Difluoromethylornithine is effective as both a preventive and therapeutic agent against the development of UV carcinogenesis in SKH hairless mice

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Targeting specific events associated with tumor development represents a rational approach to chemoprevention as well as therapeutic intervention. In this study the ability of difluoromethylornithine (DFMO) to inhibit UV-induced skin carcinogenesis when administered before or after the appearance of tumors was examined. SKH hairless mice were irradiated 3 times per week with 90 mJ/cm2; this dose was increased by 10% weekly to a maximum of 175 mJ/cm2. Mice supplied 0.4% DFMO in the drinking water continuously throughout the experiment had an average of 2.0 tumors/mouse (72% incidence) at 30 weeks while controls had an average of 8.2 tumors/mouse (100% incidence). DFMO started after 12 weeks of UV, a time prior to tumor appearance, yielded 3.6 tumors and 100% incidence at 30 weeks. Starting DFMO at 22 weeks, when an average of 2.5 tumors were present, caused regression of tumors for several weeks, followed by a slight rebound. The final tumor number at 30 weeks was 3.0 (96% incidence). Thus, DFMO has strong chemopreventive efficacy, as well as therapeutic activity, against UV-induced skin tumors. Histological and proliferative markers support this conclusion.

Introduction

Recognition that the tumorigenic process occurs by discrete stepwise changes and involves specific quantifiable events offers the opportunity of intervening and interfering with these processes in order to prevent tumor development. Thus, one of the major approaches to chemoprevention is the use of agents known to block specific pathways. While initiation events are generally irreversible, many of the processes associated with promotion and progression can be effectively inhibited or reversed, leading to a reduction in tumor incidence and severity (1).

Chronic exposure to UV radiation is the primary cause of non-melanoma and melanoma skin cancer in humans. UV light is considered a complete carcinogen in that it has characteristics of both tumor initiators and tumor promoters. UV light acts as an initiator by producing irreversible mutagenic damage and can behave as a tumor promoter by inducing epigenetic changes that cause expansion of the initiated cell population and changes in their behavior (2,3). Tumor promotion events include, but are not limited to, dermal inflammation, epidermal hyperplasia and increased ornithine decarboxylase (ODC) activity (4,5).

ODC is the first and rate-limiting enzyme in polyamine synthesis and catalyzes the synthesis of putrescine from ornithine. Putrescine is further metabolized to spermidine and spermine by S-adenosylmethionine decarboxylase. In mouse skin the induction of ODC by chemical tumor promoters, and subsequent increases in polyamine levels, has been proposed as an obligatory step in tumor promotion (6,7). The correlation between rapid proliferation and high levels of polyamines in many tissues and tumor types suggested that ODC was likely to be an excellent target for chemoprevention. This is supported by the findings that inhibitors of ODC significantly reduce skin tumor development in initiation-promotion models (8–10) and that ODC overexpression is a sufficient condition for tumor promotion (11). Additionally, the observation that in tumors ODC and polyamine levels are constitutively up-regulated independent of tumor promoter exposure suggests that inhibiting ODC in tumors may also have therapeutic efficacy (12).

D.L-α-Difluoromethylornithine (DFMO) is an analog of ornithine that irreversibly inhibits ODC activity. DFMO is decarboxylated by an enzymatic mechanism that generates an intermediate carbonionic species. With the loss of fluorine this species alkylates a nucleophilic residue near or at the active site, resulting in covalent binding of the inhibitor to the enzyme (13). This inhibitor has been found to significantly suppress tumor formation in a number of chemically induced experimental models, including colon and skin (14).

The goal of this study was: (i) to determine whether DFMO has chemopreventive activity against UV-induced skin carcinogenesis; (ii) to determine whether it has therapeutic activity such that it is efficacious when introduced late in the carcinogenic process. While continuous DFMO administration was found to have significant preventive activity, administration late in the tumor development process was also effective in reducing tumor burden. Thus, DFMO appears to have both chemopreventive and therapeutic activity against UV-induced skin cancer.

Materials and methods

Animal treatment

Female hairless SKH:HR-1-hBr mice at 4–6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and housed 10 per cage. Animals were allowed free access to food (AIN 76A diet) and water and were housed in climate controlled quarters (22 ± 1°C at 50% humidity) with a 12 h light/dark cycle. DFMO was provided by the National Cancer Institute, Division of Cancer Prevention and Control (lot no. 76494) and used at a dose of 0.4% in the drinking water. Fresh solutions of DFMO were supplied to the mice thrice weekly. Body weights were measured weekly for the duration of the experiment. For the tumor experiment mice were randomly assigned to one of four treatment groups, shown in Table I, and supplied DFMO either continuously or starting at either 12 or 22 weeks after beginning UV irradiation and continuing until after termination of the experiment.

