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Therapy of murine squamous cell carcinomas with 2-difluoromethylornithine

Yan Chen[1], Juncai Hu[1], David Boorman[1], Andres Klein-Szanto[2] and Thomas G O'Brien*[1]

Address: [1]Lankenau Institute for Medical Research 100 Lancaster Avenue, Wynnewood, PA 19096, USA and [2]Fox Chase Cancer Center 7701 Burholme Avenue Philadelphia, PA 19111, USA

Email: Yan Chen - cheny@mlhs.org; Juncai Hu - hujunc@yahoo.com; David Boorman - boormand@mlhs.org; Andres Klein-Szanto - andres.klein-szanto@fccc.edu; Thomas G O'Brien* - obrient@mlhs.org

* Corresponding author

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Abstract

Targeted overexpression of an ornithine decarboxylase (ODC) transgene to mouse skin (the K6/ODC mouse) significantly enhances susceptibility to carcinogenesis. While in most strain backgrounds the predominant tumor type resulting from initiation-promotion protocols is benign squamous papilloma, K6/ODC mice on a FVB/N background develop malignant squamous cell carcinomas (SCCs) rapidly and in high multiplicity after carcinogen treatment. We have investigated the utility of polyamine-based therapy against SCCs in this model using the ODC inhibitor 2-difluoromethylornithine delivered orally. At a 2% concentration in drinking water, DFMO caused rapid tumor regression, but in most cases, tumors eventually regrew rapidly even in the presence of DFMO. The tumors that regrew were spindle cell carcinomas, an aggressive undifferentiated variant of SCC. At 1% DFMO in the drinking water, tumors also responded rapidly, but tumor regrowth did not occur. The majority of DFMO-treated SCCs were classified as complete responses, and in some cases, apparent tumor cures were achieved. The enzymatic activity of ODC, the target of DFMO, was substantially reduced after treatment with 1% DFMO and the high SCC polyamine levels, especially putrescine, were also significantly lowered. Based on the results of BrdUrd labeling and TUNEL assays, the effect of DFMO on SCC growth was accompanied by a significant reduction in tumor proliferation with no increase in the apoptotic index. These results demonstrate that SCCs, at least in the mouse, are particularly sensitive to polyamine-based therapy.

Introduction

Targeted overexpression of ornithine decarboxylase (ODC) to mouse epidermal keratinocytes with a bovine keratin 6 (K6) promoter/regulatory region greatly increases susceptibility to skin tumor development [1]. In fact, in the K6/ODC transgenic model, treatment with exogenous tumor promoters such as 12-0 tetradecanoylphorbol-13-acetate (TPA) is dispensable: a maximal tumor response occurs after a single initiating application of a carcinogen such as 7, 12-dimethlybenz(a)anthracene (DMBA). The increased activity of the ODC transgene in this model is clearly necessary for the enhanced susceptibility phenotype as exposure of initiated mice to the ODC inhibitor 2 – difluoromethylornithine (DFMO) completely prevents papilloma development [2]. Additionally, expression of the transgene is also important for

maintenance of papilloma growth as DFMO treatment of papilloma-bearing mice causes complete tumor regression [2].

The predominant tumor type induced in most mouse models of skin cancer, especially those involving initiation/promotion protocols, is benign squamous papilloma. Squamous cell carcinomas, if they develop at all, are a small fraction of total tumors and appear with a long latency (6–12 months). Recently, a PKC-ε transgenic mouse (on an FVB background) has been reported to develop predominantly squamous cell carcinomas after a DMBA/TPA protocol [3]. Similarly, when the K6/ODC transgene was placed on a congenic FVB background, SCCs developed rapidly and in high multiplicity after DMBA exposure [4]. Finally, a bi-transgenic K6/ODC; TG.AC model [on a (FVB × B6)F1 background] develop SCCs spontaneously due to the combined effects of ODC overexpression and the v-Ha-ras gene present in the TG.AC line [5]. The common feature of all these recently developed transgenic mouse models of SCC is the FVB genetic background, indicating the presence of SCC-predisposing genes in this strain. In the FVB/N strain itself, Hennings et al reported a higher percentage of papillomas progressed to SCCs in this strain than is typical of other susceptible strains [6].

Because of the short latency and high multiplicity of SCCs in the K6/ODC (FVB) model, it could serve as a useful preclinical model of human SCC. Specifically, existing or novel therapeutic approaches against SCC could be evaluated in this model. Because of the effectiveness of DFMO as a therapy for squamous papillomas, we have evaluated this drug as single-agent therapy for SCC. Our results demonstrate that murine SCCs are remarkably sensitive to DFMO treatment, suggesting that polyamine-based therapy may be a novel approach to the treatment of human SCC.

