[CANCER RESEARCH 47, 933–935, February 15, 1987]

Antimetastatic Activity of DL-α-Difluoromethylornithine, an Inhibitor of Polyamine Biosynthesis, in Mice

Prasad S. Sunkara[1] and Andrea L. Rosenberger

Merrell Dow Research Institute, Cincinnati, Ohio 45215

ABSTRACT

Our earlier studies indicated a role for polyamines (namely, putrescine, spermidine, and spermine) not only in tumor growth but also in tumor metastases. We have observed that administration of α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, resulted in significant inhibition of visually detectable pulmonary metastases in mice implanted with Lewis lung carcinoma. The objective of the present study is to investigate the effect of DFMO on other spontaneous and experimental metastatic models and also to determine which step(s) in the tumor metastatic cascade is sensitive to DFMO. The results presented in this study with malignant mouse B16 amelanotic melanoma (B16a) showed a dose-dependent effect of DFMO on the inhibition of both tumor growth and grossly detectable pulmonary metastases. DFMO, when administered as 0.5, 1, and 2% solution in drinking water, resulted in 0, 24.5, and 60% inhibition of tumor growth, respectively, whereas at the same doses an inhibition of 55, 83, and 96% of visible metastases was observed. At treatment levels of 1 and 2% DFMO, 30 and 65% of the animals were free of metastases. DFMO, at 0.5%, did not show any effect on tumor growth, while a significant 55% inhibition of visible pulmonary metastasis was observed, suggesting a specific role for polyamines in tumor metastasis. DFMO treatment also resulted in a significant reduction of putrescine and spermidine levels with a slight increase in spermine concentration in the tumor tissue. DFMO administration did not inhibit the experimental metastases induced as a result of i.v. injection of B16 melanoma (line F10) tumor and Lewis lung carcinoma cells into the tail vein. These results provide preliminary evidence to indicate that tumor cell polyamine depletion by DFMO might affect the first step in the metastatic cascade, intravasation (*i.e.*, prevent the invasion of metastatic tumor cells into lymphatics or blood vessels), although the effect of DFMO on other steps in the metastatic cascade cannot be ruled out.

INTRODUCTION

The naturally occurring polyamines, putrescine, spermidine, and spermine, play an important role in growth and differentiation of mammalian cells (1–3). DFMO[2] is a specific irreversible inhibitor of ornithine decarboxylase (4) and causes a rapid depletion of intracellular putrescine and spermidine in a number of cells grown in culture (5–8). Inhibition of polyamine biosynthesis by DFMO results in inhibition of tumor growth in a number of transplanted and chemically induced animal tumors (9–12). Although an essential role for polyamines in tumor cell proliferation is well established, there was a lack of information on the role of polyamines in the process of tumor metastases until recently. We have reported (13) that inhibition of polyamine biosynthesis by DFMO in animals bearing Lewis lung carcinoma (3LL) resulted in a significant inhibition of visible pulmonary metastases. Similar results were also reported by Bartholeyns (14). The objective of the present study is to investigate the effect of DFMO on other spontaneous and experimental metastatic models and also to provide preliminary evidence for which step(s) in the tumor metastatic cascade is sensitive to inhibition of polyamine synthesis by DFMO. An abstract of this study appeared elsewhere (15).

Received 7/14/86; revised 10/3/86; accepted 11/7/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: DFMO, DL-α-difluoromethylornithine hydrochloride (Eflornithine); 3LL, Lewis lung carcinoma; B16a, B16a melanotic melanoma.

MATERIALS AND METHODS

Animals

C57BL/6J mice (18 to 20 g) from Charles River were used for the transplantation of the tumors. The animals were housed in stainless steel cages with free access to food and water.

Tumors

The spontaneously metastasizing tumors, Lewis lung carcinoma (3LL) and B16 amelanotic melanoma, were kindly provided by Mason Research Institute and Dr. K. Honn, respectively. The highly metastatic B16 melanoma line F10 was obtained through the courtesy of Dr. I. Fidler.