Irradiation

Two days after beginning administration of DFMO, exposure to UV irradiation was begun. The UV apparatus consisted of eight Westinghouse FS20 sunlamps,
an IL-1400 radiometer and an attached UVB photometer. The spectral irradiance for the UV lamps was 280–400 nm, providing 80% UVB and 20% UVA. The peak intensity of the light source was 297 nm. The fluence at 60 cm from the dorsal surface of the mice was 0.48–0.50 mJ/cm²/s. The mice were placed in individual compartments in an open plastic cage on a rotating base to abrogate any differences in fluence across the UV light bulbs. Mice were exposed to 90 mJ/cm² three times per week; the dose was increased by 10% per week until the dose reached 175 mJ/cm². UV treatment was stopped at 23 weeks. We have previously shown that this protocol produces papillomas and carcinomas between 10 and 20 weeks (15).

**Tumor analysis**

Tumors were counted weekly and the data expressed both as incidence and as mean number of all tumors per mouse. The diameters of all tumors were measured at the time of death and all tumors were removed, fixed in formalin and processed for microscopic evaluation of tumor type (atypic hyperplasia, papilloma, squamous cell carcinoma or spindle cell carcinoma).

**ODC activity**

Groups of six mice were placed on DFMO for at least 48 h before a single UV exposure or placed on DFMO for 2 weeks with thrice weekly irradiation. Mice were killed 24 h after the last UV exposure. ODC activity was measured using preparations of epidermis scraped from the backs of irradiated or control animals, as previously described (16). Homogenates of the scrapings were centrifuged and aliquots of the supernatant were incubated at 37°C for 1 h with 0.05 µCi L-[14C]ornithine (52.0 mCi/mmol; Amersham Pharmacia Biotech). Protein content was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). 14CO2 released from the reaction was trapped in wells containing ethanolamine and counted. The results were calculated as nmol CO₂/mg protein/h and expressed as a percentage of the control.

**Histology and labeling index (LI)**

Groups of three or four mice were placed on DFMO for at least 48 h before a single UV exposure or placed on DFMO for 2 weeks with thrice weekly irradiation. Mice were killed 24 h after the last UV exposure. One hour before death mice were injected i.p. with 2.0% 5-bromo-2'-deoxyuridine (BrdU) (Sigma Chemical Co., St Louis, MO) in phosphate-buffered saline at 0.1 mg/g body wt. Sections of exposed skin were removed, fixed in formalin and processed for paraffin embedding. Multiple sections from each mouse were stained with an anti-BrdU antibody (Novocastra Laboratories, Newcastle-upon-Tyne, UK) as previously described (17). The antigen–antibody complex was visualized with 3,3'-diaminobenzidine (Sigma Chemical Co.) by an avidin–biotin–horseradish peroxidase system (Vectorstain Elite ABC kit; Vector Laboratories), linked to affinity-purified biotin-labeled rabbit anti-rat IgG. The LI was calculated as the number of cells staining positive for BrdU per mm length of interfollicular basement membrane, using an eyepiece reticle. Three 2 cm long sections each from three mice were analyzed.

**Results**

**Tumor development**

All animals were weighed weekly for the duration of the experiment. The differences in body weights between the control and DFMO groups were <10% and not statistically significant (Figure 1). Final body weights (n = 30, mean ± SD) were 27.6 ± 0.8 (control group), 25.6 ± 0.7 (continuous DFMO group), 25.6 ± 0.6 (DFMO at 12 weeks group) and 26.3 ± 1.3 g (DFMO at 22 weeks group).

The tumor data were calculated in terms of incidence (percentage of mice bearing tumors) and multiplicity (average number of tumors per mouse). Tumors were first observed after 17 weeks of UV irradiation and continued to appear throughout the course of the experiment. Within several weeks there was a marked difference in tumor incidence between those groups receiving DFMO (continuous DFMO and DFMO at 12 weeks) and those groups (control and DFMO at 22 weeks) not receiving DFMO. These latter groups reached an incidence of ~90% at week 21, at which time both the continuous DFMO group and the group in which DFMO was not started until 12 weeks had an incidence of only 20%. By the termination of the experiment the 12 week DFMO group had reached 100% incidence (Figure 2, A).

With regard to tumor multiplicities (Figure 2, B), up to 23 weeks the control and DFMO at 22 weeks groups had similar values (~2.2 tumors/mouse), as would be expected, while at

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**Table I. Treatment schedule**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of mice</th>
<th>Drug</th>
<th>Time of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>DFMO</td>
<td>Continuous</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>DFMO</td>
<td>Beginning at 12 weeks</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>DFMO</td>
<td>Beginning at 22 weeks</td>
</tr>
</tbody>
</table>

**Fig. 1.** Chronic DFMO administration has no significant effect on body weight. DFMO was administered at a dose of 0.4% in the drinking water. Body weights were measured weekly for the duration of the experiment. The differences in body weights between the control and DFMO groups were <10% and not statistically significant.