Methods

Animals and treatments

The K6/ODC model on the FVB/N strain background instead of the original C57BL/6J background was used in all experiments. To induce SCCs, newborn (1 day old) pups were treated with a single dose of 200 nmol of 7,12-dimethylbenz[a]anthracene (DMBA) dissolved in 50 μl acetone applied to the dorsal skin. Treatment area was approximately 40–50 mm^2. SCCs typically developed on the treated area beginning 5 weeks later. SCC bearing mice with tumor volumes in the range 250–1500 mm^3 were randomized to experimental groups between 8 and 16 weeks of age.

Groups of tumor-bearing mice (n = 5–18) were treated with DFMO dissolved at 1 or 2% in the drinking water. DFMO solutions were changed every 4 days or less. Animals were observed daily for signs of distress due to tumor burden and/or DFMO treatment. Tumor volume was calculated according to the equation $V = \frac{l \times w^2}{2}$ where l = length (longest dimension) and w = width.

Tumor harvest, ODC and polyamine measurements

SCC-bearing mice were rapidly euthanized, SCCs excised, and a piece or pieces placed in Fekete's fixative for histopathologic diagnosis and the remaining tumor was placed immediately on dry ice and transferred to -80°C for subsequent biochemical determinations. Tumor-bearing mice used for these analyses were randomized by tumor size for placement into control or DFMO-treated groups. ODC activity and polyamine levels were measured in buffer extracts and 0.2 N PCA extracts of tissue, respectively [7]. Buffer extracts for ODC determination from DFMO-treated mice were dialyzed overnight vs 1000-fold excess of buffer to remove free DFMO. One unit of ODC activity corresponds to 1 nmol of $C0_2$ liberated/h. ODC specific activity is expressed as units/mg protein. Polyamine levels are expressed as nmol/mg DNA.

Measurement of apoptosis and cell proliferation in SCCs

Tumor-bearing mice were injected i.p. with BrdUrd (100 μg/g body weight) 1 hour before sacrifice. Mice were randomized into control and DFMO-treatment groups based on tumor size. Pieces of tumor were fixed overnight in Fekete's solution and processed for paraffin embedding. Assessment of apoptotic and cell proliferation indices was performed using a TUNEL-based assay and detection of incorporation of BrdUrd into nuclei, respectively, as previously described [2]. Differences in these parameters between control and treatment groups were assessed by ANOVA using StatView® (SAS Institute).

Results

Efficient induction of cutaneous SCCs in K6/ODC (FVB) mice

We have previously reported the propensity of K6/ODC mice (founder line 55/2 m) on an FVB background to develop SCCs rapidly after a single dose of 200 nmol DMBA [4]. Due to breeding problems with 55/2 m founder line, mice from a second founder line 39/1m were treated with a single dose of DMBA (either 50 or 200 nmol) to determine if SCCs are also induced in this line. [The papilloma response of these two lines on a C57BL/6J background is identical [8].] The results (Figure 1) confirm the high sensitivity of K6/ODC (FVB) mice from this founder line to SCC induction: maximal SCC multiplicities of 2.1 ± 0.3 and 4.3 ± 0.6 were observed in mice treated with 50 and 200 nmol DMBA, respectively. For the studies described, mice from both founder lines were used. The latent period for SCC appearance in this model

