polytron. These acid extracts were frozen at −70°C until assayed.

Polyamine Assay. The polyamines putrescine, spermidine and spermine were analyzed in the 1 ml of acid extract by the method of Kabra *et al.* (14). This assay was well suited to automation for efficient production of timely results. After addition of the internal standard (1,7-diaminoheptane), the samples were derivatized with dansyl chloride, and the derivatized polyamines were extracted from the samples using Bond-Elut C_{18} SPE columns. The derivatized polyamines were separated and quantitated by high-performance liquid chromatography on a Waters 8 × 10 Novapak C_{18} cartridge using a gradient of 48–100% acetonitrile against 10 mM sodium acetate buffer for a period of 30 min with fluorescent detection (excitation, 340 nm; emission, 515 nm). Polyamines were quantitated by comparison of chromatographic peak areas of each polyamine to the peak area of the internal standard. The standard curve for each polyamine was linear from 0 to 50 nmol/ml of extract ($r^2 >$ 0.99 in each case). The intraday coefficient of variation in duplicate determinations was <2% for low and high standards of each polyamine. The interday coefficient of variation was <5% for high standards of each polyamine ($n = 4$; putrescine, 10 nmol/ml; spermidine and spermine, 50 nmol/ml). The interday coefficient of variation for low standards of each polyamine was <10% for each polyamine ($n = 4$; putrescine, 0.625 nmol/ml; spermidine and spermine, 3.12 nmol/ml).

Statistical Analysis. The primary objective of this study is to investigate whether DFMO has a measurable effect on ODC activity and polyamines in 3-mm punch biopsies. The sample size of 6 per group was derived to have 80% power using a one-sided test at the 0.05 significance level to placebo, when the dose with the maximum decrease in ODC level to a placebo, when one dose was expected to result in a 75% decrease and the other was expected to result in a 50% decrease. Mean levels of ODC and polyamines were computed at days 1 and 28, as well as the mean change from day 1 to day 28. To test for differences in mean changes in ODC activity and polyamines, Wilcoxon's two-sample test was used. *P*s from Wilcoxon's test are reported

Table 1 Concomitant medications used by the subjects

Group	ASA/NSAID[a]	Prednisone	Anti-rejection[b]
1.0 g/day	3/6	5/6	6/6
0.5 g/day	4/6	6/6	6/6
Placebo	2/6	5/6	6/6

[a] ASA, aspirin; NSAID, nonsteroidal anti-inflammatory drugs.
[b] Cyclosporine, tacrolimus, mycophenolate, and azothioprine.

for comparisons of the two DFMO arms to placebo, as well as for the direct comparison of the two DFMO arms to one another. All *P*s were computed under two-sided alternative hypotheses. The results are expressed as nmol/ml, which is the exact volume into which the skin biopsies were extracted.

Results

Eighteen subjects were randomized: 13 males and 5 females with an average age of 53.7 years (range, 37.6–68.3 years). One subject had a heart transplant, 5 subjects had kidney transplants, and 12 subjects had liver transplants. Three of the 18 subjects presented with a history of multiple organ transplants. One of those individuals was status after three liver transplants and another individual had two kidney transplants. One subject had five separate liver transplants and a kidney transplant.

Table 1 summarizes the concomitant medications used by the subjects. All of them were on anti-rejection medications, most were on prednisone, and about half were on aspirin and/or nonsteroidal anti-inflammatory drug.

Only one subject had a serious adverse event, which consisted of a urinary tract infection. One subject who described muffled hearing had an audiogram. No changes were noted between the baseline audiogram and the episode. Another subject had a history of gastrointestinal reflux disease and reported nausea and vomiting. The dose was reduced by 50%, and he had no additional problems. The other side effects were distributed across all treatment arms and were not clearly related to DFMO.

No significant changes in hemoglobin or white blood count levels were seen. One subject on the 1.0-g/day dose had a decrease in platelets from 104,000 to 80,000 μl. Another subject had a slight increase in K^+ from 4.6 to 5.1 mmol/l. Two subjects had elevated alkaline phosphatase at onset on the 1.0 g/day, and the levels decreased on DFMO. Three subjects on placebo had elevated phosphatases; two improved. Overall, no consistent changes were seen.

