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DEPLETION OF INTRACELLULAR PUTRESCINE AND SPERMIDINE BY α-DIFLUOROMETHYLORNITHINE DOES NOT INHIBIT PROLIFERATION OF 9L RAT BRAIN TUMOR CELLS

Jerome Seidenfeld[2] and Laurence J. Marton[3]

Brain Tumor Research Center

Department of Neurological Surgery

University of California School of Medicine

San Francisco, CA 94143

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Incubation of 9L rat brain tumor cells with 25 mM DL-α-difluoromethylornithine inhibits cell proliferation, while treatment with 10 mM and 1 mM do not. All three concentrations cause equal degrees of depletion of intracellular putrescine and spermidine content, but have no effect on spermine content. These observations show that 9L cells can continue to proliferate in spite of significant polyamine depletion and leads one to question the role of polyamines in 9L cell replication. These observations also suggest that inhibition of 9L cell proliferation by 25 mM DL-α-difluormethylornithine is probably not due to its effect on ornithine decarboxylase or on intracellular polyamine content.

INTRODUCTION

The current interest in elucidating the intracellular role(s) of the polyamines has led to the synthesis and investigation of a number of inhibitors of L-ornithine decarboxylase (ODC[4], E.C.4.1.1.17)(1-11), the first enzyme in the polyamine biosynthetic pathway (12,13). Metcalf, et al showed (9) that DL-α-difluoromethylornithine (DFMO), first synthesized by Bey and Vevert (8), is an enzyme-activated irreversible inhibitor of mammalian ODC. Mamont, et al report (7) that DFMO treatment results in inhibition of proliferation of rat hepatoma, L1210 murine leukemia,

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[2]To whom reprint requests should be addressed.

[3]Also in the Department of Laboratory Medicine.

[4]The abbreviations used are: ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine; Pu, putrescine; Sd, spermidine; Sp, spermine; HBSS, Hank's balanced salt solution; neq, nanoequivalents; αMO, DL-α-methylornithine.

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and human prostatic adenoma cells in vitro. These authors attribute this cytostatic effect of DFMO to the observed decline in intracellular putrescine (Pu) and spermidine (Sd). DFMO was also shown (11) to prolong the mean survival time of mice incubated with L1210 leukemic cells. We report here some preliminary observations on the effects of DFMO on 9L rat brain tumor cells grown in vitro.

MATERIALS AND METHODS

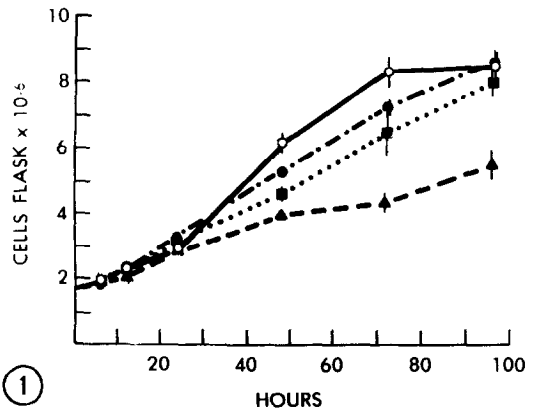
All chemicals used were of analytical grade. The DFMO used in this study was the generous gift of Merrell International Research Center, Strasbourg, France.

Rat 9L brain tumor cells were grown in vitro as previously described (14). Cells were seeded into plastic Falcon flasks (75 cm^2 surface area, 10^6 cells/flask) in 13.5 ml of medium (14) 24-26 hours before starting an experiment, to allow cells to reach exponential growth before treatment (15). Cells were treated with 1.5 ml of a 10 fold concentrated solution of DFMO in Hank's balanced salt solution (HBSS), adjusted to pH 7.2, that when added to the flask was diluted to the appropriate concentration. Control flasks were treated with 1.5 ml of HBSS. At various times after treatment the incubation medium was decanted, the cells were harvested by trypsinization (0.25% trypsin and 0.02% Na_2EDTA in Ca^{+2}-and Mg^{+2}-free HBSS for 5 min at 37 ^{0}) and suspended in 12 ml of ice cold 0.02% Na_2 EDTA in HBSS. Three flasks treated with each concentration of DFMO and three control flasks were harvested at each time point. The total number of cells/flask was measured by counting an aliquot of each cell suspension using a Royco Cell-Crit model 927-TC counter (Royco Instruments, Menlo Park, CA).