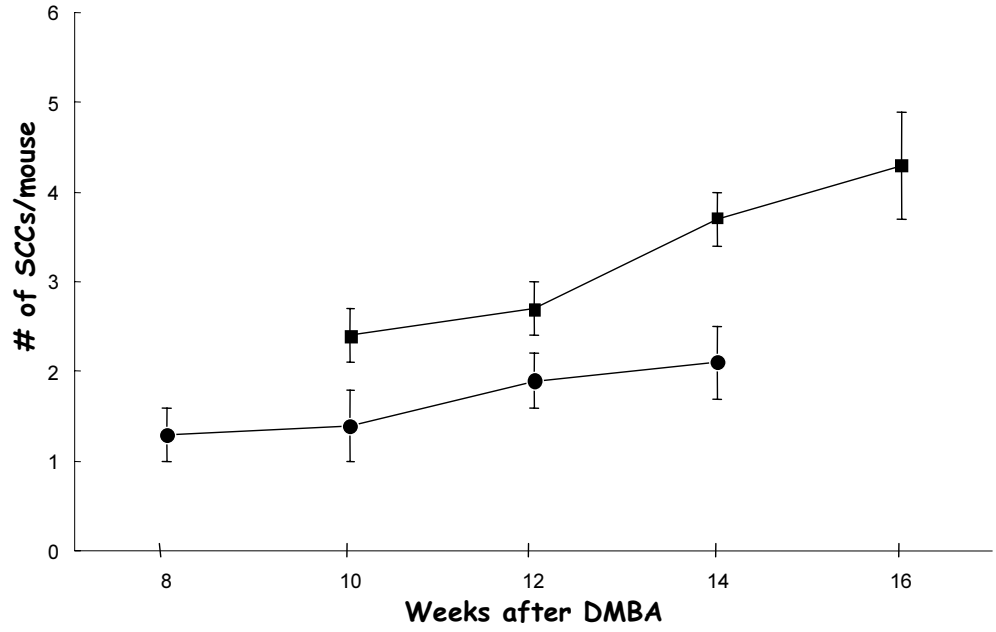

Figure 1
Dose-dependent induction of SCCs in K6/ODC(FVB) mice of founder line 39/1m. Newborn mice (1 day old) were treated with either 50 nmol (●) or 200 nmol (■) DMBA dissolved in 50 ul acetone. Visible tumors occurred as early as 5 weeks after treatment and SCCs were quantitated beginning at 8 or 10 weeks after treatment. Results are given as mean number of SCCs per mouse ± S.E. There were 10 mice in the 50 nmol group and 18 mice in the 200 nmol group.

is remarkably short, 5–6 weeks, compared to other models (typically 6 months or longer). As previously reported [4], the majority of tumors that developed were SCCs, not benign papillomas typically found in most other models.

Effect of DFMO on growth of murine SCCs

Based on the therapeutic effect of DFMO on papillomas in both C57BL/6(B6) and K6/ODC(B6) mice [2], SCC-bearing K6/ODC(FVB) mice were administered DFMO in the drinking water at a concentration of 2% (w/v). The SCCs exhibited a biphasic response to DFMO: there was a pronounced volume reduction of each tumor over the first two weeks of treatment, followed in five of six cases by a rapid regrowth of the tumor in the continued presence of DFMO (Figure 2). When the tumors that regrew were examined histologically, all were diagnosed either as spindle cell carcinomas or mixed squamous cell and spindle cell carcinoma. Immunostaining with an anti-keratin antibody confirmed the diagnosis as spindle cell carcinomas (as opposed to undifferentiated sarcomas). Spindle cell carcinomas are an anaplastic, highly aggressive, variant of SCC in the mouse [9]. A typical squamous cell carcinoma and spindle cell carcinoma are shown in Fig 3. The extremely rapid regrowth of the spindle cell carcinomas in the continued presence of DFMO raised the question whether ODC activities and polyamine levels

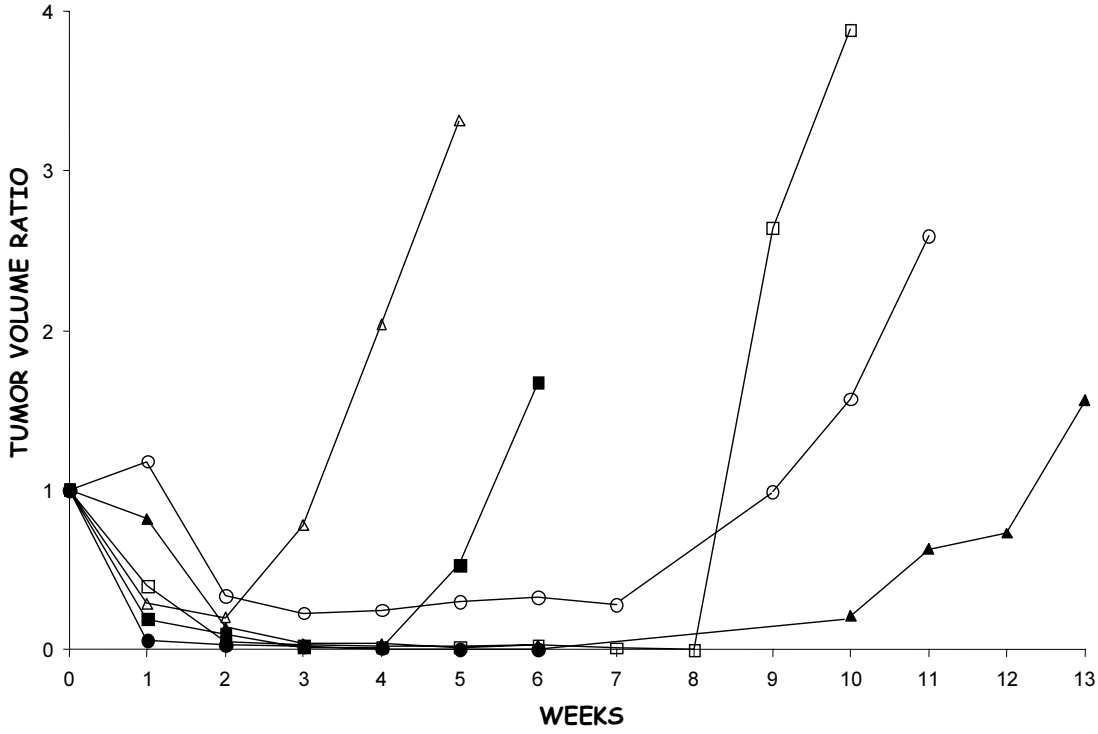

Figure 2
Response of SCCs to 2% DFMO in the drinking water. Five K6/ODC(FVB) mice of founder line 55/2m bearing 6 SCCs were administered 2% DFMO in the drinking water and tumor volumes measured weekly over the course of treatment. Individual tumors are indicated by use of different symbols. Initial tumor volumes ranged from 279–1238 mm^3. All mice were eventually sacrificed with large tumor burdens due to a rapidly growing subcutaneous tumor. Tumor volume ratio is defined as the tumor volume after DFMO treatment divided by the initial tumor volume.

