Long-Term Maintenance Therapy of Established Human Small Cell Variant Lung Carcinoma Implants in Athymic Mice with a Cyclic Regimen of Difluoromethylornithine

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ABSTRACT

We report that cyclic p.o. administration of α-difluoromethylornithine (DFMO), an inhibitor of polyamine biosynthesis, is an effective long-term (1-year) maintenance therapy for established implants of cultured human small cell lung carcinoma in athymic (nude) mice. Human small cell lung carcinoma cells, from a line which exhibited cell death in culture in the presence of DFMO, were inoculated into athymic mice and permitted to grow to palpable tumors (3–5-mm nodules with mean volume of 0.04 cm³). The animals were then randomized into untreated, continuous treatment and cyclic (3 weeks of beginning 1 week after 8 weeks continuous) treatment groups. Treatment consisted of 3% DFMO in the drinking water (5.1 g/kg/day). The tumors in the untreated group grew to 27 cm³ by 8 weeks and the animals had a median survival of 7.6 weeks. Tumor growth was inhibited by 99% (0.3 cm³) in the continuous treatment group in comparison to untreated controls. Survival was prolonged with 93% survival at 10 weeks and a 101% increase in median survival to 15.3 weeks (P < 0.05). The cyclic DFMO group had a 98.3% inhibition in tumor growth for longer than 1 year (0.56 cm³; P < 0.05). Survival was also markedly prolonged compared to the untreated group with 100% survival up to 24 weeks and a median survival of 54.3 weeks (P < 0.05). No significant toxicities were observed in the first 10 weeks of DFMO treatment even though antitumor effects were observed. With continuous DFMO treatment, the animals eventually became debilitated and developed marked weight loss and thrombocytopenia; by 20 weeks, mortality was 79%. With cyclic therapy, the animals resumed weight gain, recovered from thrombocytopenia and, at 20 weeks, had 0% mortality. By 55 weeks, mortality was 50% which, however, was not significantly different (P ~ 0.50) from mortality of a control group of nontumorous, athymic mice that had weekly body weight and skin fold measurements concurrently with the experimental, tumor-bearing animals. Thus, the observed mortality is ascribable to continuous encroachment on the normally sterile environment. These data suggest a role for DFMO in long-term therapy of sensitive human tumors such as small cell lung carcinoma, especially in patients with a low tumor burden. Furthermore, a cyclic regimen might be an important tool in maintaining clinical remissions induced by conventional combination chemotherapy.

INTRODUCTION

In previous studies, our laboratory has established the essential role of polyamine biosynthesis for the growth and survival of human SCC in cell culture (1, 2) and that the specific inhibitor of polyamine biosynthesis, DFMO markedly inhibits the growth of established implants of human SCC in athymic (nude) mice (3).

The polyamines, putrescine, spermidine, and spermine, have long been implicated in the initiation of rapid growth of cells and tissues, and in the proliferation of neoplastic cells (4–7). The first step in polyamine biosynthesis is the conversion of ornithine to putrescine via ODC (EC 4.1.1.17) (5–7). DFMO (MDL71,782A; Merrell Dow Research Institute) is a specific, enzyme-activated, irreversible inhibitor of ODC (8, 9). This drug has made possible the sustained inhibition of ODC and the subsequent depletion of polyamines in vitro and in vivo (4, 10). DFMO has been shown in vitro to suppress the early increase in ODC activity which accompanies the onset of proliferation and to retard the growth of treated cells in culture, including rat hepatoma cells, mouse mammary EMT6 sarcoma cells, L1210 leukemia cells, and human prostate adenocarcinoma cells (4, 10). We have shown that, in culture, two types of human tumor cells, HL-60 promyelocytic leukemia cells (12) and small cell lung carcinoma cells (1, 2), are unspecifically sensitive to DFMO and demonstrate not only a cessation of growth but also inability to survive during polyamine depletion.

