Eur J Cancer Clin Oncol, Vol. 19, No. 4, pp. 567–572, 1983.
Printed in Great Britain.

0277-5379/83/040567-06$03.00/0

Treatment of Metastatic Lewis Lung Carcinoma with DL-α-Difluoromethylornithine

JACQUES BARTHOLEYNS
Centre de Recherche Merrell International 16, *rue d'Ankara*, 67084 *Strasbourg-Cedex, France*

Abstract—*The effects of* DL-α-*difluoromethylornithine (DFMO), a specific, irreversible inhibitor of ornithine decarboxylase (ODC), on tumors induced in the muscle of C57BL mice by Lewis lung (LL) carcinoma cells and on the development of lung metastases have been investigated. ODC activity and putrescine, spermidine and spermine concentrations were increased both during the early phase of development of the primary LL tumor and in the lung coinciding with the development of metastases. Oral treatment with DFMO (2% aqueous solution as sole drinking fluid, equivalent to* 4 g *DFMO/*kg/day) *decreased markedly the ODC activity and the putrescine and spermidine concentrations of the primary tumor, and stimulated* S-*adenosyl-*L-*methionine decarboxylase activity. ODC activity and putrescine and spermidine concentrations were similarly markedly reduced in the metastatic lung by DFMO treatment. By comparison with untreated controls, DFMO treatment from day* 1 *after inoculation resulted in an* 81% *decrease in tumor size and a* 92% *reduction of lung metastases by day* 20 *and prolonged the mean survival time from* 20.2 *to* 28.8 days. *The same treatment regimen started* 8 days *after tumor inoculation resulted in a* 52% *inhibition of tumor growth and an* 82% *reduction of lung metastases, and prolonged the mean survival time to* 24.9 days. *The clear antitumoral effects obtained with DFMO on this animal metastatic cancer indicate its potential value in the treatment of metastases in humans.*

INTRODUCTION

THE NATURALLY occurring polyamines, putrescine, spermidine and spermine, have long been implicated in the initiation and maintenance of rapid cell growth and have important regulatory functions in the proliferation of malignant cells [1, 2]. The first and rate-limiting step in polyamine formation is the conversion of ornithine to putrescine, catalyzed by ornithine decarboxylase (ODC: EC 4.1.1.17). DL-α-Difluoromethylornithine (DFMO, MDL 71782) is a specific enzyme-activated irreversible inhibitor of ODC [3] which causes rapid depletion of intracellular putrescine and spermidine *in vitro* and, associated with this, antiproliferative effects [4, 5]. Moreover, DFMO suppresses the increase in ODC activity and the subsequent accumulation of polyamines which occurs after tumor inoculation in animals, and has antitumoral effects against several transplanted or chemically induced animal tumors [6–14].

Accepted 21 October 1982.

DFMO therefore appears to be a useful new antiproliferative agent with a mode of action entirely different from that of currently used cytostatic or cytotoxic agents. Clinically, the terminal phase of several forms of cancer is hastened by the appearance of rapidly proliferating metastases or secondary tumors. The effects of DFMO on mammalian cells include features making it specially suitable for the regulation of rapidly dividing cells and suggest its potential value in the control of early metastatic progression. To date there has been no information as to the effects of DFMO on the growth of metastases. The Lewis lung (LL) carcinoma is an animal tumor in which the growth of the primary tumor and the subsequent appearance of metastases in the lung can easily be followed. Here I report that DFMO is an effective inhibitor of the increased polyamine biosynthesis associated both with the growth of the primary LL tumor and with the development of secondary lung metastases, and that DFMO manifests clear antitumoral effects on each phase of tumor development in this model.

MATERIALS AND METHODS

Animals

Female C57BL mice (18–20 g) from Charles River were used for the transplantation of Lewis lung carcinoma. The animals were housed in metal cages with free access to food and water. Fluid intake and body weight were measured at regular intervals. Room temperature (21–23°C), humidity (45–55%) and a 12-hr light cycle (beginning at 6 a.m.) were kept constant throughout the investigations.