To measure the intracellular polyamine content 6 ml of each cell suspension was sedimented by centrifugation and the cell pellet was sonicated in 8% 5-sulfosalicylic acid (250 µl/10^6 cells). The homogenate was kept on ice for one hour to precipitate protein, centrifuged (8,000 xg for 10 min at 4^0) and 50 µl of supernatant was analyzed for polyamine content using a Durrum Instruments Inc. (Sunnyvale, CA) D-500 amino acid analyzer as previously described (16) with the following modifications. An 11.5 cm (1.75mm i.d.) stainless steel column was packed with a 16% cross-linked 10+ µm diameter sulfonated polystyrene cation exchange resin (Durrum Chemical Co., Sunnyvale, CA). Four buffers used for sequential elution of the polyamines were prepared as follows. A stock buffer (Buffer IV), 2.40N KCl and 0.09N potassium citrate (pH 5.56), to which 12 ml/l thiodiglycol and 0.5 ml/l liquified phenol were added, was diluted to prepare Buffers I-III as follows (stock:water): I, 1:4; II, 1:1; III, 4:1. The three polyamines were quantitated by fluorescense measurement of the chromophore produced by reaction with o-phthal - aldehyde.

RESULTS

The effects of 25, 10, and 1 mM DFMO on 9L cell proliferation are shown in Figure 1. At 48 and 72 hours post treatment flasks containing 25 mM DFMO had only 65% and 52% as many cells respectively as did control flasks. The lower concentrations of DFMO produced much smaller degrees of inhibition of cell



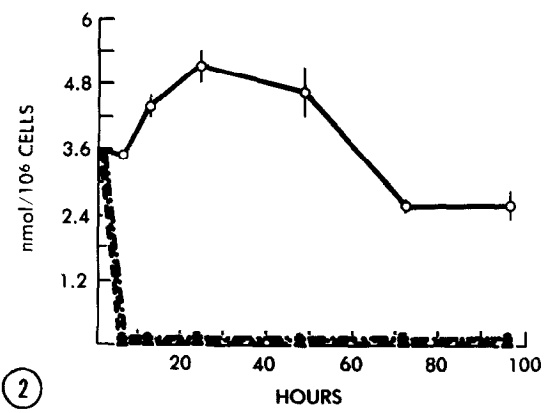


Figure 1: Effect of DFMO on 9L cell proliferation. Rat 9L brain tumor cells were incubated in the absence (◯) or in the presence of 1.0 mM (●), 10.0 mM (■), or 25.0 mM (▲) DFMO. Three flasks at each concentration were harvested by trypsinization at each time point and counted using a Royco Cell-Crit Model 927-TC cell counter. Points represent the mean (± SD) of three determinations.

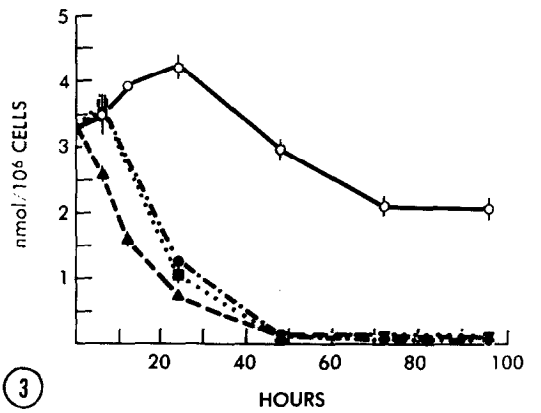
Figure 2: Effect of DFMO on 9L intracellular putrescine content. Rat 9L brain tumor cells were incubated in the absence (◯) or in the presence of 1.0 mM (●), 10.0 mM (■), or 25.0 mM (▲) DFMO. Three flasks at each concentration were harvested by trypsinization at each time point. Cells were pelleted, sonicated in 8% 5-sulfosalicylic acid, spun at 8000 x g and aliquots of the supernatants were analyzed for intracellular putrescine content with a Durrum D-500 amino acid analyzer. Points represent the mean (± SD) of three determinations.

proliferation. By 96 hours flasks with 10 or 1 mM DFMO had as many cells as did controls.