were affected in these tumors. Prior to treatment, SCCs express abundant levels of ODC and polyamine levels, especially putrescine, are extraordinarily high (Table 1). In contrast, the spindle cell carcinomas express, in general, low levels of ODC and greatly reduced polyamine levels. The one spindle cell carcinoma with moderately elevated ODC and polyamine levels was actually a mixed tumor composed of both squamous cell carcinoma and spindle cell carcinoma components, suggesting the former contributes most of the measured ODC activity and polyamines. These results indicate that excessively elevated ODC and polyamines are not required for growth of spindle cell carcinomas.

A second, larger, study was conducted to determine the response of SCCs to 1% DFMO. Compared to control SCC-bearing mice, after a one-week lag period, tumor-bearing mice administered 1% DFMO exhibited a rapid reduction in tumor volume (figure 4). The response of individual tumors to 1% DFMO is shown in Table 2: of the 16 SCCs, 15 responded with at least an 80% volume reduction while 1 tumor was non-responsive (17A). Of 8 tumors evaluable after 10 weeks of observation, (2 mice

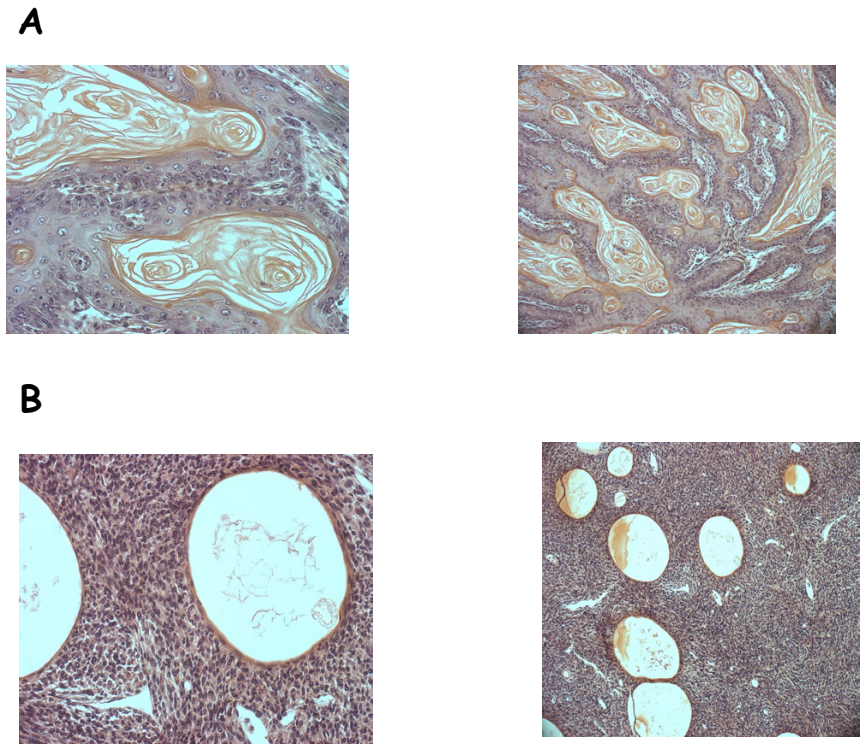

Figure 3
Histology of SCCs vs spindle cell carcinomas. (A) A typical well-differentiated SCC is shown. (B) Morphology of a spindle cell carcinoma that regrew in the presence of 2% DFMO (figure 2). Note the small, tightly packed fibroblastic cells with loss of epithelial features in the spindle cell carcinoma. The prominent epithelial cysts are a feature of normal K6/ODC skin [1].

Table 1: ODC and polyamine levels in SCCs vs. spindle cell carcinomas. Portions of 5 histologically confirmed SCCs from 5 different mice and the 5 spindle cell carcinomas from Figure 2 were harvested for measurement of ODC activity and polyamine levels as described in Methods. Results shown are mean ± S.E. Abbreviations: Pu, Putrescine; Spd, Spermidine; Sp, Spermine

	ODC Specific Activity units/mg protein			Polyamines nmol/mg DNA		
Tumor type	Mean ± S.E.	Range	Pu	Spd	Sp	Total
SCC	126 ± 38	16.7 – 251	1543 ± 604	306 ± 68	79 ± 35	1928
Spindle Cell Carcinomas	1.6 ± 1.2	0.11 – 7.1	14.1 ± 2.9	68.7 ± 25	54.7 ± 18	138

with 8 SCCs died unexpectedly after 6–7 weeks on DFMO), five tumors (62.5%) exhibited complete responses and 1 responded with a >95% reduction in tumor volume. For 2 of the tumors that responded completely, (14A and 20A) a follow-up period of 5–6 weeks indicated no tumor regrowth after cessation of DFMO therapy. Thus, in contrast to the results with 2% DFMO (Figure 2), we observed no outgrowth of highly aggressive spindle cell carcinomas after treatment with 1% DFMO, and in fact some SCCs appear to have been cured by this therapy. However, studies involving a longer follow-up period than 5–6 weeks are required to reach definite conclusions regarding tumor cures after DFMO therapy.