Cells and tumors

The 3LL cells were kindly provided by Ms A. L. Van Lansweerde, I.C.P. Brussels, Belgium. Lewis lung carcinoma (3LL tumors, LL57 B004-005) were propagated and maintained *in vivo* in C57 black mice. The tumors were cut into small pieces in 10 vol. of isotonic saline, homogenized by 2 strokes of a Potter–Elvehjem homogenizer at 500 revs/min and filtered on gauze. Tumors were induced in mice by injection of 5×10^5 cells contained in 0.05 ml of 0.15 M NaCl into the femoral muscle. The tumor could be detected from day 8.

Animals were examined every week for tumor growth, and the diameter of their legs at the tumor level was measured with calipers in 2 perpendicular directions (breadth and width). The tumoral cross-section was considered an ellipse, and the following formula was used to calculate its size:

$$\frac{\pi}{4} \times \left[(t_1 \times t_2) - (c_1 \times c_2)\right],$$

where t_1 and t_2 are the perpendicular axes of the tumoral left leg and c_1 and c_2 are the perpendicular axes of the control right leg.

Mice bearing tumors were killed after 21 days and their lungs examined under a magnifying lens for the presence of macroscopic metastases.

Biochemistry

ODC and S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50, SAMDC) activities were measured according to Prakash *et al.* [6] on aliquots of freshly homogenized tumors in 9 vol. of ice-cold phosphate buffer (0.1 M, pH 7.2) containing 1 mM dithiothreitol, 0.1 mM disodium EDTA and 10 μM pyridoxal phosphate. Further aliquots of the homogenate were deproteinized by mixing immediately with an equal volume of 0.4 M perchloric acid, centrifuged and analyzed for polyamines by published procedures [5].

Drugs

DL-α-Difluoromethylornithine (DFMO, RMI 71782) was synthesized in our laboratories [3]. It was dissolved in tap water at a concentration of 2% for administration via the drinking water.

RESULTS

Changes in ODC and SAMDC activities and in putrescine, spermidine and spermine concentrations in the primary tumor and in the lung of mice inoculated i.m. with 3LL cells. Effect of DFMO

ODC activity was normally undetectable in samples of femoral muscle removed from C57BL mice. Five days after intramuscular inoculation of LL carcinoma cells ODC activity began to increase, and reached a peak between days 7 and 10, when the tumor could be clearly detected. Tumor mass was 0.36 g and the cross-section was 0.58 cm^2 at day 8. Thereafter the tumoral ODC activity slowly decreased (Fig. 1). Lung ODC activity remained very low until day 20 following inoculation of the tumor, when it markedly increased. The activity of SAMDC, the enzyme which provides the propylamine moiety for the spermidine and spermine synthase reactions, tended to decrease somewhat with time in both the tumor and the lung (Fig. 1). In mice receiving DFMO 2% as the sole drinking fluid starting on day 3 following inoculation of the tumor (mean daily intake, 4 g/kg) ODC activity was markedly