Spontaneous Metastases. The tumors were propagated and maintained *in vivo* by serial transfers of dissociated tumor cells in C57BL/6J mice. Rapidly dividing 2-wk-old tumors were excised from the animals and trypsinized. The resulting cell suspension was passed through sterile gauze. The viability of the tumor cells in the supernatant was determined by the trypan blue dye exclusion method. Tumors were induced in mice by s.c. injection of 1×10^5 viable cells at the interscapular region. Tumors started to appear within a wk. Mice bearing tumors were killed on the 18th day for 3LL and 25th day in the case of B16a tumors. The primary s.c. tumor was excised, and a portion of the tissue was frozen at −70°C for polyamine analysis. In the case of 3LL the lungs were examined for metastases by injecting diluted India ink into the trachea before fixation of the whole lung (16). Metastases appeared as white nodules against black normal lung. The lungs of B16a tumor-bearing mice were fixed in Bouins solution, and the white metastatic nodules over yellow normal lung tissue were counted.

Experimental Metastases. C57BL/6J mice were given injections in the lateral tail vein with 1×10^5 tumor cells/animal in a volume of 0.1 ml in minimal essential medium. At the end of 2 wk animals from different groups were sacrificed, and the number of visible metastatic foci was determined.

DFMO Administration

DL-α-Difluoromethylornithine as the hydrochloride monohydrate salt (eflornithine) was synthesized in our laboratories (4). DFMO was administered in the drinking water as an aqueous solution at different concentrations.

Polyamine Analysis

The tumor tissues were homogenized in 0.4 M perchloric acid, and the supernatants obtained after centrifugation were used for polyamine determination of initial dansylation according to the procedure of Seiler (17) and separation by reversed-phase high-pressure liquid chromatography analysis as described below.

Briefly, 1 g of tumor tissue was homogenized in 0.5 ml of $HClO_4$ (0.4 N) for 5 min at 4°C. Following centrifugation, an aliquot (0.3 ml) of the supernatant was combined with 3 N Na_2CO_3 (0.15 ml) and 1% (w/v) dansyl chloride (Sigma) in acetone (0.9 ml). This mixture was allowed to stand in the dark for 18 h at room temperature. Excess dansyl chloride was subsequently removed with 0.075 ml of proline (Sigma) solution (150 mg of proline/ml of 3 N Na_2CO_3). Acetone was then removed by evaporation, and the sample was dissolved in water (0.6 ml). Following the addition of 2 N NaOH (0.1 ml), the samples

were extracted with toluene (1.0 ml). An aliquot (0.7 ml) of the toluene layer was evaporated to dryness, and the dansylated polyamines were redissolved in 1.0 ml of mobile Solvent B.

A Waters gradient high-pressure liquid chromatographic system, equipped with two Model 510 pumps, a Model 680 automated gradient controller, and a Model 710B WISP autoinjector, was used for polyamine analysis. The fluorescence detector was a LDC/Milton Roy FluoroMonitor III, with a 370-nm excitation filter and a 418-nm emission filter. The separation was achieved on a Resolve 5-μm C_{18} column (Waters Associates) equipped with a Brownlee RP-18 guard column. Data were collected by an HP 1000 computer using CALS software.

The final conditions for analysis by high-pressure liquid chromatography were a flow of 1.0 ml/min and 25-μl injection volume. Mobile Solvent A was 20/80 acetonitrile/0.002 M sodium phosphate buffer, pH 7.0. Mobile Solvent B was 80/20 acetonitrile/0.001 M sodium phosphate buffer, pH 7.0. The gradient was run from 0 to 100% mobile Solvent B over 25 min using a convex curve (No. 4), followed by 13-min isocratic at 100% before returning to initial conditions.

The peaks of interest were identified and quantitated using polyamine standards (Sigma) at known concentrations. The elution order of the polyamine derivatives was determined to be putrescine, spermidine, and then spermine. Polyamine levels were detectable to levels of 1 pmol injected onto the column.