Effect of DFMO on ODC activity and polyamine levels

In order to determine the effect of DFMO on its only known target, we measured ODC activities in tumors following 1% DFMO treatment for 1–8 days. This period was

Table 2: Response of individual tumors to treatment with 1% DFMO. SCC-bearing mice were administered 1% DFMO in the drinking water until all tumors had completely regressed, then transferred to regular drinking water to evaluate tumor recurrence. The values in bold represent the DFMO treatment period for each mouse. Tumor volume ratio is defined as the tumor volume after DFMO treatment divided by the initial pretreatment volume. There were 8 concurrent control mice with 13 total tumors, all of which increased in tumor volume throughout the experimental period (data not shown).

	TUMOR VOLUME RATIO Weeks after start of treatment									
Tumor #	1	2	3	4	5	6	7	8	9	10
14A	**0.18**	**0**	**0**	**0**	0	0	0	0	0	0
14B	**0.12**	**0.02**	**0.01**	**0.01**	0.01	0.01	0.03	0.04	0.08	0.03
16A[a]	**0.08**	**0.02**	**0.02**	**0**	**0.03**	**0.06**				
16B	**1.19**	**0.31**	**0.16**	**0.05**	**0.02**	**0.02**				
16C	**0.06**	**0.08**	**0.01**	**0**	**0**	**0**				
16D	**1.60**	**0.21**	**0.02**	**0.01**	**0.02**	**0.02**				
16E	**1.21**	**0.14**	**0.04**	**0**	**0**	**0**				
16F	**1.43**	**0.15**	**0.19**	**0.04**	**0.09**	**0.03**				
16G	**0.31**	**0.11**	**0.08**	**0.05**	**0.08**	**0.08**				
17A	**2.03**	**1.90**	**2.19**	**2.25**	**2.29**	**2.74**	**1.40**	**1.69**	**1.35**	**2.07**
18A	**0.83**	**0.10**	**0.02**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0**
19A[b]	**3.28**	**1.92**	**0.76**	**0.31**	**0.33**	**0.09**	**0**			
20A[c]	**0.77**	**0.03**	**0.02**	**0**	**0**	**0**	**0**	0	0	0
21A	**0.54**	**0.10**	**0.01**	**0.03**	**0.01**	**0.02**	**0.01**	**0**	**0**	**0**
21B	**0.77**	**0.25**	**0.20**	**0.15**	**0.17**	**0.19**	**0.37**	**0.58**	**0.97**	**0.97**
21C	**1.18**	**0.34**	**0.07**	**0.01**	**0**	**0**	**0**	**0**	**0**	**0**

[a]Mouse died after 6 weeks of treatment [b]Mouse died after 7 weeks of treatment [c]Mouse was followed for 12 weeks with no recurrence of tumor

chosen based on previous results in squamous papillomas [2]. Control SCCs expressed very high levels of ODC, albeit with a substantial variability from tumor to tumor (Table 3). DFMO treatment led to a rapid and substantial decrease in SCC ODC activity; as early as 24 hours after the start of DFMO therapy, the mean ODC activity was reduced by 91% (Table 3). By 8 days after DFMO, ODC activity was reduced by greater than 96%. Despite very high levels of pretreatment ODC activity, DFMO administered p.o. was able to rapidly normalize enzyme levels to values typical of K6/ODC epidermis [4].

We also measured polyamine levels in the same tumors used for ODC analyses. As expected based on previous studies of benign tumors in this model [2], putrescine is the most abundant polyamine in untreated SCCs, presumably due to the high level of ODC expression compared to downstream enzymes in putrescine metabolism (S-adenosyl-L-methionine decarboxylase, spermidine synthase). After 1% DFMO treatment, SCC polyamine levels, especially putrescine, declined rapidly (Table 3). As early as 24 hours after drug treatment, putrescine levels were reduced 71%. After 8 days of treatment, putrescine levels were reduced 88% from untreated SCC levels. Spermidine and spermine levels also declined by approximately 50% over the 8-day treatment period.