ODC Activity. DFMO is a specific inhibitor of ornithine decarboxylase. Our hypothesis has been that measuring TPA-induced ODC in a skin biopsy from subjects on DFMO represents a biological marker of its effect. This assay measures the increase in ODC activity after TPA stimulation of skin *in vitro*. The TPA-induced ODC activity was significantly lowered by DFMO at 0.5 and 1.0 g/day (Table 2). At the 1.0-g/day dose, 5 of the 6 subjects had TPA-induced ODC activity that showed stimulation compared with baseline. One subject did not have an increase after TPA, making the ODC after DFMO not evaluable. In the other 5 subjects, the post-DFMO levels were all suppressed by >50%. At the 0.5-g/day dose, again 5 of 6 subjects had the appropriate stimulation after TPA. All of these subjects had suppression on DFMO of TPA-induced ODC activity. In the placebo group, the pre-DFMO baseline TPA-induced ODC activity skin biopsy was lost for one subject and could not be assayed. However, in these subjects, the TPA-induced ODC activity before and after treatment were not affected in any of the subjects.

In Table 2, we see that the mean ODC activity levels in the 1-g/day group and the 0.5-g/day group decreased by 80 and 67%, respectively. The mean decrease for both groups was highly significant when compared with the placebo group.

Polyamine Levels. The levels of putrescine, spermine, and spermidine are shown in Table 3. The values can be summarized as follows. In the 1.0- and 0.5-g/day groups, putrescine levels were decreased in 4 of 6 and 5 of 6 subjects. In the placebo group only 1 of 6 subjects had decreases in putrescine. The mean change in putrescine levels in the 0.5-g/day group was significantly lower than the mean change in the placebo group (Table 3A). No statistically significant differences among the treatment groups were seen in spermine and spermidine levels.

DFMO Levels. In the 1.0-g/day group, the levels were about two times the level of the 0.5-g/day group, 27.2 $\pm$ 8.2 μM, compared with 12.2 μM $\pm$ 3.0 (mean $\pm$ SE). The measurements were made in a blinded fashion for all subjects. No detectable levels of DFMO were seen in the placebo group.

Discussion

Skin cancer is the most common malignancy arising in the posttransplantation setting (15). Multiple factors contribute to the high risk for cutaneous carcinomas. With prolonged allograft function and subject survival, the majority of solid-organ transplant recipients will eventually develop skin cancer. Highly susceptible subjects may develop hundreds of squamous cell carcinomas, which may be life threatening. In Australian heart transplant recipients, the cumulative incidence of skin cancer was 31% at 5 years and 43% at 10 years, with a squamous cell carcinoma:basal cell carcinoma ratio of 3:1 (16). Caucasian origin, increasing age at transplantation, and duration of follow-up were significantly associated with skin cancer. Skin cancer accounted for 27% of 41 deaths occurring after the fourth year. The squamous cell carcinomas in organ transplants tend to be multiple and may have a life-threatening course (17). The only management approaches are regular examination and aggressive surgery.

The frequency and multiplicity of these skin cancers not only present a problem but also an opportunity to test new chemoprevention agents. The frequency and multiplicity of new skin cancers creates a high-risk situation that allows end points to be achieved quickly. However, the challenge is not to interfere with the organ transplant (and the patient's) survival. In addition, because of the complex medications and immunosuppression drugs, the effects of chemoprevention drugs may be abrogated.

The effect of retinoids had no significant benefit on squamous or basal cell skin cancers in the high-risk subjects on the SKICAP-S/B trial. Daily retinol was effective in preventing squamous cell cancers in moderate risk subjects (18). High-dose 13-*cis*-retinoic acid also has achieved significant activity in preventing invasive carcinomas of the skin. High-dose 13-*cis*-retinoic acid, however, is not ideal for widespread chemoprevention approaches because of its toxicity. The toxicity-to-risk balance is delicate and complicated (19). Other agents being evaluated as potential chemopreventive agents include the green tea catechin epigallocatechin gallate, the limonene derivative perillyl alcohol, the ornithine decarboxylase inhibitor DFMO, selenium, retinoids, and salicylates (20).