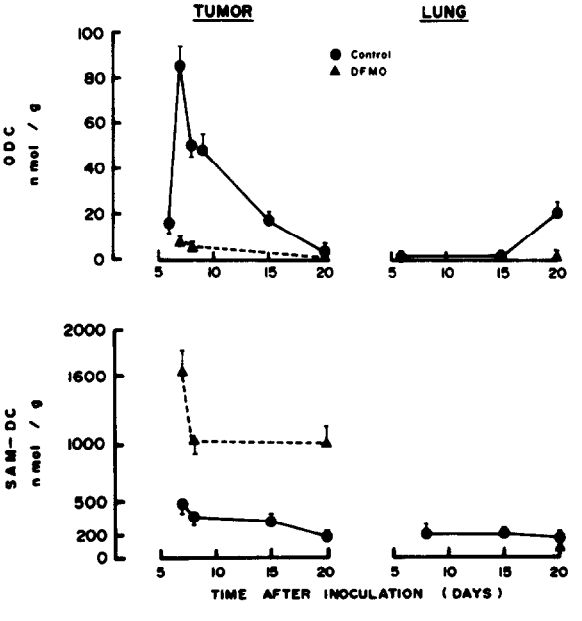

Fig. 1. Effect of DFMO on ODC and SAMDC activities in the primary tumor and in the lung of C57BL mice inoculated i.m. with Lewis lung carcinoma cells. ● Control mice, ▲ mice receiving 2% DFMO in water as sole drinking fluid beginning 3 days after inoculation. The tumors taken between days 6 and 8 following inoculation were contaminated with muscle tissue; lungs taken at day 20 were invaded by metastases. Means and S.E.M of 5 animals are presented.

reduced, both in the tumor and in the lung. The activity of SAMDC was increased about 3-fold in the tumor but remained unchanged in the lung (Fig. 1).

Putrescine concentrations in the tumor paralleled the changes in ODC activity, showing a peak at day 8 following inoculation. The tumor spermidine concentrations increased in a similar manner to those of putrescine but remained elevated between days 7 and 15 following inoculation. Spermine levels also increased during this time, although the increase was delayed relative to that of spermidine (Fig. 2). Lung putrescine and spermidine levels were little changed until day 20 when, in parallel to the change in ODC activity and coincident with the development of metastases, they showed an increase. The spermine concentration of the lung did not change significantly (Fig. 2). In mice given 2% DFMO in water as drinking fluid putrescine and spermidine levels were decreased in the primary tumor and in the lung at day 20, while spermine was only slightly affected (Fig. 2).

Effect of DFMO on the development of the primary LLC tumor and on the appearance of lung metastases

A discrete tumor could be detected in the muscle 8 days after inoculation of Lewis lung carcinoma cells and it grew rapidly thereafter. DFMO treatment resulted in a marked reduction in tumor growth (Fig. 3). If the treatment was started one day after tumor inoculation an 81% decrease in tumor size was observed at day 20. DFMO was also active when treatment was started 8 days after inoculation, the inhibition of growth being 52% at day 20 (Fig. 3).

DFMO treatment markedly reduced the development of lung metastases 20 days after intramuscular inoculation of the carcinoma cells. The number of tumoral nodules in the lung was reduced by 92 and 82% respectively after DFMO treatment started on days 1 and 8 (Table 1). Consistent with this observation was the fact that the weight of the lungs taken from mice 20 days after tumor inoculation increased by 27% compared to animals not inoculated (mean lung weight from animals not bearing tumors = 0.141 g ± 0.004, $n = 5$), whilst the weight of the lungs from the animals receiving DFMO remained close to this value.

The lifespan of the mice treated with DFMO was significantly prolonged. The increases in the mean survival time were 43 and 23% respectively for the mice treated from 1 and 8 days following tumor inoculation (Table 1). Interpretation of the weight changes in the animals during DFMO treatment was difficult due to the variable contribution arising from the tumor mass; the weight gain in DFMO-treated mice was 1.17 g/mouse/week, compared with 1.30 g for control animals. No obvious signs of toxicity or emaciation related to DFMO could be detected; subtle signs of toxicity like loose stools previously seen in rats treated with DFMO were not observed in mice.