Effect of DFMO on tumor proliferation and apoptotic index

To investigate the mechanism of DFMO-induced SCC regression, we measured the proliferation index and fraction of cells undergoing apoptosis in untreated and 1% DFMO-treated tumor-bearing mice. Consistent with previous results with benign squamous papillomas [2], DFMO treatment did not cause an increase in the number of cells undergoing apoptosis in SCCs. (Table 4). Instead, at 48 hours and later after treatment, a significant reduction in apoptotic cells was observed. These results indicate that an induction of a large number of apoptotic cells after DFMO treatment is not involved in the early response of SCCs to DFMO, although we can not rule out a role for apoptosis at later times following DFMO therapy.

In marked contrast to the apoptotic index, DFMO had a rapid effect on tumor proliferation (Table 5). The percentage of BrdUrd-positive cells was decreased as early as 24 hours after DFMO therapy, reached a maximum at 48 hours (73% inhibition), and was significantly decreased through 8 days of treatment. As described earlier, polyamine levels, especially putrescine, are decreasing precipitously throughout this 8-day period. These results are consistent with our previous studies in squamous skin papillomas indicating a regulatory role for polyamines, and in particular putrescine, in neoplastic growth [2].

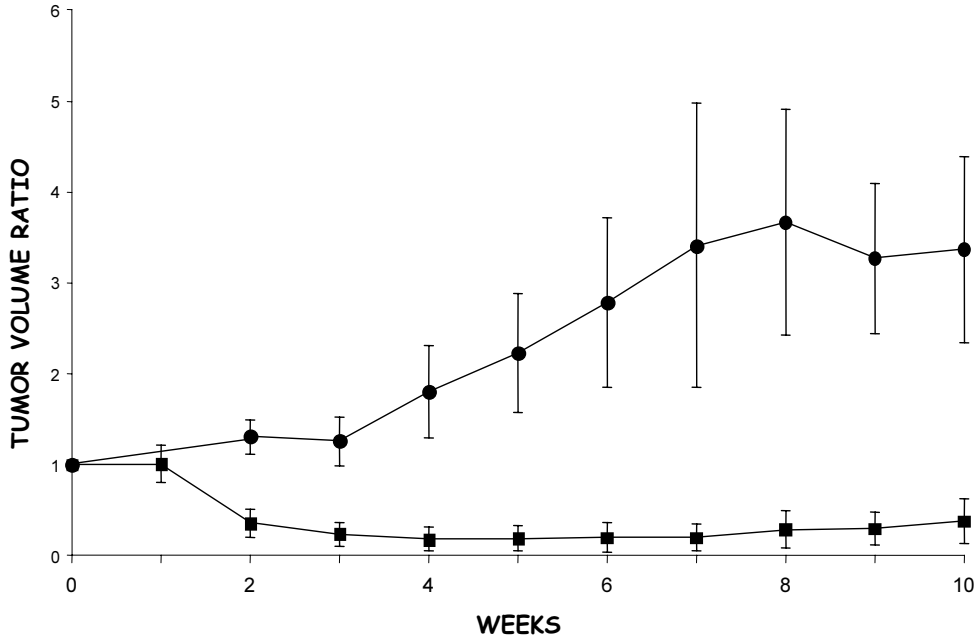

Figure 4
Response of SCCs to 1% DFMO in the drinking water. Seven K6/ODC(FVB) mice of founder line 39/1m bearing 16 SCCs were administered 1% DFMO (■) in their drinking water for 10 weeks (or less if complete tumor regression was observed). Control mice (●) (n = 8, bearing 13 total tumors) received regular drinking water. The tumor volume ratio was measured weekly. Two mice with 8 SCCs with very low tumor burden died unexpectedly at 6 and 7 weeks of DFMO treatment. For individual tumor responses, see Table 2.

Table 3: Effect of DFMO on SCC ODC activity and polyamine levels. Untreated (control) SCC-bearing mice or mice administered 1% DFMO for the indicated times were sacrificed and portions of their SCCs quickly frozen and started at -80°C. Mice from founder line 39/1m were used. Subsequently tumor extracts were prepared for measurement of ODC activity and polyamine levels as described in Methods. There were 6 control SCCs and 3–4 SCCs in the DFMO groups. Results are expressed as mean ± S.E. Control values for ODC activity and polyamine levels are taken from Table 1. Abbreviations: Pu, Putrescine; Spd, Spermidine; Sp, Spermine.

Treatment	ODC Specific Activity units/mg	P value[b]	Polyamine nmol/mg DNA Pu	P value	Spd	P value	Sp
Control	126 ± 38.2	-	1543 ± 04	-	306 ± 68	-	79 ± 35
DFMO, 24 hrs.	11.3 ± 1.2	0.07	445 ± 164	0.07	267 ± 29	0.65	38 ± 3.5
DFMO, 92 hrs.	7.2 ± 3.1	0.06	310 ± 121	0.06	172 ± 40	0.17	37 ± 15
DFMO, 192 hrs.	4.5[a]	-	190 ± 47	0.03	153 ± 58	0.09	36 ± 9.9

[a]Two SCCs were used for ODC determination. [b]Compared to control value (ANOVA).