DFMO is an agent that has been shown to have tumor-suppressive properties in a variety of experimental situations

Table 2 TPA-induced ODC activity levels (pmol/h/skin biopsy)

Treatment group	Mean (SE)		Mean Δ (SE) Day 28 to Day 1	*P* for Δ *vs.* placebo	*P vs.* 0.5 g
	Day 1	Day 28			
1 g/day	31.25 (6.20)	6.19 (0.83)	−25.01 (6.32)	0.0087	1
0.5 g/day	33.44 (7.35)	11.06 (1.97)	−22.38 (5.96)	0.0152	
Placebo	31.81 (7.00)	33.93 (4.73)	2.12 (3.71)		

Table 3 Tissue polyamines

Treatment group	Mean (SE)		Mean Δ (SE) Day 28 to Day 1	*P* for Δ *vs.* placebo	*P vs.* 0.5 g
	Day 1	Day 28			
A. Putrescine levels (nmol/skin biopsy)					
1 g/day	0.049 (0.026)	0.062 (0.034)	0.013 (0.041)	0.3095	0.3095
0.5 g/day	0.032 (0.006)	0.020 (0.008)	−0.012 (0.007)	0.0411	
Placebo	0.012 (0.004)	0.028 (0.006)	0.016 (0.009)		
B. Spermidine levels (nmol/skin biopsy)					
1 g/day	0.268 (0.024)	0.313 (0.038)	0.046 (0.047)	0.8182	0.2972
0.5 g/day	0.282 (0.055)	0.254 (0.048)	−0.027 (0.013)	0.2972	
Placebo	0.208 (0.035)	0.263 (0.017)	0.055 (0.041)		
C. Spermine levels (nmol/skin biopsy)					
1 g/day	0.939 (0.088)	0.989 (0.135)	0.050 (0.107)	0.6991	0.3939
0.5 g/day	0.861 (0.140)	1.038 (0.187)	0.176 (0.072)	0.6991	
Placebo	0.910 (0.144)	1.132 (0.072)	0.222 (0.188)		

(21). DFMO inhibits ODC activity, a key enzyme involved in the biosynthesis of polyamines, required for cell growth and cell division. The polyamines are increased during tumor promotion, and polyamine inhibition inhibits neoplasia. DFMO has not proven to be an anticancer agent when the disease is clinically apparent. Several trials at the University of Wisconsin Comprehensive Cancer Center have demonstrated its safety at low doses that inhibit TPA-induced ODC activity. Two Phase III trials are under way, supported by National Cancer Institute and ILEX Oncology, Inc., in nonmelanoma skin cancers and superficial bladder cancers. The prevention of skin cancers in subjects with organ transplants represents a unique challenge. This Phase I study was designed to explore whether measurements of TPA-induced ODC activity and resultant polyamines in skin biopsies were applicable in this population (22). In addition, short-term safety (1 month) was assessed.

Our studies showed that DFMO was tolerated with no acute major short-term toxicities. The placebo, 0.5-g/day, and 1.0-g/day dosage patterns of side effects were not different. The numbers of subjects were small, and toxicities of lower frequencies would not be picked up. Larger Phase II trials need to be done. We also noted that the levels of DFMO in the blood were about twice as high as expected in normal subjects based on our prior studies. Comparing these results to our prior studies using 0.5 g/m^2 (~0.9 g/day), the mean levels were 14.5 ± 5.2 μM (23) and 13.0 5.1 μM (24) for DFMO. The predicted level for 1.0 g/day would be 14.0 μM and 0.5 g/day would be 6.1 μM. These increased levels may be attributable to changes in renal clearance of the drugs because of interference by other drugs the subjects are on or mild renal impairment because of decreased renal function. DFMO is excreted primarily unchanged in the urine (25).

The effect of DFMO on skin biopsies TPA-induced ODC activity at both doses indicated a significant inhibition of ODC activity, a finding consistent with our earlier DFMO trials (22, 24). The polyamine levels, particularly putrescine, were also suppressed by DFMO. Levels of spermine and spermidine were not affected. This is because of the fact that the levels of spermidine and spermine are regulated by acetylation. Acetylated spermidine and spermine are readily excreted to maintain the intracellular levels. Spermidine and spermine are also converted to putrescine by acetylation and oxidation (26).

A logical next step would be to do a larger trial: placebo controlled, randomized to the lowest dose of DFMO (0.5 g/day) used in this study as well as one dose lower. The high rate at which OTR patients develop nonmelanoma skin cancers should allow the efficacy of DFMO to be determined by relatively small numbers of patients in Phase II or III trials. The duration of therapy should be longer, at least 1 year. The safety of DFMO in transplant patients needs to be monitored carefully. In summary, DFMO was safe and well tolerated in OTR patients, and inhibition of the biological target was documented in this study.

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