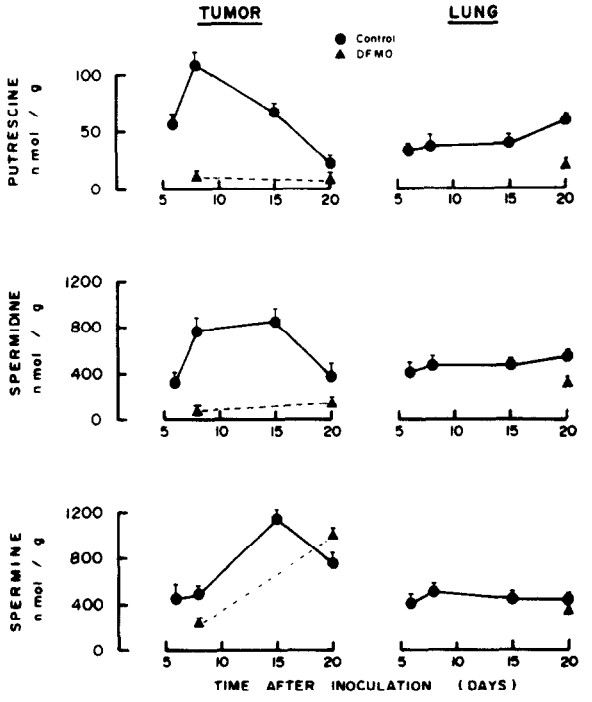

Fig. 2. Effect of DFMO on polyamine levels in the primary tumor and in the lung of C57BL mice inoculated i.m. with Lewis lung carcinoma cells. Same experiments as in Fig. 1.

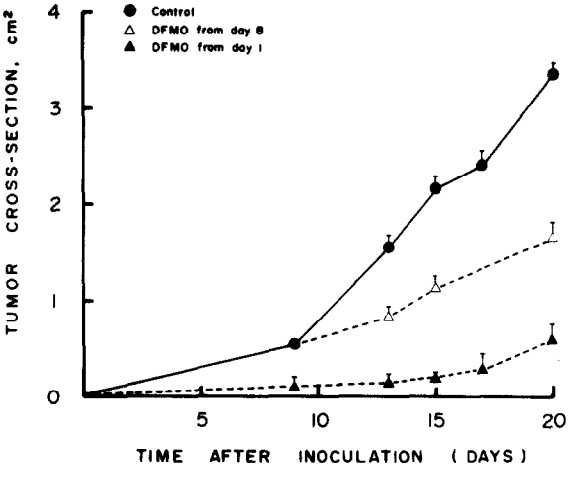

Fig. 3. Effect of DFMO on the growth of Lewis lung carcinoma inoculated in the leg of C57BL mice. ● Control mice, ▲ mice receiving 2% DFMO in water beginning 1 day after tumor inoculation, △ mice receiving DFMO in water beginning 8 days after tumor inoculation. Means and S.E.M of 5 animals are presented.

Table 1. Antitumoral effect of DFMO on the growth of Lewis lung carcinoma implanted in mice

Treatment	Control mice	Mice + 2% DFMO in drinking water from day 1 following inoculation	Mice + 2% DFMO in drinking water from day 8 following inoculation
Cross-section of the primary tumor (cm^2)	3.35 ± 0.07	0.62 ± 0.15 ($P < 0.001$)	1.60 ± 0.17 ($P < 0.001$)
Weight of the lung	0.187 ± 0.006	0.141 ± 0.006 ($P < 0.001$)	0.145 ± 0.008 ($P < 0.001$)
No. of lung metastases	18.7 ± 2.3	1.5 ± 0.8 ($P < 0.001$)	3.4 ± 1.4 ($P < 0.001$)
Mean survival time of the animals (days)	20.2 ± 0.6	28.8 ± 2-1 ($P < 0.001$)	24.9 ± 1.6 ($0.05 < P < 0.1$)

Means of 5 animals and S.E.M. are presented, except for the survival time, where 10 animals were used. Tumor size and lung metastases were measured at day 20. Statistical evaluation was by Student's *t* test.

DISCUSSION

Depletion of intracellular putrescine and spermidine by DFMO has been shown to slow the growth of several transplanted or chemically induced tumors in animals [6, 7, 10, 11, 14]. The animal tumor model used here has the advantage of allowing investigation of the effect of DFMO not only on the intramuscular primary tumor but also on the secondary lung metastases. In agreement with earlier findings [7, 10], the early growth of the primary tumor was clearly associated with an active polyamine metabolism (see time sequence in Figs 1 and 2). That these changes are essential to the tumorigenic process is suggested by the fact that treatment with DFMO, an entirely selective inhibitor of ODC, suppressed these changes and clearly inhibited growth of the primary tumor (Figs 2 and 3, Table 1).