Table 4: Effect of DFMO on apoptotic index of SCCs. Tumor-bearing K6/ODC transgenic mice of founder line 39/1m were administered regular drinking water or water containing 1% DFMO. At the indicated time, animals in each group were sacrificed, and 3–5 representative tumors were harvested for measurement of apoptotic frequency as described in Methods. Control mice (n = 4) were sacrificed at various time throughout the 8d experimental period. Results represent > 800 cells counted in multiple sections from each tumor.

Group	No. of Cells Counted	% Apoptotic Cells (mean ± SE)	P value*
Control	5311	2.7 ± 0.4	
DFMO, 1d	4100	2.6 ± 0.1	0.81
DFMO, 2d	3429	1.4 ± 0.5	0.035
DFMO, 4d	5831	1.4 ± 0.2	0.026
DFMO, 8d	4189	1.1 ± 0.1	0.013

* vs control value (ANOVA)

Table 5: Effect of DFMO on proliferative index of SCCs. Tumor-bearing K6/ODC transgenic mice of founder line 39/1m were administered 1% DFMO in their drinking water and sacrificed at the indicated times after the start of treatment. Control animals (n = 4) received regular drinking water and were sacrificed at various times throughout the 8 day experimental period. One hour before sacrifice, mice were injected i.p. with BrdUrd (100 μg/g body weight), and tumors and surrounding skin were processed for immunocytochemistry as described in Methods. Results represent counts of > 800 cells/section with 3–5 tumors per group.

Group	No. of Cells Counted	% of Nuclei, BrdUrd-Positive (mean ± SE)	P Value*
Control	4375	28.7 ± 3.6	
DFMO, 1d	4696	21.2 ± 3.9	0.13
DFMO, 2d	4339	7.8 ± 1.0	0.007
DFMO, 4d	6202	13.3 ± 1.9	0.004
DFMO, 8d	2651	14.4 ± 1.9	0.009

*vs control value (ANOVA)

Discussion

On all inbred strain backgrounds examined to date, expression of the K6/ODC transgene increases susceptibility of skin to tumor development [1,4] and unpublished results]. On most strain backgrounds, the great majority of tumors induced in this model after a single low dose of a carcinogen such as DMBA are benign squamous papillomas. On the FVB/N background however, DMBA induces predominantly squamous cell carcinomas (Figure 1 and reference [4]). The predisposition to SCC development of K6/ODC (FVB) mice is consistent with the results of others using different models on an FVB strain background [3,5,6]. A unique feature of the K6/ODC(FVB) model is the very short latency of tumor development (5–6 weeks) and high tumor multiplicity (Figure 1). These properties favor its utility as an autochthonous mouse model of SCC development, amenable to rapid preclinical testing of new therapeutic modalities.

Based on previous studies showing efficacy of the ODC inhibitor DFMO against squamous papillomas in K6/ODC B6 mice, we asked whether this drug would be effective against SCCs. The answer is clearly yes, although significant differences exist in outcomes depending on the dose of DFMO used. At a 2% dose level, all SCCs responded initially with a substantial volume reduction, but in the great majority of cases (83%) tumors rapidly regrew in the continuous presence of DFMO. Interestingly, these DFMO-resistant tumors were of a different histologic type (spindle cell carcinoma or mixed spindle cell/squamous cell carcinoma) and had markedly reduced ODC and polyamine levels. It appears that this high concentration of DFMO selects for a "new" tumor type that does not require high polyamine levels for growth. The mechanism responsible for this selection process is not known. However, since most DMBA-induced skin tumors in K6/ODC (and other) mice contain a mutant *c-Ha-ras* allele, a possible mechanism involves loss of the wild-type *c-Ha-ras* allele as suggested by others [10]. This possibility is currently under investigation.

Lowering the dose of DFMO to 1% greatly improved the eventual therapeutic outcome: the early response of SCCs to this dose was similar to mice treated with 2% DFMO, but no spindle cell carcinomas emerged. It is presently not clear why reducing the DFMO dose from 2% to 1% elim-

inates the high rate of conversion of SCCs to spindle cell carcinomas. However, the absence of such a transition allowed us to evaluate the effect of long-term DFMO treatment on SCCs. In the 1% DFMO treatment group, we achieved a complete response rate of 62.5% (5/8) and a partial response of 12.5%. Of the complete responders, at least 2 tumors were apparently cured, based on the lack of tumor regrowth over a 5–6 week period after cessation of DFMO. To our knowledge, there are no published reports of curative therapy using DFMO in any preclinical tumor model. Looking forward to potential human trials in SCC patients it should be emphasized that oral dose levels of 1–2 % used in this study are roughly 10–20 times higher than has ever been administered to humans in early therapeutic trials [11]. Ongoing studies are evaluating lower doses of DFMO (more relevant to tolerable human doses) to determine efficacy in this preclinical SCC model.