RESULTS

The data presented in Table 1 show a dose-dependent inhibition of tumor growth and metastases in B16 amelanotic melanoma by DFMO treatment. The greatest inhibition of tumor growth (60%) was observed at 2% DFMO in drinking water. At the same dose about 65% of the animals were free from visible metastases, and in the remainder there was a 96% inhibition of metastases compared to animals in the control group. DFMO when administered at 0.5% in drinking water did not show any effect on tumor growth. However, a 55% inhibition of visible tumor metastases was observed. The inhibition of visible tumor growth and metastases by DFMO correlated well with depletion of putrescine and spermidine but not spermine in tumor tissues (Table 2). At higher concentrations of DFMO (1 and 2%) only spermidine was further decreased, suggesting an important role for this polyamine in tumor growth and metastases.

The experiments described in Table 3 were performed to answer two questions relating to the effect of DFMO on (*a*) experimental metastases and (*b*) to provide preliminary evidence on the mechanism of inhibition of tumor metastases by DFMO. The results indicate that DFMO treatment did not inhibit experimental metastases induced by i.v. injection of tumor cells directly into the tail vein. As can be seen, DFMO treatment did not inhibit the formation of viable metastatic foci in the case of the B16 melanoma (F10) line. The control group had a mean foci value of 104.2 ± 16.6 compared to the DFMO-treated group (132.0 ± 11.1). Although polyamine-depleted cells when injected into the tail vein caused about 30% fewer foci (71.6 ± 16.8) compared to cells from tumors obtained from control animals (104.2 ± 16.6), no significant decrease was observed upon further treatment of these animals with DFMO (86.6 ± 14.8). Pretreatment of animals for 1 wk with 2% DFMO in the drinking water and then injecting the tumor cells i.v. did not inhibit the metastatic spread. Similar results were obtained with Lewis lung tumor cells (Table 3).

Table 1 *Effect of DFMO on the growth and pulmonary metastases of B16 amelanotic melanoma (B16a) in mice*

B16a cells (1×10^5) were injected s.c. in the interscapular region of C57/BL mice on Day 0. Animals were divided into 4 groups. One group was put on normal drinking water and served as control. The other 3 groups were allowed to drink water containing 0.5, 1, or 2% DFMO starting on Day 1. At the end of 25 days the animals were sacrificed, and tumors were dissected and weighed. A portion of the tumor was frozen at −70°C for polyamine determinations. The lungs were fixed in Bouin's fixative for determining the extent of pulmonary metastases.

Treatment	Tumor wt (g)	% of inhibition	No. of animals showing visible metastases	Metastatic foci	% of inhibition
Control	4.99 ± 0.29[a]		20/20	48.3 ± 5.3	
DFMO (0.5)[b]	5.13 ± 0.50[c]	0	12/12	21.5 ± 5.6[d]	55
DFMO (1)	3.77 ± 0.32[d]	24.5	14/23	8.4 ± 2.1[d]	83
DFMO (2)	2.03 ± 0.18[d]	60.0	6/17	2.2 ± 0.9[d]	96

[a] Mean ± SE.
[b] Numbers in parentheses, percentage.
[c] Not significant compared to control.
[d] $P < 0.01$ compared to control.

Table 2 *Effect of DFMO on the polyamine levels of B16 amelanotic melanoma in mice*

The experimental details are as described in Table 1.

Treatment	Polyamine concentration (nmol/g)		
	Putrescine	Spermidine	Spermine
Control	26.2 ± 4.6[a]	398 ± 8.2	298 ± 46
DFMO (0.5)[b]	8.9 ± 4.1	164 ± 69	427 ± 139
DFMO (1)	7.1 ± 4.1	109 ± 24	420 ± 73
DFMO (2)	9.5 ± 2.3	103 ± 21	354 ± 90

[a] Mean ± SD ($n = 6$).
[b] Numbers in parentheses, percentage.

DISCUSSION

The results presented in this study suggest an essential role for polyamines not only in the growth of primary tumor but also in the process of tumor metastasis. Our earlier studies in the Lewis lung tumor model could not determine whether the effect of DFMO on tumor metastases is primarily due to its effect on the primary tumor growth. However, in the present study at a dose of 0.5% DFMO in the drinking water (approximately 0.75 g/kg/day), no significant effect on the growth of primary tumor was observed, while a significant (55%) inhibition of visible tumor metastases was observed. Further these effects of DFMO correlated well with a significant decrease in putrescine and spermidine levels in the tumor tissue (Table 2). These data clearly indicate an important role of polyamines, especially spermidine, in the process of tumor metastases.