In vivo, DFMO suppresses the appearance of several transplanted or chemically induced animal tumors and the engraftment and growth of transplanted animal tumors in rats (13–20). Our laboratory has shown that DFMO can exert antitumor therapeutic effects on established human SCC xenografts in athymic (nude) mice (3). However, even though DFMO produced a 99% inhibition in tumor growth and prolonged survival by 53%, significant host toxicities were observed after 8 to 10 weeks of treatment. The toxicity manifested as weight loss, debilitation, and thrombocytopenia with evidence of increased bleeding tendencies and contributed to mortality after tumor burden was reduced (3). Since our in vitro studies with SCC cells suggested that removal of DFMO did not result in a resumption of proliferation for at least 3 to 5 days, we designed the present study to assess whether cyclic DFMO therapy might diminish the toxicity of DFMO while maintaining a growth-inhibitory effect on the established tumor implants. The results indicate that growth inhibition of this human tumor can indeed be maintained long-term (>1 year) in this animal model and provide relationships between drug effects, tumor burden, and host toxicity which may prove important for guiding investigations of the therapeutic efficacy of DFMO in patients with SCC and other neoplasms.

MATERIALS AND METHODS

We used a previously described and well-established and characterized human SCC cell line, NCI-H82 (1, 21). The seeding, growth, and design of the cell growth experiments were as previously described (1–3).

Female BALB/c-nu/nu athymic mice (Harlan Sprague-Dawley, Walkersville, MD), 7 weeks old and weighing 25 to 30 g, were used for animal studies. The experimental protocols, including animal care, tumor cell inoculation, DFMO administration, body weight, and tumor size measurements, were as described previously (3).
Animals were divided into 5 groups, 2 control groups and 3 tumor groups. To estimate the effect of weekly manipulation and encroachment of the sterile environment on the survival of the athymic mice in the tumor groups, 2 control groups that received no tumor implants were used. One group was the untreated control group that received only routine biweekly care of cage, food, and water changes. The other group was the manipulated control group, which had additional weekly body weights and skin fold and thigh diameter measurements done, to approximate the degree of manipulation received by the tumor groups. The athymic mice were housed 4 to a cage in individual sterilized filter cages in a shared animal facility. Biweekly handling was done with sterile surgical gloves, but without sterile gowning, in a shared laminar-flow hood.

The tumor groups were inoculated with $10^7$ H-82 tumor cells as described previously (3). After tumor engraftment, the tumor-bearing animals were randomized into 3 groups: untreated; continuous DFMO; and cyclic DFMO treatment. The continuous DFMO treatment group received DFMO as a 3% solution in the sterile drinking water continuously. The DFMO solution was placed in special drip-proof and spill-proof bottles. The cyclic DFMO treatment group received DFMO continuously for 8 weeks and then 3 weeks of 4 beginning 1 week later. DFMO was supplied as DL-a-difluoromethylornithine monohydrate (eflornithine hydrochloride, MDL 71.782A) by the Merrell Dow Research Institute, Cincinnati, OH. Fluid intake was similar in all groups, and the mean daily DFMO intake was 5.1 g/kg body weight, calculated from the average weekly intake per cage (3).

Analysis of variance was used to estimate the statistical significance of differences in tumor volumes and mortality between the different groups (22).

RESULTS

Cell Studies

Similar to previous studies (1, 2), when DFMO was added on day 5, after spheroidal aggregates had formed, the cells showed a failure to continue exponential growth and a disruption of their multicellular suspended aggregates within 3 to 4 days after treatment with 5 mM DFMO; cell loss then ensued exponentially (Fig. 1).

After the initial 14-day continuous administration of DFMO, inhibition of cell growth and disruption of the multicellular suspended aggregates were maintained when DFMO was administered intermittently for 10 days during every 2-week period (Fig. 1). These results confirmed our earlier results (1, 3) that the growth and survival of established SCC cell aggregates could be altered by DFMO, and more important, that growth inhibition could be maintained even if DFMO was administered intermittently during a 2-week cycle. This suggested that cyclic DFMO might be effective in suppressing the growth of H82 cells long term in vivo, even after tumors were already established. The results also suggested that cyclic DFMO therapy should begin only after tumor growth inhibition was achieved.

Animal Studies

Control (Nontumorous) Animals. Untreated control animals had an 8% mortality after 1 year. In the manipulated control animals, the median survival was 62 weeks, a decrease of greater than 35% from untreated controls (Table 1). This established that biweekly manipulation in a shared animal facility contributed to mortality of the athymic mice. At autopsy, there was evidence of skin and blood colonization by bacteria.