As opposed to human tumor xenografts [12-14] there have been relatively few studies of the efficacy of DFMO as a therapeutic agent against autochthonous rodent tumors. Zhang, et al, demonstrated that 3% DFMO in the drinking water retarded the growth of small colon tumors (probably adenomas) in azoxymethane-treated rats [15]. These tumors eventually began to regrow in the presence of DFMO, reminiscent of the current results with 2% DFMO. Previous results from this laboratory demonstrated complete regression of benign squamous papillomas after 1% DFMO treatment in both C57BL/6J and K6/ODC. C57BL/6J mice [2]. Finally, Lan, et al [16] reported nearly complete regression of keratoacanthomas (a form of SCC) after 1% DFMO in a model similar, in many respects, to ours (bitransgenic K6/ODC; TG.AC mice on a (B6 × FVB)F1 background); in this model spontaneous SCC development is driven by the combined effect of a *v-Ha-ras* oncogene and ODC overexpression. Taken together, our results and those of Lan, et al, indicated that autochthonous squamous tumors, both benign and malignant, appear to be particularly responsive to single agent therapy with DFMO. In a study relevant to human SCCs of the head and neck, DFMO at 1.5% inhibited both the growth of SCC-derived cell lines in vitro and SCC xenografts in athymic mice [17].

As expected, DFMO treatment rapidly inhibited the enzymatic activity of its only cellular target, ODC, and reduced polyamine levels. A reduction in these parameters was observed as early as 24 hours after DFMO administration in the drinking water. In contrast to normal tissues, putrescine accumulates to extraordinary levels in SCCs and is the most abundant polyamine in these tumors. We have speculated that putrescine, rather than spermidine and/or spermine, is the regulatory polyamine for modulating the neoplastic phenotype [2], but this question is difficult to answer in a complex in vivo model. DFMO over an 8-day period was able to reduce putrescine levels by 88%, with lesser reductions in spermidine and spermine levels. Over the same time period, the tumor proliferation index was reduced by 50%. If the rate of cell loss due to terminal differentiation remains the same during the course of DFMO therapy, tumor volume reduction could be explained simply by this effect of DFMO on tumor proliferation. In this model of SCC, DFMO did not cause an increase in apoptosis over the same period in which polyamine levels are decreasing rapidly, tumor cell proliferation is reduced, and tumor volume is shrinking. In contrast, the apoptotic index actually decreased after DFMO therapy. There are conflicting reports on the effect of DFMO on apoptosis in various in vivo models. In a rat model of esophageal squamous cell carcinoma driven by zinc deficiency, Fung et al [18] reported an induction (approximately 2.5 fold) of apoptosis after 1% DFMO treatment in the esophageal epithelium. Similar results have been reported in rat colonic adenomas [19] and human gastric carcinoma xenographs [20]. In the bitransgenic K6/ODC; TG.AC model of SCC, very similar to the model used in our studies, Lan et al [16] found that 1% DFMO treatment increased the apoptotic index in both small and large squamous tumors in the same time period we have found a decrease in apoptotic index in SCCs (Table 4). While the reason for these discordant results in two very similar models are not readily apparent, there are at least two significant differences between the models that might explain the results. The SCCs in the K6/ODC model were induced by DMBA, which causes a variety of single point mutations in the *c-Ha-ras* and *c-Ki-ras* genes [21]. In the bitransgenic K6/ODC; TG.AC model, tumors developed spontaneously due to combined expression of a strong, doubly-mutated *v-Ha-ras* oncogene and the K6/ODC transgene. The second difference in the two models is genetic background: FVB for the K6/ODC model and (B6 × FVB)F1 for the bitransgenic model, raising the intriguing possibility of modifier alleles in the B6 strain capable of modifying the apoptotic response of SCCs to DFMO. In human actinic keratoses, premalignant squamous lesion of the skin, topical DFMO (10%) treatment causes lesion regression [22]. This effect of DFMO occurred without change in the apoptotic index [23], consistent with our previous results in mouse squamous papillomas. Taken together, these results suggest that the apoptotic response of a tissue or tumor to DFMO may depend on the complex interplay of several factors, including tissue type, degree of malignancy, nature of the genetic alterations present, and possibly genetic background.

In summary, autochthonous mouse SCCs appear to be very susceptible to therapy with DFMO. However, at the highest dose tested (2%) the initial positive response of SCCs was followed by the emergence of a histologically

different tumor, spindle cell carcinoma, which is resistant to DFMO. This selection for DFMO-resistant tumor growth did not occur at 1% dose level. While early (reviewed in [24]) and more recent [25,26] trials of DFMO against advanced cancers have been generally disappointing, there are no published reports of the use of DFMO against SCCs at any organ site. Upon further development, human SCCs should be evaluated as candidates for polyamine-based therapy using DFMO, either with or without standard chemotherapy regimens.

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