Figure 2 shows that all three concentrations of DFMO depleted intracellular Pu content to less than 0.1 nmol/10^6 cells by 12 hours after treatment. This is the lower limit of detection of our preparative and analytical methods. It is also less than 2% of control cells' Pu content at the same time. The Pu content of treated cells did not increase during the remainder of the 96 hour study.

Treatment with DFMO also resulted in depletion of intracellular Sd content (Fig. 3) which was maximal (to less than 4% of control levels) at 48 hours post treatment for all three concentrations studied. The rate of decline of Sd content was faster for cells treated with 25 mM DFMO than for those incubated with lower concentrations.

Figure 4 shows that there were no statistically significant differences in spermine (Sp) content between control cells and cells treated with 25, 10, or 1 mM



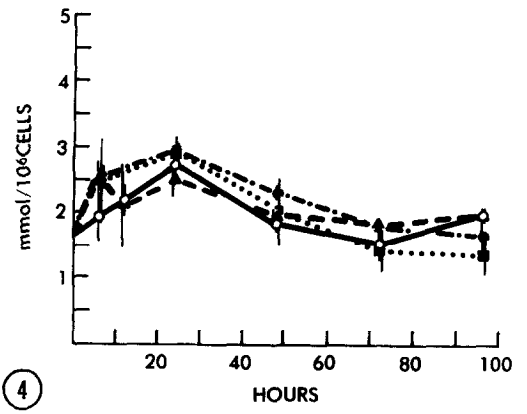


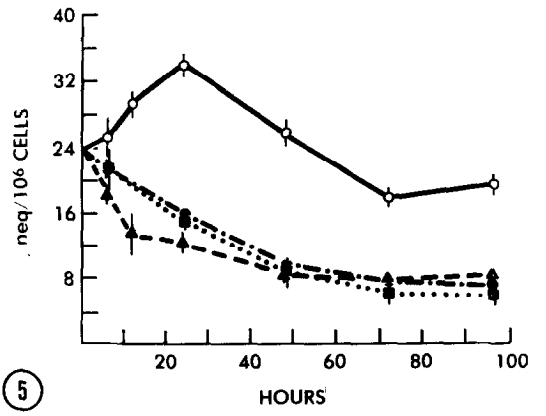
Figure 3: Effect of DFMO on 9L intracellular spermidine content. Rat 9L brain tumor cells were incubated in the absence (◯) or in the presence of 1.0 mM (●), 10.0 mM (■), or 25.0 mM (▲) DFMO. Three flasks at each concentration were harvested by trypsinization at each time point. Cells were pelleted, sonicated in 8% 5-sulfosalicylic acid, spun at 8000 x g and aliquots of the suspernatants were analyzed for intracellular spermidine content with a Durrum D-500 amino acid analyzer. Points represent the mean (± SD) of three determinations.

Figure 4: Effect of DFMO on 9L intracellular spermine content. Rat 9L brain tumor cells were incubated in the absence (◯) or in the presence of 1.0 mM (●), 10.0 mM (■), or 25.0 mM (▲)DFMO. Three flasks at each concentration were harvested by trypsinization at each time point. Cells were pelleted, sonicated in 8% 5-sulfosalicylic acid, spun at 8000 x g, and aliquots of the supernatant were analyzed for intracellular spermine content with a Durrum D-500 amino acid analyzer. Points represent the mean (± SD) of three determinations.

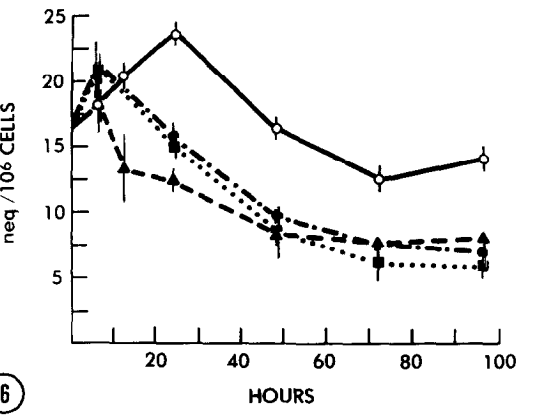
DFMO. The total polyamine content (in nanoequivalents (neq)/10^6 cells) and the sum of Sd content plus Sp content (in the same units), for cells treated with the above-mentioned concentrations of DFMO, are shown in Figures 5 and 6 respectively.