In the present study we have also investigated which stages in the metastatic cascade are most sensitive to DFMO treatment. The metastatic cascade involves a number of stages (18). The first is intravasation, *i.e.*, the release of tumor cells from the primary tumor and their invasion into circulatory systems (lymphatics or blood vessels). These tumor cells then interact with the host cells and are transported into distant organs. Tumor cells can induce platelet aggregation, and this in turn is thought to facilitate attachment of tumor cells to the endothelium. The second stage of the metastatic cascade is extravasation, *i.e.*, the process of endothelial cell retraction and degradation of basement membrane by proteolytic enzymes released from the tumor cells and their entry into the secondary site. The final stage is the establishment of these metastatic cells in the distant organ and growth into a viable metastatic foci. The above cascade of events can reoccur, resulting in a disseminated metastasis of the secondary tumors.

The results presented in the study suggest that DFMO inhibits its tumor metastasis in the first stage, *i.e.*, intravasation. The introduction of tumor cells into blood vessels by i.v. inoculation and treatment of these animals thereafter with DFMO did not result in inhibition of metastases (Table 3). Once the intrava-

Table 3 *Effect of DFMO administration on the experimental metastases of Lewis lung carcinoma (3LL) and B16 (F10) melanoma in mice*

Tumor cells (1×10^5/animal) of both B16 (F10) melanoma and Lewis lung carcinoma (3LL) were injected s.c. at the interscapular region. The animals were divided into 2 groups of 5 animals each. One group was put on normal drinking water, and the other group on 2% DFMO as the sole source of drinking fluid (3 g/kg/day). At the end of 18 days the tumors from both groups were dissected, and single cell suspensions were obtained by mincing and mild trypsinization. For B16 (F10) melanoma about 5×10^4 cells obtained from both control and DFMO-treated tumors were injected i.v. through the tail vein in a volume of 0.1 ml. In the case of Lewis lung carcinoma, 1×10^5 cells/animal were injected i.v. through the tail vein. The animals were further divided into 2 groups for each tumor type. One group was allowed to drink normal drinking water, and the other group was given 2% DFMO as the sole drinking fluid. At the end of 2 wk the animals were sacrificed, and the number of metastatic foci was quantitated.

Tumor	Pretreatment	Treatment	Metastatic foci
B16 melanoma	Control drinking water	Control water	104.2 ± 16.6[a]
		DFMO (2%) in drinking water	132.0 ± 11.1[b]
	DFMO (2%) in drinking water	Control water	71.6 ± 16.8
		DFMO (2%) in drinking water	86.6 ± 14.8
Lewis lung	Control drinking water	Control water	13.0 ± 0.8
		DFMO (2%) in drinking water	7.4 ± 1.5[b]
	DFMO (2%) in drinking water	Control water	8.6 ± 2.8
		DFMO (2%) in drinking water	6.6 ± 0.8[b]

[a] Mean ± SE ($n = 10$).
[b] Not significant compared to control group.

sation occurs, DFMO did not show any effect. However, if the cells are depleted of polyamines and then are inoculated into the blood vessels, there were consistently fewer (about 30% compared to control) pulmonary foci, suggesting a minor role for polyamines in either the extravasation or implantation of these cells to form metastatic foci.

The results of this present study suggest that inhibition of polyamine biosynthesis by DFMO significantly reduces metastatic spread at doses where no effect on primary tumor growth was observed. The data also suggest that DFMO might interfere at the intravasation stage in the metastatic cascade, *i.e.*, the initial spread of the metastatic cells from the primary tumor and their entry into blood vessels. Further, the results presented in this and other studies (13, 14) indicate that specific inhibitors of polyamine biosynthesis, such as DFMO, merit consideration as potential therapeutic agents in the clinical management of neoplasia.

ACKNOWLEDGMENTS

The authors would like to thank Greg Davis for technical assistance and Sue Treadway for secretarial assistance.