The novel finding from the present experiments concerns the growth and development of metastases from the primary site in the lung. The changes in pulmonary ODC activity and putrescine and spermidine concentrations which we observed on day 20 correspond to the appearance of metastases and suggest a role for ODC and polyamines in the metastatic process. The marked suppression of metastases following treatment with DFMO provides evidence directly in support of this suggestion.

The question arises as to whether metastases are suppressed because their secondary implantation and development is impaired, because the primary tumor does not develop to the point where metastatic cells are released or because DFMO stops cells from leaving the primary tumor. The first of these possibilities seems the most likely explanation for the finding since DFMO was still able to suppress metastatic development when administered from day 8, at which time resection of the primary tumor as such does not delay the appearance of metastases [15].

The effects of DFMO treatment are particularly impressive in this vigorously growing tumor which is relatively resistant to therapy. Lewis lung metastatic carcinoma cannot usually be effectively treated by classical chemotherapy alone, particularly after intramuscular implantation. For example, systemic treatment with cytotoxic drugs like cyclophosphamide or methyl-CCNU has to be combined with early surgery of the primary tumor (before day 6) to obtain long-term survivors [15]. The effects of combining such cytotoxic agents with DFMO is currently being studied.

In agreement with earlier observations [6–14], treatment with DFMO resulted in an increase in the tumor SAMDC activity, which is thought to reflect the changes in spermidine concentration [4, 7]. It was paradoxical that the lack of an increase in ODC activity in the lung at day 20 following DFMO treatment was not accompanied by the usual increase in SAMDC activity. This can probably be explained by the fact that DFMO treatment delayed the development of metastases to the extent that the greater part of the lungs was free of tumor cells at day 20. It seems likely that the decrease in spermidine seen in the lung after DFMO treatment was not sufficient to trigger the induction of SAMDC.

In conclusion, DFMO has been shown to inhibit effectively the appearance and development of metastases arising from LL carcinoma in mice. The similarities which exist between the LL carcinoma model and the human small cell lung

carcinoma indicate that we can tentatively compare our results obtained *in vivo* in mice with the results obtained by Luk *et al.* [9, 16] on human small cell and non-small cell carcinoma grown *in vitro* in the presence of DFMO. DFMO markedly reduced the growth and the viability of small cell lung carcinoma *in vitro* [9] and blocked the proliferation but not the viability of human non-small cell lung cancer cells [16]. These data suggest that DFMO could be of value as adjuvant therapy in the treatment of certain forms of human metastatic cancer. In this context it seems important to emphasise that DFMO retains activity when given late in therapy, when the primary tumor can be readily detected. Moreover, since in a number of animal models additional antitumoral effects are regularly obtained when DFMO is combined with a variety of cytotoxic agents (BCNU, cyclophosphamide, cytosine-arabinoside, adriamycin, vinca alkaloids, MGBG) with minimal increase in toxicity [7, 10, 12–14, 17], the potential of these combined regimes for the chemotherapy of human metastatic cancer may be considerable.

Acknowledgements—I thank Dr J. Grove for the determination of polyamines, Dr J. Fozard for helpful suggestions during preparation of the manuscript, Mrs C. Kratz for secretarial assistance and Mrs M. Nagy and M. Host for their excellent technical assistance.