DISCUSSION

Within 48 hours of incubation of 9L cells with 10 or 1 mM DFMO the cells lost more than 98% and 96% of their normal Pu and Sd content respectively (Figs 2 and 3). Nevertheless, the parallel growth curves obtained show that from 48 to 72 hours post treatment these depleted cells grew at the same rate as controls (Fig. 1). We believe this is the first report of a wild-type cell line that continues to proliferate at the same rate as controls in spite of depletion of more than 95% of its normal complement of two of the three polyamines. In addition, the lack of



(5)



(6)

Figure 5: Effect of DFMO on 9L intracellular total polyamine content. Rat 9L brain tumor cells were incubated in the absence (○) or in the presence of 1.0 mM (●), 10.0 mM (■) or 25.0 mM (▲) DFMO. Data shown in Figures 2, 3, and 4 were used to calculate total polyamine content in neq/10^6 cells as (2 x Pu content) + (3 x Sd content) + (4 x Sp content). Points represent the mean (± SD) of three determinations.

Figure 6: Effect of DFMO on 9L intracellular Sd plus Sp content. Tumor cells were incubated in the absence (○) or in the presence of 1.0 mM (●), 10.0 mM (■), or 25.0 mM (▲) DFMO. Data shown in Figures 3 and 4 were used to calculate total Sd plus Sp content in neq/10^6 cells as (3 x Sd content) + (4 x Sp content). Points represent the mean (± SD) of three determinations.

correlation of the degree of polyamine depletion with the degree of cytostatic action for the three concentrations of DFMO studied implies that inhibition of proliferation by DFMO may not be due to effects on polyamine content of 9L cells, but rather to some non-ODC specific action.

Previous work on the effects of α-methylornithine (αMO) on 9L cell proliferation and polyamine content (17) showed that 10 mM αMO, which did not have a cytostatic effect on 9L cells, depleted Pu and Sd to less than 5% and 33% of control levels. However, 10 mM αMO increased Sp content to 150% of controls. We hypothesized (17) that the failure of 10 mM αMO to inhibit 9L cell proliferation, in spite of Pu and Sd depletion, could be due to the compensatory increase in Sp and proposed that the cell's ability to replicate might possibly be determined by the total number of neq/10^6 cells of Sd and Sp. This parameter did not differ for controls and cells treated with 10 mM αMO although total neq/10^6 cells of all three polyamines did decline for treated cells.

The results we report here, however, clearly argue against our previous hypothesis. Figures 5 and 6 show that both 10 and 1 mM DFMO significantly decrease both total polyamine content and total Sd plus Sp content of 9L cells. This is due to both the greater Sd depletion and the absence of increased Sp content resulting from DFMO treatment. Nonetheless, cells treated with 10 or 1 mM DFMO proliferate at the same rate as controls. In addition, in spite of the loss of more than 2/3 of the cells' normal complement of polyamines, or more than half their normal complement of Sd plus Sp, there were as many cells present in flasks containing 10 or 1 mM DFMO as in control flasks at 96 hours post treatment.

Similar results were recently reported by Mamont, et al (18) for a variant clone of the hepatoma tissue culture cell line produced by the selective pressure of continual incubation with αMO. These cells, designated HMO_a, can proliferate at their normal growth rate for several generations even in the presence of ODC inhibitors. While these authors refer to the HMO_a clone as "resistant to the anti-proliferative effect of ODC inhibitors," we feel it may be more appropriately described as resistant to the effects of polyamine depletion. The data reported by these authors show that incubation of HMO_a with αMO or DFMO does result in almost immediate loss of greater than 90% of control HMO_a Pu and Sd content, indicating that ODC inhibitors do act on HMO_a cells. Their observation of normal rates of cellular proliferation in spite of significant polyamine depletion is, to our knowledge, the first such report. The cell line used in that study, however, was a variant clone produced by selective pressure rather than a wild-type cell line such as 9L.

As we have discussed previously (17), 9L may be unique among cell lines used to study the role(s) of polyamines in cell proliferation and division. For example, the intracellular Pu content of unperturbed 9L cells is particularly high relative to intracellular Sd levels. The results we report here may indicate that polyamines do not play a crucial role in 9L cell replication or may, alternatively, imply that unperturbed 9L cells produce nearly two orders of magnitude more Pu and Sd than needed to support their continued proliferation. Either of these explanations for our observations may also be a characteristic unique to 9L cells.

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