Untreated Tumor-bearing Animals. The engraftment and growth of tumors in untreated tumor-bearing animals (Fig. 2) was similar to previous studies (3). The animals became markedly debilitated by week 8. Eleven of the 22 animals had died by week 8 (Fig. 2). The remainder of the untreated tumor-bearing animals were sacrificed at week 11, when they became debilitated and had marked weight loss (40% weight loss compared to non-tumor-bearing controls). The median survival was thus 7.6 weeks, and there was a 77% mortality at 8 weeks (Table 1).

Tumor volume was calculated from the tumor measurement just before death or sacrifice and the tumor weight was then measured. There was a significant correlation between the measured tumor volume and the measured tumor weight for all ranges of tumor sizes ($r = 0.95$; $P < 0.01$). Thus, because tumor volume was used to calculate tumor weight, net body weight could be estimated (Fig. 3).

Continuous DFMO Treatment. Antitumor effects of continuous DFMO treatment were similar to previous studies (3). Suppression of tumor growth was evident during the first week of treatment ($P < 0.05$), complete arrest was achieved by week 6, and tumor size gradually shrank beginning in week 7 (Fig. 2). The median survival was 15.3 weeks, and the mortality at 10 weeks was 7%, as compared to a 77% 10-week mortality for the untreated tumor group ($P < 0.01$ (Table 1)).

The continuous treatment had two phases with respect to tumor response versus host toxicity. The animals began to manifest loss of net body weight (total body weight minus calculated tumor weight) beginning in week 6, but their weight loss was not markedly different from those of the untreated tumor-bearing mice (Fig. 3). Thus, drug toxicity was not apparent during the first 6 weeks, at a time when tumor growth was already suppressed by greater than 90% from controls. From 6 weeks on, there was a marked thrombocytopenia (platelets decreased...
Fig. 2. Antitumor effects of continuous and cyclic DFMO treatment on established human SCC tumor implants in athymic nude mice. Untreated tumor-bearing animals received no treatment (A, n = 14). Cyclic DFMO animals were given 3% DFMO continuously for 8 weeks beginning with tumor engraftment at week 0, and then 3 of 4 weeks beginning at week 9 (O, n = 12). Tumor volume was calculated from the formula for an ellipsoid, $\pi/4(\ell \times \pi)^2$. Numbers above symbols, the number of spontaneous deaths within the immediately preceding week; numbers in parentheses, number of animals sacrificed. The SE is less than 20% of the mean for all data shown.

Fig. 3. Effects of continuous and cyclic DFMO treatment on net body weights of athymic nude mice carrying human SCC tumor implants. Untreated control mice did not have tumor implants and received no treatment (C, n = 12); the other 3 groups all carried tumor implants. The untreated tumor group received no treatment (A, n = 22). Continuous DFMO animals received 3% DFMO continuously for 8 weeks beginning with tumor engraftment at week 0 (A, n = 14). Cyclic DFMO animals received 3% DFMO continuously for 8 weeks beginning at week 0, and then 3 of 4 weeks beginning at week 9 (O, n = 12). The net body weight of animals was calculated as total body weight less the calculated tumor weight, calculated from the tumor volume. Tumor weight was calculated from tumor volume by the linear regression formula described in “Results.”

DISCUSSION

The results from our present study document that cyclic administration of DFMO can markedly and chronically inhibit the growth of established implants of cultured human SCC in athymic nude mice with minimal host toxicities. These in vivo data have two important parallels to the in vitro effects of DFMO on cultured human SCC cells. First, the SCC cells in vivo maintain their extreme sensitivity to the antiproliferative effects of DFMO which they exhibit in vitro (1–3). Thus, it is apparent that adequate inhibition of polyamine biosynthesis can be achieved in an in vivo setting for these human tumor cells.

Second, the in vivo results are very similar to the in vitro findings in terms of continued antiproliferative response to intermittent DFMO. The in vitro growth-inhibitory effects of DFMO on SCC cells persist when the drug is administered to cells intermittently. However, it is important to note that DFMO was administered continuously to produce an initial antiproliferative and a subsequent cytotoxic effect before intermittent stoppage of DFMO. These effects are, as we have previously noted, unusual responses of tumor cells to inhibition of polyamine biosynthesis (1–3). The established tumor implants of SCC in athymic mice also show a distinct relationship between antiproliferative response to DFMO and tumor burden. As in culture, the onset of growth inhibition is achieved much more rapidly when palpable tumors are treated early during growth, as was done during our previous and current study (3).