**Fig. 2.** DFMO inhibits UV-elicited skin tumor development. Using groups of 30 SKH hairless mice, DFMO was administered either continuously throughout the experiment or beginning at either week 12 or 22. UV treatment was stopped at week 23 but DFMO continued until the end of the experiment. Tumor incidence was calculated as the percentage of animals having tumors in each treatment group (A). Tumors were counted weekly and tumor yield or multiplicity was calculated as the average number of tumors per mouse for each treatment group (B).
**Di**fluoromethylornithine and UV carcinogenesis

Table II. Distribution of tumors by type

<table>
<thead>
<tr>
<th>Group</th>
<th>Percent of each tumor typea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Papilloma</td>
</tr>
<tr>
<td>UV only</td>
<td>73 (66)b</td>
</tr>
<tr>
<td>UV + DFMO continuous</td>
<td>71 (17)</td>
</tr>
<tr>
<td>UV + DFMO 12 weeks</td>
<td>92 (49)</td>
</tr>
<tr>
<td>UV + DFMO 22 weeks</td>
<td>88 (50)</td>
</tr>
</tbody>
</table>

aAt the termination of the tumor experiment all tumors were removed and processed for histological analysis. Hematoxylin and eosin stained tumor sections were classified as papilloma, squamous cell carcinoma (SCC) or spindle cell carcinoma.

bValues are percent of total; the number of tumors are shown in parentheses.

ments were carried out. As shown in Figure 4 (A), a single UV treatment caused an ~10-fold induction in ODC activity at 24 h. DFMO reduced this activity by >60%. DFMO administered for 2 weeks (Figure 4, B), during which time the mice were irradiated six times, reduced UV-induced ODC activity to control levels. ODC activity after six treatments with UV is less than after a single exposure because the skin becomes refractory to repeated exposures. DFMO by itself slightly reduced, but not in a statistically significant manner, ODC activity in non-irradiated animals.

DFMO was also found to reduce UV-induced increases in keratinocyte proliferation. BrdU labeling was used to calculate the LI. As shown in Figure 5, a single UV treatment raised the LI by 370%. DFMO reduced the UV-induced LI by 33%. More striking, however, was the effect of continuous DFMO administration on chronic (six times) UV irradiation, where DFMO reduced the LI to non-irradiated control levels. DFMO alone had no effect on LI.

**Discussion**

The original observations that there is a strong association between high levels of polyamines and rapid cell proliferation were made more than 25 years ago (18–20). The development of DFMO by the Merrell Research Institute definitively demonstrated that polyamines derived from ornithine are necessary for maximum cell growth (21,22). Suppression of polyamine synthesis, however, generally does not cause the death of normal mammalian cells (23,24). An additional observation is that polyamine levels are usually elevated in rodent and human neoplasms, relative to surrounding normal tissue. This has been well documented in human and mouse colon and in mouse epidermis (12,25–27). Because polyamine metabolism is believed to be an integral component of the mechanism of epithelial carcinogenesis, it was expected that agents that reduced polyamine synthesis would inhibit tumor development. Inhibitors of ODC were subsequently shown to suppress tumor formation in experimental models of chemically induced bladder, breast, colon and skin cancer (8–10,28–30).

In this study we have demonstrated that oral DFMO has significant chemopreventive effects on UV-induced skin tumorigenesis. Takigawa et al. (8) found that DFMO applied to mouse skin or administered in the drinking water (1% w/v) during phorbol ester promotion of chemical carcinogen-initiated mice inhibited the formation of papillomas by 50% and 90%, respectively. Using a multistage promotion protocol (limited 12-O-tetradecanoylphorbol-13-acetate followed by mezerein), Weeks et al. (10) found that DFMO was a better

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**Fig. 3.** Effects of DFMO on tumor size distribution. At the time of death the diameters of all tumors were measured and recorded. For each size group data are expressed as a percentage of the total (100%).
Fig. 5. DFMO reduces UV-induced increases in keratinocyte proliferation. Groups of six mice were placed on DFMO for at least 48 h before a single UV (1× UV) exposure or placed on DFMO for 2 weeks with thrice weekly (6× UV) irradiation. Mice were killed 48 h after the last UV exposure; 1 h before death mice were injected i.p. with BrdU. Sections of exposed skin were fixed and processed for immunohistochemical staining for BrdU incorporation. The LI was calculated as the number of cells staining positive for BrdU per mm length of interfollicular basement membrane.

that overexpress ODC in the epidermis, showed that 1% DFMO in the drinking water completely prevented the development of papillomas in carcinogen-treated mice.