REFERENCES

1. JÄNNE J, PÖSÖ M, RAINA A. Polyamines in rapid growth and cancer. *Biochim Biophys Acta* 1978, **473**, 241–293.
2. WILLIAMS-ASHMAN HG, CANELLAKIS ZN. Polyamines in mammalian biology and medicine. *Perspect Biol Med* 1979, **22**, 421–453.
3. METCALF BW, BEY P, DANZIN C, JUNG MJ, CASARA P, VEVERT JP. Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C. 4.1.1.17) by substrate and product analogues. *J Am Chem Soc* 1978, **100**, 2551–2553.
4. MAMONT PS, DUCHESNE MC, GROVE J, BEY P. Antiproliferative properties of DL-α-difluoromethylornithine on cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. *Biochem Biophys Res Commun* 1978, **81**, 58–66.
5. DANZIN C, JUNG MJ, GROVE J, BEY P. Effect of α-difluoromethylornithine, an enzyme activated irreversible inhibitor of ornithine decarboxylase, on polyamine levels in rat tissues. *Life Sci* 1979, **24**, 519–524.
6. PRAKASH NJ, SCHECHTER PJ, GROVE J, KOCH-WESER J. Effect of α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, on L1210 leukemia in mice. *Cancer Res* 1978, **38**, 3059–3062.
7. PRAKASH NJ, SCHECHTER PJ, MAMONT PS, GROVE J, KOCH-WESER J, SJOERDSMA A. Inhibition of EMT6 tumor growth by interference with polyamine biosynthesis; effects of α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase. *Life Sci* 1980, **26**, 181–194.
8. FOZARD JR, KOCH-WESER J. Pharmacological consequences of inhibition of polyamine biosynthesis with DL-α-difluoromethylornithine. *Trends Pharmacol Sci* 1982, **3**, 107–110.
9. LUK GD, GOODWIN G, MARTON LJ, BAYLIN SB. Polyamines are necessary for the survival of human small-cell lung carcinoma in culture. *Proc Natl Acad Sci USA* 1982, **78**, 2359–2398.
10. BARTHOLEYNS J, KOCH-WESER J. Effects of α-difluoromethylornithine alone and combined with adriamicin or vindesine on L1210 leukemia in mice, EMT6 solid tumors in mice, and solid tumors induced by injection of hepatoma tissue culture cells in rats. *Cancer Res* 1981, **41**, 5158–5161.
11. KELLEN JA, CHAN AV, MIRIAKIAN A. Effect of α-difluoromethylornithine on the transplantable rat hepatoma, *Res Commun Chem Pathol Pharmacol* **30**, 377–380.
12. MARTON LJ, LEVIN VA, HERVATIN SJ, KOCH-WESER J, MCCANN PP, SJOERDMA A. Potentiation of the antitumoral therapeutic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea by α-difluoromethylornithine, an ornithine decarboxylase inhibitor. *Cancer Res* 1981, **41**, 4426–4431.
13. JÄNNE J, ALHONEN-HONGISTO L, SEPPÄNEN P, SIIMES M. Use of polyamines antimetabolites in experimental tumors and in human leukemia. *Med Biol* 1981, **59**, 448–457.
14. FOZARD JR, PRAKASH NJ. Effects of DL-α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, on the rat mammary tumor induced by 7,12-dimethylbenz(α)anthracene. *Naunyn Schmiedebergs Arch Pharmacol* **320**, 72–77.

15. MAYO JG, LASTER WR, ANDREWS CM, SCHABEL FM. Success and failure in the treatment of solid tumors. Cure of metastatic Lewis lung carcinoma with methyl-CCNU and surgery chemotherapy. *Cancer Chem Rep* 1972, **56**, 183–195.
16. LUK GD, GOODWIN G, GAZDAR A-F, BAYLIN SB. Growth inhibitory effects of DL-α-difluoromethylornithine in the spectrum of human lung carcinoma cells in culture. *Cancer Res* 1982, **42**, 3070–3073.
17. SIIMES M, SEPPÄNEN P, ALHONEN-HONGISTO L, JÄNNE J. Synergistic action of two polyamine antimetabolites leads to rapid therapeutic response in childhood leukemia. *Int J Cancer* 1981, **28**, 567–570.