As opposed to the distinct antiproliferative effects discussed above, it is difficult to perceive in the present athymic mouse studies whether DFMO causes the same type of secondary “cytotoxic” response in SCC cells in vivo as occurs in vitro (Fig. 1). Certainly, in the continuous treatment group, both an antiproliferative effect and a subsequent shrinkage of tumor size (Fig. 2) occurred within the first 6 weeks. The animals appeared healthy during this time period and we presume that the loss in tumor volume resulted from a direct effect of DFMO on
tumor cells rather than from a toxic effect on the host.

The toxic effects of DFMO on the normal host bear special comment. The antiproliferative effects of this drug on certain parasites and neoplastic cells in vitro and in vivo have obviously created great interest in the clinical potential of the drug (20, 23). Recent clinical studies have suggested potential therapeutic effects in acute leukemia, trypanosomiasis, and Pneumocystis carinii (20, 23), and phase I and II trials of DFMO p.o. have recently been completed at our institution (24, 25). The dose-limiting toxicity of the drug appears to be thrombocytopenia, which occurred in about 50% of the patients (24, 25). All patients who developed thrombocytopenia had received prior chemotherapy. In rats, this finding is a uniform one and occurs within 3 to 4 weeks of treatment (26). The platelet counts in our current study document that this toxic manifestation of DFMO could have been a major factor in the deterioration after 12 weeks in our continuous DFMO treatment group. However, no evidence of gross hemorrhage was observed in any animals, and the precise mechanism of DFMO toxicity in the athymic mice must be further studied.

It should also be emphasized that the human SCC cells used in this study are unusually sensitive to DFMO. Thus results obtained with other tumors could be much less striking than the ones reported in this study. Such comparative studies using other less sensitive cell lines might now be indicated (1). In summary, our studies in athymic mice are encouraging for considering the use of DFMO to treat patients with SCC and provide data which suggest means to enhance the therapeutic response of tumor cells in general and in SCC cells in particular to treatment with DFMO in vivo: (a) we showed that animal model systems can be established for studying the in vivo effects of DFMO on human tumor cells, (b) early continuous treatment at a time of low tumor burden appears to be essential for achieving maximal antiproliferative effects; (c) after tumor growth inhibition is achieved, long-term maintenance cyclic DFMO therapy appears to be effective in maintenance therapy with minimal toxicity. In fact, the recent data that DFMO retards tumor formation in the classic tumor promotion models (27, 28) and blocks tumor engraftment in our studies and those of others (3, 13, 17, 18) suggest that DFMO could have a "chemoprophylactic" therapeutic role in patients at high risk for developing cancer (i.e., familial polyposis, multiple endocrine neoplasia syndromes, progressive bronchial epithelial atypia in smokers, etc.). In chemoprophylaxis studies, it would appear that cyclic DFMO administration might be the appropriate regimen; (d) the response of established SCC tumors to DFMO, in vivo, takes 3 to 4 weeks to occur. Thus, adequate initial treatment time with adequate doses of DFMO will be essential to exert a full antiproliferative effect. Clinical studies with P. carinii suggest that an initial high dose of i.v. DFMO may be most efficacious (23). This "lag" time for treatment effect will have to be carefully balanced with the effects of DFMO on normal cells. Thus, the fall in platelets now observed in human trials (24, 25) and rat studies (26) will have to be titrated against therapeutic response.

Finally, our previous results (1–3) and the present in vivo studies strongly suggest that DFMO could play an important role in the treatment of human SCC. DFMO appears to be most effective in the setting of low tumor burden. SCC is usually very responsive to combination chemotherapy initially (29, 30). When disease recurs, however, chemotherapy resistance is almost a universal finding and SCC remains a highly resistant tumor in virtually all patients (29, 30). In this situation where an initial reduction in tumor burden can usually be achieved, consolidation or maintenance therapy with DFMO following such initial tumor reduction should be strongly considered. The athymic mouse model presented should allow for further testing of this hypothesis and also for studies of DFMO in combination with other effective agents in the treatment of this virulent form of lung cancer.

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REFERENCES


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