While concomitant administration of DFMO with exposure to carcinogens/promoters are used to study the prevention of malignant lesions, it has been recognized that this 'preventive' agent may also have therapeutic efficacy. With regard to rodent skin models, using the K14-HPV16 transgenic mice, which develop spontaneous skin lesions, Arbeit et al. (31) showed that treatment with DFMO from 28 to 32 weeks of age, at which time visible lesions were present, caused a marked regression of dysplastic papillomas of the ear and reduced severity of lesions on the chest. Peralta Soler et al. (33) demonstrated that DFMO caused a rapid regression of epidermal papillomas induced by 7,12-dimethylbenz[a]anthracene in transgenic mice overexpressing ODC. In the UV study described here, beginning DFMO treatment at a time (22 weeks) in which UV-induced tumors were well developed also caused regression of many tumors. This regression was not complete and after several weeks tumor multiplicities began to increase. Whether this represents de novo tumor development, which was occurring in the control group, or the reappearance of regressed tumors due to refractoriness to DFMO is unknown.

Generally, human clinical trials have not shown DFMO alone to be highly efficacious and at doses 3 g/m²/day side effects, cancers, administered DFMO (1% w/v) at distinct stages of neoplastic progression. Those groups receiving DFMO for 32 weeks showed complete inhibition of visible or microscopic malignancies compared with control animals, which had a 21% skin cancer incidence. The greater inhibition seen by Arbeit et al. (31), using K14-HPV16 transgenic mice, which develop spontaneous epidermal cancers, administered DFMO (1% w/v) at distinct stages of neoplastic progression. That observation that DFMO reduced tumor multiplicities by ~75% is in concordance with an earlier study by Gensler (32) in which 1% DFMO reduced UV-induced skin cancer incidence from 38 to 9%. Peralta Soler et al. (33), using transgenic animals

stage II than stage I inhibitor. Arbeit et al. (31), using K14-HPV16 transgenic mice, which develop spontaneous epidermal cancers, administered DFMO (1% w/v) at distinct stages of neoplastic progression. Those groups receiving DFMO for 32 weeks showed complete inhibition of visible or microscopic malignancies compared with control animals, which had a 21% skin cancer incidence. The greater inhibition seen by Arbeit et al. (31) compared with the UV carcinogenesis model presented here may be due to either the higher dose of DFMO in the Arbeit study (1.0 versus 0.4%) or to differences in the ‘strength’ of the ODC-inducing signals. Our observation that 0.4% DFMO reduced tumor multiplicities by ~75% is in concordance with an earlier study by Gensler (32) in which 1% DFMO reduced UV-induced skin cancer incidence from 38 to 9%. Peralta Soler et al. (33), using transgenic animals

Fig. 4. DFMO inhibits UV-elicited increases in ODC activity. Groups of six mice were placed on DFMO for at least 48 h before a single UV (1× UV) exposure or placed on DFMO for 2 weeks with thrice weekly (6× UV) irradiation and were killed 24 h after UV exposure. Epidermal homogenates were used to measure ODC activity, which was calculated as nmol CO₂/mg protein/h. The data are expressed as a percent of control.
bearing tumors were administered 1% DFMO in the drinking water, which caused a rapid reduction in tumor cell proliferation (with concomitant tumor regression) but no reduction in proliferation of normal keratinocytes, even though putrescine levels were reduced (33). This selective cytotoxicity of DFMO has been attributed to the presence of a mutated ras gene in the skin chemical carcinogenesis model, such that polyamines are possibly required for either inhibition of cell death factors or formation of cell survival factors (14). Several other events, or genes, have been shown to be regulated by polyamines, including the matrix metalloproteinases, which are critical for tumor cell invasion (39). Recently DFMO was shown to cause apoptosis in proliferating endothelial cell in vitro and to have anti-angiogenic effects in vivo (40). This suppression of angiogenesis is likely to contribute to the tumor-specific cytotoxicity of DFMO. Additionally, depletion of polyamines inhibits DNA repair following UV irradiation or treatment with alkylating agents, suggesting both a role for polyamines in repair and the possible therapeutic use of DFMO to confer greater sensitivity to DNA-reactive chemotherapeutic drugs (41,42).

In summary, we have shown that a low dose of DFMO can have strong chemopreventive efficacy against UV-induced carcinogenesis. Importantly, significant protection is conferred when administration is begun either at the time tumor development is anticipated or after tumors have developed.

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References


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