REFERENCES

1. Janne, J., Poso, H., and Raina, A. Polyamines in rapid growth and cancer. Biochim. Biophys. Acta, *473:* 241–293, 1978.
2. Pegg, A. E., and McCann, P. P. Polyamine metabolism and function: a review. Am. J. Physiol., *243:* 212–221, 1982.
3. Sunkara, P. S., and Prakash, N. J. Inhibitors of polyamine biosynthesis as antitumor and antimetastatic agents. *In:* P. S. Sunkara (ed.), Novel Approaches to Cancer Chemotherapy, pp. 93–125. New York: Academic Press, 1984.
4. Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P., and Vevert, J. P. Catalytic irreversible inhibition of mammalian ornithine decarboxylase by substrate and product analogues. J. Am. Chem. Soc., *100:* 2551–2553, 1978.
5. Mamont, P. S., Duchesne, M. C., Grove, J., and Bey, P. Antiproliferative properties of D,L-α-difluoromethylornithine in cultured cells. A consequence of irreversible inhibition of ornithine decarboxylase. Biochem. Biophys. Res. Commun., *81:* 58–66, 1978.
6. Luk, G. D., Goodwin, G., Marton, L. J., and Baylin, S. B. Polyamines are necessary for the survival of human small-cell lung carcinoma in culture. Proc. Natl. Acad. Sci. USA, *78:* 2355–2358, 1981.
7. Seidenfeld, J., Gray, J. W., and Marton, L. J. Depletion of 9L rat brain tumor cell polyamine content by treatment with D,L-α-difluoromethylornithine inhibits proliferation and the G_1 to S transition. Exp. Cell Res., *131:* 209–216, 1981.
8. Sunkara, P. S., Chang, C. C., Prakash, N. J., and Lachmann, P. J. Effect of inhibition of polyamine biosynthesis by DL-α-difluoromethylornithine on the growth and melanogenesis of B16 melanoma *in vitro* and *in vivo*. Cancer Res., *45:* 4067–4070, 1985.
9. Luk, G. D., Abelhoff, M. L., Griffin, C. A., and Baylin, S. B. Successful treatment with DL-α-difluoromethylornithine in established human small cell variant lung carcinoma implants in athymic mice. Cancer Res., *43:* 4239–4243, 1983.
10. Marton, L. J., Levin, V. A., Hervatin, S. J., Koch-Weser, J., McCann, P. P., and Sjoerdsma, A. Potentiation of the antitumor therapeutic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea by α-difluoromethylornithine, an ornithine decarboxylase inhibitor. Cancer Res., *41:* 4426–4431, 1981.
11. Prakash, N. J., Schechter, P. J., Mamont, P. S., Grove, J., Koch-Weser, J., and Sjoerdsma, A. Inhibition of EMT6 tumor growth by interference with polyamine biosynthesis: effects of α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase. Life Sci., *26:* 181–194, 1980.
12. Sunkara, P. S., Prakash, N. J., Mayer, G. D., and Sjoerdsma, A. Tumor suppression with a combination of α-difluoromethylornithine and interferon. Science (Wash. DC), *219:* 851–853, 1983.
13. Sunkara, P. S., Prakash, N. J., and Rosenberger, A. L. An essential role for polyamines in tumor metastases. FEBS Lett., *150:* 397–399, 1982.
14. Bartholeyns, J. Treatment of metastatic Lewis lung carcinoma with DL-α-difluoromethylornithine. Eur. J. Cancer Clin. Oncol., *19:* 567–572, 1983.
15. Sunkara, P. S., Rosenberger, S. L., and Davis, G. F. Further studies on the antimetastatic activity of α-difluoromethylornithine, an inhibitor of polyamine biosynthesis. Proc. Am. Assoc. Cancer Res., *26:* 59, 1985.
16. Wexler, H. J. Accurate identification of experimental pulmonary metastasis. Natl. Cancer Inst. Monogr., *36:* 641–645, 1966.
17. Seiler, N. Use of dansyl reaction in biochemical analysis. Methods Biochem. Anal., *18:* 259–334, 1970.
18. Poste, G., and Fidler, I. J. The pathogenesis of cancer metastasis. Nature (Lond.), *283:* 239–246, 1980.